These findings have fostered the current view of schizophrenia as a disorder of connectivity (3,4) and of the synapse (5). Although the mechanism underlying the neurodevelopmental/neurodegenerative process is still unclear, a way forward is provided by the recent identification of several putative susceptibility genes, such as *Neuregulin 1* (6), *Dysbindin* (7), *G72* (8), Catechol-*O*-methyltransferase (*COMT*) (9–11) and others (12,13). For none of these genes, however, has a causative allele or the mechanism by which it predisposes to schizophrenia been identified.

Disrupted-in-schizophrenia 1 (*DISC1*) was first described as a strong candidate gene in a large Scottish family in which a balanced chromosomal translocation segregates with schizophrenia and other psychiatric disorders (12,14,15). The translocation mutation may result in loss of DISC1 function via haploinsufficiency or dominant-negative effects of a predicted mutant DISC1 truncated protein product. *DISC1* has been implicated in neurodevelopment, including maturation of the cerebral cortex (16).

DISC1 interacts with several proteins, including NudE-like (NUDEL) (17–19), lissencephaly-1 (LIS1, also called PAFAH1B1) (20), fasciculation and elongation protein zeta 1 (FEZ1) (21) and phosphodiesterase 4B (PDE4B) (16). Recently, we identified several novel DISC1-interacting molecules, including 14-3-3epsilon, Kinesin family 5A of Kinesen1 (KIF5A) and Growth factor receptor bound protein 2 (Grb2) by affinity column chromatography (22,23). Furthermore, we confirmed that DISC1 regulates the localization of the NUDEL/LIS1/14-3-3epsilon complex or Grb2 into axons as a cargo receptor (22,23) and it also regulates Neurotrophin-induced axon elongation by Grb2 (23).

In this study, we screened for the genetic association of DISC1-interacting molecules—*NUDEL* (17p13.1, OMIM: *607538), *LIS1* (17p13.3, OMIM: #607432), 14-3-3epsilon (17p13.3, OMIM: *605066), *Grb2* (17p24-q23, OMIM: *108355) and *KIF5A* (12q13, OMIM: *602821)—with schizophrenia, and identified the gene encoding 14-3-3epsilon (*YWHAE*) as a possible susceptibility gene. Our results show that a SNP of *YWHAE*, which influence the expression of 14-3-3epsilon RNA and protein, is associated with schizophrenia and seems to work protectively. We also investigated the behavioral phenotype of mice with ~50% reduction in 14-3-3epsilon protein expression and found that these mice displayed weak phenotypes consistent with some aspects of human schizophrenia.

RESULTS

Screening analysis of *DISC1*-related genes and identification of *YWHAE* as a possible susceptibility gene for schizophrenia

To investigate whether novel DISC1-interacting molecules such as NUDEL, LIS1, YWAHE, GRB2 and KIF5A are associated with schizophrenia, we performed genetic association analyses using a Japanese population.

We failed to develop the genotyping of three SNPs in *LIS1* (*rs8082331*, *rs12938775* and *rs4790348*) and one SNP in *GRB2* (*rs16967795*), therefore a total of 25 SNPs were assessed in this analysis.

Though genotype distributions of two SNPs significantly deviated from Hardy–Weinberg Equilibrium (HWE, $P_{HWE} = .0143$: *rs4789172* in case sample, and $P_{HWE} = .0171$: *rs11172247* in control sample), those of the other markers were in HWE. Six tagging SNPs in *YWHAE* were significantly associated with schizophrenia and also *YWHAE* showed gene-wide significance (permutation $P = 0.0021$), whereas we found no association of tagging SNPs in *NUDEL*, *LIS1*, *GRB2* or *KIF5A* (Table 1).

Since six tagging SNPs in *YWHAE* located in the intron region, we performed denaturing high-performance liquid chromatography (dHPLC) analysis in 5′ flanking regions and entire exon regions of *YWHAE* to identify the possible causal polymorphism, and detected two SNPs: one in the 5′ flanking region (−261 bp from the initial exon: *rs28365859*) and the other one in the 3′-UTR (*rs9393*). Since the 5′ flanking region SNP might have a functional effect due to its position, we focused on this SNP in the following analysis [linkage disequilibrium (LD) structure of first-set samples in *YWHAE* can be seen in Fig. 1).

First, to examine the association of this SNP, we expanded the sample size (1065 cases and 1386 controls in a second set of confirmation samples, for a total of 1429 cases and 1728 controls including the first set of screening samples, call rates were 100%), and significant association was obtained ($P_{allele} = 1.01 \times 10^{-5}$ and $P_{genotype} = 4.08 \times 10^{-5}$). Furthermore, the significance could be detected in either set independently (Table 2). The commonly observed feature of these analyses was that the minor allele frequencies (MAFs) of this SNP were higher in controls than in schizophrenia patients. There was no discrepancy out of 380 randomly selected samples (190 cases and 190 controls) genotyped in duplicate and by another method (TaqMan Assay: C12125119) for this marker, suggesting it is unlikely that genotyping error had occurred.

Functional analysis of the promoter SNP in *YWHAE*: *in vitro* and *in vivo* expression assays

We first investigated the influence of *rs28365859* on *YWHAE* expression by dual-luciferase assay, although there is no evidence that the region where this SNP is located on is evolutionally conserved and that any regions in *YWHAE* are match as a core promoter by in silico promoter detection software. As shown in Fig. 2, a trend for significance in a promoterless vector and significance in a promoter vector were obtained in the different cell lines. The constructs containing a minor allele (C allele) showed higher expression in the promoter vector, suggesting that the C allele plays a possible enhancer role in these cell lines.

Next, to examine the role of this SNP in peripheral blood of healthy control subjects, real-time RT–PCR and western blot analysis were performed. Similar to the luciferase assays, heterozygous and homozygous minor allele (G/C and C/C genotype) subjects showed higher expression levels of 14-3-3epsilon than did homozygous major allele (G/G genotype) subjects (one-way analysis of variance, ANOVA, $P = 0.0251$ and 0.0014 in real-time RT–PCR and western blot analysis, respectively). Experimental analysis were performed to examine the differences under an additive model (G/G

Table 1. Screening analysis of DISC1-related genes

Gene	SNPs		Position[a]	Missing rate (%)	MAF Cases	Controls	*P*-value Allele	Genotype
NUDEL	rs3744652	C>T	8280008	0.3	33.0	35.9	0.250	0.274
	rs8064655	C>T	8301185	0	33.2	36.3	0.228	0.246
LIS1	rs1266474	A>G	2481460	0.4	9.72	12.4	0.110	0.0876
	rs4790356	G>A	2532979	0	10.6	11.7	0.528	0.730
	rs7212450	C>G	2538690	0	42.3	41.7	0.821	0.907
YWHAE	rs34041110	C>T	1193642	0	48.9	42.5	**0.0166**	**0.00563**
	rs9393	A>G	1195142	0	27.3	27.9	0.805	0.868
	rs8064578	C>T	1201625	0	48.5	43.4	0.0562	0.117
	rs7224258	G>C	1202252	2.1	15.0	20.3	**0.0102**	**0.0342**
	rs3752826	G>T	1211814	0	48.6	42.1	**0.0139**	**0.0175**
	rs7214541	T>C	1220072	0	44.6	49.4	0.0725	0.107
	rs11655548	A>G	1230748	2.3	29.3	38.3	**0.000418**	**0.00162**
	rs2131431	A>C	1241645	0.3	13.2	18.5	**0.00598**	**0.0176**
	rs1873827	A>G	1247690	0	42.4	49.6	**0.00732**	**0.0136**
	rs12452627	C>T	1249222	0	17.7	19.6	0.367	0.662
GRB2	rs7219	T>C	70826963	0	9.07	6.85	0.125	0.239
	rs8079197	C>G	70828274	0.6	8.45	6.60	0.190	0.308
	rs4789172	C>T	70853307	0.6	24.9[c]	26.1	0.617	0.659
	rs2053156	T>G	70890035	0	6.04	4.53	0.206	0.344
	rs930296	G>A	70915763	0	5.91	4.66	0.298	0.432
KIF5A	rs11172247	C>G	56232777	0	39.4	38.3[b]	0.676	0.609
	rs11172254	G>A	56255005	0.3	19.5	21.2	0.422	0.679
	rs775250	C>A	56263307	0	20.8	21.7	0.672	0.690
	rs775251	C>T	56265007	0.4	27.7	32.2	0.0713	0.129
	rs1678536	C>G	56265457	0.1	47.9	47.4	0.833	0.644

YWHAE showed gene-wide significance (permutation $P = 0.0021$).
Bold numbers represent significant *P*-values (<0.05).
[a]Based on HapMap database release#21.
[b]deviated from Hardy–Weinberg equilibrium.
MAF, minor allele frequency.

versus G/C+C/C), again significant associations were obtained.

Furthermore, haplotype trend regression test was applied to check the effects of haplotypes of *rs28365859* and other four SNPs in intron 1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*), which might also be in an enhancer region. This showed significant association in either analysis ($P = 0.0282$ and 0.0186 in real-time RT–PCR and western blot analysis, respectively), however, each SNP in intron 1 was not correlated with the expression level (data not shown).

Effect of reduction of 14-3-3epsilon protein on the cognitive functions of mice

14-3-3 proteins are highly conserved across species, from bacteria to humans, and bind to phosphoserine/phosphothreonine motifs in a sequence-specific manner (24–28). Previously we reported that 14-3-3epsilon binds to CDK5/p35-phosphorylated NUDEL and maintains NUDEL phosphorylation. To examine the protective effect of 14-3-3epsilon on schizophrenia using mice, we should investigate whether overexpression of 14-3-3epsilon results in resistance for the onset of schizophrenic symptoms. However, an assay system to evaluate the effect of a gene on the onset of schizophrenia in mice has not yet been developed. Thus, in support of a role for *YWHAE* in schizophrenia, we investigated *Ywhae* knockout mice. Null mice of *Ywhae* gene ($Ywhae^{-/-}$) show a severe cell migration defect in both the cortex and the hippocampus, whereas $Ywhae^{+/-}$ mice, in which the expression level of 14-3-3epsilon protein is reduced to ~50% compared with their wild-type littermates, show a milder migration defect (29). Because most $Ywhae^{-/-}$ mice die at birth as previously reported (29), $Ywhae^{+/-}$ mice and their wild-type littermates were analyzed by a comprehensive behavioral test battery to investigate whether the reduction in 14-3-3epsilon protein affects behavior (30,31). $Ywhae^{+/-}$ mice appeared normal, healthy and fertile (Table 3).

To examine whether reduction in 14-3-3epsilon was associated with cognitive deficits, we analyzed $Ywhae^{+/-}$ mice and their wild-type littermates in working memory and reference memory tasks (Table 3). To assess working memory of $Ywhae^{+/-}$ mice, we used a spatial working memory version of the 8-arm radial maze task (32,33). The mice were trained for 26 trials. During training, both control and mutant mice improved their performance and no significant difference was observed ($P = 0.3325$) (Fig. 3A). The number of revisiting errors of $Ywhae^{+/-}$ mice was significantly more than their wild-type littermates during trials with a delay of 300 s ($P = 0.0229$) (Fig. 3C). The number of different arms chosen during the first eight choices, which is considered a measure of working memory that is relatively independent of locomotor activity levels and the total number of choices, was not significantly affected by the deficit of 14-3-3epsilon protein during training and trials with 30, 120 and 300 s of delay ($P = 0.3325$, 0.8972, 0.6476 and 0.5077, respectively) (Fig. 3B and D). These results

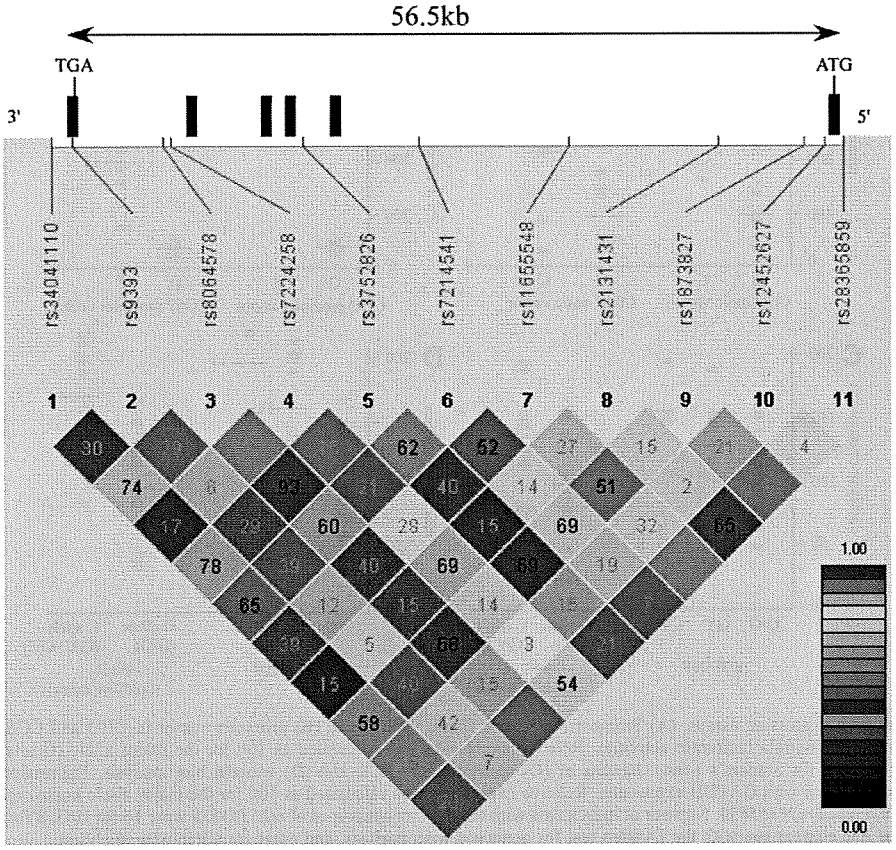

Figure 1. Tagging SNPs and LD evaluation of *YWHAE* for first-set screening samples. rs28365859 was included. Vertical bars represent exons. Numbers in boxes represent r^2 values, which should be expressed as decimals. r^2 values of 1.0 are not shown. Color scheme was based on GOLD format. Additional information is provided at the Haploview website.

Table 2. Association analysis of promoter SNP in YWHAE (rs28365859)

Samples[a]	Phenotype	*n*	Genotype G/G	G/C	C/C	MAF (%)	*P*-values HWE[b]	Allele	Genotype
Combined	Cases	1429	921	457	51	19.6	0.537	1.01×10^{-5}	4.08×10^{-5}
	Controls	1728	1000	620	108	24.2	0.366		
First-set	Cases	364	245	106	13	18.1	0.715	0.00108	0.00545
	Controls	342	192	127	23	25.3	0.748		
Second-set	Cases	1065	676	351	38	20.0	0.359	0.00123	0.00280
	Controls	1386	808	493	85	23.9	0.399		

First-set samples were identical to those used in screening analysis.
Second-set samples were independent set of samples to increase the sample size.
[a]Combined samples = first-set+second-set samples.
HWE, Hardy–Weinberg equilibrium.

suggest that *Ywhae*$^{+/-}$ mice show weak defects in working memory.

Next, we analyzed reference memory of *Ywhae*$^{+/-}$ mice, using the left–right discrimination test version of the T-maze. *Ywhae*$^{+/-}$ mice and their wild-type littermates were trained for 6 trials; then the correct side was reversed. The next 6 trials were performed under the reversal-learning condition. No significant difference was observed in the percentage of correct choices at the sixth trial (*Ywhae*$^{+/+}$, 80.647%; *Ywhae*$^{+/-}$, 77.157%; $P = 0.7516$), and no significant difference was observed under the reversal-learning condition ($P = 0.4567$) (Table 3). These results suggest that a decrease in the 14-3-3epsilon protein results in weak defects, specifically in spatial working memory.

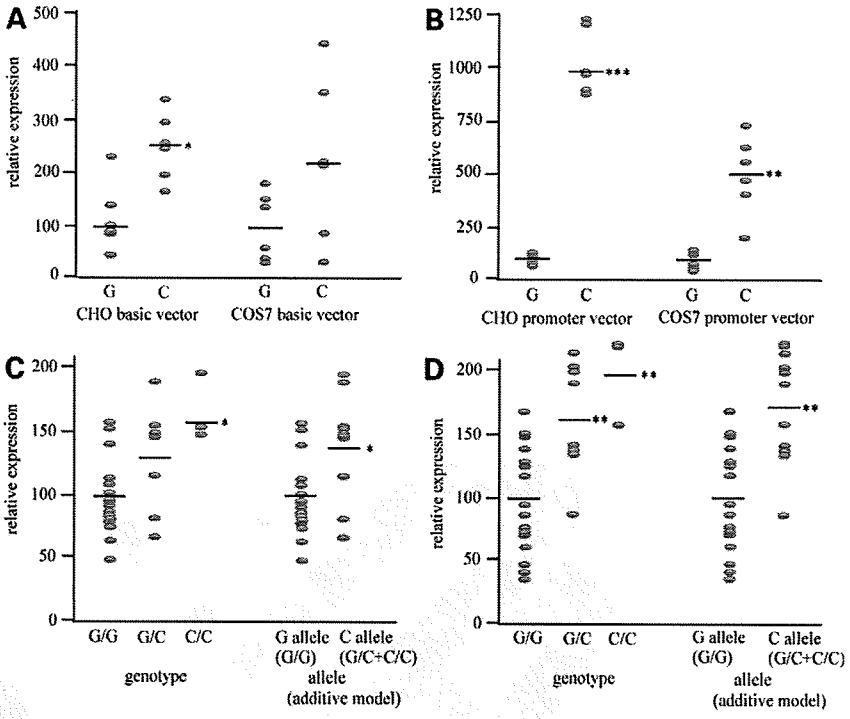

Figure 2. *In vitro* and *in vivo* expression assays. (**A**) Promoterless vector (basic vector) and (**B**) promoter vector in CHO and COS7 cells. Firefly luciferase activities were normalized with Renilla luciferase activities. Relative expression was calculated as 100 for the major allele (G allele) of *rs28365839*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (in Student's *t*-test). Results of (**C**) real-time RT–PCR and (**D**) western blot analysis. Expression levels of 14-3-3epsilon RNA and protein were normalized with GAPDH expression. Relative expression was calculated as 100 for the major allele homozygous genotype (G/G genotype) or major allele (G allele) of *rs28365839*. Number of individuals with the distinct genotypes of *rs28365839* were 16 for G/G, 8 for G/C and 3 for C/C. $^{*}P < 0.05$, $^{**}P < 0.01$ (in *post hoc* comparison with the Dunnett test for genotype-wise analysis, and *t*-test for allele-wise analysis).

Moderately enhanced anxiety-like behavior in *Ywhae*$^{+/-}$ mice in the elevated plus-maze test

To examine the effect of 14-3-3epsilon deficit on anxiety-like behavior, *Ywhae*$^{+/-}$ mice and their wild-type littermates were analyzed in light/dark transition and elevated plus-maze tests. In light/dark transition, no significant difference was observed between *Ywhae*$^{+/-}$ mice and their wild-type littermates (Table 3). In the elevated plus-maze test, *Ywhae*$^{+/-}$ mice showed a smaller number of total entries ($P = 0.0075$) (Fig. 3E), increased time spent on closed arms ($P = 0.0195$) (Fig. 3F) and decreased time spent on center area ($P = 0.0012$) (Table 3). A significant difference was not observed in the number of entries onto open arms, total distance travelled or time spent on open arms ($P = 0.2044$, 0.1071, 0.3798, respectively) (Table 3). Thus, it is conceivable that *Ywhae*$^{+/-}$ mice have moderately enhanced anxiety-like behavior that could be detected only by the elevated plus-maze test but not by the light/dark transition or by the open-field tests.

DISCUSSION

Association between *YWHAE* and schizophrenia

In this study, we have identified *YWHAE*, the gene encoding 14-3-3epsilon, which forms a complex with DISC1 *in vivo*, as a possible susceptibility gene for schizophrenia. Genetic and expression evidence indicates that the SNP in 5′ flanking region (*rs28365859*) is associated with schizophrenia through influencing the expression level of *YWHAE*. Subjects with the C allele of *rs28365859* were thought to have a reduced risk of schizophrenia [odds ratio of combined subjects = 0.76 (95% confidence interval: 0.68-0.86)]. Our sample size was relatively large (3157samples consisting of 706 first-set and 2451 second-set samples: 1429 schizophrenics and 1728 controls), making our results reliable. In addition, another research assessing the genetic association of *YWHAE* with suicide victims [two of SNPs (*rs3752826* and *rs9393*) are identical SNPs in our study and another SNP (*rs1532976*) can be captured by *rs3752826* using HapMap information] can support our results, since it showed the same trends in the distributions of MAFs (MAFs of these SNPs were higher in controls than in cases) (34). However, a couple of limitations should be outlined. First, our results that show statistical significances may be derived from unknown population stratification, since Genomic Control was not included in this analysis. Secondly, there could be a possible effect of differential age distribution between cases and controls in the association analysis.

The *in vitro* luciferase assay suggests that the C allele might act as an enhancer, since significant luciferase induction could not be seen with the use of a promoterless vector, but luciferase activity (LA) could be assayed from the vector containing a promoter. Further, *in vivo* expression assays of RNA and

Table 3. Comprehensive behavioral test battery

Test		*Ywhae*$^{+/+}$	*Ywhae*$^{+/-}$	*P*-value	*F* value
General health					
Weight (g)		28.6	29.682	0.0262*	1.335
Rectal temperature (°C)		37.033	36.688	0.0435*	4.406
Pain test					
Hot plate (latency, s)		6.206	5.053	0.1142	2.633
Motor tests					
Grip strength (*n*)		1.044	1.085	0.2825	1.194
Wire hang (latency to fall, s)		60	50	0.0234*	5.65
Rotarod (latency to fall, s; average of six trials)		161.759	182.618	0.3391	0.941
Anxiety-like behavior					
Light/dark transition					
Distance travelled (cm)	Light side	484.983	617.782	0.0728	3.434
	Dark side	1095.389	1099.288	0.97	0.001
Stay time in light side (s)		214.972	231.176	0.6043	0.274
Transitions (times)		35.111	33.588	0.6827	0.17
Latency to light side (s)		31.444	34.941	0.6683	0.187
Elevated plus-maze					
Number of entries (times)		32.556	25.118	0.0075**	8.126
Entries onto open arms (%)		31.824	26.648	0.2044	1.677
Distance travelled (cm)		1323.722	1194.329	0.1071	2.744
Time on open arms (%)		15.269	12.971	0.3798	0.793
Time on closed arms (%)		50.87	63.196	0.0195*	6.034
Time on center area (%)		35.034	23.283	0.0012*	12.495
Depression model					
Porsolt forced swim (immobility, %)	Day 1	59.369	65.648	0.0661	3.614
	Day 2	77.026	78.564	0.6256	0.243
Tail suspension (immobility, %)		26.194	22.774	0.6267	0.241
Locomotor activity					
Open field					
Total distance travelled (cm)		8745.222	9258.941	0.5822	0.309
Vertical activity (times)		208.722	393.824	0.047*	4.259
Center time (s/min)		1.432	1.107	0.6505	0.209
Stereotypic counts (times)		7260.944	6124.118	0.2251	1.528
Sensory motor gating					
Acoustic startle response		3.021	2.704	0.32	1.02
Prepulse inhibition (startle stimulus, %)					
110-dB startle		48.887	47.697	0.8496	0.037
120-dB startle		13.566	16.6	0.5617	0.344
Working memory					
8-arm radial maze					
Training					
Different arm choice in first 8 entries (times)		6.209	6.016	0.3325	0.967
Revisiting errors (times)		6.12	7.613	0.1557	2.11
Delay 30 s					
Different arm choice in first 8 entries (times)		6.5	6.471	0.8972	0.017
Revisiting errors (times)		3.417	3.676	0.7599	0.095
Delay 120 s					
Different arm choice in first 8 entries (times)		6	5.882	0.6476	0.213
Revisiting errors (times)		4.944	6.735	0.1715	1.954
Delay 300 s					
Different arm choice in first 8 entries (times)		6.167	5.971	0.5077	0.448
Revisiting errors (times)		3.778	6.294	0.0229*	5.698
Reference memory					
T-maze (correct, %)	Training	80.648	77.157	0.0696	3.519
	Reverse	61.759	59.314	0.4567	0.567
Cued and contextual fear conditioning					
Conditioning (freezing, %)		28.324	29.29	0.7581	0.096
Context testing (freezing, %)		50.998	46.611	0.5754	0.32
Cued testing with altered context freezing, %)		53.641	52.926	0.8342	0.045
Social interaction					
Total duration of contact (s)		118.386	153.383	0.1239	2.776
Number of contacts (times)		49.429	53.333	0.4968	0.494
Total duration of active contacts (s)		14.257	18.733	0.0809	3.693
Mean duration/contact		2.443	3.017	0.2889	1.241
Distance travelled (cm)		2789.357	2882.167	0.7206	0.135

Behavioral test battery was performed in the following order: general health/neurological screen, wire hang, grip strength test, light/dark transition, open field, elevated plus-maze, hot plate, social interaction (novel environment), rotarod, prepulse inhibition, Porsolt forced swim, eight arm radial maze, T-maze, cued and contextual fear condition test, latent inhibition, tail suspension test.
*$P < 0.05$, **$P < 0.01$.

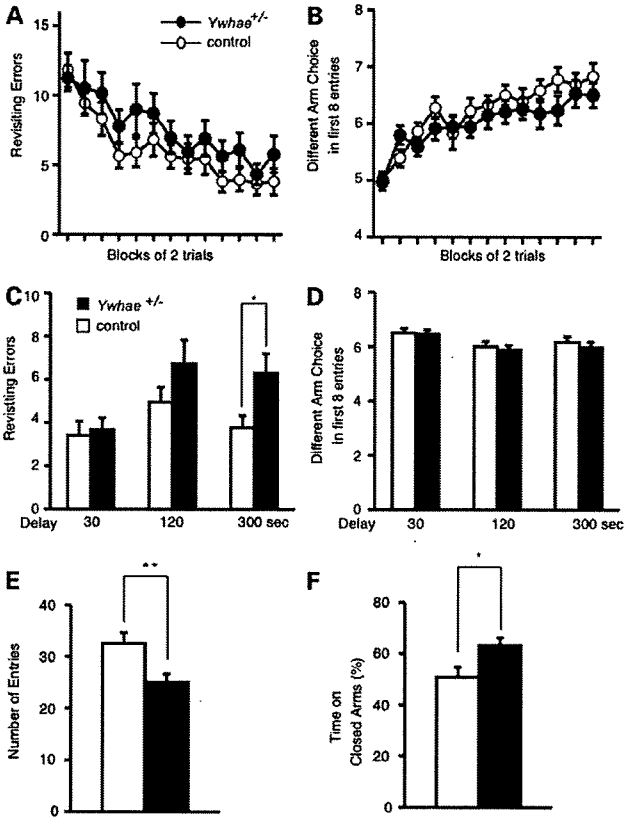

Figure 3. Behavioral abnormality of *Ywhae*$^{+/-}$ mice. (**A**–**D**) Working memory test of *Ywhae*$^{+/-}$ mice in the 8-arm radial maze (A and B). Total number of revisiting errors (A) and the number of different arms chosen in the first 8-arm visits (B) across training were counted. Data are presented as 2-day/trial averages. (C and D) Total number of revisiting errors (C) and the number of different arms chosen in the first 8-arm visits (D) of mice after training; exposure to delays of 30, 120 or 300 s after four pellets had been taken were counted (see *Materials and Methods*). (**E** and **F**) Anxiety-like behavior test of *Ywhae*$^{+/-}$ mice in the elevated plus-maze. (E) Number of total entries. (F) Time spent on closed arms. Number of total entries was lower and time spent on closed arms were greater in *Ywhae*$^{+/-}$ mice than in controls. Asterisks indicate a difference from the values of control mice. $^{*}P < 0.05$, $^{**}P < 0.01$.

protein in peripheral blood samples clarified the functional relevance of this SNP: Subjects who were heterozygous and homozygous with the C allele had higher expression of 14-3-3epsilon. Of note, our samples were control subjects not on medication; therefore, we could avoid the bias related to drug effects, which may be seen when studying schizophrenia subjects.

Also, haplotype trend regression analysis showed that the haplotypes consisted of five SNPs located in 5′ flanking region (*rs28365859*) and intron1 (*rs11655548*, *rs2131431*, *rs1873827* and *rs12452627*) were correlated with the expression level of *YWHAE*, whereas each SNP in intron 1 was not correlated with the expression. Therefore, we speculate that this significant result in haplotype-wise analysis may be derived mainly from the effects of *rs28365859*.

We analyzed for the homology of genome sequence between human and mice *Ywhae* gene using 500 bp upstream region from start ATG. About 200 bp upstream region from start ATG shows high identity, however, a region containing *rs2836589* SNP does not show homology. This result suggests that this SNP is not evolutionally conserved. We searched for functional motif on the sequence in the 5′ upstream region of *YWHAE* including *rs28365859* using TESS: Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess). In minor allele (C allele), ubiquitously expressed cellular upstream stimulatory factor (USF) -interacting motif 'CCACGT' was detected in this in silico analysis. This result may explain a possible functional effect of this SNP, an upregulation of 14-3-3epsilon in C allele-harboring people, however, further analysis would be needed to provide definitive conclusion.

Role of 14-3-3epsilon in neuronal development

Several observations of the postmortem brain suggest that alterations in neuronal cell migration, and synaptic, dendritic and axonal organizations occur in schizophrenia patients (35,36). *Ywhae*$^{+/-}$ mice show milder migration defects in both the cortex and the hippocampus, whereas *Ywhae*$^{-/-}$ mice display severe neuronal migration defects (29). Primary hippocampal neurons from *Ywhae*$^{-/-}$ mice display shorter axons and a defect in accumulation of the NUDEL/LIS1 complex in the distal part of axons (29). We confirmed that knockdown of 14-3-3epsilon by RNAi impairs not only the NUDEL/LIS1 complex transport into axons but also axon elongation (data not shown). Previously, we identified 14-3-3epsilon as an interacting molecule of *DISC1* (22). DISC1 is required to transport the NUDEL/LIS1/14-3-3epsilon complex into axons (22). Of note, depletion of endogenous DISC1 by RNAi results in a severe neuronal migration defect in the developing neocortex via regulation of the dynein complex (37). These results and reports suggest that both DISC1 and 14-3-3epsilon are required for neuronal development via transport of the NUDEL/LIS1 complex. To clarify the functional relationship between 14-3-3 epsilon and DISC1 on neuronal development via transport of the NUDEL/LIS1 complex, further genetic analysis using knockout mice will be required.

Cognitive dysfunction of *Ywhae*$^{+/-}$ mice

Ywhae$^{+/-}$ mice, in which the expression of 14-3-3epsilon protein was reduced to ~50% compared with their wild-type littermates, showed weak cognitive dysfunction specifically in working memory (Table 3). Interestingly, missense mutant mice of the *DISC1* gene show defects in working memory (38). Reduction of DISC1 or 14-3-3epsilon results in developmental defects of hippocampal neurons. These results and reports suggest that impairment of DISC1 or 14-3-3epsilon cause neuronal developmental defects, that result in cognitive dysfunction. Interestingly, impairment of working memory is one of the prominent features of schizophrenia symptomatology (39–41). Non-synonymous polymorphism of *DISC1* that consists of a serine to cysteine substitution at codon 704 (*DISC1*Ser704Cys) is reported to correlate with variations in hippocampal size and cognitive function including working memory, and is associated with

schizophrenia (42). Although relation between 14-3-3epsilon and cognitive function in human is not known, 14-3-3epsilon could be implicated in cognitive function that is associated with DISC1. Another prominent feature of schizophrenia symptomatology, prepulse inhibition (43), did not differ in *Ywhae*$^{+/-}$ mice compared with their wild-type littermates (Table 3). Schizophrenia is a complex disorder with a variety of pathology and risk factor genes. It is a reasonable assumption that modification of a single gene does not mimic all features of schizophrenia symptomatology. We think that our results using *Ywhae*$^{+/-}$ mice partly support our genetic data. However, further analysis would be required to clarify a role of 14-3-3epsilon on cognitive functions and functional relationship between *YWHAE* and *DISC1*.

YWHAE as a possible susceptibility gene for schizophrenia

In this study, we found that a SNP of *YWHAE* that correlates the expression of 14-3-3epsilon is associated with schizophrenia, and that this SNP would reduce the risk of schizophrenia. Perhaps, increased 14-3-3epsilon expression in humans affected by the identified SNP is protective, whereas decreased 14-3-3epsilon expression due to 50% reduction by heterozygous knockout in mice results in behavioral deficits. At this point, we do not know why higher expression levels of 14-3-3epsilon reduce the risk of schizophrenia, or why lower expression levels of this gene result in increase of the risk in human and behavioral changes in mice. By its susceptibility genes, schizophrenia seems to be a complex disorder with multiple symptoms and genetic risk factors. We predict that schizophrenia would be divided into several classes by its susceptibility genes. Each class would have its own molecular/signaling pathway that plays important roles in the pathogenesis. DISC1 and its interacting molecules are required in neuronal developments and adult neurongenesis (44), and would play critical roles in pathogenesis of specific classes of schizophrenia. In other classes of schizophrenia, the DISC1-pathway would not be implicated in the pathogenesis. Some genes could have redundant functions. 14-3-3epsilon is a member of adaptor proteins that interact with phosphorylated serine or threonine residue of target proteins. More than 100 of 14-3-3-binding partners involved in signal transduction, cell cycle regulation, apoptosis, stress responses and malignant transformation have been identified (45). Proteomic analysis of synapse revealed that 14-3-3epsilon forms a complex with NMDA receptor (46). Placing these results and reports in the context of the pathogenesis of schizophrenia, 14-3-3epsilon could be a susceptibility gene of not only DISC1-implicated, but also wide range of schizophrenia because of its wide variety of interacting partners. 14-3-3epsilon would be a key molecule to understand molecular mechanisms of susceptibility genes for schizophrenia.

MATERIALS AND METHODS

Subjects in genetic association analyses

In the genetic association analyses, two independent sets of subjects were examined. The first screening analysis included 364 patients with schizophrenia (188 male and 176 female; mean age $\pm$ SD 42.5 $\pm$ 14.8 years) and 342 healthy controls (191 male and 151 female; 35.0 $\pm$ 13.6 years). Patients for the second confirmation analysis included 1065 patients with schizophrenia (562 male and 503 female; 48.9 $\pm$ 14.7 years) and 1386 controls (714 male and 672 female; 42.6 $\pm$ 14.6 years). All subjects were unrelated to each other and reported to be of Japanese ethnicity. Forty patients with schizophrenia were used as subjects for a mutation search; these subjects were also included in the first-set screening scan. The schizophrenia patients were diagnosed according to criteria in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition* after at least two experienced psychiatrists reached consensus on the diagnosis on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened on the basis of unstructured interviews; to exclude subjects with any brain disorder, or psychotic disorder, or who had first-degree relatives with psychotic disorders, trained psychiatrist interviewed them to assess current and/or past mental states (psychotic, mood, anxiety, obsessive-compulsive symptoms) and family history. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, and Nagoya University Graduate School of Medicine.

SNP selection and genotyping

For LD-based association analysis using the first set of screening samples, we first consulted the HapMap and dbSNP databases to pick-up 'tagging SNPs'. From the HapMap database (Data Release #21: population JPT: MAF of >0.05: regions 8275000.8320000 for NUDEL, 2440000.2537000 for *LIS1*, 1193000.1256000 for *YWHAE*, 70823000.70917000 for *GRB2*, 56227000.56266000 for *KIF5A*), we selected a total of 27 tagging SNPs (one SNP for *NUDEL*, six SNPs for *LIS1*, nine SNPs for *YWHAE*, six SNPs for *GRB2* and five SNPs for *KIF5A*) with a threshold criterion of $r^2 > 0.8$ in pairwise tagging mode using Tagger software (47). Two SNPs (one for *NUDEL*, rs3744652 and one for *YWHAE*, rs34041110) were added for denser mapping.

All SNPs were genotyped by TaqMan assays, primer extension using dHPLC and polymerase chain reaction–restriction fragment length polymorphism assays as described previously (48). More detailed assay information can be found in Supplementary Material, Table S1.

Mutation search

After we detected significant association of *YWHAE* in screening samples, we used dHPLC analysis for a mutation search, the details of which are described in a previous paper (48). Primer pairs (Supplementary Material, Table S2) were designed with the use of information from the GenBank sequence (accession number: NT 010718.15) into 10 amplified regions, which covered all the coding regions, the branch sites and the 5′ flanking region 1026 bp upstream from the initial exon of *YWHAE*.

In vivo and *in vitro* expression assays

We used a dual-luciferase assay, real-time RT–PCR and western blot analysis to examine the influence of SNP *rs28365859* in the 5′ flanking region on expression levels of *YWHAE*. For the dual-luciferase assay, 497-bp fragments that included *rs28365859* were PCR amplified (Supplementary Material, Table S1). Genomic DNAs with identified genotypes were used as templates, and PCR products of either genotypes were cloned into a pGL3-basic vector and a pGL3-promoter vector (Promega, WI). These vectors with both alleles, the Renilla luciferase vector and the phRL-TK vector, were transiently transfected into Chinese hamster ovary (CHO) cells and COS-7 cells with the use of Lipofectamine 2000™ (Invitrogen, CA). All inserts were sequenced to confirm the containing alleles. After 48 h, cell extracts were prepared and assayed for firefly LA (LA_F) and Renilla LA (LA_R) as described by the manufacturer (Pikka-Gene Dual SeaPansy™ Luminescence Kit, Tokyo Ink, Japan) on a Fluoroskan Ascent FL (Thermo Labsystems, Finland).

For *in vitro* assays (real-time RT–PCR and western blot analysis), we processed and analyzed a total of 27 peripheral blood samples from normal control subjects to determine the amount of *YWHAE* transcript or protein: 16 subjects with homozygous major alleles (G/G genotype: 7 male and 9 female; 32.6 ± 6.4 years) in *rs28365859*; 8 subjects with heterozygous major alleles (G/C genotype: 4 male and 4 female; 33.5 ± 7.7 years) and 3 subjects with homozygous minor alleles (C/C genotype: 1 male and 2 female; 51.3 ± 17.0 years). These subjects were healthy controls who had not received any medication within at least 1 month before the collection of RNA and protein.

In the real-time RT–PCR assay, total RNA was isolated with the use of a QIAamp RNA Blood Mini Kit (QIAGEN, Inc., CA). Complementary DNA was generated with the use of a High-Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR constituents were 50 ng DNA, 2× TaqMan Universal Master Mix and 20× primer/probe mix (Hs00356749_g1, Applied Biosystems) in a 50-μl final volume. The amplification was done according to the manufacturer's instructions, and signals were recorded during PCR with the use of an ABI PRISM 7900 instrument. All gene expression results were normalized to the expression of glyceryl aldehyde-3-phosphate dehydrogenase (*GAPDH*).

In the western blot analysis, lymphocytes were purified (Axis-Shield, Oslo, Norway) and protein concentrations were determined with bovine serum albumin as the reference protein. The antibody against 14-3-3epsilon and alpha-tubulin were purchased (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Proteins were subjected to SDS–PAGE, followed by immunoblot analyses with anti-14-3-3epsilon or anti-alpha-tubulin antibody. The amount of 14-3-3epsilon was detected by chemiluminescence in a linear range using serial dilutions of standards and was estimated with Densitograph (ATTO, Tokyo, Japan). Alpha-tubulin was used as the standard for quantification. The results of these *in vivo* and *in vitro* expression assays were representative of three independent experiments.

Animals and experimental design

Ywhae$^{+/-}$ mice and their wild-type littermates were obtained as previously reported (29). Genetic background of mice is mixed 129/S6 × NIH Black Swiss. All behavioral tests (8-arm radial maze test, elevated plus-maze test, T-maze test, light/dark transition test and startle response/prepulse inhibition tests) were carried out with male mice that were 9–10 weeks old at the start of the testing. Heterozygous knockout mice and wild-type littermates were compared in experiments. Mice were housed in a room with a 12-h light/dark cycle (lights on at 7:00 a.m.) with access to food and water ad libitum. Behavioral testing was performed between 9:00 a.m. and 6:00 p.m. After the tests, all apparatus was cleaned with super hypochlorous water to prevent a bias on the basis of olfactory cues with the apparatus. Detailed description of each behavioral test (neurological screen, neuromascular strength, rotarod test, open-field test, light/dark transition test, elevated plus-maze test, hot plate test, startle response/prepulse inhibition tests, social interaction test in a novel environment, sociability and social novelty preference test, social interaction test in home cage, T-maze test and contextual and cued fear conditioning) can be seen in Supplementary methods.

Statistical analysis

Tests for HWE and marker-trait association were evaluated by χ^2 test (SAS/Genetics, release 8.2, SAS Institute Japan Inc., Tokyo, Japan). Gene-wide significance of single-SNP test was estimated by permuting phenotype status to generate 10 000 data set of SNPs in each gene under null hypothesis of no association (49). Differences in relative expression between alleles (for luciferase assay) and genotypes (for real-time PCR and western blot) were evaluated by a two-tailed Student's *t*-test and one-way ANOVA, respectively (JMP5.1J, SAS Institute Japan Inc.). When a significant difference was obtained in ANOVA, *post hoc* comparison with the Dunnett test [with homozygous major alleles (G/G genotype) set as controls] was used to identify specific group differences. Also to check the effects of haplotypes on gene expression, haplotype trend regression test with permutation (10 000 times) was applied (Power Marker V3.25 by Jack Liu, www://powermarker.net/). In behavior analysis, statistical analysis was conducted by using STATVIEW (SAS Institute, Cary, NC). Data were analyzed by ANOVA or repeated-measures ANOVA. Values in graphs were expressed as mean ± SEM. The level of significance was set at 0.05.

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SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* Online.

Conflict of Interest statement: None declared.

REFERENCES

1. Shenton, M.E., Dickey, C.C., Frumin, M. and McCarley, R.W. (2001) A review of MRI findings in schizophrenia. *Schizophr. Res.*, **49**, 1–52.
2. Pantelis, C., Yucel, M., Wood, S.J., Velakoulis, D., Sun, D., Berger, G., Stuart, G.W., Yung, A., Phillips, L. and McGorry, P.D. (2005) Structural brain imaging evidence for multiple pathological processes at different stages of brain development in schizophrenia. *Schizophr. Bull.*, **31**, 672–696.
3. Stephan, K.E., Baldeweg, T. and Friston, K.J. (2006) Synaptic plasticity and dysconnection in schizophrenia. *Biol. Psychiatry*, **59**, 929–939.
4. Paul, L.K., Brown, W.S., Adolphs, R., Tyszka, J.M., Richards, L.J., Mukherjee, P. and Sherr, E.H. (2007) Agenesis of the corpus callosum: genetic, developmental and functional aspects of connectivity. *Nat. Rev. Neurosci.*, **8**, 287–299.
5. Frankle, W.G., Lerma, J. and Laruelle, M. (2003) The synaptic hypothesis of schizophrenia. *Neuron*, **39**, 205–216.
6. Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T.T. *et al.* (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.*, **71**, 877–892.
7. Straub, R.E., Jiang, Y., MacLean, C.J., Ma, Y., Webb, B.T., Myakishev, M.V., Harris-Kerr, C., Wormley, B., Sadek, H., Kadambi, B. *et al.* (2002) Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. *Am. J. Hum. Genet.*, **71**, 337–348.
8. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P. *et al.* (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. *Proc. Natl. Acad. Sci. USA*, **99**, 13675–13680.
9. Egan, M.F., Goldberg, T.E., Kolachana, B.S., Callicott, J.H., Mazzanti, C.M., Straub, R.E., Goldman, D. and Weinberger, D.R. (2001) Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc. Natl. Acad. Sci. USA*, **98**, 6917–6922.
10. Bilder, R.M., Volavka, J., Czobor, P., Malhotra, A.K., Kennedy, J.L., Ni, X., Goldman, R.S., Hoptman, M.J., Sheitman, B., Lindenmayer, J.P. *et al.* (2002) Neurocognitive correlates of the COMT Val(158)Met polymorphism in chronic schizophrenia. *Biol. Psychiatry*, **52**, 701–707.
11. Shifman, S., Bronstein, M., Sternfeld, M., Pisante-Shalom, A., Lev-Lehman, E., Weizman, A., Reznik, I., Spivak, B., Grisaru, N., Karp, L. *et al.* (2002) A highly significant association between a COMT haplotype and schizophrenia. *Am. J. Hum. Genet.*, **71**, 1296–1302.
12. Craddock, N., O'Donovan, M.C. and Owen, M.J. (2005) The genetics of schizophrenia and bipolar disorder: dissecting psychosis. *J. Med. Genet.*, **42**, 193–204.
13. Harrison, P.J. and Weinberger, D.R. (2005) Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. *Mol. Psychiatry.*, **10**, 40–68.
14. Millar, J.K., Wilson-Annan, J.C., Anderson, S., Christie, S., Taylor, M.S., Semple, C.A., Devon, R.S., Clair, D.M., Muir, W.J., Blackwood, D.H. *et al.* (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum. Mol. Genet.*, **9**, 1415–1423.
15. Blackwood, D.H., Fordyce, A., Walker, M.T., St Clair, D.M., Porteous, D.J. and Muir, W.J. (2001) Schizophrenia and affective disorders—cosegregation with a translocation at chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. *Am. J. Hum. Genet.*, **69**, 428–433.
16. Millar, J.K., Pickard, B.S., Mackie, S., James, R., Christie, S., Buchanan, S.R., Malloy, M.P., Chubb, J.E., Huston, E., Baillie, G.S. *et al.* (2005) DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science*, **310**, 1187–1191.
17. Ozeki, Y., Tomoda, T., Kleiderlein, J., Kamiya, A., Bord, L., Fujii, K., Okawa, M., Yamada, N., Hatten, M.E., Snyder, S.H. *et al.* (2003) Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc. Natl. Acad. Sci. USA*, **100**, 289–294.
18. Millar, J.K., Christie, S. and Porteous, D.J. (2003) Yeast two-hybrid screens implicate DISC1 in brain development and function. *Biochem. Biophys. Res. Commun.*, **311**, 1019–1025.
19. Morris, J.A., Kandpal, G., Ma, L. and Austin, C.P. (2003) DISC1 (Disrupted-In-Schizophrenia 1) is a centrosome-associated protein that interacts with MAP1A, MIPT3, ATF4/5 and NUDEL: regulation and loss of interaction with mutation. *Hum. Mol. Genet.*, **12**, 1591–1608.
20. Brandon, N.J., Handford, E.J., Schurov, I., Rain, J.C., Pelling, M., Duran-Jimeniz, B., Camargo, L.M., Oliver, K.R., Beher, D., Shearman, M.S. *et al.* (2004) Disrupted in Schizophrenia 1 and Nudel form a neurodevelopmentally regulated protein complex: implications for schizophrenia and other major neurological disorders. *Mol. Cell. Neurosci.*, **25**, 42–55.
21. Miyoshi, K., Honda, A., Baba, K., Taniguchi, M., Oono, K., Fujita, T., Kuroda, S., Katayama, T. and Tohyama, M. (2003) Disrupted-in-Schizophrenia 1, a candidate gene for schizophrenia, participates in neurite outgrowth. *Mol. Psychiatry*, **8**, 685–694.
22. Taya, S., Shinoda, T., Tsuboi, D., Asaki, J., Nagai, K., Hikita, T., Kuroda, S., Kuroda, K., Shimizu, M., Hirotsune, S. *et al.* (2007) DISC1 regulates the transport of the NUDEL/LIS1/14-3-3 complex through Kinesin-1. *J. Neurosci*, **27**, 15–26.
23. Shinoda, T., Taya, S., Tsuboi, D., Hikita, T., Matsuzawa, R., Kuroda, S., Iwamatsu, A. and Kaibuchi, K. (2007) DISC1 regulates neurotrophin-induced axon elongation via interaction with Grb2. *J. Neurosci*, **27**, 4–14.
24. Muslin, A.J., Tanner, J.W., Allen, P.M. and Shaw, A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.
25. Muslin, A.J. and Xing, H. (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.*, **12**, 703–709.
26. Yaffe, M.B., Rittinger, K., Volinia, S., Caron, P.R., Aitken, A., Leffers, H., Gamblin, S.J., Smerdon, S.J. and Cantley, L.C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell*, **91**, 961–971.
27. Fu, H., Subramanian, R.R. and Masters, S.C. (2000) 14-3-3 proteins: structure, function, and regulation. *Annu. Rev. Pharmacol. Toxicol.*, **40**, 617–647.
28. Tzivion, G. and Avruch, J. (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J. Biol. Chem.*, **277**, 3061–3064.
29. Toyo-oka, K., Shionoya, A., Gambello, M.J., Cardoso, C., Leventer, R., Ward, H.L., Ayala, R., Tsai, L.H., Dobyns, W., Ledbetter, D. *et al.* (2003) 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller–Dieker syndrome. *Nat. Genet.*, **34**, 274–285.
30. Crawly (2000) *What's Wrong with My Mouse? Behavioral Phenotyping of Transgenic and Knockout Mice*, Wiley, New York.
31. Takao, K. and Miyakawa, T. (2006) Investigating gene-to-behavior pathways in psychiatric disorders: the use of a comprehensive behavioral test battery on genetically engineered mice. *Ann. N. Y. Acad. Sci.*, **1086**, 144–159.
32. Eckerman, D.A., Gordon, W.A., Edwards, J.D., MacPhail, R.C. and Gage, M.I. (1980) Effects of scopolamine, pentobarbital, and amphetamine on radial arm maze performance in the rat. *Pharmacol. Biochem. Behav.*, **12**, 595–602.
33. Okaichi, H. and Jarrard, L.E. (1982) Scopolamine impairs performance of a place and cue task in rats. *Behav. Neural. Biol.*, **35**, 319–325.
34. Yanagi, M., Shirakawa, O., Kitamura, N., Okamura, K., Sakurai, K., Nishiguchi, N., Hashimoto, T., Nushida, H., Ueno, Y., Kanbe, D. *et al.* (2005) Association of 14-3-3 epsilon gene haplotype with completed suicide in Japanese. *Hum. Mol. Genet.*, **50**, 210–216.

35. Harrison, P.J. (1999) The neuropathology of schizophrenia. A critical review of the data and their interpretation. *Brain*, **122** (Pt 4), 593–624.
36. Lewis, D.A. and Levitt, P. (2002) Schizophrenia as a disorder of neurodevelopment. *Annu. Rev. Neurosci.*, **25**, 409–432.
37. Kamiya, A., Kubo, K.I., Tomoda, T., Takaki, M., Youn, R., Ozeki, Y., Sawamura, N., Park, U., Kudo, C., Okawa, M. *et al.* (2005) A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. *Nat. Cell Biol.*, **7**, 1167–1178.
38. Clapcote, S.J., Lipina, T.V., Millar, J.K., Mackie, S., Christie, S., Ogawa, F., Lerch, J.P., Trimble, K., Uchiyama, M., Sakuraba, Y. *et al.* (2007) Behavioral phenotypes of Disc1 missense mutations in mice. *Neuron*, **54**, 387–402.
39. Goldman-Rakic, P.S. (1994) Working memory dysfunction in schizophrenia. *J. Neuropsychiatry Clin. Neurosci.*, **6**, 348–357.
40. Elvevag, B. and Goldberg, T.E. (2000) Cognitive impairment in schizophrenia is the core of the disorder. *Crit. Rev. Neurobiol.*, **14**, 1–21.
41. Callicott, J.H., Egan, M.F., Mattay, V.S., Bertolino, A., Bone, A.D., Verchinksi, B. and Weinberger, D.R. (2003) Abnormal fMRI response of the dorsolateral prefrontal cortex in cognitively intact siblings of patients with schizophrenia. *Am J. Psychiatry*, **160**, 709–719.
42. Callicott, J.H., Straub, R.E., Pezawas, L., Egan, M.F., Mattay, V.S., Hariri, A.R., Verchinski, B.A., Meyer-Lindenberg, A., Balkissoon, R., Kolachana, B. *et al.* (2005) Variation in DISC1 affects hippocampal structure and function and increases risk for schizophrenia. *Proc. Natl. Acad. Sci. USA*, **102**, 8627–8632.
43. Braff, D.L. and Geyer, M.A. (1990) Sensorimotor gating and schizophrenia. Human and animal model studies. *Arch. Gen. Psychiatry*, **47**, 181–188.
44. Duan, X., Chang, J.H., Ge, S., Faulkner, R.L., Kim, J.Y., Kitabatake, Y., Liu, X.B., Yang, C.H., Jordan, J.D., Ma, D.K. *et al.* (2007) Disrupted-in-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell*, **130**, 1146–1158.
45. Darling, D.L., Yingling, J. and Wynshaw-Boris, A. (2005) Role of 14-3-3 proteins in eukaryotic signaling and development. *Curr. Top. Dev. Biol.*, **68**, 281–315.
46. Collins, M.O., Husi, H., Yu, L., Brandon, J.M., Anderson, C.N., Blackstock, W.P., Choudhary, J.S. and Grant, S.G. (2006) Molecular characterization and comparison of the components and multiprotein complexes in the postsynaptic proteome. *J. Neurochem,.*, **97** (Suppl 1), 16–23.
47. de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J. and Altshuler, D. (2005) Efficiency and power in genetic association studies. *Nat. Genet.*, **37**, 1217–1223.
48. Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Inada, T., Ujike, H. and Ozaki, N. (2005) Association analysis of chromosome 5 GABAA receptor cluster in Japanese schizophrenia patients. *Biol. Psychiatry*, **58**, 440–445.
49. Dudbridge, F. (2003) Pedigree disequilibrium tests for multilocus haplotypes. *Genet Epidemiol*, **25**, 115–121.

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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	.181	
HAP_{ICE}#2	rs4513929	.846	.384	.196
HAP_{ICE}#3	SNP8NRG221533	.188	.397	.620
HAP_{ICE}#4	rs10096573	.200	.414	.462
HAP_{ICE}#5	rs4733263	.310	.616	.267
HAP_{ICE}#6	rs4733263	.274	.399	.578
HAP_{ICE}#7	SNP8NRG241930	.724	.113	.326
HAP_{ICE}#8	SNP8NRG243177	.288	.520	.492
HAP_{ICE}#9	rs4733267	.769	.889	.190
HAP_{ICE}#10	rs13277456	.862	.736	.847
HAP_{ICE}#11	rs13274954	.457	.670	.255
HAP_{ICE}#12	rs12677942	.312	.271	.128
HAP_{ICE}#13	rs4403369	.0803	.268	.548
HAP_{ICE}#14	rs4566990	.625	.628	.525
HAP_{ICE}#15	rs13270788	.541	.730	.699
HAP_{ICE}#16	rs1503491	.813	.866	.0960
HAP_{ICE}#17	rs2202262	.704	.324	.0653
HAP_{ICE}#18	rs10087212	.682		
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		
C#2	rs7016691	.231	.0603	.349
C#3	rs11782671	.472	.371	.296
C#4	rs10103930	.168	.474	.508
C#5	rs10503917	.699	.322	.0935
C#6	rs10107065	.765	.628	**.0244**
C#7	rs6468118	.138	.138	.174
C#8	rs7000590	.0939	.154	.158
MS1	rs7820838	.110	.107	.181
MS2	rs7834206	.149	.142	.145
C#9	rs4236709	.0786	.0879	.352
C#10	rs13260545	.0994	.187	.403
C#11	rs4316112	.948	.248	.0984
C#12	rs2439305	.196	.144	.132
C#13	rs7826814	.715	.130	.129
C#14	rs2466064	.690	.851	.436
MS3	rs3924999	.162	.313	.0699
C#15	rs10954864	.803	.113	.602
C#16	rs2439281	.965	.969	.301
C#17	rs9642729	.0680	.0725	.137
C#18	rs12547858	.0801	.0988	.523
C#19	rs10098373	.801	.457	.654
C#20	rs10095694	.380	.835	.872
MS4	rs3735774	.762	.727	.718
C#21	rs2466058	.372	.727	.587
C#22	rs2466052	.379	.526	.509
C#23	rs2466046	.187	.286	.431
C#24	rs10503923	.546	.372	.203
C#25	rs2466084	.310	.473	.197
C#26	rs2976515	.253	.551	.563
C#27	rs4445183	.702	.654	.500
C#28	rs2919377	.151	.484	.455
C#29	rs2919375	.819	.341	.182
MS5	rs3735776	.740	.222	.129
C#30	rs7007436	.711	.758	.866
C#31	rs3757934	.758	.815	.562
MS7	rs4733376	.379	.421	.357
C#32	rs4360253	.357	.336	.789
C#33	rs7005288	.864	.893	.738
C#34	rs6992642	.569	.812	
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

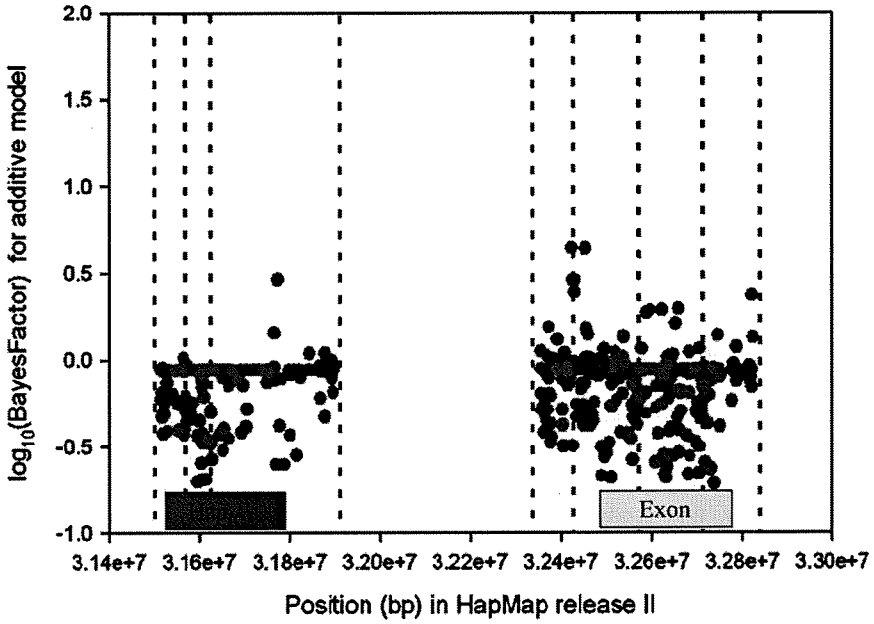

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate 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.

References

Badner, J.A., Gershon, E.S., 2002. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. Mol. Psychiatry 7 (4), 405–411.

Balding, D.J., 2006. A tutorial on statistical methods for population association studies. Nat. Rev., Genet. 7 (10), 781–791.

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21 (2), 263–265.

Corfas, G., Roy, K., Buxbaum, J.D., 2004. Neuregulin 1-erbB signaling and the molecular/cellular basis of schizophrenia. Nat. Neurosci. 7 (6), 575–580.

Corvin, A.P., Morris, D.W., McGhee, K., Schwaiger, S., Scully, P., Quinn, J., Meagher, D., Clair, D.S., Waddington, J.L., Gill, M., 2004. Confirmation and refinement of an 'at-risk' haplotype for schizophrenia suggests the EST cluster, Hs.97362, as a potential susceptibility gene at the Neuregulin-1 locus. Mol. Psychiatry 9 (2), 208–213.

de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J., Altshuler, D., 2005. Efficiency and power in genetic association studies. Nat. Genet. 37 (11), 1217–1223.

Dudbridge, F., 2003. Pedigree disequilibrium tests for multilocus haplotypes. Genet. Epidemiol. 25 (2), 115–121.

Fukui, N., Muratake, T., Kaneko, N., Amagane, H., Someya, T., 2006. Supportive evidence for neuregulin 1 as a susceptibility gene for schizophrenia in a Japanese population. Neurosci. Lett. 396 (2), 117–120.

Harrison, P.J., Law, A.J., 2006. Neuregulin 1 and schizophrenia: genetics, gene expression, and neurobiology. Biol. Psychiatry 60 (2), 132–140.

Harrison, P.J., Weinberger, D.R., 2005. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol. Psychiatry 10 (1), 40–68. image 5.

Ikeda, M., Iwata, N., Suzuki, T., Kitajima, T., Yamanouchi, Y., Kinoshita, Y., Inada, T., Ujike, H., Ozaki, N., 2005. Association analysis of chromosome 5 GABAA receptor cluster in Japanese schizophrenia patients. Biol. Psychiatry 58 (6), 440–445.

Iwata, N., Suzuki, T., Ikeda, M., Kitajima, T., Yamanouchi, Y., Inada, T., Ozaki, N., 2004. No association with the neuregulin 1 haplotype to Japanese schizophrenia. Mol. Psychiatry 9 (2), 126–127.

Lewis, C.M., Levinson, D.F., Wise, L.H., DeLisi, L.E., Straub, R.E., Hovatta, I., Williams, N.M., Schwab, S.G., Pulver, A.E., Faraone, S.V., Brzustowicz, L.M., Kaufmann, C.A., Garver, D.L., Gurling, H.M., Lindholm, E., Coon, H., Moises, H.W., Byerley, W., Shaw, S.H., Mesen, A., Sherrington, R., O'Neill, F.A., Walsh, D., Kendler, K.S., Ekelund, J., Paunio, T., Lonnqvist, J., Peltonen, L., O'Donovan, M.C., Owen, M.J., Wildenauer, D.B., Maier, W., Nestadt, G., Blouin, J.L., Antonarakis, S.E., Mowry, B.J., Silverman, J.M., Crowe, R.R., Cloninger, C.R., Tsuang, M.T., Malaspina, D., Harkavy-Friedman, J.M., Svrakic, D.M., Bassett, A.S., Holcomb, J., Kalsi, G., McQuillin, A., Brynjolfson, J., Sigmundsson, T., Petursson, H., Jazin, E., Zoega, T., Helgason, T., 2003. Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. Am. J. Hum. Genet. 73 (1), 34–48.

Marchini, J., Howie, B., Myers, S., McVean, G., Donnelly, P., 2007. A new multipoint method for genome-wide association studies by imputation of genotypes. Nat. Genet. 39 (7), 906–913.

Neale, B.M., Sham, P.C., 2004. The future of association studies: gene-based analysis and replication. Am. J. Hum. Genet. 75 (3), 353–362.

Pritchard, J.K., 2001. Are rare variants responsible for susceptibility to complex diseases? Am. J. Hum. Genet. 69 (1), 124–137.

Purcell, S., Cherny, S.S., Sham, P.C., 2003. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19 (1), 149–150.

Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S., Ivarsson, O., Chou, T.T., Hjaltason, O., Birgisdottir, B., Jonsson, H., Gudnadottir, V.G., Gudmundsdottir, E., Bjornsson, A., Ingvarsson, B., Ingason, A., Sigfusson, S., Hardardottir, H., Harvey, R.P., Lai, D., Zhou, M., Brunner, D., Mutel, V., Gonzalo, A., Lemke, G., Sainz, J., Johannesson, G., Andresson, T., Gudbjartsson, D., Manolescu, A., Frigge, M.L., Gurney, M.E., Kong, A., Gulcher, J.R., Petursson, H., Stefansson, K., 2002. Neuregulin 1 and susceptibility to schizophrenia. Am. J. Hum. Genet. 71 (4), 877–892.

Stefansson, H., Sarginson, J., Kong, A., Yates, P., Steinthorsdottir, V., Gudfinnsson, E., Gunnarsdottir, S., Walker, N., Petursson, H., Crombie, C., Ingason, A., Gulcher, J.R., Stefansson, K., St Clair, D., 2003. Association of neuregulin 1 with schizophrenia confirmed in a Scottish population. Am. J. Hum. Genet. 72 (1), 83–87.

Walss-Bass, C., Liu, W., Lew, D.F., Villegas, R., Montero, P., Dassori, A., Leach, R.J., Almasy, L., Escamilla, M., Raventos, H., 2006. A novel missense mutation in the transmembrane domain of neuregulin 1 is associated with schizophrenia. Biol. Psychiatry 60 (6), 548–553.

Williams, N.M., Green, E.K., Macgregor, S., Dwyer, S., Norton, N., Williams, H., Raybould, R., Grozeva, D., Hamshere, M., Zammit, S., Jones, L., Cardno, A., Kirov, G., Jones, I., O'Donovan, M.C., Owen, M.J., Craddock, N., 2006. Variation at the DAOA/G30 locus influences susceptibility to major mood episodes but not psychosis in schizophrenia and bipolar disorder. Arch. Gen. Psychiatry 63 (4), 366–373.

Yang, J.Z., Si, T.M., Ruan, Y., Ling, Y.S., Han, Y.H., Wang, X.L., Zhou, M., Zhang, H.Y., Kong, Q.M., Liu, C., Zhang, D.R., Yu, Y.Q., Liu, S.Z., Ju, G.Z., Shu, L., Ma, D.L., Zhang, D., 2003. Association study of neuregulin 1 gene with schizophrenia. Mol. Psychiatry 8 (7), 706–709.

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Relationship between three serotonin receptor subtypes (*HTR3A*, *HTR2A* and *HTR4*) and treatment-resistant schizophrenia in the Japanese population

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Abstract

The proportion of treatment-resistant schizophrenia (TRS) has been estimated as 20–40% in the schizophrenic patients. Genetic factors are considered to be involved in the development of this condition. Serotonin subtypes are hypothesized to be the candidate genes. In the present study, single marker and haplotype analyses between several mutations of serotonin receptor subtypes (*HTR2A*, *HTR3A* and *HTR4*) and TRS (TRS = 101, NON-TRS = 239) were performed to determine a possible relationship with the development of TRS. Additionally, we also compared the daily neuroleptic dosage among each genotype. No significant association was observed between TRS and each allele, genotype, and haplotype. However, the daily neuroleptic dosage that patients had been receiving during their maintenance therapy was significantly higher in patients with the T/T genotype of *HTR3A* polymorphism (rs1062613, $p = 0.041$). The present results support further research to examine the relationship between *HTR3A* polymorphism and the development of TRS in the Japanese population.

Keywords: *HTR*; Serotonin; Antipsychotic drug; Refractory; Gene; Haplotype

The proportion of treatment-resistant schizophrenia (TRS) has been estimated as 20–40% in the schizophrenic patients, and this unfortunate situation in the clinical psychiatric field still remain unchanged even after the introduction of several atypical antipsychotic agents [27]. Among the atypical antipsychotics, only clozapine has been reported to be effective for 30–60% of schizophrenic patients refractory to typical and atypical antipsychotics [20,28]. Clozapine is known to provide antipsychotic effects through binding to the several serotonin receptor subtypes (5-HT) [3] although the actual mechanism of clozapine for TRS has not been elucidated yet. In order to clarify this mechanism several researches investigated the predictable genetic factors for the clinical response to clozapine, as a result a significant association with the 5-HT receptor subtypes has been reported in a number of studies as follows.

Clozapine has a high affinity for 5-HT2A receptor [21] and produces a significant downregulation of cortical 5-HT2A receptor in the radioligand binding studies [2]. In addition, two PET studies have shown that the systemic administration of clozapine to schizophrenic patients produces an 84–90% occupation of cortical 5-HT2A receptor [23,6]. A couple of researches have reported the association between 5-HT2A receptor gene (*HTR2A*) polymorphism and TRS [13,7] or response to clozapine [17], although no association study has been reported in the Japanese subjects with TRS.

Since the 5-HT3A receptor has been reported to have potential anxiolytic and antipsychotic properties from animal studies, 5-HT3A receptor antagonists are being explored as therapeutic agents for a variety of behavioral disorders [5]. Additionally, 5-HT3A receptor gene (*HTR3*) is located on 11q23.1, where linkage with schizophrenia has been suggested in several studies [19,16]. These results suggest that *HTR3* may be related to

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the treatment response in the schizophrenic patients. Gutierrez have reported no association between *HTR3A* polymorphism and clozapine response [8], however, this study did not take haplotype block structure into consideration and did not cover whole genomic region of *HTR3A*.

5-HT4 receptor gene (*HTR4*) also has been reported to be associated with schizophrenia in the Japanese population [25]. Therefore, this gene could also be a candidate gene for TRS.

Thus, the *HTR2A*, *3A*, *4* could be considered as plausible genes related to the development of the TRS. Therefore, in the present study, we performed linkage disequilibrium (LD) analysis of *HTR3A*, followed by the case-control association studies between *HTR3A* polymorphisms and TRS using single-marker association analyses and haplotype analyses. In addition, the association was also examined among *HTR2A* polymorphism, *HTR4* polymorphism and TRS.

This study was initiated after the approval by the Ethics Committee of the Nagoya University School of Medicine. Written informed consent was obtained from all subjects at study entry.

A total of 340 patients with schizophrenia (male = 200; female = 140; age: 54 ± 12.8; duration of illness: 33.6 ± 12.4 years; daily neuroleptic dosage: 1021 ± 1857 mg/day) who had been diagnosed using the criteria of DSM-IIIR [1] were selected in this study. All patients were Japanese descent and had been hospitalized and receiving antipsychotic drugs for more than 1 year.

The definition of TRS is described elsewhere in the previous study [11]. Briefly, information about the neuroleptic therapy that the schizophrenic patients had been receiving was obtained from their clinical records. The daily neuroleptic dosage was calculated from the recent 1-year neuroleptic prescription history. Schizophrenic patients were diagnosed as having TRS when they had been hospitalized for more than 1 year and had been receiving antipsychotic therapy at dosages of at least 1000 mg/day chlorpromazine equivalents for more than 1 year.

Using the information obtained from the HapMap Database and the dbSNP Database, two single nucleotide polymorphisms – rs1062613 and rs1176713 – were selected as haplotype tag SNPs (htSNPs) that covered the whole coding region, 5′ flanking region upstream 500 bp, and 3′ UTR region downstream 500 bp of *HTR3A*. The LD block was defined using HAPLOVIEW Version 3.0 (http://www.broad.mit.edu/mpg/haploview/) as a region of $D' > 0.8$. In each LD block, haplotype frequency was estimated by the expectation-maximization (EM) algorithm and htSNPs were selected using the same program. Additionally, a SNP (rs6313) of *HTR2A* and two SNPs (rs2278392, rs3734119) of *HTR4* which have been reported to be associated with schizophrenia in the previous study [25] were selected. Genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism assays or direct sequence assays for each SNP. Sequences of each primer pairs are available on request.

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by Chi-square test. Single-marker and haplotype analyses were performed using SPSS Version 11.0J (Tokyo, Japan) and Cocaphase 2.403 (http://www.rfcgr.mrc.ac.uk/~fdudbrid/software/unphased/), respectively. Comparison of the daily neuroleptic dosage among each genotype was performed using Mann–Whitney *U*-test. Power calculation was performed by Power Calculator (http://calculators.stat.ucla.edu/powercalc/). The level of significance for all statistical tests was set at 0.05.

A total of 101 schizophrenic patients were identified as the TRS (TRS: male = 67, female = 34, age = 50 ± 10.5, onset age = 20 ± 5.3; NON-TRS: male = 133, female = 106, age = 56 ± 13.1, onset age = 23.5 ± 8.2). The male ratio tended to be higher in the TRS patients ($p < 0.1$), and the age at onset was significantly younger in this group ($p = 0.009$). However, no significant difference was observed in the incidence of any psychiatric symptom between the two groups, such as delusion and hallucination, bizarre behavior, disorganization, and negative symptoms at their first episode, as reported in our previous report [12]. The genotype distributions of the polymorphisms did not deviate significantly from the HWE in each study group for any polymorphism. The genotype and allele frequencies of three kinds of serotonin receptor genes in TRS and NON-TRS groups are shown in Table 1.

No significant association was observed in the single marker analysis of *HTR2A*, *HTR3A*, and *HTR4*, and in haplotype analysis of *HTR3A* and *HTR4* (Table 1).

The characteristics of neuroleptic treatment among the three subgroups showing each SNP polymorphism are shown in Table 2. In rs1062613 of *HTR3A*, the daily neuroleptic dosage during maintenance therapy was significantly higher in patients with the T/T genotype than the others ($p = 0.041$).

When the proportion of TRS was set to be 30% [9], we obtained more than 80% power to detect an association with the SNPs of which the minor frequency is more than 10%.

The results presented here suggest that *HTR3A* may be involved in the development of TRS in the Japanese population. In this study, significant difference in the daily neuroleptic dosage received during maintenance therapy was observed in schizophrenic patients with the T/T genotype of *HTR3A* polymorphism (rs1062613).

The SNP rs1062613 is located on the promoter region of *HTR3A* and has been reported to regulate the expression of this gene [22]. Since presynaptic 5-HT3A receptors modulate the release of several neurotransmitters in various brain regions [15,26], the abnormal expression of *HTR3A* might increase the dopamine concentration in the synaptic cleft. This may lead to increase the therapeutic antipsychotic doses in the schizophrenic patients with this mutation.

Additionally, several antipsychotic drugs reduce the dopaminergic neurotransmission by antagonizing the 5-HT3A receptor [24]. Therefore, reduction in the expression of 5-HT3A receptor may weaken the therapeutic effect of antipsychotics through this pathway; even higher dose of most antipsychotic drugs may not reduce the dopaminergic neurotransmission.

Furthermore, this SNP has been reported to have a critical role in the amygdala activity leading to the facial expression recognition [10], and the defect of facial expression recognition has been reported to be a specific symptom to the schizophrenia including TRS [4,18]. Therefore, this SNP may have a role in