Table 3 Haplotype analysis of tagging SNPs in *TSNAX*

TSNAX common haplotypes rs1630250-rs766288-rs6662926	Phenotype[a]	Individual haplotype frequency	Individual *P* value[b]	Phenotype[a]	Global *P* value
C–C–G	Control	0.263			
	MDD	0.236	0.280		
	BP	0.252	0.712		
C–T–G	Control	0.296		MDD	0.143
	MDD	0.266	0.258	BP	0.724
	BP	0.321	0.421		
G–C–C	Control	0.441			
	MDD	0.498	**0.0481**		
	BP	0.427	0.677		

[a] *MDD* Major depressive disorder, *BP* bipolar disorder

[b] Bold numbers represent significant *P* value

Table 4 Tagging SNPs and association analysis of *TSNAX* by sex

SNP ID[a]	Phenotype[b]	MAF	*N*	Genotype distribution			*P* value[c,d]			Corrected *P* value[d,e]	
				M/M	M/m	m/m	HWE	Genotype	Allele	Genotype	Allele
rs1630250	Male controls	0.440	352	107	180	65	0.482				
5′ flanking region	Male MDD	0.455	155	46	77	32	0.983	0.848	0.669		
C>G	Male BP	0.469	81	20	46	15	0.207	0.567	0.506		
	Female controls	0.443	459	138	235	86	0.425				
	Female MDD	0.494	159	39	83	37	0.577	0.286	0.120		
	Female BP	0.422	77	23	43	11	0.204	0.607	0.623		
rs766288	Male controls	0.362	352	148	153	51	0.266				
Intron 4	Male MDD	0.368	155	62	72	21	0.989	0.822	0.866		
C>T	Male BP	0.364	81	29	45	7	0.0726	0.110	0.962		
	Female controls	0.362	459	192	202	65	0.315				
	Female MDD	0.277	159	79	72	8	0.0980	**0.00661**	**0.00586**	**0.0397**	**0.0352**
	Female BP	0.377	77	28	40	9	0.351	0.429	0.721		
rs6662926	Male controls	0.492	352	92	174	86	0.835				
3′ flanking region	Male MDD	0.471	155	41	82	32	0.442	0.630	0.547		
C>G	Male BP	0.444	81	25	40	16	1.00	0.561	0.280		
	Female controls	0.501	459	115	228	116	0.889				
	Female MDD	0.456	189	46	81	32	0.735	0.363	0.166		
	Female BP	0.494	77	19	40	18	0.731	0.920	0.862		

[a] Major allele > minor allele

[b] *MDD* Major depressive disorder, *BP* bipolar disorder, *MAF* minor allele frequency, *M* major allele, *m* minor allele

[c] Hardy–Weinberg equilibrium

[d] Bold represents significant *P* value

[e] Calculated by Bonferroni's correction

Discussion

We first performed a gene-based association analysis between *TSNAX* and mood disorders including BP and MDD in the Japanese population. We found almost no association between *TSNAX* and mood disorders. However, we detected a significant association between *TSNAX* and Japanese female MDD in the Japanese population. This significant association remained after Bonferroni's correction was used to control inflation of the type I error rate due to multiple testing. *TSNAX* was associated with impaired spatial working memory, increased reaction time to visual targets, and reduced gray matter predominantly in the superior and middle frontal gyri (Cannon et al. 2005). This evidence may be involved in the pathophysiology of female MDD.

Table 5 Haplotype analysis of tagging SNPs in *TSNAX* by sex

TSNAX common haplotypes rs163 0250–rs766288–rs6662926	Phenotype[a]	Individual haplotype frequency	Individual *P* value[b]	Phenotype[a]	Global *P* value
C–C–G	Male controls	0.282			
	Male MDD	0.227	0.134		
	Male BP	0.258	0.596		
	Female controls	0.249		Male MDD	0.751
	Female MDD	0.245	0.903	Male BP	0.751
	Female BP	0.246	0.937		
C–T–G	Male controls	0.284		Female MDD	0.0744
	Male MDD	0.304	0.508	Female BP	0.885
	Male BP	0.317	0.477		
	Female controls	0.304			
	Female MDD	0.231	**0.0400**		
	Female BP	0.325	0.628		
G–C–C	Male controls	0.424			
	Male MDD	0.469	0.393		
	Male BP	0.425	0.864		
	Female controls	0.447			
	Female MDD	0.524	**0.0488**		
	Female BP	0.429	0.703		

[a] *MDD* Major depressive disorder, *BP* bipolar disorder

[b] Bold numbers represent significant *P* value

Table 6 Genotype and allele distributions of *TSNAX* in both definition groups

SNP ID[a]	Phenotype[b]	MAF	*N*	Genotype distribution			*P* value[c]		
				M/M	M/m	m/m	HWE	Genotype	Allele
rs1630250	Responders	0.500	61	15	31	15	0.898		
5′ flanking region	Nonresponders	0.551	59	14	25	20	0.270	0.507	0.430
C>G	Remission	0.510	47	12	22	13	0.664		
	Nonremission	0.534	73	17	34	22	0.584	0.942	0.721
rs766288	Responders	0.311	61	26	32	3	0.0815		
Intron 4	Nonresponders	0.271	59	31	24	4	0.823	0.429	0.492
C>T	Remission	0.266	47	23	23	1	0.0824		
	Nonremission	0.308	73	34	33	6	0.608	0.380	0.482
rs6662926	Responders	0.426	61	20	30	11	0.966		
3′ flanking region	Nonresponders	0.339	59	18	29	12	0.959	0.936	0.720
C>G	Remission	0.404	47	16	24	7	0.680		
	Nonremission	0.459	73	22	35	16	0.768	0.628	0.405

[a] Major allele > minor allele

[b] *MDD* Major depressive disorder, *BP* bipolar disorder, *M* major allele, *m* minor allele, *MAF* minor allele frequency

[c] Hardy–Weinberg equilibrium

It is known that there are sex differences in the pathophysiology of mood disorders (Currier et al. 2006; Faraone et al. 1987). We detected an association between rs766288 in intron 4 in *TSNAX* and female Japanese MDD patients. Several other investigations have also reported sex differences in associations between *TSNAX* and psychiatric disorders. Thomson et al. (2005) showed an association between several SNPs, including rs766288, in *TSNAX* and male Scottish BP patients in a haplotype analysis. Palo et al. (2007) reported that *TSNAX* (single marker association analysis: rs1655285 and haplotype analysis, including rs1655285) was associated with female psychotic disorder. However, because SNP composites for these haplotypes were "MAFs = 0" or raw data were not presented in the

HapMap database, we did not perform an association analysis for this SNP in the current study. Other genes have demonstrated gender differences in association to mood disorders. Szczepankiewicz et al. reported an association between a diagnosis of BP II in females and the glycogen synthase kinase-3 β gene (*GSK3B*) (Szczepankiewicz et al. 2006). Our previous study reported an association between prokineticin 2 receptor gene (*PROKR2*) and female BP and MDD (Kishi et al. 2009c). Several sex differences are observed in mood disorders, with the prevalence of MDD being higher in females. Hormonal variations in females, such as during the menstrual cycle, pregnancy and menopause, affect the onset and course of mood disorders, and it is possible that the etiology of mood disorders differs somewhat in females and males. Since our findings show significant associations between *TSNAX* and MDD in female Japanese patients, our results may support the supposition that the etiology of mood disorders differs somewhat in females and males.

According to HapMap database, rs1630250's MAFs in the Japanese population appear to be smaller than in Caucasians. On the other hand, rs6662926's MAFs in Caucasians were smaller than in Japanese. Also, rs766288 was the almost same MAFs in both Japanese and Caucasians. Schosser et al. (2009) reported that rs766288 was not associated with BP or MDD in the UK population. Although they selected only one SNP (rs766288) in *TSNAX*, their study was a case–control study using larger samples than our study (Schosser et al. 2009). Hennah et al. haplotype transmission analysis showed that SNPs in intron 4 in *TSNAX* (rs1615344, rs1615409, and rs766288) was associated with schizophrenia. Zhang et al. reported that *TSNAX* was not associated with Japanese schizophrenic patients. In this study, they selected rs1630250, rs1621135, and rs1655284 in *TSNAX*. We used rs1630250, and considered other SNPs that were in LD with tagging SNPs according to the HapMap database (release#23a/phase II, March 2008, www.hapmap.org, population: Japanese Tokyo). For MAF of rs1630250, our findings were almost same as those of Zhag and et al.' study (2005). In the Finnish population, Kilpinen et al. (2008) did not detect an association between *TSNAX* and autism or Asperger syndrome.

Because testing for HWE is commonly used for quality control in large-scale genotyping and is one of the few ways to identify systematic genotyping errors in unrelated individuals (Wittke-Thompson et al. 2005), we estimated HWE and confirmed the genotyping quality in this study. Genotype frequencies were in HWE for the SNPs in this study.

A few points of caution should be mentioned with respect to our results. Firstly, an association of *TSNAX* with female MDD patients may be due to biased samples, such as small sample sizes or unmatched age. In the power analysis, we obtained power of more than 80% for the detection of association when we set the genotype relative risk at 1.26–1.30 and 1.41–1.48 in MDD and BP, respectively, under a multiplicative model of inheritance. Because our samples were small, the statistical errors are possible in the results of these statistical association analyses. On average, the controls were much younger than the patients. This means that a number of young controls may go on to develop one of these disorders, most likely MDD, since the incidence of major depression is as high as 5% or more. Our subjects did not undergo structured interviews, and it is reported that MDD patients who are not diagnosed by structured interview may develop bipolar disorder in the future (Bowden 2001; Kishi et al. 2008b, 2009a; Stensland et al. 2008). However, in this study patients were carefully diagnosed according to DSM-IV criteria with consensus of at least two experienced psychiatrists on the basis of a review of medical records. In addition, when we found a patient who had been misdiagnosed, we promptly excluded the misdiagnosed case to maintain the precision of our sample (Kishi et al. 2008a, b, 2009b). Secondly, we did not perform a mutation scan of *TSNAX*. Because we consider it to be difficult to evaluate the association of extremely rare variants from the viewpoint of statistical power, a replication study using a larger sample will be required for conclusive results. Thirdly, HapMap data has been updated to release #27 to date.

In conclusion, our results suggest that *TSNAX* probably plays a role in female MDD in the Japanese population. However, because our samples are small, it will be important to replicate and confirm these findings in other independent studies using larger samples.

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The *chitinase 3-like 1* gene and schizophrenia: Evidence from a multi-center case–control study and meta-analysis

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ABSTRACT

The *chitinase 3-like 1* (*CHI3L1*) gene acts as a cellular survival factor in response to several environmental and psychosocial stresses. The expression level of *CHI3L1* was increased in the hippocampus and prefrontal cortex regions of patients with schizophrenia. Genetic variants of the *CHI3L1* gene have been significantly associated with schizophrenia in two distinct ethnic groups, the Chinese and Irish populations. The aims of this study are to confirm the association between the *CHI3L1* gene and schizophrenia in a Japanese population using the largest sample size to date (1463 cases and 1795 controls) and perform a meta-analysis of the combined samples (3005 cases, 3825 controls and 601 trios). We found significant associations between single nucleotide polymorphism (SNP) 4/rs4950928 ($p=0.009$), which is located in the promoter region of the *CHI3L1* gene, and haplotypes including this SNP and schizophrenia (the most significant global $p<0.001$). As the meta-analysis of the combined samples showed significant heterogeneity among studies of SNP3/rs10399805 ($p=0.026$) and SNP4 ($p<0.001$), we performed meta-analyses separately in the Japanese (2033 cases and 2365 controls) and Chinese populations (412 cases, 464 controls and 601 trios), the major groups analyzed in association studies of the *CHI3L1* gene. The meta-analysis in Japanese populations showed stronger evidence for the association of schizophrenia with SNP4 ($p=0.003$), while the meta-analysis in Chinese populations showed an association with a different variant (SNP3) ($p=0.003$). We conclude that the genetic variants in the *CHI3L1* gene have ethnic heterogeneity and confer a susceptibility to schizophrenia in Asian populations.

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1. Introduction

Schizophrenia (OMIM 181500) is a common and complex psychiatric disease. The lifetime morbidity rate is 0.5–1.0% across distinct populations. Family, twin and adoption studies of schizophrenia have indicated that there are strong genetic factors with an estimated heritability of approximately 80% (Cardno and Gottesman, 2000). Many genes have been implicated in the pathogenesis of schizophrenia (Sun et al., 2008).

The *chitinase 3-like 1* gene [*CHI3L1*, (OMIM 601525)] consists of 10 exons and spans approximately 8 kb of genomic DNA. The protein was named YKL-40 based on its three N-terminal amino acids, tyrosine (Y), lysine (K) and leucine (L), and its molecular mass of 40 kDa (Johansen et al., 1992). The protein has several names, including chitinase 3-like 1, human cartilage glycoprotein-39 (HC gp39), breast regressing protein 39 (brp-39), 38-kDa heparin-binding glycoprotein (gp38k), chondrex and 40-kDa mammary gland protein (MGP-40). In this study, to avoid confounding these terms, the gene is referred to as *CHI3L1* and the protein is referred to as YKL-40.

This gene acts as a cellular survival factor in responses to a variety of adverse environments, including various types of physiologic stress, such as inflammation, hypoxia and nutrient deprivation. These stresses may induce high expression of *CHI3L1* (Junker et al., 2005; Recklies et al., 2005). YKL-40 is secreted by activated macrophages and neutrophils in different tissues during inflammation and during increased remodeling of the extracellular matrix (Kirkpatrick et al., 1995; Rehli et al., 1997; Volck et al., 1998). YKL-40 initiates mitogen-activated protein (MAP) kinase and phosphoinositide 3-kinase (PI3K) signaling cascades in fibroblasts. Signaling leads to the phosphorylation of both the extracellular signal-regulated kinase (ERK)-1/2 MAP kinase- and the protein kinase B (AKT)-mediated signaling cascades, which are associated with the control of mitogenesis (Recklies et al., 2002). The PI3K pathway and the downstream phosphorylation of AKT in particular are strongly associated with cell survival (Bakkenist and Kastan, 2004), which suggests a role for YKL-40 as an anti-apoptotic protein.

The synthesis of YKL-40 is induced by the inflammatory cytokines IL-1, IL-6 and TNF-α (Ling and Recklies, 2004; Recklies et al., 2005; Johansen et al., 2006). The genetic variants of the *CHI3L1* gene and high serum levels of YKL-40 are associated with several inflammatory diseases, including sarcoidosis, asthma and inflammatory bowel diseases (Kruit et al., 2007; Kucur et al., 2007; Ober et al., 2008). The role of YKL-40 in the nervous system is unclear. YKL-40 is elevated in the cerebrospinal fluid (CSF) of patients with spinal diseases in which the neural tissue has been damaged or stressed, including cervical myelopathy, lumbar canal stenosis and lumbar disc herniation (Tsuji et al., 2002). High levels of YKL-40 in the CSF have also been reported in patients with purulent meningitis (Ostergaard et al., 2002). The *CHI3L1* gene expression analyses demonstrated higher postmortem mRNA levels in the hippocampus and prefrontal cortex of patients with schizophrenia than in the respective tissues of controls (Chung et al., 2003; Arion et al., 2007). It has been hypothesized that YKL-40 protects cells from undergoing apoptosis and plays a role in inflammatory processes in patients with schizophrenia.

The *CHI3L1* gene is located on chromosome 1q32.1 and shows evidence of modest linkage with schizophrenia (Shaw et al., 1998; Jang et al., 2007), although recent genome-wide association studies have not identified any variant of this gene that is associated with schizophrenia (O'Donovan et al., 2008). Zhao et al. (2007) have detected genetic associations between schizophrenia and three single nucleotide polymorphisms (SNPs; rs6691378, rs10399805 and rs4950928) within the promoter region of *CHI3L1* in two independent Chinese cohorts. They found that an allele at rs4950928 impaired MYC/MAX-regulated transcriptional activation of *CHI3L1* by altering the transcription factor consensus sequences. Yang et al. (2008) subsequently indicated significant associations between schizophrenia and two SNPs in an Irish cohort. One was the same SNP (rs10399805) in the promoter that was reported in the original study and the other SNP (rs2275351) was within the gene at intron 7. These findings suggest that the *CHI3L1* gene is likely involved in predisposition to schizophrenia. However, the two studies were not replicated in two more recent studies, one conducted with Chinese trio samples and Japanese case–control samples (Yamada et al., 2008) and the other studying a small Bulgarian population (Betcheva et al., 2009). To further investigate this controversial issue, we first investigated whether the *CHI3L1* gene is associated with schizophrenia in a large Japanese population. Second, we performed meta-analyses on the overall population and separately in Japanese and Chinese populations.

2. Methods

2.1. Subjects

The subjects in our genetic association study consisted of 1463 unrelated patients with schizophrenia [54.6% males (799/664), mean age ± SD; 47.3 ± 15.0 years] and 1795 unrelated healthy controls [51.3% males (920/875), mean age ± SD; 45.5 ± 20.1 years]. The sex ratio did not differ significantly between groups ($\chi^2 = 3.7$, $p = 0.06$), while the mean age differed significantly between groups ($z = -5.1$, $p < 0.001$). These subjects were independent of those used by Yamada et al. (2008). All subjects were biologically unrelated Japanese and were recruited at three geographic regions in Japan: Osaka, Aichi and Tokushima (Yamaguchi-Kabata et al., 2008; Ohi et al., 2009). Cases were recruited from both outpatients and inpatients at university hospitals and psychiatric hospitals. Each schizophrenic research subject had been diagnosed and assessed by at least two trained psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) criteria, based on an unstructured clinical interview. Controls, including the hospital and institutional staff, were recruited through local advertisements. Psychiatrically healthy controls were evaluated using unstructured interviews to exclude individuals who had current or past contact with psychiatric services. Written informed consent was obtained for all subjects after the procedures had been fully explained. This study was carried out in accordance with World Medical Association's Declaration of Helsinki and approved by the Research Ethical Committee of Osaka University, Fujita Health University, Nagoya University and Tokushima University.

2.2. SNP selection and SNP genotyping

We designed our replication study by selecting six SNPs in the *CHI3LI* gene and the flanking regions. Five of the six SNPs were identical to the SNPs used in the original study: rs2364574 (SNP1), rs6691378 (SNP2), rs10399805 (SNP3), rs4950928 (SNP4) and rs880633 (SNP5). The designations of these SNPs in parentheses are according to Zhao et al. (2007). The remaining SNP (rs2275351) was chosen from the following study as it showed evidence for association with schizophrenia (Yang et al., 2008). Venous blood was collected from the subjects and genomic DNA was extracted from whole blood according to standard procedures. These SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay (Applied Biosystems, Foster City, California, USA), as described previously (Hashimoto et al., 2006, 2007; Ohi et al., 2009). Detailed information on the PCR conditions is available upon request. Genotyping call rates were 99.0% (SNP1), 95.0% (SNP2), 99.2% (SNP3), 99.6% (SNP4), 99.8% (SNP5) and 97.7% (rs2275351). SNP2 was excluded from the present study because this variant was not clearly discriminated as a result of a lower call rate. No deviation from Hardy–Weinberg equilibrium (HWE) in the examined SNPs was detected in the controls ($p>0.05$), while the genotypic frequencies of two SNPs deviated from HWE in the schizophrenia patients (SNP1; $p=0.016$, rs2275351; $p<0.001$). The positions of the five SNPs analyzed in the present study are indicated in Fig. 1.

2.3. Power analysis

We performed power calculations using the Power Calculator for Two Stage Association Studies [http://www.sph.umich.edu/csg/abecasis/CaTS/; (Skol et al., 2006)]. Power estimates were based on allele frequencies in patients ranging from 0.17 (SNP4) to 0.29 (SNP3), odds ratios ranging from 1.29 (SNP3) to 1.49 (SNP4) for each associated SNP, as indicated by Zhao et al. (2007), and an alpha level of 0.05. Power was calculated under a prevalence of 0.01 using a multiplicative model, assuming varying degrees of the marker allele frequency and the odds ratio.

2.4. Meta-analysis of the CHI3L1 association studies

The studies included in the meta-analysis were selected using the Schizophrenia Research Forum (http://www.schizophreniaforum.org) and PubMed with the search terms "CHI3L1" and "Schizophrenia." The analyzed data encompass all publications up to May 2009.

2.5. Statistical analyses

Statistical analyses were performed using SNPAlyze V5.1.1 Pro software (DYNACOM, Yokohama, Japan) and SPSS 16.0J software (SPSS Japan Inc., Tokyo, Japan). Differences in clinical characteristics between patients and controls were

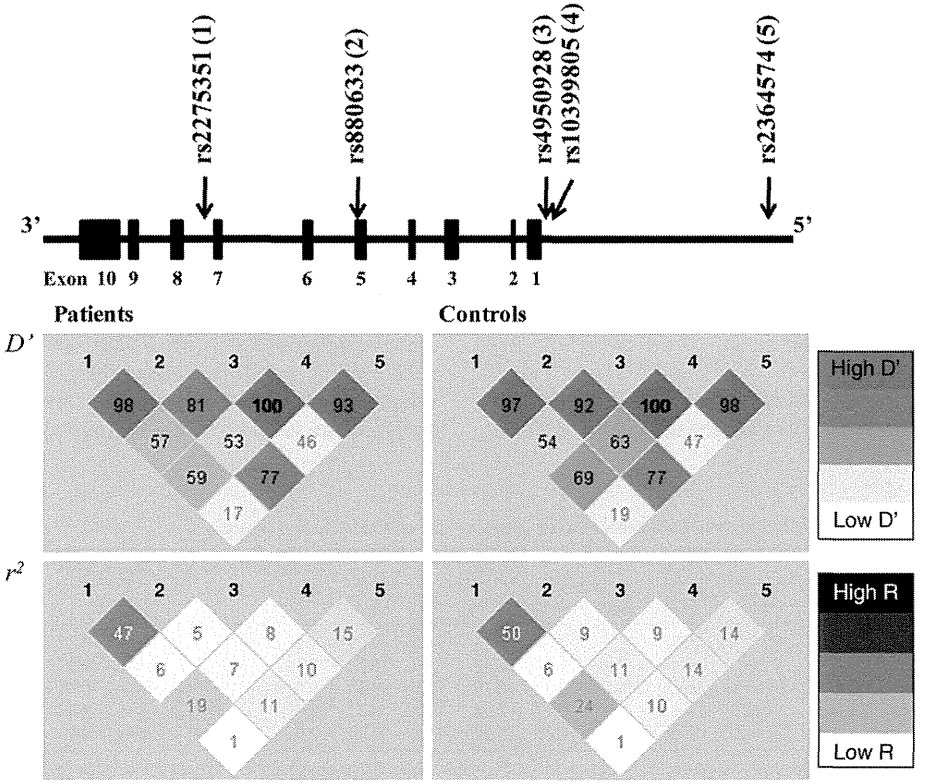

Fig. 1. Genomic structure of *CHI3L1*, including locations of the five SNPs studied, and linkage disequilibrium of these five SNPs in the patient and control groups. Based on an entry in the Entrez Gene database (National Center for Biotechnology Information), the genomic structure of *CHI3L1* is shown above. The locations of SNPs analyzed in this study are indicated by arrows. Numbers indicated in parentheses refer to numbering of the SNPs in the linkage disequilibrium (LD) diagram. The distances of exons–introns and intermarkers are drawn to scale. The LD between pairwise SNPs, using D' and r^2 values, are shown at the bottom of the map of gene structure separately for cases and controls. High levels of LD are represented by red (D') and black (r^2) coloring with increasing color intensity from 0 to 100, as shown by color bars.

analyzed using χ^2 tests for sex and the Mann–Whitney *U*-test for age. Deviation from HWE was tested separately in cases and controls using χ^2 tests for goodness of fit. The allelic and genotypic distributions of *CHI3L1* polymorphisms between patients and controls were analyzed using χ^2 tests. The number of effective independent SNPs assayed was estimated by the spectral decomposition method of Nyholt using SNPSpD software (Nyholt, 2004). Pairwise linkage disequilibrium (LD) analyses, expressed by D' and r^2, were applied to detect the intermarker relationship in each group using Haploview 4.1 software (http://www.broad.mit.edu/mpg/haploview/contact.php). Haplotype frequencies were estimated by the method of maximum likelihood using genotyping data through the use of the expectation–maximization algorithm. Rare haplotypes found in less than 3% of both patients and controls were excluded from the haplotypic association analysis. We performed 10,000 permutations for most significant tests to determine empirical significance. We used a 2- to 5-window fashion analysis.

The meta-analyses were performed using the case–control and TDT meta-analysis package (catmap) for the R-project program (Version 2.8.0, http://www.r-project.org.), which implements fixed- and random-effect pooled estimates for case–control and the transmission disequilibrium method, allowing for the use of genetic association data across study types (Nicodemus, 2008). Cochran's χ^2 based Q statistical test was performed in order to assess possible heterogeneity among the individual studies and, thus, to ensure that each group of studies was suitable for meta-analysis. The catmap was configured so that the random-effect model described by DerSimonian and Laird was applied in the presence of heterogeneity of the genetic effects ($p \leq 0.32$), while the fixed-effect model described by Mantel–Haenszel was applied in the absence of heterogeneity ($p > 0.32$). The significance of the pooled ORs and the heterogeneity of the group of ORs were assessed using a χ^2 test. The significance level for statistical tests was set at two-tailed $p < 0.05$.

3. Results

3.1. Genetic association analysis

Our study size of 1463 cases and 1795 controls had sufficient power (>0.98) to detect an effect at an odds ratio of 1.29 or larger, as described in the initial report, for each SNP (Zhao et al., 2007). The genotype and allele frequencies of five SNPs located in the *CHI3L1* gene and the flanking regions are summarized in Table 1. Significant differences in the genotype and allele frequency between patients and controls were observed in SNP4, which is located within the promoter region (genotype; $\chi^2 = 7.9$, $p = 0.019$, allele; $\chi^2 = 6.7$, $p = 0.009$). The significant association remained even after SNPSpD correction for multiple tests (the effective number of independent marker loci: 4.47; $p = 0.040$). The G-allele frequency at SNP4 was higher in patients (85.9%) than in controls (83.6%). There was no allelic or genotypic association with schizophrenia for the other four SNPs. Haplotype analysis showed several significant associations with schizophrenia (the most significant global $p < 0.001$, SNP3–SNP4–SNP5 and SNP3–SNP4–SNP5–rs2275351) (Table 2). This evidence for association remained positive after correction for multiple tests (10 independent global tests, the haplotypic association: $p = 0.0010$ after Bonferroni correction). The differences in detailed haplotype frequencies between cases and controls are provided in Supplementary Table 1. The LD relationships between markers are provided in Fig. 1. The LD pattern observed in our controls was nearly identical to that among our patients, the previously reported Chinese samples and JPT HapMap samples, but was different from those reported for the CEU and YRI HapMap samples. The moderate LD patterns observed between SNP5–rs2275351 were observed in both groups ($0.25 < r^2 \leq 0.50$).

3.2. Meta-analysis

We selected four studies using the Schizophrenia Research Forum and MEDLINE (Zhao et al., 2007; Yamada et al., 2008; Yang et al., 2008; Betcheva et al., 2009). The four studies and the present study (five case–control studies and two family-based studies) included 3005 patients, 3825 controls and 601 trios. The demography of the combined studies is shown in Table 3. No association between any SNP and schizophrenia was revealed in the overall population (Table 4). There was no heterogeneity among studies in SNP1 or SNP5 in the overall population. We found evidence of heterogeneity among studies of SNP3 ($p = 0.026$), SNP4 ($p = 0.00035$) and rs2275351 ($p = 0.048$). Heterogeneity in the meta-analysis refers to variation in study outcomes among studies. Thus, we analyzed two subdivided ethnic groups, Japanese populations

Table 1
Genotype and allele distributions for SNPs in the *CHI3L1* gene between patients with schizophrenia and controls.

Marker				SCZ			CON			Genotypic *p*-value (*df*=2)	SCZ	CON	Allelic *p*-value (*df*=1)	OR
SNP IDs[a]	Position[b]	M/m[c]	Gene	M/M	M/m	m/m	M/M	M/m	m/m		MAF			
SNP1	201426329	T/C	5′	0.57	0.36	0.08	0.58	0.36	0.06	0.14	0.26	0.24	0.13	1.09
SNP3	201422621	C/T	5′	0.45	0.44	0.11	0.46	0.42	0.12	0.73	0.33	0.33	0.71	1.02
SNP4	201422505	G/C	5′ UTR	0.74	0.23	0.02	0.70	0.28	0.03	**0.019**	0.14	0.16	**0.009**	0.83
SNP5	201419424	A/G	Exon 5	0.43	0.45	0.13	0.42	0.45	0.13	0.89	0.35	0.36	0.65	1.02
rs2275351	201416696	G/A	Intron 7	0.30	0.45	0.25	0.27	0.48	0.25	0.08	0.47	0.49	0.22	0.94

SCZ, patients with schizophrenia; CON, healthy controls; m, minor allele; M, major allele; MAF, minor allele frequency; OR, odds ratio. Significant p values are shown as bold face and underline.

[a] The db SNP IDs equivalent to the SNP IDs designed by Zhao et al. (2007) are the following: SNP1 (rs2364574), SNP3 (rs10399805), SNP4 (rs4950928), SNP5 (rs880633).

[b] db SNP build 129.

[c] The first shown alleles are major allele. All the alleles are represented according to the minus strand DNA sequence to make them comparable with the previous published data.

Table 2
Haplotype analysis of *CHI3L1* gene between patients and controls.

	Haplotypic global *p* values			
	Window level			
db SNP IDs[a]	2	3	4	5
rs2364574 (SNP1)				
	0.25			
rs10399805 (SNP3)		**0.018**		
	0.037		**0.0037**	
rs4950928 (SNP4)		**0.00010**		**0.0040**
	0.0038		**0.00010**	
rs880633 (SNP5)		**0.0017**		
	0.18			
rs2275351				

Haplotypes with frequencies <3% in each group are excluded. Significant *p* values are shown as bold face and underline.
[a] The db SNP IDs equivalent to the SNP IDs designed by Zhao et al. (2007) are shown in parentheses.

Table 3
Demography of the combined studies.

Authors	Ethnicities	Patients	Controls
Case–control studies			
Zhao et al. (2007)	Chinese	412	464
Yang et al. (2008)	Irish	375	812
Yamada et al. (2008)	Japanese	570	570
Betcheva et al. (2009)	Bulgarian	185	184
Ohi et al. (present study)	Japanese	1463	1795
Family-based studies			
Zhao et al. (2007)	Chinese	308 probands	
Yamada et al. (2008)	Chinese	293 probands	

(2033 patients and 2365 controls) and Chinese populations (412 patients, 464 controls and 601 trios), which were major groups across the five studies (Table 4). There was no heterogeneity among studies for these SNPs in Japanese and Chinese populations individually, expect for SNP4 in Chinese populations ($p=0.012$). We detected a significant association between SNP4 and schizophrenia in Japanese populations [$p=0.003$, OR = 0.84 (0.75–0.94)], while we detected a significant association between SNP3 and schizophrenia in Chinese populations [$p=0.003$, OR = 0.85 (0.76–0.95)]. These results remained significant even after Bonferroni correction (independent tests of the four SNPs, SNP4; corrected $p=0.012$, SNP3; corrected $p=0.012$).

4. Discussion

In this study, we found that SNP4 in the *CHI3L1* gene was associated with schizophrenia in a large Japanese population. Second, we performed a meta-analysis of the overall combined populations of several studies. In the meta-analysis, significant heterogeneity among studies was observed in SNP3 and SNP4. Because of the significant heterogeneity, we stratified the studies by ethnicity. We found that schizophrenia was associated with distinct SNPs in the *CHI3L1* gene in the Japanese and the Chinese populations.

We revealed a significant association of the G-allele of SNP4, which is located in the promoter region of the *CHI3L1* gene, with schizophrenia in a Japanese cohort (patients 85.9% vs. controls 83.6%). Our meta-analysis indicated a stronger association between SNP4 and schizophrenia in Japanese populations. Despite similar allele frequencies between cases and controls in the two Japanese cohorts, Yamada et al. (2008) reported no association between SNP4 and schizophrenia (patients 85.9% vs. controls 83.7%). This discrepancy might be attributed to the type II error for their small sample size (570 vs. 570) compared with our large sample size (1463 vs. 1795). In the meta-analysis of the overall combined population (Caucasian, Chinese and Japanese subjects), we found no association between these SNPs in the *CHI3L1* gene and schizophrenia. This result can be explained by the fact that the LD patterns in the HapMap data are different among each of these populations. For SNPs with heterogeneity among studies, we separately analyzed their association with schizophrenia in Japanese and Chinese populations. The meta-analyses showed that schizophrenia was associated with different variants (SNP3 and SNP4) in each population. Although the LD patterns between Asian populations were similar, the risk allele differed between Japanese and Chinese populations. It is unclear whether the difference resulted from subtle differences in LD patterns or allelic heterogeneity. It seems that an SNP might exist in this region that is more strongly associated with schizophrenia. This possibility could be addressed by re-sequencing or genotyping dense SNP mapping in this region and evaluating the association with schizophrenia.

It has been suggested that YKL-40 might be a potential biomarker for a cellular survival factor in an adverse microen-

Table 4
Meta-analysis of the genetic association studies for each SNP.

		Overall			Japanese			Chinese		
SNP ID	M/m	OR (95% CI)	$p(\chi)$	$p(Q)$	OR (95% CI)	$p(\chi)$	$p(Q)$	OR (95% CI)	$p(\chi)$	$p(Q)$
SNP1 (C)	T/C	(4)[a] 1.07 (0.99–1.15)	0.11[b]	0.74	(2)[a] 1.07 (0.97–1.18)	0.16[b]	0.53	(2)[a] 1.05 (0.93–1.19)	0.41[b]	0.37
SNP3 (T)	C/T	(6) 0.90 (0.80–1.01)	0.06	**0.026**	(2) 1.03 (0.94–1.12)	0.56[b]	0.79	(3) 0.85 (0.76–0.95)	**0.003**[b]	0.41
SNP4 (C)	G/C	(7) 1.03 (0.86–1.24)	0.72	**0.00035**	(2) 0.84 (0.75–0.94)	**0.003**[b]	0.90	(3) 1.29 (0.93–1.79)	0.13	**0.012**
SNP5 (G)	A/G	(4) 1.01 (0.94–1.08)	0.75[b]	0.80	(2) 1.00 (0.91–1.09)	0.98[b]	0.40	(2) 1.03(0.92–1.16)	0.58[b]	0.80
rs2275351 (A)	G/A	(2) 0.84 (0.65–1.09)	0.19	**0.048**	(1)–	–	–	(0)–	–	–

$p(\chi)$: chi-square test used determines the significance of the overall OR. Multiple testing corrections were not performed. Significant *p* values are shown as bold face and underline.
$p(Q)$: Cochran's Q test used to assess the heterogeneity. Random-effect model was applied in the presence of heterogeneity of the genetic effects ($p \leq 0.32$), while fixed-effect model was applied in the absence of heterogeneity ($p > 0.32$).
[a] The number of studies included in each meta-analysis is indicated in parentheses.
[b] This analysis was performed by fixed-effect model.

vironment because increased YKL-40 expression is found upon genotoxic and microenvironmental stress (i.e., hypoxia and ionizing radiation). It has been considered that a number of environmental stresses, such as fetal hypoxia and infection, in addition to genetic contributions, might induce susceptibility to schizophrenia (Palomo et al., 2004; Mittal et al., 2008). Patients with schizophrenia have shown increased levels of IL-6, IL-1RA and sIL-2R and a decrease in IL-2 (Potvin et al., 2008). YKL-40 is stimulated by IL-6 (Johansen et al., 2006), a multifunctional cytokine with varied system functions that plays a role in inflammatory processes and induces cell differentiation (Trikha et al., 2003). Cytokines play important roles in infection and inflammation and are crucial mediators of cross-talk between the brain and the immune system. Schizophrenia might be associated with an imbalance in inflammatory cytokines.

Elevated expression of the *CHI3L1* gene has been indicated in the hippocampus and prefrontal cortex in independent postmortem studies of patients who had schizophrenia (Chung et al., 2003; Arion et al., 2007). The G-allele at SNP4 has been associated with higher transcriptional activity according to a luciferase reporter assay and with higher *CHI3L1* mRNA levels in peripheral blood cells in patients with schizophrenia (Zhao et al., 2007). Interestingly, higher serum YKL-40 levels are involved in several inflammatory processes and tissue remodeling (Vind et al., 2003; Bergmann et al., 2005; Nordenbaek et al., 2005; Johansen, 2006; Kucur et al., 2007; Nojgaard et al., 2008). The G-allele at SNP4 occurred at a higher frequency in patients with asthma than in controls and was associated with higher serum YKL-40 levels (Ober et al., 2008). Our results suggest that the G-allele, which is enriched in patients with schizophrenia compared with controls, has a role in the etiology of schizophrenia. The risk *CHI3L1* genotype might be associated with serum YKL-40 levels in patients with schizophrenia. Further study of the possible association of *CHI3L1* genotype in patients with schizophrenia is required.

As schizophrenia is sensitive to environmental and psychological stresses (Leff, 1994; Howes et al., 2004), higher *CHI3L1* gene expression in patients with schizophrenia may be due to an excessive response to various stressors. SNP4, which is located within the promoter of the *CHI3L1* gene, might play a role in altering the expression and serum levels of YKL-40. In conclusion, we suggest that SNPs in the *CHI3L1* gene have ethnic heterogeneity and might contribute to the pathogenesis of schizophrenia in Asian populations. Further replication studies in other ethnic populations are required to confirm the possible relationship between *CHI3L1* and schizophrenia.

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Contributors

R. Hashimoto supervised the entire project, collected the data, wrote the manuscript, was critically involved in the design, analysis and interpretation of the data and was responsible for performing the literature review. K. Ohi was critically involved in the collection and analysis of the data, and contributed to the editing of the final manuscript and contributed intellectually to the interpretation of the data. Y. Yasuda, T. Yoshida, H. Takahashi, N. Iike, M. Iwase, K. Kamino, R. Ishii, H. Kazui, M. Fukumoto, H. Takamura, H. Yamamori, M. Azechi, K. Ikezawa, H. Tanimukai, S. Tagami, T. Morihara, M. Okochi, K. Yamada, S. Numata, M. Ikeda, T. Tanaka, T. Kudo, S. Ueno, T. Yoshikawa, T. Ohmori, N. Iwata, N. Ozaki and M. Takeda were heavily involved in the collection of the majority of the data and contributed intellectually to the interpretation of the data. All authors contributed to and have approved the final manuscript.

Conflict of interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary table

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.12.002.

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The *dopamine D3 receptor* (*DRD3*) gene and risk of schizophrenia: Case–control studies and an updated meta-analysis

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ABSTRACT

The dopamine D3 receptor (DRD3) has been suggested to be involved in the pathophysiology of schizophrenia. *DRD3* has been tested for an association with schizophrenia, but with conflicting results. A recent meta-analysis suggested that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may confer protection against schizophrenia. However, almost all previous studies of the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. To assess whether *DRD3* is implicated in vulnerability to schizophrenia, we conducted case–control association studies and performed an updated meta-analysis. In the first population (595 patients and 598 controls), we examined 16 genotyped single nucleotide polymorphisms (SNPs), including tagging SNPs selected from the HapMap database and SNPs detected through resequencing, as well as 58 imputed SNPs that are not directly genotyped. To confirm the results obtained, we genotyped the SNPs rs7631540–rs1486012–rs2134655–rs963468 in a second, independent population (2126 patients and 2228 controls). We also performed an updated meta-analysis of the haplotype, combining the results obtained in five populations, with a total sample size of 7551. No supportive evidence was obtained for an association between *DRD3* and schizophrenia in our Japanese subjects. Our updated meta-analysis also failed to confirm the existence of a protective haplotype. To draw a definitive conclusion, further studies using larger samples and sufficient markers should be carried out in various ethnic populations.

1. Introduction

The dopamine D3 receptor (DRD3) has been suggested to be involved in the pathophysiology of schizophrenia (for a

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review, Schwartz et al., 2000). DRD3 has relatively strong affinity for both first- and second-generation antipsychotics (Sokoloff et al., 1990). Postmortem studies have revealed changes in the mRNA and protein levels of DRD3 in the brains of patients with schizophrenia (Gurevich et al., 1997; Meador-Woodruff et al., 1997; Schmauss et al., 1993). Altered levels of *DRD3* mRNA in blood lymphocytes of patients with schizophrenia have also been reported (Ilani et al., 2001; Vogel et al., 2004). *DRD3* is located on 3q13.3 where some linkage analyses have suggested a region of susceptibility to schizophrenia (Brzustowicz et al., 2000; Kaneko et al., 2007). Therefore, *DRD3* is a promising functional and positional candidate gene for schizophrenia.

More than 60 studies have tested an association between *DRD3* and schizophrenia (Allen et al., 2008). The most extensively investigated *DRD3* polymorphism is Ser9Gly (rs6280) in exon 2 resulting in a serine to glycine substitution at codon 9. This polymorphism has been reported to be associated with altered dopamine binding affinity, suggesting that the Ser9Gly polymorphism may be functional (Lundstrom and Turpin, 1996). An initial study reported an association between homozygosity of this polymorphism and schizophrenia (Crocq et al., 1992). Some studies showed an association of the Ser allele with schizophrenia (Ishiguro et al., 2000; Shaikh et al., 1996), whereas others reported that the Gly allele was over-represented in patients with schizophrenia (Kennedy et al., 1995; Utsunomiya et al., 2008). However, two recent large meta-analyses did not provide evidence for an association between the Ser9Gly polymorphism and schizophrenia (Allen et al., 2008; Ma et al., 2008). Therefore, if *DRD3* is implicated in genetic susceptibility to schizophrenia, this cannot be wholly accounted for by the Ser9Gly polymorphism. This view has been supported by two studies using tagging single nucleotide polymorphisms (SNPs) based on linkage disequilibrium (LD) (Domínguez et al., 2007; Talkowski et al., 2006). A recent meta-analysis showed that the second most common haplotype (T–T–T–G) for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was less frequent in patients with schizophrenia than in control subjects, suggesting that this haplotype may confer protection against schizophrenia (Costas et al., 2009).

Almost all previous studies on the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. Here, we tried to increase the power by increasing the sample size and testing more markers, including tagging SNPs selected from the HapMap database and SNPs detected through resequencing of whole exon regions of *DRD3*. First, we conducted a moderate-scale case–control association study (595 patients and 598 controls) using 16 genotyped SNPs and 58 imputed SNPs that have not been directly genotyped. Second, we carried out an independent large-scale case–control association study (2126 patients and 2228 controls) to confirm the results of the first study, specifically to test the association of the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 with schizophrenia. Third, we performed an updated meta-analysis of this haplotype to assess the collective evidence across individual studies.

2. Materials and methods

The present study was approved by the Ethics Committee of each participating institute, and written informed consent was obtained from all participants. All participants were unrelated Japanese subjects.

2.1. Subjects

The first population consisted of 595 patients with schizophrenia (313 men and 282 women; mean age, 40.2 [SD 14.1] years) and 598 control subjects (311 men and 287 women; mean age, 38.1 [SD 10.5] years). These subjects partially overlapped with those in the report of Tanaka et al. (1996). Case and control groups were matched for sex ($p = 0.836$). Although the mean age of the patients was significantly higher than that of the control subjects ($p = 0.004$), the difference in mean age between the groups was relatively small (2.1 years). The second population consisted of 2126 patients with schizophrenia (1137 men and 989 women; mean age, 47.3 [SD 14.3] years) and 2228 control subjects (1189 men and 1039 women; mean age, 46.6 [SD 13.9] years). Case and control groups were matched for sex ($p = 0.940$) and age ($p = 0.083$).

We conducted a psychiatric assessment of every participant, as described previously (Nunokawa et al., 2007). In brief, the patients were diagnosed according to the *Diagnostic and Statistical Manual of Mental Disorders Fourth Edition* (DSM-IV) criteria by at least two experienced psychiatrists, on the basis of all available sources of information, including unstructured interviews, clinical observations and medical records. The control subjects were mentally healthy subjects with no self-reported history of psychiatric disorders; they showed good social and occupational skills, but were not assessed using a structured psychiatric interview.

The subjects for resequencing of exon regions were six patients with schizophrenia from a Japanese single multiplex schizophrenia pedigree. In this pedigree, our previous linkage analysis revealed that 3q is one of the candidate regions for schizophrenia (Kaneko et al., 2007). These patients were diagnosed according to the DSM-IV criteria by two experienced psychiatrists, on the basis of all available sources of information, including direct interviews using the Structured Clinical Interview for DSM-IV Axis I disorders and Axis II disorders, medical records, and information from reliable relatives and psychiatric professionals.

2.2. Tagging SNP selection

Tagging SNPs for *DRD3*, covering gene region and the 5′ and 3′ flanking regions (chr3:115307882..115402406), were selected from the HapMap database (release#22, population: Japanese in Tokyo [JPT], minor allele frequency [MAF]: more than 0.05). We applied the criterion of an r^2 threshold greater than 0.8 in the '*aggressive tagging: use 2- and 3-marker haplotype*' mode using the 'Tagger' program (de Bakker et al., 2005), as implemented in Haploview v4.0 (Barrett et al., 2005); rs6280 (Ser9Gly) was forced to be selected as a tagging SNP. To confirm the existence of a common protective haplotype (Costas et al., 2009), we also included rs963468.

2.3. Resequencing of exon regions

All seven exons of *DRD3* were screened for polymorphisms using direct sequencing of PCR products. The sequences

of primers used for amplification are listed in Supplementary Table 1. Detailed information on amplification conditions is available upon request. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

2.4. SNP genotyping

All SNPs were genotyped using the TaqMan 5′-exonuclease assay (Supplementary Table 2), as described previously (Watanabe et al., 2006).

2.5. Statistical analysis

Deviation from the Hardy–Weinberg equilibrium (HWE) was tested using the χ^2 test for goodness-of-fit. LD blocks defined in accordance with Gabriel's criteria (Gabriel et al., 2002) and haplotype frequencies were determined using Haploview v4.1. The allele, genotype and haplotype frequencies of the patients and control subjects were compared using the χ^2 test or Fisher's exact test. Permutation tests were performed to calculate corrected p values for multiple testing using Haploview v4.1.

We imputed the genotype distributions of 59 SNPs in *DRD3* (chr3:115307882..115402406) using the observed SNPs and the HapMap database (release#24, Han Chinese in Beijing [CHB] + JPT), using MACH 1.0 (Li and Abecasis, 2006). We adopted imputed SNPs with an Rsq (which estimates the squared correlation between imputed and true genotypes) greater than 0.3 as recommended (Li and Abecasis, 2006).

Power calculation was performed using Genetic Power Calculator (Purcell et al., 2003). Power was estimated with an α of 0.05, assuming a disease prevalence of 0.01 and the risk allele frequencies to be the values observed in control subjects.

2.6. Meta-analysis

To assess whether the haplotype T–T–T–G for SNPs rs7631540–rs1486012–rs2134655–rs963468 confers protection against schizophrenia, we performed an updated meta-analysis combining the results of three previous studies (Costas et al., 2009; Domínguez et al., 2007; Talkowski et al., 2006) and our current study, as described previously (Watanabe et al., 2007). First, we explored whether heterogeneity was present using Q statistics. Second, a fixed effects model meta-analysis was performed within groups of homogeneous odds ratios (ORs). The significance of the pooled OR was determined using a Z-test. Third, publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry (Egger et al., 1997).

3. Results

Twelve SNPs were selected as tagging SNPs for *DRD3* from the HapMap database. We also included rs963468, as described above. By resequencing the exon regions of *DRD3*, we detected four SNPs: rs6280 (Ser9Gly), rs3732783 (Ala17Ala), rs3732791 (His359His), and g.−6664T>G. Three of these SNPs had previously been reported: rs6280 (Ser9Gly) in exon 2, rs3732783 (Ala17Ala) in exon 2 and rs3732791 (His359His) in exon 7. The SNP g.−6664T>G in exon 1 (GenBank accession no. NG_008842.1; position 5146) was previously unidentified.

A total of 16 SNPs (12 tagging SNPs, rs963468 and three SNPs detected) were genotyped in the first population (Table 1). Their order and physical locations are shown in Fig. 1A. The genotype distributions of all SNPs did not deviate significantly from the HWE in both groups, with the exception of rs17605608 in patients ($p=0.033$). None of the genotype or allele frequencies of the SNPs examined differed significantly between patients and control subjects. In *DRD3*, five LD blocks were defined (Fig. 1B). There were no significant associations between common haplotypes of these LD blocks and schizophrenia (Table 2).

Table 1
Genotype and allele frequencies of 16 SNPs in the first population.

SNP #	dbSNP ID	Allele[a]	Patients						Controls						p	
			n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	Genotype	Allele
1	rs9288990	C/T	594	0.143	267	274	53	0.320	597	0.694	255	267	75	0.349	0.126	0.129
2	rs10934251	A/G	593	0.437	446	134	13	0.135	593	0.216	449	130	14	0.133	0.948	0.904
3	rs7631540	C/T	595	0.975	218	284	93	0.395	596	0.570	201	296	99	0.414	0.570	0.333
4	rs1486012	A/T	595	0.623	174	301	120	0.455	596	0.944	156	297	143	0.489	0.221	0.092
5	rs3732790	T/A	593	0.228	262	274	57	0.327	596	0.366	278	265	53	0.311	0.683	0.405
6	rs3732791	G/A	595	0.820	584	11	0	0.009	597	0.724	580	17	0	0.014	0.255	0.258
7	rs2134655	C/T	595	0.944	287	253	55	0.305	596	0.962	277	259	60	0.318	0.793	0.496
8	rs963468	G/A	593	0.882	257	268	68	0.341	593	0.485	260	271	62	0.333	0.856	0.696
9	rs9880168	A/G	595	0.366	439	141	15	0.144	597	0.211	431	157	9	0.147	0.297	0.842
10	rs2630350	C/T	594	0.299	500	92	2	0.081	598	0.971	485	107	6	0.100	0.191[c]	0.111
11	rs167771	A/G	595	0.829	397	179	19	0.182	597	0.322	374	202	21	0.204	0.338	0.174
12	rs3732783	T/C	594	0.723	577	17	0	0.014	598	0.647	576	22	0	0.018	0.428	0.432
13	rs6280	T/C	594	0.510	301	239	54	0.292	595	0.815	306	243	46	0.282	0.700	0.569
14	g.−6664T>G	T/G	595	0.820	584	11	0	0.009	597	0.756	582	15	0	0.013	0.433	0.435
15	rs17605608	G/A	595	0.033	364	191	40	0.228	596	0.791	357	210	29	0.225	0.257	0.866
16	rs16822440	C/T	595	0.585	513	78	4	0.072	598	0.259	501	95	2	0.083	0.343[c]	0.337

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.
[a] Major/minor allele.
[b] Genotypes, major and minor alleles are denoted by 1 and 2, respectively.
[c] Calculated using Fisher's exact test.

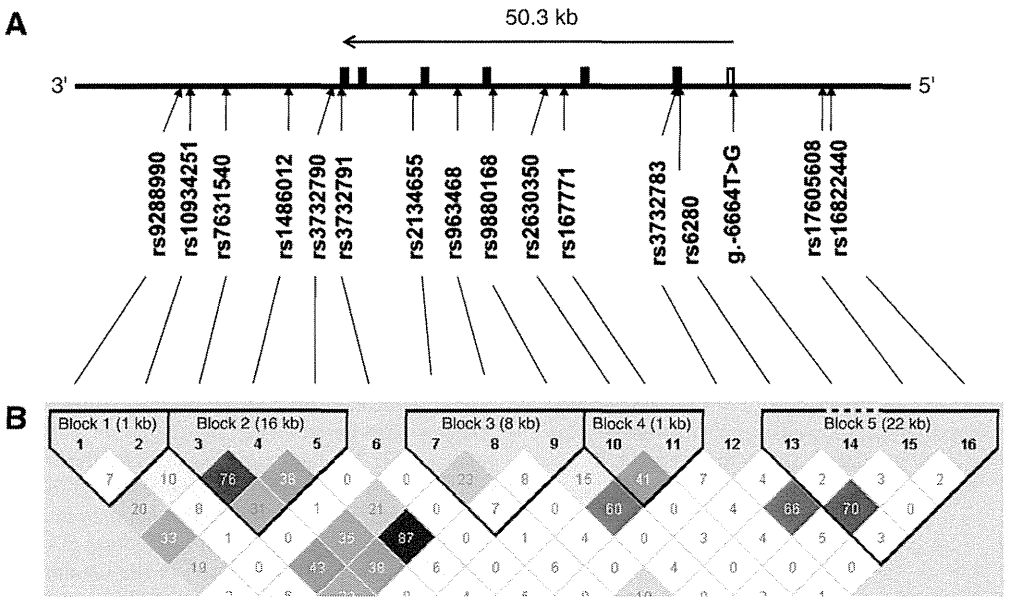

Fig. 1. Genomic structure and linkage disequilibrium (LD) of *DRD3*. (A) Genomic structure of *DRD3* and the locations of the single nucleotide polymorphisms (SNPs) analyzed in the present study. *DRD3* has seven exons (rectangles) and spans approximately 50.3 kb. Black and white rectangles represent coding and untranslated regions, respectively. The horizontal arrow and vertical arrows indicate the transcriptional orientation and locations of SNPs, respectively. (B) LD between markers of *DRD3*. A block is defined in accordance with Gabriel's criteria using Haploview v4.1. Each box represents the r^2 value corresponding to each pair-wise SNP.

Out of the 59 SNPs included for imputation analysis, we adopted 58 imputed SNPs with an Rsq greater than 0.3 (Supplementary Table 3). We found no significant associations between any of these imputed SNPs and schizophrenia after correction for multiple comparisons.

To assess whether the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 confers protection against schizophrenia (Costas et al., 2009), we conducted haplotype analyses of these SNPs (Table 3). Although the haplotype T–T–T–G was less frequent in patients than in control subjects (26.5% vs. 28.6%), this difference did not reach statistical significance ($p=0.261$).

To further test for such a haplotype association, we genotyped these four SNPs in the second population (Table 4). In the patient group, the genotype distributions of rs7631540 and rs963468 significantly deviated from the HWE ($p=0.046$ and 0.043, respectively). There were no significant associations between any of the four SNPs examined and schizophrenia in the second population. We also could not confirm an association of the haplotype T–T–T–G with schizophrenia, even in our large sample (Table 5).

We then performed an updated meta-analysis of the haplotype T–T–T–G (Table 6). The total sample sizes for the patients and control subjects from five independent populations were 3585 and 3966, respectively. We did not observe significant heterogeneity among ORs ($Q=8.22$, $df=4$, $p=0.084$). Our updated meta-analysis failed to provide

Table 2
Haplotype analyses of LD blocks.

Haplotype	Patients	Controls	*p*
Block 1 (SNP #1–2)			0.290[a]
CA	0.546	0.517	0.166
TA	0.320	0.349	0.125
CG	0.135	0.133	0.924
Block 2 (SNP #3–4–5)			0.346[a]
TTT	0.395	0.412	0.386
CAA	0.323	0.301	0.265
CAT	0.223	0.210	0.433
CTT	0.055	0.068	0.200
Block 3 (SNP #7–8–9)			0.882[a]
CAA	0.341	0.334	0.722
TGA	0.305	0.318	0.491
CGA	0.211	0.202	0.595
CGG	0.144	0.146	0.856
Block 4 (SNP #10–11)			0.265[a]
CA	0.818	0.796	0.177
CG	0.101	0.105	0.784
TG	0.081	0.099	0.116
Block 5 (SNP #13–15–16)			0.637[a]
TGC	0.634	0.635	0.927
CAC	0.225	0.223	0.907
TGT	0.071	0.081	0.346
CGC	0.066	0.057	0.335

LD, linkage disequilibrium; SNP, single nucleotide polymorphism.
[a] Global *p* values.

Table 3
Haplotype analyses of the SNPs rs7631540–rs1486012–rs2134655–rs963468 in the first population.

Haplotype	Patients	Controls	*p*[a]
C–A–C–A	0.332	0.318	0.461
T–T–T–G	0.265	0.286	0.261
C–A–C–G	0.181	0.171	0.525
T–T–C–G	0.126	0.123	0.807
C–T–C–G	0.047	0.056	0.353
C–A–T–G	0.033	0.022	0.119

SNP, single nucleotide polymorphism.
[a] Global $p=0.438$.

Table 4
Genotype and allele frequencies of four SNPs in the second population.

SNP #	dbSNP ID	Allele[a]	Patients						Controls						p	
			n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	Genotype	Allele
3	rs7631540	C/T	2080	0.046	722	969	389	0.420	2177	0.206	731	1036	410	0.426	0.727	0.555
4	rs1486012	A/T	2079	0.211	567	1010	502	0.484	2213	0.984	560	1106	547	0.497	0.342	0.240
7	rs2134655	C/T	2089	0.209	971	888	230	0.323	2195	0.928	999	965	231	0.325	0.613	0.811
8	rs963468	G/A	2093	0.043	955	887	251	0.332	2197	0.558	997	957	243	0.328	0.577	0.736

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.
[a] Major/minor allele.
[b] Genotypes, major and minor alleles are denoted by 1 and 2, respectively.

sufficient evidence for the existence of a protective haplotype (pooled OR = 0.93, 95% CI = 0.87–1.00, $Z = 1.90$, $p = 0.058$). A linear regression analysis showed significant funnel plot asymmetry ($t = -4.48$, $p = 0.021$; Supplementary Fig. 1).

4. Discussion

We carried out a moderate-scale case–control association study using 16 genotyped SNPs (12 tagging SNPs from the HapMap database, rs963468 and three SNPs detected through resequencing) and 58 imputed SNPs that are not directly genotyped. However, we could not obtain supportive evidence for an association between *DRD3* and schizophrenia in the Japanese population. Almost all previous studies of the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. Specifically, the most extensively investigated SNP is the Ser9Gly polymorphism. Our study is in line with two recent large meta-analyses reporting no association of this polymorphism with schizophrenia (Allen et al., 2008; Ma et al., 2008). Interestingly, several studies have reported associations of the Ser9Gly polymorphism with promising endophenotypes for schizophrenia, including the intensity of eye movement (Rybakowski et al., 2001), executive functions (Bombin et al., 2008; Szekeres et al., 2004), event-related P300 potentials (Mulert et al., 2006) and prepulse inhibition of the acoustic startle reflex (Roussos et al., 2008), whereas other studies failed to find these associations (Rybakowski et al., 2005; Tsai et al., 2003). Taken together, these findings indicate that the Ser9Gly polymorphism does not contribute to genetic susceptibility to schizophrenia, but may have effects on the endophenotypes for schizophrenia.

Table 5
Haplotype analyses of the SNPs rs7631540–rs1486012–rs2134655–rs963468 in the second population.

Haplotype	Patients	Controls	p[a]
C–A–C–A	0.306	0.299	0.454
T–T–T–G	0.278	0.276	0.793
C–A–C–G	0.172	0.161	0.178
T–T–C–G	0.128	0.130	0.815
C–T–C–G	0.050	0.058	0.087
C–A–T–G	0.032	0.035	0.374
T–T–C–A	0.009	0.012	0.241

SNP, single nucleotide polymorphism.
[a] Global $p = 0.337$.

Two recent studies identified common haplotypes of *DRD3* associated with schizophrenia using different sets of tagging SNPs based on LD (Domínguez et al., 2007; Talkowski et al., 2006). The haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was significantly less frequent in patients with schizophrenia than in control subjects (25% vs. 31%) in the Galician population (Domínguez et al., 2007). This was not replicated in the Catalonian population (23% vs. 26%) (Costas et al., 2009). In the Catalonian population, the protective haplotype reported by Domínguez et al. (2007) was always associated with the haplotype A–T–G–A for the SNPs rs10934254–rs2134655–rs324030–rs324029, and vice versa (Costas et al., 2009). This haplotype was less frequent in patients with schizophrenia than in control subjects (26% vs. 31%) in a U.S. Caucasian population (Talkowski et al., 2006). A meta-analysis combining the results of these three previous studies showed that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was less frequent in patients with schizophrenia than in control subjects (Costas et al., 2009). Our moderate- and large-scale case–control studies did not confirm this finding in Japanese populations (27% vs. 29% in the first population; 28% vs. 28% in the second population).

To assess the collective evidence across individual studies, an updated meta-analysis with a total sample size of 7551 was performed. The results suggested that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may not confer protection against schizophrenia (pooled OR = 0.93, 95% CI = 0.87–1.00). However, considering a limited number of studies and the existence of publication bias, the findings of our meta-analysis should be interpreted with caution. There is the possibility that this haplotype may be regarded as a protective haplotype in Caucasian populations, but not in Asian populations. The frequencies of the haplotype T–T–T–G in control subjects ranged from 26% to 31% among ethnic groups (Supplementary Table 4). The third most common haplotype among Spanish populations is T–T–C–G, whereas, among Japanese populations, the third most common haplotype is C–A–C–G. Three major haplotypes accounted for approximately 0.9 of the total chromosomes in Spanish populations, but less than 0.8 in Japanese populations. These differences in haplotype structures among ethnic groups may account for the inconsistent results between Costas et al.'s and our meta-analyses. To draw any conclusion, further studies using larger samples are required in various ethnic populations.

We recognize some limitations of the present study. First, the sample size of the first population constitutes one of the

Table 6
Meta-analysis of the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468.

Study	Ethnicity	Patients		Controls		T–T–T–G vs. the others	
		n	T–T–T–G	*n*	T–T–T–G	OR	95% CI
Talkowski et al. (2006)	U.S. Caucasian	331	0.26	274	0.31	0.78	0.61–1.00
Domínguez et al. (2007)	Spanish	260	0.25	354	0.31	0.73	0.56–0.94
Costas et al. (2009)	Spanish	273	0.23	512	0.26	0.86	0.67–1.10
Current study (I)	Japanese	595	0.27	598	0.29	0.91	0.76–1.08
Current study (II)	Japanese	2126	0.28	2228	0.28	1.00	0.91–1.10
Pooled [a]		3585		3966		0.93	0.87–1.00

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.
[a] $Q = 8.22$, $df = 4$, $p = 0.084$ for heterogeneity.

largest samples examined for an association of *DRD3* with schizophrenia, but may not have sufficient power to detect associations between schizophrenia and SNPs with low minor allele frequencies and small effects. A power calculation showed that, when the genotypic relative risk was set to 1.69 for homozygous risk allele carriers under the multiplicative model of inheritance, the power was 0.12–0.90. Second, our subjects were not assessed using a standardized structured interview. However, the diagnosis of schizophrenia was assigned on the basis of all available sources of information. To the best of our knowledge, there were no control subjects who were likely to develop schizophrenia at their present stage of life. Thus, it is unlikely that our failure to find a significant association is attributable to misdiagnosis.

In conclusion, we obtained no supportive evidence for an association between *DRD3* and schizophrenia in our Japanese subjects. The findings of our updated meta-analysis also suggest that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may not confer protection against schizophrenia. However, to draw a definitive conclusion, further studies using larger samples and sufficient markers should be carried out in various ethnic populations.

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Contributors

Author Nunokawa designed the study, conducted resequencing and undertook statistical analyses. Author Watanabe designed the study, performed the TaqMan assays and undertook statistical analyses. Author Kaneko conducted resequencing. Author Sugai designed the study. Author Yazaki performed the TaqMan assays. Authors Arinami, Ujike, Inada, Iwata, Kunugi, Sasaki, Itokawa, Ozaki and Hashimoto managed sample collection. Author Someya supervised the study. All authors contributed to and have approved the final manuscript.

Conflict of interest

None of the authors have a conflict of interest to declare.

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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.2009.10.016.

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ARTICLE

CINP

Galantamine ameliorates the impairment of recognition memory in mice repeatedly treated with methamphetamine: involvement of allosteric potentiation of nicotinic acetylcholine receptors and dopaminergic-ERK1/2 systems

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Abstract

Galantamine, a drug used to treat Alzheimer's disease, inhibits acetylcholinesterase (AChE) and allosterically modulates nicotinic acetylcholine receptors (nAChRs) resulting in stimulation of catecholamine neurotransmission. In this study, we investigated whether galantamine exerts cognitive-improving effects through the allosteric modulation of nAChRs in an animal model of methamphetamine (Meth) psychosis. The mice treated with Meth (1 mg/kg.d) for 7 d showed memory impairment in a novel object recognition test. Galantamine (3 mg/kg) ameliorated the memory impairment, and it increased the extracellular dopamine release in the prefrontal cortex (PFC) of Meth-treated mice. Donepezil, an AChE inhibitor (1 mg/kg) increased the extracellular ACh release in the PFC, whereas it had no effect on the memory impairment in Meth-treated mice. The nAChR antagonist, mecamylamine, and dopamine D_1 receptor antagonist, SCH 23390, blocked the ameliorating effect of galantamine on Meth-induced memory impairment, whereas the muscarinic AChR antagonist, scopolamine, had no effect. The effects of galantamine on extracellular dopamine release were also antagonized by mecamylamine. Galantamine attenuated the defect of the novelty-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2). The ameliorating effect of galantamine on recognition memory in Meth-treated mice was negated by microinjection of an ERK inhibitor, PD98059, into the PFC. These results suggest that the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1 receptor-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could be a useful therapeutic agent for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Key words: Allosteric potentiation of nicotinic acetylcholine receptors, cognitive impairment, dopamine, extracellular signal-regulated kinase 1/2, galantamine, methamphetamine.

Introduction

Galantamine, a potent allosteric potentiating ligand (APL) and a drug approved for treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nicotinic acetylcholine receptors (nAChRs) (Eisele *et al.* 1993; Santos *et al.* 2002). We have found that galantamine has ameliorating effects on the impairment of performance in the novel object recognition (NOR) and/or conditioned fear learning tasks caused by a single intracerebroventricular infusion of amyloid-β peptide

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(Aβ) fragment (as an animal model of Alzheimer's disease) (Wang *et al.* 2007*a*) and by repeated treatment with a non-competitive *N*-methyl-D-asparatate receptor antagonist, phencyclidine (PCP) (as an animal model of schizophrenia) (Wang *et al.* 2007*b*). It increases the extracellular dopamine release in the hippocampus and prefrontal cortex (PFC) of Aβ_{25-35}-infused and PCP-treated mice, respectively. The ameliorating effects of galantamine on Aβ_{25-35}- and PCP-induced cognitive impairment are mediated through the augmentation of dopaminergic neurotransmission following activation of nAChRs (Wang *et al.* 2007*a*, *b*). These studies provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the hippocampus/PFC through the allosteric activation of nAChRs. Thus, galantamine shows potential as a novel therapeutic agent for cognitive impairments associated with schizophrenia, as well as Alzheimer's disease, although the molecular mechanism of action remains to be determined in detail.

Methamphetamine (Meth) is a highly addictive drug of abuse, and addiction to Meth has increased to epidemic proportions worldwide (Cretzmeyer *et al.* 2003; Rawson *et al.* 2002). Chronic Meth users show psychotic signs such as hallucinations and delusions, which are indistinguishable from paranoid schizophrenia (Sato *et al.* 1983; Srisurapanont *et al.* 2003; Yui *et al.* 2002). Recent studies have suggested that chronic use of Meth causes long-term cognitive deficits (Kalechstein *et al.* 2003; Nordahl *et al.* 2003; Simon *et al.* 2000). We have found that repeated Meth treatment in mice impairs long-term recognition memory after withdrawal, which is associated with the dysfunction of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the PFC, and that Meth-induced cognitive impairment is reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei *et al.* 2006). Meth-induced cognitive impairment in mice may be a useful animal model for cognitive deficits in Meth abusers and/or schizophrenia patients.

The present study was designed to test the hypothesis that galantamine improves cognitive deficit in the Meth-treated animal model of Meth psychosis and/or schizophrenia (Kamei *et al.* 2006), and that such cognitive-improving effects are mediated via activation of nAChR-dopaminergic-ERK1/2 pathways. We attempted to investigate: (1) whether cognitive-improving effects of galantamine are mediated via nAChRs in Meth-treated mice and (2) whether galantamine augments dopamine neurotransmission in the PFC by activation of nAChRs.

Methods

Animals

Male mice of the ICR strain (Japan SLC Inc., Japan), aged 6 wk at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Japan) and water *ad libitum*, and were maintained on a 12-h light/dark cycle (lights on 08:00 hours). Behavioural experiments were performed in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were conducted blind to treatment and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Drugs

Galantamine hydrobromide (4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro [3a,3,2-ef] benzazepin-6-ol hydrobromide) was supplied by Janssen Pharmaceutica (Tokyo, Japan). Galantamine, donepezil hydrochloride (Toronto Research Chemicals Inc., Canada), methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd, Japan), mecamylamine hydrochloride (Sigma-Aldrich, USA), (−)scopolamine hydrobromide (Sigma-Aldrich) and R(+)-SCH 23390 hydrochloride [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride] (Sigma-Aldrich) were dissolved in saline. PD98059 (Sigma-Aldrich) was dissolved in 60% dimethylsulfoxide (DMSO) saline.

Drug treatment

The mice were administered Meth (1 mg/kg.d s.c.) or saline once a day for 7 consecutive days (Kamei *et al.* 2006). The NOR test and microdialysis experiment were started 1 d and 3 d, respectively after the withdrawal of Meth treatment. The saline- or Meth-treated mice were administered galantamine (3 mg/kg p.o.) or donepezil (1 mg/kg p.o.) 1 h before the training session of the NOR test, or immediately after baseline collections in the microdialysis experiment. Mecamylamine (3 mg/kg s.c.), scopolamine (0.1 mg/kg s.c.) and SCH 23390 (0.02 mg/kg s.c.) were injected 20, 20 and 30 min, respectively, after treatment with galantamine. The doses of galantamine and donepezil used in the present study were as determined in previous experiments (Wang *et al.* 2007*a*, *b*) and in the report by Geerts *et al.* (2005), in which donepezil is