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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.

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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.

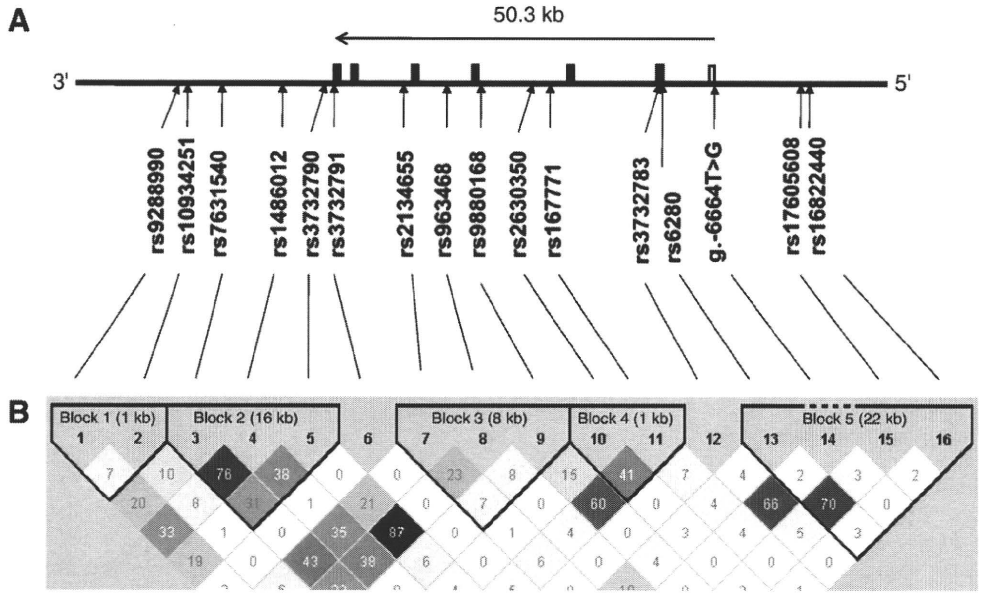

Fig. 1. Genomic structure and linkage disequilibrium (LD) of *DRD3*. (A) Genomic structure of *DRD3* and the locations of the single nucleotide polymorphisms (SNPs) analyzed in the present study. *DRD3* has seven exons (rectangles) and spans approximately 50.3 kb. Black and white rectangles represent 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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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

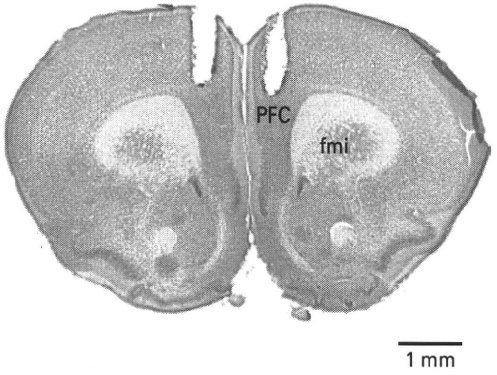

Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

3–15 times more potent than galantamine in inhibiting brain AChE *in vivo*. The doses of antagonists were selected based on our previous publications (Kamei *et al.* 2006; Wang *et al.* 2007*a*, *b*). All compounds except for PD98059 were systemically administered at a volume of 0.1 ml/10 g body weight. Control mice received the same volume of saline.

For local microinjection into the PFC, mice were anaesthetized with diethyl ether and fixed on the stereotactic apparatus (Narishige, Japan) 30 min before the training session. An L-shaped injection cannula (27 gauge) with a bevel tip at its short end was grasped with forceps and implanted into the PFC (+0.3 mm mediolateral from the midpoint on the line linking the two rear canthi, −2.5 mm in depth). PD98059 at a dose of 2 μg/1 μl/bilateral or vehicle (60% DMSO/2 μl/bilateral) was infused into the PFC for 45 s using a Hamilton microsyringe connected to the cannula via a Teflon tube, and the connection was maintained for another 45 s after the injection. After the behavioural experiments, the mice were decapitated, and the brains were removed. The brains were transversely cut along the direction of the vertical insertion of the cannula to confirm the injection site, which was obvious due to its dark red colour, and easily recognized as shown in Fig. 1. Misinjected mice were excluded from subsequent data analysis.

NOR test

The task was carried out on days 1–3 after the final injection of Meth in accordance with the method of Kamei *et al.* (2006) with a minor modification. The experimental apparatus consisted of a Plexiglas open-field box (40 × 40 × 29 high cm), the floor of which was covered with paper bedding. The apparatus was placed in a sound-isolated room. A light bulb, located in the upper part of the room and which could not be seen directly by the mice, provided constant illumination of about 40 lx at the level of the task apparatus.

The NOR task procedure consisted of three sessions: habituation, training, retention. Each mouse was individually habituated to the box, with 10 min exploration in the absence of objects on day 1 (habituation session). During the training session on day 2, two objects (A and B) were placed in the back corner of the box, 10 cm away from the side wall. A mouse was then placed in the middle front of the box and the total time spent in exploring the two objects was recorded for 10 min by the experimenter using two stopwatches. Exploration of an object was defined as directing the nose to the object at a distance of <2 cm and/or touching it with the nose. During the retention session on day 3, the animals were returned to the same box 24 h after the training session, in which one of the familiar objects (e.g. object A) used during the training session was replaced by a novel object C. The animals were then allowed to explore freely for 10 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a balanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function, e.g.

training session: A or B/(B+A) × 100 (%),

retention session: B or C/(B+C) × 100 (%).

Determination of extracellular acetylcholine (ACh) and dopamine levels in the PFC

In-vivo microdialysis was performed 3 d after the final injection of Meth. One day before microdialysis, mice were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and a guide cannula (MI-AG-6; Eicom Corp., Japan) was implanted into the mPFC (+1.9 mm anteroposterior, +1.0 mm mediolateral from bregma, −1.5 mm dorsoventral from the skull, +15° angle from vertical) according to the atlas of Franklin & Paxinos (1997). One day after the operation, the dialysis probe of ACh (A-I-4-02; 2 mm membrane length; Eicom Corp.) and dopamine (A-I-6-01; 1 mm membrane length; Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a flow rate of 1 μl/min (Mouri *et al.*

2006) and 1.2 μl/min (Shintani *et al.* 1993), respectively. The outflow fractions of ACh and dopamine were collected every 20 min and 10 min, respectively. When the difference of each fraction was <20%, we considered this a stable baseline. Following the collection of three stable baseline fractions of ACh and dopamine, mice were treated with donepezil, galantamine and/or mecamylamine, and then dialysates of ACh and dopamine were collected every 20 min for 120 min and every 10 min for 90 min, respectively. ACh and dopamine levels in the dialysates were analysed using an HPLC system equipped with an electrochemical detector (Mouri *et al.* 2007, 2006).

Western blotting

We examined activation of ERK1/2 in the brain of mice that were exposed to the novel objects during the training session. Phosphorylation of ERK1/2 was examined by Western blotting as described previously (Kamei *et al.* 2006; Mizoguchi *et al.* 2004). Immediately after a training session, the mice were sacrificed by decapitation, and the brain was immediately removed. The PFC was rapidly dissected out on an ice-cold plate, frozen, and stored at −80 °C until required. Tissue samples from the PFC were homogenized by sonication at 4 °C in a lysis buffer composed of 20 mM Tris–HCl, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1% SDS, 1% sodium deoxycholate, 0.5 mM dithiothreitol, 10 mM sodium pyrophosphate decahydrate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (pH 7.4). The homogenate was centrifuged at 13 000 *g* for 20 min and the supernatant was used. The protein concentration of tissue extracts was determined using a DC Protein Assay kit (Bio-Rad, USA). Samples (20 μg protein) were boiled in a sample buffer [0.125 M Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 0.002% Bromphenol Blue, and 5% 2-mercaptoethanol], applied onto a 10% polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corporation, USA) or a nitrocellulose membrane (GE Healthcare Biosciences, USA), and blocked with a Detector Block kit (Kirkegaard and Perry Laboratories, USA). Membranes were incubated with anti-phospho-ERK1/2 [phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr^{202}/Tyr^{204}) Antibody no. 9101] (1:1000 dilution; Cell Signaling Technology Inc., USA) and washed with Tris-buffered saline (TBS)-Tween 20 [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 1% Tween 20] three times for 10 min each. After incubation with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (secondary antibody) for 1 h, membranes were washed with TBS-Tween 20 three times for 10 min each. The immune complex was detected using ECL Western blotting detection reagents (GE Healthcare Biosciences). The same membranes were stripped with a stripping buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM disodium hydrogen phosphate, 12-water, 1.5 mM potassium dihydrogen phosphate, and 0.2% 2-mercaptoethanol) at 55 °C for 30 min, incubated with anti-ERK1/2 (1:1000 dilution, p44/42 MAPK Antibody no. 9102, Cell Signaling Technology Inc.), and treated as described above.

Statistical analysis

Statistical significance was determined using a one-way analysis of variance (ANOVA) or a two-way ANOVA with repeated measures, followed by Bonferroni's test for multigroup comparisons. Statistical differences between two sets of groups were determined with the Student's *t* test. *p* values <0.05 were taken to indicate statistically significant differences.

Results

Effect of galantamine on Meth-induced impairment of recognition memory in mice

We examined whether Meth-induced cognitive impairment was reversed by galantamine. One day after the cessation of repeated Meth (1 mg/kg.d s.c.) treatment for 7 d, mice were subjected to the NOR test. Galantamine (3 mg/kg p.o.) was acutely administered 1 h before the training session.

As shown in Fig. 2, repeated Meth treatment significantly reduced the exploratory preference for a novel object in the retention session ($p<0.01$) (Fig. 2*a*). Treatment with galantamine significantly improved cognitive impairment in Meth-treated mice ($p<0.01$) (Fig. 2*a*). Galantamine affected neither the level of exploratory preference for the objects in the training session [$F(3,36)=1.188$, $p=0.328$] (Fig. 2*a*) nor the total exploration time in either the training [$F(3,36)=1.241$, $p=0.309$] or retention [$F(3,36)=2.396$, $p=0.084$] sessions in Meth-treated mice (Fig. 2*b*).

Effect of donepezil on the extracellular ACh levels of the PFC and the impairment of recognition memory in Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment

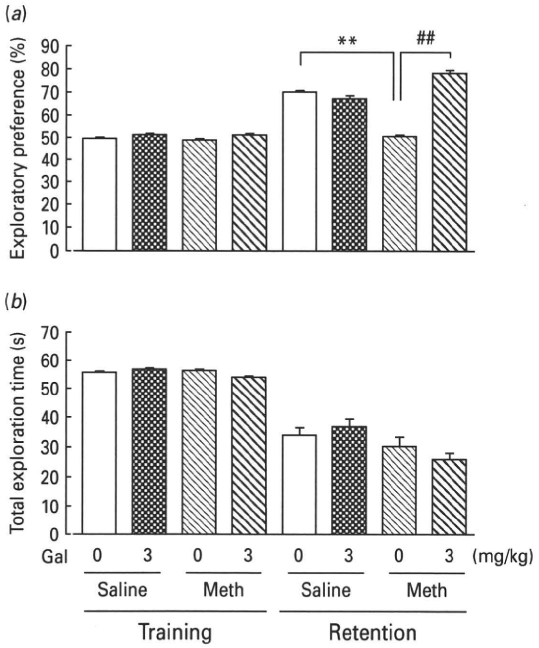

Fig. 2. Effect of galantamine on methamphetamine (Meth)-induced impairment of recognition memory in mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (3 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean ± S.E. ($n=10$). One-way ANOVA, (*a*) training: $F(3,36)=1.188$, $p=0.328$; retention: $F(3,36)=63.849$, $p<0.01$; (*b*) training: $F(3,36)=1.241$, $p=0.309$; retention: $F(3,36)=2.396$, $p=0.084$. ** $p<0.01$ compared to saline + saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth + saline-treated group (Bonferroni's test).

are due to increase of ACh levels caused by inhibition of AChE, we examined the effect of donepezil, an AChE inhibitor, on the impairment of cognition in Meth-treated mice.

Donepezil at a dose of 1 mg/kg caused about a 2-fold increase in the levels of extracellular ACh in the PFC of Meth-treated mice [$F(1,35)=14.042$, $p<0.01$] (Fig. 3*a*). However, donepezil (1 mg/kg) had no effect on the level of exploratory preference for the objects in the retention sessions in Meth-treated mice (Fig. 3*b*). It also affected neither the level of exploratory preference for the objects in the training session [$F(2,40)=0.159$, $p=0.854$] (Fig. 3*a*) nor the total exploration time in either the training [$F(2,40)=0.296$, $p=0.746$] or retention [$F(2,40)=0.160$, $p=0.215$] sessions in Meth-treated mice (Fig. 3*c*).

Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated via nAChRs, but not muscarinic AChRs (mAChRs), we examined the antagonism by using mecamylamine, a nAChR antagonist and scopolamine, a mAChR antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

In the training session of the NOR task, there were no differences in exploratory preference for the objects in any of the groups (Fig. 4*a*,*c*). The nAChR antagonist, mecamylamine (3 mg/kg) significantly and completely prevented the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice ($p<0.01$) (Fig. 4*a*). In saline-treated mice, mecamylamine alone at the dose used had no effect on the NOR performances (Fig. 4*a*). The antagonistic effect of mecamylamine on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(4,57)=0.516$, $p=0.725$; retention: $F(4,57)=2.403$, $p=0.060$] (Fig. 4*b*).

Scopolamine at a dose of 0.1 mg/kg impaired the performance of saline-treated mice in the NOR task (Fig. 4*c*). However, scopolamine failed to prevent the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice (Fig. 4*c*). Treatment with any compound did not affect the total exploration time in either the training [$F(6,77)=2.193$, $p=0.053$] or retention [$F(6,77)=1.919$, $p=0.088$] sessions (Fig. 4*d*).

Effects of galantamine on the levels of extracellular dopamine in the PFC of Meth-treated mice

We examined whether galantamine at a dose of 3 mg/kg, which improved the cognitive deficit in Meth-treated mice, facilitated dopamine release in the PFC of Meth-treated mice.

There were no differences in the basal levels of extracellular dopamine in the PFC in any of the groups (Fig. 5 insert). As shown in Fig. 5, galantamine (3 mg/kg) caused a marked increase in the levels of extracellular dopamine in the PFC of Meth-treated mice (Fig. 5). The significant increase in the levels of extracellular dopamine was observed from 30 min after galantamine administration ($p<0.01$ by *post hoc* test, Fig. 5). When mecamylamine (3 mg/kg) was injected into Meth-treated mice 20 min after galantamine administration, galantamine-induced elevation of extracellular dopamine levels was significantly diminished

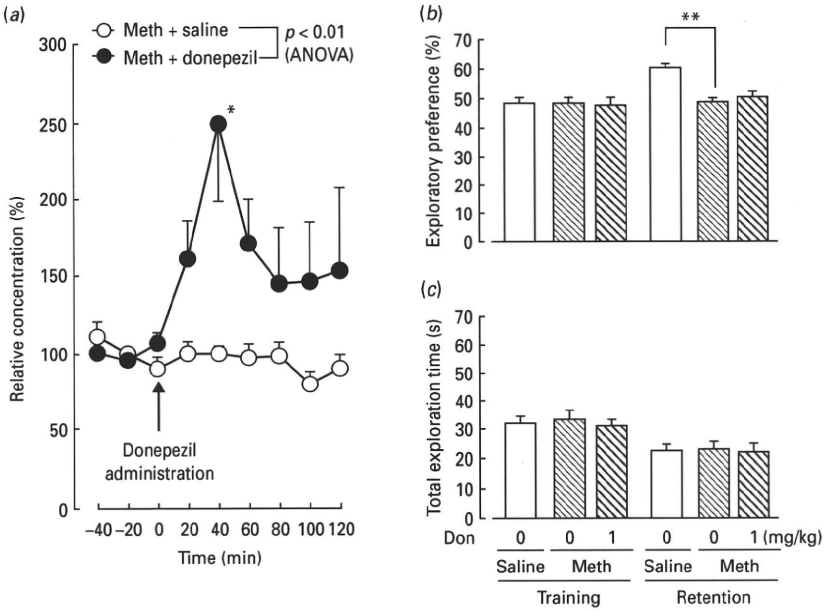

Fig. 3 Effect of donepezil on the extracellular acetylcholine (ACh) levels of the prefrontal cortex (PFC) and the impairment of recognition memory in methamphetamine (Meth)-treated mice. (*a*) Extracellular ACh levels of PFC in microdialysis. *In-vivo* microdialysis was performed 3 d after the final injection of Meth (1 mg/kg s.c.) treatment for 7 d. Donepezil (1 mg/kg p.o.) was administered to the Meth-treated mice (●, Meth + donepezil). In the control group, an equivalent amount of saline was given to the Meth-treated mice (○, Meth + saline). Values indicate the mean ± S.E. ($n = 4$–5). Results with the repeated ANOVA were: time [$F(5, 35) = 1.111$, $p = 0.37$]; treatment [$F(1, 35) = 14.042$, $p < 0.01$]; time × treatment interaction [$F(5, 35) = 0.677$, $p = 0.64$]. * $p < 0.05$ compared to Meth + saline-treated group (Bonferroni's test). The basal levels of ACh in the PFC of the Meth + saline- and Meth + donepezil-treated mice were 0.17 ± 0.05 and 0.12 ± 0.06 pmol/20 μl per 20 min, respectively. (*b*) Exploratory preference in novel object recognition (NOR) test. (*c*) Total exploration time in NOR test. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the NOR test. Donepezil (1 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean ± S.E. ($n = 13$–15). One-way ANOVA, (*b*) training: $F(2, 40) = 0.159$, $p = 0.854$; retention: $F(2, 40) = 9.400$, $p < 0.01$; (*c*) training: $F(2, 40) = 0.296$, $p = 0.746$; retention: $F(2, 40) = 0.160$, $p = 0.215$. ** $p < 0.01$ compared to saline + saline-treated group (Bonferroni's test).

(Fig. 5). However, mecamylamine alone did not affect the extracellular dopamine levels in saline-treated mice (data not shown).

Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on Meth-treated mice

Previous studies have shown that the ERK1/2 signalling pathway linked to dopamine D_1 receptors (D_1Rs) (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in Meth-associated contextual memory in rats (Mizoguchi *et al.* 2004) and that repeated Meth treatment induces cognitive impairment in the NOR test in mice, which is accompanied by dysfunction of the dopamine D_1R-ERK1/2 pathway in the PFC (Kamei *et al.* 2006). To clarify whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated through the activation of dopamine D_1Rs, we investigated the antagonism by using SCH 23390, a dopamine D_1R antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

SCH 23390 (0.02 mg/kg) significantly and completely prevented the improving effects of galantamine on Meth-induced cognitive impairment without affecting the exploratory preference for the objects in the training session (Fig. 6*a*). In saline-treated mice, SCH 23390 alone had no effect on NOR performance (Fig. 6*a*). SCH 23390 also had no effect on the total exploration time in either the training [$F(4, 50) = 1.520$, $p = 0.211$] or retention [$F(4, 55) = 1.943$, $p = 0.116$] sessions of Meth-treated mice (Fig. 6*b*).

Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of Meth-treated mice

Kamei *et al.* (2006) have demonstrated novelty-induced ERK1/2 activation in the PFC when mice are

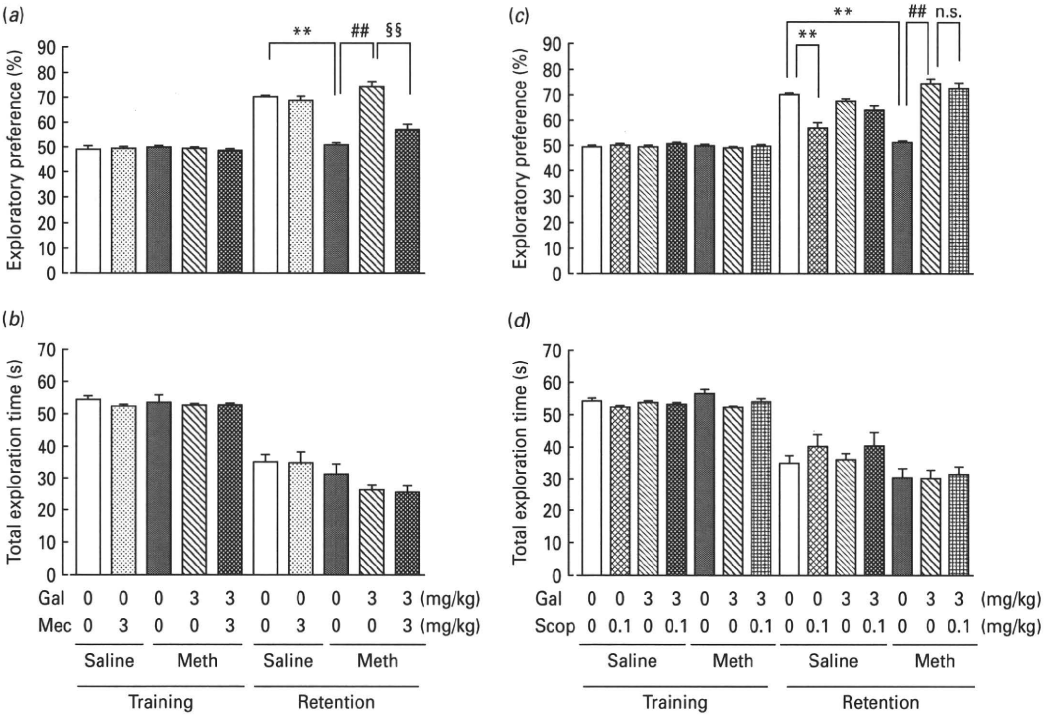

Fig. 4. Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.), mecamylamine (Mec; 3 mg/kg s.c.) and/or scopolamine (Scop; 0.1 mg/kg s.c.) were administered to saline- or Meth-treated mice 1 h, 40 min and/or 40 min, respectively, before the training session. Values indicate the mean ± S.E. ($n = 10$–15). One-way ANOVA, (*a*) training: $F(4, 57) = 0.255$, $p = 0.906$; retention: $F(4, 57) = 28.901$, $p < 0.01$; (*b*) training: $F(4, 57) = 0.516$, $p = 0.725$; retention: $F(4, 57) = 2.403$, $p = 0.060$; (*c*) training: $F(6, 77) = 0.429$, $p = 0.858$; retention: $F(6, 77) = 20.277$, $p < 0.01$; (*d*) training: $F(6, 77) = 2.193$, $p = 0.053$; retention: $F(6, 77) = 1.919$, $p = 0.088$. ** $p < 0.01$ compared to saline + saline/saline-treated group (Bonferroni's test). ## $p < 0.01$ compared to Meth + saline/saline-treated group (Bonferroni's test). §§ $p < 0.01$ compared to Meth + galantamine/saline-treated group (Bonferroni's test). n.s., Not significant.

exposed to novel objects, leading to the formation of long-lasting object recognition memory. Further, memory impairment in Meth-treated mice was associated with dysfunction of ERK1/2 signalling in the PFC. In order to examine the mechanism by which galantamine ameliorates the impairment of recognition memory in Meth-treated mice, we examined the effect of galantamine on ERK1/2 phosphorylation in the PFC of Meth-treated mice when they were exposed to novel objects.

A significant increase in phosphorylation of ERK1/2 levels was observed in the PFC of saline-treated mice immediately after a 10-min exposure to novel objects (Fig. 7*a*, *b*) ($p < 0.01$ *vs.* baseline in saline-treated mice, Student's *t* test), and repeated Meth treatment abolished novelty-induced ERK1/2 activation in the PFC in accord with the previous study (Kamei *et al.* 2006) ($p < 0.01$) (Fig. 7*a*). Galantamine (3 mg/kg) significantly recovered the defect of novelty-induced activation of ERK1/2 in the PFC of Meth-treated mice ($p < 0.01$) (Fig. 7*a*). SCH 23390 (0.02 mg/kg) significantly blocked the improving effects of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC ($p < 0.01$) (Fig. 7*a*). SCH 23390 alone had no effect on the levels of phosphorylation and total ERK1/2 in either the baseline or exposure of saline-treated mice (Fig. 7*b*). The levels of total ERK1/2 did not differ in the exposed groups examined [$F(3, 16) = 1.629$, $p = 0.222$].

Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on Meth-treated mice

We confirmed that PD98059 (2 μg/1 μl/bilateral) has no effect on the phosphorylation of ERK1/2 in the PFC and hippocampus of naive mice (data not shown). Then, we examined the effect of PD98059 (2 μg/1 μl/bilateral) administered before the training session on

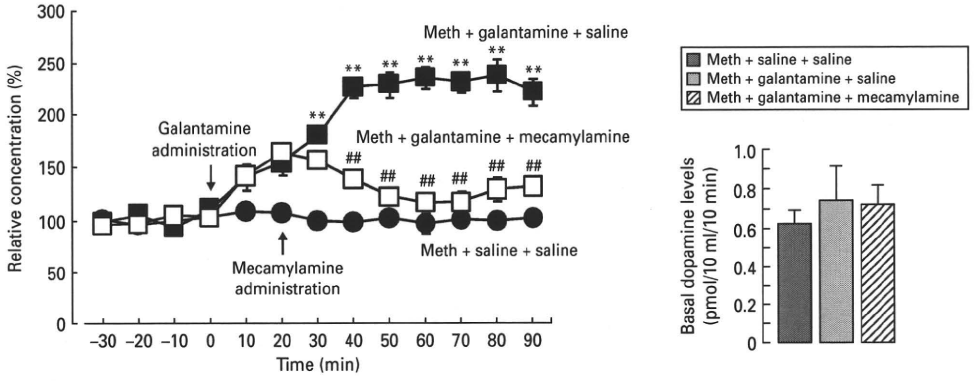

Fig. 5. Effects of galantamine on the levels of the extracellular dopamine in the PFC of methamphetamine (Meth)-treated mice. Meth (1 mg/kg, s.c.) was injected for 7 d, and 3 d after withdrawal, extracellular levels of dopamine were measured in the PFC by *in-vivo* microdialysis. Galantamine (3 mg/kg p.o.) was administered to the Meth-treated mice (■, Meth + galantamine + saline). In the control group, an equivalent amount of saline was given (●, Meth + saline + saline) to the Meth-treated mice. Mecamylamine (3 mg/kg s.c.) was injected 20 min after galantamine (□, Meth + galantamine + mecamylamine) to Meth-treated mice. The basal levels of dopamine in the PFC of the Meth + saline + saline (■)-, Meth + galantamine + saline (▩)- and Meth + galantamine + mecamylamine (▨)-treated mice were 0.62 ± 0.08, 0.74 ± 0.18 and 0.72 ± 0.10 pmol/10 μl per 10 min, respectively (right-hand panel). Values indicate the mean ± S.E. ($n = 3$). Results with the repeated ANOVA were time [$F(9, 54) = 8.063$, $p < 0.01$], treatment [$F(2, 6) = 73.188$, $p < 0.01$], and time × treatment interaction [$F(18, 54) = 10.802$, $p < 0.01$]. ** $p < 0.01$ compared to Meth + saline + saline-treated group (Bonferroni's test). ## $p < 0.01$ compared to Meth + galantamine + saline-treated group (Bonferroni's test).

the cognitive-improving effect of galantamine in Meth-treated mice to determine the involvement of ERK1/2 activation in the mechanism of action of galantamine.

In the training session, bilateral microinjections of PD98059 into the PFC (1 μg/side) of saline-treated mice did not affect the exploratory preference for the objects (Fig. 8*a*). In the retention session, the level of exploratory preference in PD98059-treated mice was significantly increased as for vehicle-treated mice ($p < 0.01$, Fig. 8*a*), but it was significantly decreased compared to that in vehicle-treated mice ($p < 0.05$, Fig. 8*a*). PD98059 had no effect on the total exploration time in either the training or retention sessions of saline-treated mice (Fig. 8*b*).

In Meth-treated mice, PD98059 completely blocked the ameliorating effect of galantamine on the impairment of exploratory preference for a novel object in the retention session [$F(2, 25) = 27.986$, $p < 0.01$] (Fig. 8*c*). The antagonistic effect of PD98059 on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(2, 25) = 0.399$, $p = 0.676$; retention: $F(2, 25) = 0.015$, $p = 0.985$] (Fig. 8*d*).

Discussion

We have reconfirmed that Meth-treated mice show impairments to their novelty discrimination ability in the NOR test that is consistent with previous reports (Ito *et al.* 2007; Kamei *et al.* 2006). It is unlikely that the impairment in performance of Meth-treated mice in learning and memory tasks is due to changes in motivation, although various motivations are involved in the behavioural task. The fact that Meth reduced the exploratory preference for the objects in the retention session could be interpreted as neophobia. However, the possible involvement of motivation and/or neophobia can be excluded because Meth treatment had no effect on total exploration time of novel objects during the training session. Therefore, it is likely that impairment of performance in Meth-treated mice is due to learning and memory deficits.

Galantamine, a drug approved for the treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nAChR as a potent APL (Eisele *et al.* 1993; Santos *et al.* 2002). We have recently reported that galantamine reverses the impairment of object recognition in $A\beta_{25-35}$-infused mice as an animal model of Alzheimer's disease and in repeated PCP-treated mice as an animal model of schizophrenia (Wang *et al.* 2007*a*, *b*). In accord with these findings, in the present study, galantamine significantly ameliorated the cognitive impairments induced by Meth in the NOR test. Galantamine at a dose of 3 mg/kg had no effect on the total exploration time in the training session of the NOR test in Meth-treated

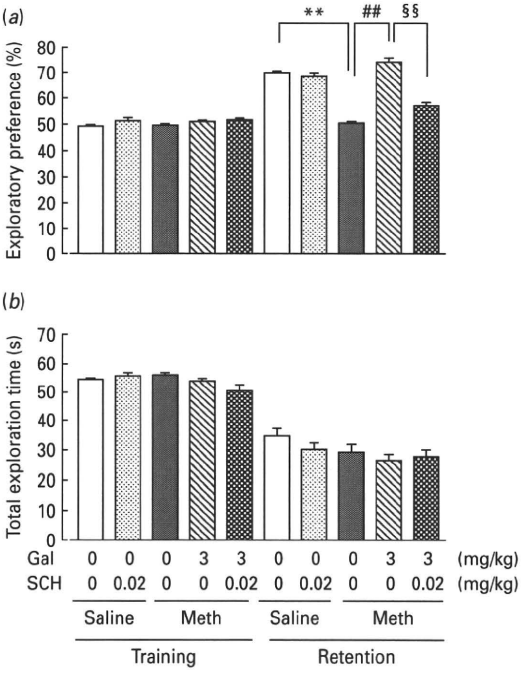

Fig. 6. Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and SCH 23390 (SCH; 0.02 mg/kg s.c.) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean ± S.E. ($n = 10$–15). One-way ANOVA, (*a*) training: $F(4, 50) = 1.422$, $p = 0.240$; retention: $F(4, 55) = 40.622$, $p < 0.01$; (*b*) training: $F(4, 50) = 1.520$, $p = 0.211$; retention: $F(4, 55) = 1.943$, $p = 0.116$. ** $p < 0.01$ compared to saline + saline/saline-treated group (Bonferroni's test). ## $p < 0.01$ compared to Meth + saline/saline-treated group (Bonferroni's test). §§ $p < 0.01$ compared to Meth + galantamine/saline-treated group (Bonferroni's test).

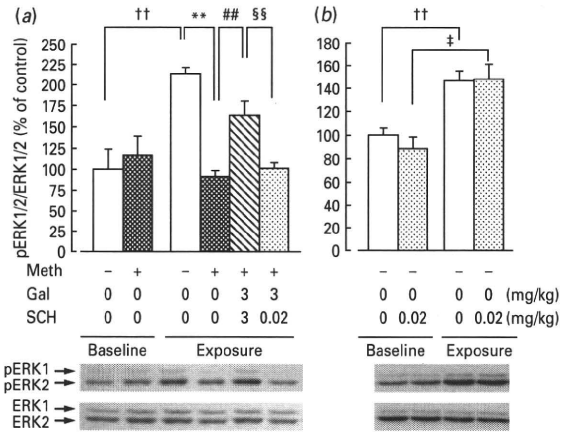

Fig. 7. Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of methamphetamine (Meth)-treated mice. One hour before exposure to novel objects, galantamine (Gal; 3 mg/kg p.o.) or saline was administered to mice that had been previously treated with either saline or Meth (1 mg/kg s.c.) for 7 d. SCH 23390 (SCH; 0.02 mg/kg s.c.) was administered 30 min before exposure to novel objects. Values indicate the mean ± S.E. ($n = 4$–5). †† $p < 0.01$ compared to saline + saline/saline-treated group that was not exposed to novel objects (baseline) (Student's t test). ‡ $p < 0.05$ compared to saline + saline/SCH23390-treated group that was not exposed to novel objects (baseline) (Student's t test). One-way ANOVA: $F(3, 16) = 28.286$, $p < 0.01$. ** $p < 0.01$ compared to saline + saline/saline-treated group (exposure) (Bonferroni's test). ## $p < 0.01$ compared to Meth + saline/saline-treated group (exposure) (Bonferroni's test). §§ $p < 0.01$ compared to Meth + galantamine/saline-treated group (exposure) (Bonferroni's test).

mice. Therefore, it is unlikely that the observed improvement in performance in the task brought about by galantamine is due to changes in motivation in Meth-treated mice, and it is apparently true that galantamine ameliorates learning and memory deficits caused by repeated Meth treatment in mice. The improving effects of galantamine on the performance of Meth-treated mice were prevented by treatment with mecamylamine, a nAChR antagonist, at a dose that did not significantly affect the performance of saline-treated mice. These findings support the notion that galantamine improves Meth-induced cognitive impairment via activation of nAChRs. Alternatively, the roles of mAChRs in the effects of galantamine were also investigated in the present study. The effects of galantamine on the performance of Meth-treated mice in the NOR task were not blocked by scopolamine at the dose that impaired the performance of saline-treated mice. Although mAChR agonists improve cognitive dysfunctions in patients with Alzheimer's disease and schizophrenia (Friedman, 2004), the present result indicated that mAChRs have little influence on the effects of galantamine for this particular cognitive task. On the other hand, the activation of nAChRs may be due to an increase in the levels of ACh caused by AChE inhibition of galantamine. We investigated the effect of donepezil, which is 3–15 times more potent in AChE inhibition than that of galantamine *in vivo* (Geerts *et al.* 2005), on Meth-induced cognitive impairment. Although donepezil at 1 mg/kg caused about a 2-fold increase from basal extracellular ACh levels in the PFC of Meth-treated mice, it had no effect on behavioural performance in Meth-treated mice. From the

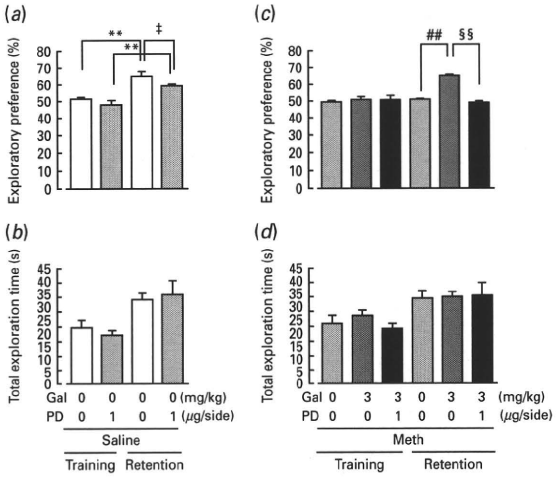

Fig. 8. Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and PD98059 (PD; 1 μg/0.5 μl per side) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean ± S.E. (*a*, *b*; $n=8$) (*c*, *d*; $n=9$–10). One-way ANOVA, (*c*) training: $F(2, 25)=0.309$, $p=0.737$; retention: $F(2, 25)=27.986$, $p<0.01$; (*d*) training: $F(2, 25)=0.399$, $p=0.676$; retention: $F(2, 25)=0.015$, $p=0.985$. ** $p<0.01$ compared to corresponding saline-treated training group (Student's *t* test). ‡ $p<0.05$ compared to saline + saline/vehicle-treated retention group (Student's *t* test). ## $p<0.01$ compared to Meth + saline/vehicle-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/vehicle-treated group (Bonferroni's test).

present results and a report that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts *et al.* 2005), our conclusion is that galantamine induces the ameliorating effect on impairment of memory mainly by allosterically modulating the function of nAChRs, but not by AChE inhibition. However, further experiments are needed to exclude the involvement of AChE inhibition by galantamine in the ameliorating effect of it on cognitive impairment in Meth-treated mice, since the allosteric potentiating effect of nAChRs can be detected at lower doses (Geerts *et al.* 2005).

Accumulating evidence suggests that the dopaminergic system in the PFC is involved in cognitive function. For instance, disruption of dopamine transmission in the PFC by infusions of dopamine D_1R antagonists or by excitotoxic lesions impairs the performance of object retrieval-detour tasks, as well as delayed response tasks in non-human primates (Dias *et al.* 1996*a*, *b*; Sawaguchi & Goldman-Rakic, 1991). A previous study with functional magnetic resonance imaging has shown that dysfunction in the PFC of Meth abusers is related to cognitive impairment (Paulus *et al.* 2002). Accordingly, cognitive impairment in Meth abusers may be associated with deficits in dopamine transmission in the PFC. Our previous findings in *in-vivo* microdialysis experiments demonstrated that galantamine increases the extracellular dopamine release in the hippocampus and PFC and that the increasing effects of galantamine on dopamine release in the hippocampus are potentiated by nicotine and antagonized by mecamylamine (Wang *et al.* 2007*a*). The present *in-vivo* microdialysis experiment show that galantamine significantly increased extracellular dopamine release in the PFC of Meth-treated mice. The effects of galantamine on increasing dopamine release were antagonized by mecamylamine. These results strongly suggest that galantamine ameliorates Meth-induced learning and memory deficits by activating nAChRs, and thereby stimulates release of dopamine in the PFC. Further, we found that the improving effects of galantamine were prevented by SCH 23390, a dopamine D_1R antagonist. Galantamine enhances dopaminergic neurotransmission *in vivo* via allosteric potentiation of nAChRs. These findings provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. The present results are supported by the results published by Schilström *et al.* (2007) that effects of galantamine on dopamine cell firing are mediated by allosteric potentiation of nAChRs. Taken together, our results suggest that the PFC-dependent behaviour task was impaired due to dysfunction of dopaminergic systems induced by Meth, since the PFC is involved in object recognition behaviour (Kamei *et al.* 2006). In fact, Kamei *et al.* (2006) have already demonstrated that repeated administration of Meth in mice induces object recognition impairment, which is associated with the dopamine D_1Rs, but not dopamine D_2Rs in the PFC. However, the object recognition memory is ascribed to the perirhinal cortex and its interactions with the hippocampus (Winters *et al.* 2008). We will investigate the functional role of the perirhinal cortex in Meth-induced cognitive deficits, in the ameliorating effects of galantamine and D_1R/ERK signalling in the NOR test.

Previous studies have demonstrated that the ERK1/2 signalling pathway linked to dopamine D_1Rs (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in the rewarding effects induced by Meth (Mizoguchi *et al.* 2004) and the behavioural sensitization and

rewarding effects induced by cocaine (Valjent *et al.* 2000). Regarding the mechanism underlying the repeated Meth-induced memory impairment, Kamei *et al.* (2006) have already demonstrated dysfunction of the ERK1/2 pathway in the PFC. Hyperphosphorylation of ERK1/2 was found in the PFC when control mice were exposed to novel objects, whereas this activation was abolished in repeated Meth-treated mice. Inhibition of ERK1/2 by the microinjection of PD98059 (4 μg/mouse/bilateral), a selective MEK inhibitor, into the PFC resulted in cognitive impairment (Kamei *et al.* 2006). Ito *et al.* (2007) have also found that another MEK1/2 inhibitor, SL327 (30 and 50 mg/kg i.p.), significantly impairs long-term recognition memory 24 h after a training session in naive mice. In this study, galantamine ameliorated the Meth-induced defect of ERK1/2 hyperphosphorylation in the PFC of mice exposed to novel objects. In addition, the ameliorating effect of galantamine on Meth-induced object recognition impairment was completely blocked by pretreatment with the ERK inhibitor PD98059 at the dose used, slightly affecting the performance of saline-treated mice. Accordingly, these results suggest that the ameliorating effect of galantamine on Meth-induced cognitive impairment is related to the activation of ERK1/2 in the PFC.

As discussed above, our findings suggest that dopamine D_1R-ERK1/2 systems are required for the effects of galantamine. Since dopamine the D_1R antagonist and ERK inhibitor impaired recognition memory based on phosphorylation of ERK in the PFC of normal mice (Kamei *et al.* 2006), dopamine D_1R-ERK1/2 systems are critical in recognition memory. If the action site of galantamine is downstream of dopamine D_1R-ERK1/2 systems, dopamine D_1R antagonists or the ERK inhibitor would fail to reverse the effect of galantamine. Accordingly, our data suggest that galantamine acts upstream of dopamine D_1R-ERK1/2 systems.

In conclusion, the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1R-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could prove to be a useful therapeutic drug for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Statement of Interest

None.

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Rapid communication

The expression of HMGA1a is increased in lymphoblastoid cell lines from schizophrenia patients

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ABSTRACT

The high-mobility group A protein 1a (HMGA1a) is a well-documented DNA-binding protein acting as an architectural transcription regulator. Recently, HMGA1a protein has been identified as a hypoxia-inducible RNA-binding *trans*-acting factor for aberrant splicing of presenilin-2 (PS2) pre-mRNA observed in the brains of sporadic Alzheimer's disease. Interestingly, this aberrant splicing of PS2 was also observed in the brains of bipolar disorder and schizophrenia. Many downstream genes under the control of HMGA1a could be associated with schizophrenia. On the other hand, many gene transcripts are aberrantly spliced in schizophrenia. Therefore, we examined the expression at the mRNA and protein levels of this DNA- and RNA-binding factor HMGA1a in the lymphoblastoid cell lines obtained from 16 schizophrenia patients with age-matched controls. We observed markedly higher HMGA1a mRNA and the increased HMGA1a protein in the nuclear fractions of schizophrenia patients. In contrast, there were no significant differences in the expression levels of HMGA1b, which is an alternatively spliced isoform of HMGA1a. The present study is the first to report a significant upregulation of HMGA1a in schizophrenia, suggesting its potential roles in both transcription and splicing of target genes linked with schizophrenia.

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High-mobility group A protein 1a (HMGA1a) is a non-histone DNA-binding architectural transcription factor that regulates the expressions of many target genes, and thus serving as a central 'hubs' of nuclear functions (reviewed in Reeves, 2001). However, we have identified HMGA1a as an RNA-binding factor responsible for aberrant exon 5 skipping of presenilin-2 (PS2) pre-mRNA, which produces a deleterious protein variant PS2V in the brains of sporadic Alzheimer disease (AD) patients. (Sato et al., 1999, 2001; Higashide et al., 2004; Manabe et al., 2002, 2003, 2007b). HMGA1a expression and subsequent production of PS2V are stimulated by oxidative stresses such as hypoxia, aluminum and radicals in neuroblastoma cell lines (Sato et al., 1999, 2001; Manabe et al., 2003; Matsuzaki et al., 2004), which is mediated by the neuron-specific transcription factor N-Myc (Yanagita et al., 2005). PS2V protein increases amyloid-β (Sato et al., 2001) and causes conformational changes of tau proteins (Nishikawa et al., 2004) in neuroblastoma cell lines by impairing the signaling pathway of the unfolded-protein response in the endoplasmic reticulum (Sato et al., 2001), suggesting that HMGA1a-induced aberrant splicing of the PS2 pre-mRNA is involved in neurodegenerative disorders including sporadic AD. Most recently, the definitive mechanism of HMGA1a-induced exon skipping has been elucidated (Ohe and Mayeda, in press).

Schizophrenia is one of the mental disorders that chronically show various symptoms of delusion and illusion among others. Various risk factors are known to be associated with the onset of schizophrenia, which indicates complex cause of this serious disease. Nevertheless, a large number of reports support its close implications with the neural development abnormalities involved

Abbreviations: HMGA1a, high-mobility group A protein 1a; HMGA1b, high-mobility group A protein 1b; LCL, lymphoblastoid cell line; PS2V, presenilin-2 splice variant (lacking exon 5); MIF-1, melanocyte-stimulating hormone release inhibiting factor-1; SELEX, systematic evolution of ligands by exponential enrichment.

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