Hashimoto, 2007]. Glutathione (GSH), one of the major non-protein antioxidants and redox regulators, detoxifies reactive oxidative stress (ROS) and thus plays a major role in protecting neural tissues [Dringen, 2000; Schulz et al., 2000]. A number of papers have demonstrated the neuroprotective effects of GSH or its related compounds such as *N*-acetyl-L-cysteine on METH-induced dopaminergic neuronal damage [Choi et al., 2002; Fukami et al., 2004; Hashimoto et al., 2004]. Given the role of GSH in the antioxidative process, the genes encoding the proteins known as polymorphic glutathione *S*-transferases (GSTs: Enzyme Commission (EC) number 2.5.1.18), glutathione cysteine ligase (GCL: EC 6.3.2.2), and glutathione peroxidase (GPX: EC 1.11.1.9) are clearly worthy of investigation [Thomas et al., 1990; Smythies and Galzigna, 1998; Anema et al., 1999; Nakamura et al., 2002; McIlwain et al., 2006]. The GSTs are a family of multifunctional enzymes that catalyze the conjugation of reduced GSH with electrophilic groups of a wide variety of compounds, including carcinogens, environmental contamination, and products of oxidative stress [Mannervik, 1985; Hayes and Strange, 2000; Hayes et al., 2005]. We reported that the functional polymorphisms of the GSTP1 and GSTM1 genes are associated with METH abuse and METH-induced psychosis [Koizumi et al., 2004; Hashimoto et al., 2005], suggesting that the GSTP1 and GSTM1 genes play a role in the pathogenesis of METH abuse.

Both GSTM1 and GSTT1 contain gene deletions, resulting in no enzymatic activity for that isozyme [McLellan et al., 1997; Sprenger et al., 2000]. The single nucleotide polymorphisms (SNPs) related to amino acid substitution among GSTs were shown in GSTT2 (G > A, Met139Ile, rs1622002) and GSTO1 (C > A, Ala140Asp, rs4925) [Yoshimura et al., 2003; Li et al., 2006]. The SNPs in the promoter regions of the GSTA1 gene contain three linked base substitutions (−567T/G, −69C/T, and −52G/A), and affect the gene expression [Ambrosone et al., 2006]. Furthermore, glutathione peroxidase 1 (GPX1), which belongs to a family of selenium-dependent peroxidases, protects cells by eliminating hydrogen peroxides and a wide range of organic peroxides by using GSH as a reducing substrate [Schweizer et al., 2004]. One functional polymorphism of the GPX1 gene is a substitution at codon 198 (Pro198Leu), and the leucine allele is less responsive to added selenium than the proline allele [Hu and Diamond, 2003]. Human glutamate cysteine ligase (GCL) is a rate-limiting enzyme for GSH synthesis, and GCL modifier (GCLM) is one of two subunits composing GCL [Huang et al., 1993]. The SNP (−588C/T) in the promoter region of the GCLM gene was associated with higher promoter activity [Nakamura et al., 2002], and an SNP (G > A, rs2301022) in the intron was demonstrated to be associated with the pathogenesis of schizophrenia [Tosic et al., 2006].

Given these findings, it is of great interest to study the association between the gene polymorphisms in GSH-related enzymes and METH abusers. The present study was undertaken to examine the association between the genetic polymorphisms among GSH-related enzymes (GSTT1, GSTT2, GSTA1, GSTO1, GPX1, GCLM) and METH use disorder in a Japanese population.

MATERIALS AND METHODS

Subjects

The subjects included 218 patients (176 males and 42 females, age: 36.9 ± 12.0 years (mean ± SD), age range: 18–69 years) with METH dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) and who were outpatients or inpatients of psychiatric hospitals affiliated with the Japanese Genetics Initiative for Drug Abuse (JGIDA), and 233 age-, gender-, and geographical-origin-matched normal controls (187 males and 46 females, age: 38.7 ± 12.6 years (mean ± SD), age range: 19–73 years) with no past history and no family history of drug dependence or psychotic disorders (Table I). The age of the normal subjects did not differ from that of the METH abusers (Table I). The research was performed after approval was obtained from the Ethics Committees of each institute of the JGIDA, and all subjects provided written informed consent for the use of their DNA samples as part of this study.

Background of METH Abusers

Diagnoses were made by two trained psychiatrists based on interviews and available information, including hospital records. Subjects were excluded if they had a clinical diagnosis of schizophrenia, another psychotic disorder, or an organic mental syndrome. All subjects were Japanese, and were born and living in restricted areas of Japan, including northern Kyushu, Setouchi, Chukyo, Tokai, and Kanto. The patients were divided into subgroups based on their characteristic clinical features (Table I). We excluded the subjects for whom there was insufficient clinical data to analyze the genetic polymorphisms in the subgroups of METH abusers. One hundred fifty-two patients had abused both METH and other drugs in the present or the past. After METH, organic solvents and marijuana were the most frequently used substances. Cocaine and heroin were rarely abused in this sample of subjects.

Clinical Course of METH Psychosis

Prognosis of psychosis. The prognosis of METH psychosis varied among patients, some of whom showed continued psychotic symptoms, even after METH discontinuance, as previously reported [Sato et al., 1983, 1992]. Accordingly, the patients were categorized by prognosis into two groups, a transient type and a prolonged type, based on the duration of the psychotic state after METH discontinuance. The transient type of patient was defined as a patient whose symptoms improved within 1 month after METH discontinuance and the

TABLE I. Characteristics of Control Subjects and METH Abusers

Variable	Controls	Abusers	*P* values
Sex, male/female	187/46	176/42	0.906*
Age, mean ± SD, y	38.7 ± 12.6 (19–73)	36.9 ± 12.0 (18–69)	0.131**
METH psychosis		191	
Transient type		104	
Prolonged type		87	
Spontaneous relapse			
Positive		91	
Negative		100	

*The comparison between two groups was performed using the χ^2 test.
**The comparison between two groups was performed using the *t*-test.

start of treatment with neuroleptics, and the prolonged type was defined as a patient whose psychosis continued for more than 1 month after METH discontinuance and the start of treatment with neuroleptics. In this study, there were 104 transient-type and 87 prolonged-type patients with METH psychosis (Table I). One of the issues in categorizing was the difficulty of distinguishing patients who coincidentally developed schizophrenia. Therefore, we excluded cases in which the predominant symptoms were of the negative and/or disorganized type, in order to maintain the homogeneity of the subgroup.

Spontaneous relapse. It has been well documented that once METH psychosis has developed, patients in a state of remission are susceptible to spontaneous relapse without re-consumption of METH [Sato et al., 1983, 1992]. It has thus been postulated that a sensitization phenomenon induced by the repeated consumption of METH develops in the brains of patients with METH psychosis, which provides a neural basis for an enhanced susceptibility to relapse. Therefore, the patients in this study were divided into two groups according to the presence or absence of spontaneous relapse. In this study, 91 patients underwent a spontaneous relapse, and 100 patients did not (Table I).

Genotyping of Identified Polymorphisms

A multiplex polymerase chain reaction (PCR) technique that detects homozygous deletion of GSTT1 was used, including primers for the β-globin gene (forward, 5′-CAA CTT CAT CCA CGT TCA CC-3′; reverse; 5′-GAA GAG CCA AGG ACA GGT AC-3′) as an internal control, with an annealing temperature of 60°C. For GSTT1, primers forward (5′-TTC CTT ACT GGT CCT CAC ATC TC-3′) and reverse (5′-TCA CCG GAT CAT GGC CAG CA-3′) were used. The PCR products were separated on 2% agarose gel stained with ethidium bromide.

The absence of amplified GSTT1 product (in the presence of β-globin as a PCR product) indicated the respective "null" genotype. The individuals in whom the GSTT1 gene was present were genotyped as "non-deletion" referring to previous studies [Sreelekha et al., 2001; Ambrosone et al., 2006]. The genotype of GSTA1 (GSTA1*A-69C and GSTA1*B-69T) was determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) according to Coles et al. [2001]. Briefly, the primers used in the PCR were a forward primer (5′-TGT TGA TTG TTT GCC TGA AAT T-3′) and a reverse primer (5′-GTT AAA CGC TGT CAC CCG TCC T-3′). The amplification was performed by denaturing at 94°C for 5 min, followed by 36 cycles at 94°C for 20 sec, annealing at 65°C for 20 sec, and extending at 72°C for 20 sec. The amplification products (10 μl) were digested by 6 U of restriction endonuclease Earl (New England Biolabs, Inc., Beverly, MA) at 37°C for 6 hr.

Table II shows the following SNPs information. For genotyping of GSTT2 (G > A, rs1622002), GSTO1 (C > A, rs4925), GPX1 (C > T, rs1050450), GCLM-588 (C > T), and GCLM (G > A, rs2301022), we used TaqMan SNP Genotyping Assays to score SNPs with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Thermal cycling conditions for PCR were 1 cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 sec and 60°C for 1 min. The data were analyzed using the Allelic Discrimination Program (Applied Biosystems).

Statistical Analysis

Genotype deviation from the Hardy–Weinberg equilibrium was evaluated Chi-square test. The differences between groups were evaluated by Fisher's exact test. The odds ratio and 95% confidence intervals (CI) between two variables were calculated as an estimate of risk. We estimated the power of association for our sample size using *Genetic Power Calculator Software* [Purcell et al., 2003] with an α of 0.05 and a disease prevalence of 0.01. Differences were considered significant at $P < 0.05$.

RESULTS

The genotypic and allelic frequencies of the seven polymorphisms are presented in Table III. For confirmation of the appropriate genotyping, the genotypic frequencies of all variants (GSTT2 (rs1622002): $P = 0.168$, GSTA1 (−69C/T): $P = 0.688$, GSTO1 (rs4925): $P = 0.168$, GPX1 (rs1050450): $P = 0.374$, GCLM (−588C/T): $P = 0.380$, GCLM (rs2301022): $P = 0.371$) in the all subjects including METH abuser samples and control samples were found to be in Hardy–Weinberg equilibrium.

We found significantly different frequencies of genotype between METH abusers and controls in GSTT1 (Table III). There was a significant difference in GSTT1 genotype frequency between METH abusers and controls ($P = 0.037$). The frequency (50.0%) of the GSTT1 null genotype among METH abusers was significantly higher ($P = 0.037$, odds ratio: 1.51, 95% CI 1.04–2.19) than that (39.9%) in controls. There was also a significant difference in GSTT1 genotype frequency between patients with METH psychosis and controls ($P = 0.039$). The frequency (50.3%) of the GSTT1 null genotype among patients with METH psychosis was also significantly higher ($P = 0.039$, odds ratio: 1.52, 95% CI 1.03–2.24) than that (39.9%) in controls. We examined the association between the clinical features of patients with METH psychosis (i.e., transient-type or prolonged-type psychosis, with or without spontaneous relapse) and controls in GSTT1 genotype frequency (Table III). The frequency (58.6%) of the GSTT1 null genotype among patients who were METH abusers with

TABLE II. TaqMan Primers and Probes for Genotyping Assays

SNP	Primer sequences forward, F, or reverse, R, primer (5′–3′)	Probe sequences reporter VIC or FAM probe (5′–3′)
GSTT2 (rs1622002, G > A)	F: GAGAAGGTGGAACGCAACAG	VIC-CTGCCATGGACCAGG-MGB
	R: TTGTCCTCCAGCCATTGCA	FAM-CTGCCATAGACCAGG-MGB
GSTO1 (rs4925, C > A)	F: GCCATCCTTGGTAGGAAGCTTTAT	VIC-TCTTTTAGGCCAGCATAGT-MGB
	R: GGAGAAATAATTACCTCCTCTAGCTTGGT	FAM-TTCTTTTAGGCCATCATAGT-MGB
GPX1 (rs1050450, C > T)	F: CATCGAAGCCCTGCTGTCT	VIC-ACAGCTGGGCCCTT-MGB
	R: CACTGCAACTGCCAAGCA	FAM-ACAGCTGAGCCCTT-MGB
GCLM −588C/T (C > T)	F: GCCCTTTAAAGAGACGTGTAGGAA	VIC-CTCCCGGCGTTCAG-MGB
	R: CCGCCTGGTGAGGTAGAC	FAM-TCTCCCAGCGTTCAG-MGB
GCLM (rs2301022, G > A)	F: CAGAGTCACACACCACAGTTTGTA	VIC-CAAAGGACTAATTCTGG-MGB
	R: GTTTTATCCTACTGTTATGAAGCACCCTAA	FAM-CAAAGGACTAGTTCTGG-MGB

TABLE III. Frequencies of Genotype and Allele of GSH-Related Gene Polymorphisms Between Controls and METH Abusers

		Genotype			
GSTT1	n	Non-deletion	Null	Odds ratios (95%CI)	*P*
Controls	233	140 (60.1%)	93 (39.9%)		
Abusers	218	109 (50.0%)	109 (50.0%)	1.51 (1.04–2.19)	**0.037***
Prognosis of psychosis	191	95 (49.7%)	96 (50.3%)	1.52 (1.03–2.24)	**0.039***
Transient	104	59 (56.7%)	45 (43.3%)	1.15 (0.72–1.83)	0.632
Prolonged	87	36 (41.4%)	51 (58.6%)	2.13 (1.29–3.52)	**0.0036****
Spontaneous relapse					
Positive	91	41 (45.1%)	50 (54.9%)	1.84 (1.13–2.99)	**0.018***
Negative	100	54 (54.0%)	46 (46.0%)	1.28 (0.80–2.06)	0.333

		Genotype				Allele		
GSTT2 (rs1622002)	n	G/G	G/A	A/A	*P*	G	A	*P*
Controls	233	157 (67.4%)	73 (31.3%)	3 (1.3%)		387 (83.0%)	79 (17.0%)	
Abusers	218	149 (68.3%)	63 (28.9%)	6 (2.8%)	0.501	361 (82.8%)	75 (17.2%)	0.930
Prognosis of psychosis	191	133 (69.6%)	52 (27.2%)	6 (3.2%)	0.298	318 (83.2%)	64 (16.8%)	1
Transient	104	71 (68.3%)	30 (28.8%)	3 (2.9%)	0.536	172 (82.7%)	36 (17.3%)	0.912
Prolonged	87	62 (71.3%)	22 (25.3%)	3 (3.4%)	0.256	146 (83.9%)	28 (16.1%)	0.905
Spontaneous relapse								
Positive	91	63 (69.2%)	27 (29.7%)	1 (1.1%)	0.912	153 (84.1%)	29 (15.9%)	0.815
Negative	100	70 (70.0%)	25 (25.0%)	5 (5.0%)	0.080	165 (82.5%)	35 (17.5%)	0.911

		Genotype				Allele		
GSTA1 −69C/T	n	C/C	C/T	T/T	*P*	C	T	*P*
Controls	233	180 (77.3%)	50 (21.4%)	3 (1.3%)		410 (88.0%)	56 (12.0%)	
Abusers	218	158 (72.5%)	56 (25.7%)	4 (1.8%)	0.508	372 (85.3%)	64 (14.7%)	0.241
Prognosis of psychosis	191	144 (75.4%)	44 (23.0%)	3 (1.6%)	0.916	332 (86.9%)	50 (13.1%)	0.677
Transient	104	79 (76.0%)	23 (22.1%)	2 (1.9%)	0.811	181 (87.0%)	27 (13.0%)	0.706
Prolonged	87	65 (74.7%)	21 (24.1%)	1 (1.2%)	0.852	151 (86.8%)	23 (13.2%)	0.686
Spontaneous relapse								
Positive	91	66 (72.5%)	23 (25.3%)	2 (2.2%)	0.552	155 (85.2%)	27 (14.8%)	0.360
Negative	100	78 (78.0%)	21 (21.0%)	1 (1.0%)	1	177 (88.5%)	23 (11.5%)	0.897

		Genotype				Allele		
GSTO1 (rs4925)	n	C/C	C/A	A/A	*P*	C	A	*P*
Controls	233	166 (71.2%)	55 (23.6%)	12 (5.2%)		387 (83.0%)	79 (17.0%)	
Abusers	218	163 (74.8%)	53 (24.3%)	2 (0.9%)	**0.033***	379 (86.9%)	57 (13.1%)	0.114
Prognosis of psychosis	191	140 (73.3%)	49 (25.7%)	2 (1.0%)	0.057	329 (86.1%)	53 (13.9%)	0.253
Transient	104	74 (71.1%)	29 (27.9%)	1 (1.0%)	0.140	177 (85.1%)	31 (14.9%)	0.573
prolonged	87	66 (75.9%)	20 (23.0%)	1 (1.1%)	0.295	152 (87.4%)	22 (12.6%)	0.223
Spontaneous relapse								
Positive	91	64 (70.3%)	27 (29.7%)	0 (0.0%)	**0.042***	155 (85.2%)	27 (14.8%)	0.556
Negative	100	76 (76.0%)	22 (22.0%)	2 (2.0%)	0.404	174 (87.0%)	26 (13.0%)	0.246

		Genotype				Allele		
GPX1 (rs1050450)	n	C/C	C/T	T/T	*P*	C	T	*P*
Controls	233	207 (88.8%)	23 (9.9%)	3 (1.3%)		437 (93.8%)	29 (6.2%)	
Abusers	218	189 (86.7%)	29 (13.3%)	0 (0.0%)	0.142	407 (93.3%)	29 (6.7%)	0.892
Prognosis of psychosis	191	165 (86.4%)	26 (13.6%)	0 (0.0%)	0.185	356 (93.2%)	26 (6.8%)	0.780
Transient	104	90 (86.5%)	14 (13.5%)	0 (0.0%)	0.457	194 (93.3%)	14 (6.7%)	0.865
Prolonged	87	75 (86.2%)	12 (13.8%)	0 (0.0%)	0.403	162 (93.1%)	12 (6.9%)	0.720
Spontaneous relapse								
Positive	91	82 (90.1%)	9 (9.9%)	0 (0.0%)	0.796	173 (95.1%)	9 (4.9%)	0.710
Negative	100	83 (83.0%)	17 (17.0%)	0 (0.0%)	0.105	183 (91.5%)	17 (8.5%)	0.318

		Genotype				Allele		
GCLM −588C/T	n	C/C	C/T	T/T	*P*	C	T	*P*
Controls	233	172 (73.8%)	59 (25.3%)	2 (0.9%)		403 (86.5%)	63 (13.5%)	
Abusers	218	150 (68.8%)	62 (28.4%)	6 (2.8%)	0.214	362 (83.0%)	74 (17.0%)	0.164
Prognosis of psychosis	191	131 (68.6%)	55 (28.8%)	5 (2.6%)	0.241	317 (83.0%)	65 (17.0%)	0.177
Transient	104	73 (70.2%)	29 (27.9%)	2 (1.9%)	0.594	175 (84.1%)	33 (15.9%)	0.474
Prolonged	87	58 (66.7%)	26 (29.9%)	3 (3.4%)	0.143	142 (81.6%)	32 (18.4%)	0.134
Spontaneous relapse								
Positive	91	60 (65.9%)	29 (31.9%)	2 (2.2%)	0.217	149 (81.9%)	33 (18.1%)	0.141
Negative	100	71 (71.0%)	26 (26.0%)	3 (3.0%)	0.343	168 (84.0%)	32 (16.0%)	0.400

(*Continued*)

TABLE III. (*Continued*)

GCLM (rs2301022)	n	Genotype G/G	Genotype G/A	Genotype A/A	P	Allele G	Allele A	P
Controls	233	125 (53.7%)	90 (38.6%)	18 (7.7%)		340 (73.0%)	126 (27.0%)	
Abusers	218	126 (57.8%)	76 (34.9%)	16 (7.3%)	0.682	328 (75.2%)	108 (24.8%)	0.448
Prognosis of psychosis	191	109 (57.1%)	66 (34.5%)	16 (8.4%)	0.694	284 (74.3%)	98 (25.7%)	0.696
Transient	104	61 (58.7%)	34 (32.7%)	9 (8.6%)	0.587	156 (75.0%)	52 (25.0%)	0.636
Prolonged	87	48 (55.2%)	32 (36.8%)	7 (8.0%)	0.925	128 (73.6%)	46 (26.4%)	0.920
Spontaneous relapse								
Positive	91	52 (57.1%)	34 (37.4%)	5 (5.5%)	0.790	138 (75.8%)	44 (24.2%)	0.488
Negative	100	57 (57.0%)	32 (32.0%)	11 (11.0%)	0.403	146 (73.0%)	54 (27.0%)	1

Bold shows a significant difference.
*$P<0.05$, **$P<0.01$ as compared to control group.

prolonged-type psychosis was significantly higher ($P=0.0036$, odds ratio: 2.13, 95% CI 1.29–3.52) than that (39.9%) of controls, although there was no difference in GSTT1 genotype frequency between METH abusers with transient-type psychosis and controls (Table III). The frequency (54.9%) of the GSTT1 null genotype among METH abusers with spontaneous relapse was significantly higher ($P=0.018$, odds ratio: 1.84, 95% CI 1.13–2.99) than that (39.9%) of controls, although there was no difference in GSTT1 genotype frequencies between METH abusers without spontaneous relapse and controls (Table III). Furthermore, to examine the association between GSTT1 gene polymorphism and the clinical course of METH psychosis, we analyzed the frequency of the GSTT1 genotype among patients with METH psychosis. As shown in Table IV, we classified patients with METH psychosis into four subgroups based on the course of METH psychosis (i.e., transient or prolonged METH psychosis with or without spontaneous relapse of psychotic symptoms). There was a significant difference in GSTT1 genotype frequency between prolonged-type METH psychotic patients with spontaneous relapse and transient-type METH psychotic patients without spontaneous relapse ($P=0.025$). The frequency (66.0%) of the GSTT1 null genotype among prolonged-type METH psychotic patients with spontaneous relapse was significantly higher ($P=0.025$, odds ratio: 2.43, 95% CI 1.13–5.23) than that (44.4%) of transient-type METH psychotic patients without spontaneous relapse (Table IV).

No significant differences were found in the allelic and genotypic frequencies of the SNPs of GSTT2 (rs1622002), GSTA1 (−69C/T), GPX1 (rs1050450), GCLM −588C/T, and GCLM (rs2301022) between METH abusers and controls (Table III). Although there was a significant difference in the genotype frequency ($P=0.033$) of GSTO1 (rs4925) between METH abusers and controls, no difference in terms of allele frequency ($P=0.114$, odds ratio: 0.74, 95% CI 0.51–1.07) was detected between the two groups. There was also no difference in the allele frequency ($P=0.556$) of GSTO1 between METH abusers with spontaneous relapse and controls, though a significant difference in genotype frequency ($P=0.042$) of GSTO1 was detected between the two groups.

DISCUSSION

The present study showed that the polymorphism of the GSTT1 gene was associated with METH psychosis in a Japanese population. The finding supports the hypothesis that oxidative stress mechanisms including GSH-related enzymes might play a role in the pathogenesis of METH psychosis. In this study, we found that the frequency of the GSTT1 null genotype was significantly higher among patients with METH psychosis than among controls. This finding was consistent with our previous report that the frequency of the GSTP1 gene with the 105 valine allele, which results in low activity of GST [Pemble et al., 1994; Watson et al., 1998], was significantly higher among patients with METH psychosis than among controls [Hashimoto et al., 2005].

It has been suggested that the ROS and dopamine (DA) quinones generated by the administration of METH covalently conjugate with the sulfhydryl group of cysteine on functional proteins such as dopamine transporter (DAT), leading to METH-induced neuronal damage in the brain [Smythies and Galzigna, 1998; Whitehead et al., 2001; Asanuma et al., 2003; Miyazaki et al., 2006; Hashimoto, 2007]. GSTs are considered to play a role in the protective effect against oxidative stress by catalyzing the conjugation of electrophilic substrates such as ROS and DA quinones [Smythies and Galzigna, 1998; Whitehead et al., 2001]. In addition, recent studies reported that the mRNA expressions of both GSTT1 and GSTP1 were detected in

TABLE IV. Analysis of GSTT1 Polymorphisms in Patients with METH Psychosis

	n	Age, mean ± SD	Genotype Non-deletion	Genotype Null	Odds ratios (95% CI)	P
Transient-type psychosis						
spontaneous relapse						
Negative	63	38.9 ± 11.7	35 (55.6%)	28 (44.4%)		
Positive	41	37.1 ± 10.5	24 (58.5%)	17 (41.5%)	0.89 (0.40–1.96)	0.84
Prolonged-type psychosis						
spontaneous relapse						
Negative	37	33.4 ± 13.8	19 (51.4%)	18 (48.6%)	1.18 (0.52–2.67)	0.84
Positive	50	38.1 ± 12.0	17 (34.0%)	33 (66.0%)	2.43 (1.13–5.23)	**0.025***

P-value as compared to transient METH psychotic patients without spontaneous relapse (Fisher's exact test).
Bold shows a significant difference.
*$P<0.05$ as compared to transient METH psychotic patients without spontaneous relapse.

the human brain [Nishimura and Naito, 2006], and that GSTT1 and GSTP1 were induced under conditions of oxidative stress [Strange et al., 2000; Brind et al., 2004]. Therefore, these findings suggest that the gene polymorphisms related to low activity of GST may lead to an excess of oxidative products (e.g., ROS and DA quinones) induced by the administration of METH, and might lead to METH-induced neuronal damage in the human brain, and consequently METH-related psychiatric symptoms such as METH psychosis.

In the present study, we found that the polymorphism of GSTT1 gene was associated with prolonged, but not transient, METH psychosis, and with spontaneous relapse of a psychotic state. It is of great interest to find an association between GSTT1 gene polymorphism and the clinical course of METH psychosis. Patients with prolonged-type psychosis, that is, psychosis continuing for more than 1 month even after METH discontinuance, or patients with spontaneous relapse of psychotic symptoms are categorized as having a "severe clinical course of METH psychosis," while patients with transient-type psychosis, defined as improvement of the psychotic state within 1 month, or patients without spontaneous relapse are categorized as having a "mild clinical course" [Ujike et al., 2003; Ujike and Sato, 2004]. Our findings suggest that the GSTT1 null genotype may be associated with the severe clinical course of METH psychosis. Given that GSTs are considered to play a role in the protective effect against oxidative stress induced by METH in the brain, METH users with the GSTT1 null genotype may be more vulnerable to METH-induced neurotoxicity than those with the GSTT1 non-deletion genotype. Taken all together, the findings of the present study suggest that the polymorphism of the GSTT1 gene might be a genetic risk factor for the development of METH psychosis. Furthermore, it is also likely that a genetic polymorphism in the GSTT1 gene might serve as a molecular marker for monitoring the clinical course among patients with METH psychosis.

In this study, we found no associations between the gene polymorphisms of the GSH-related enzymes, including GSTT2 (rs1622002), GSTA1 (−69C/T), GSTO1 (rs4925), GPX1 (rs1050450), GCLM −588C/T, and GCLM (rs2301022), and METH abusers in a Japanese population, although the genotype frequency of the GSTO1 gene was significantly different between METH abusers and controls. Therefore, the GSH-related genes, including GSTT2, GSTA1, GSTO1, GPX1, and GCLM, may have no major genetic effects on the pathogenesis of METH abuse in the Japanese population. In contrast, a post-mortem brain study showed that GST activity in the putamen of METH users with severe DA loss in the caudate was decreased, although the activities of both GPX and glutamate-cysteine ligase were not changed [Mirecki et al., 2004], a finding that supports the possibility that oxidative stress plays a role in the pathophysiology of patients who engage in METH abuse.

Some limitations of the present study are as follows. First, the sample size is small. Because of the small number of subgroups, we cannot rule out type I or type II errors. Using the genetic statistics package Genetic Power Calculator prepared by Purcell et al. [2003], the genetic power of the present association analysis has been estimated to be as high as 30%. Further studies with larger samples are clearly needed to verify the present findings. Second, there is sample collection in this study. The strengths of the sample collection is the geographically matched sample of cases and controls, since we matched these cases and controls based on geographic data. The weakness of the sample collection is the small sample size of METH users without psychosis, since we collected the sample from subjects at a psychiatric hospital. Therefore, it will be very useful to collect a sample of METH users without psychosis.

In conclusion, the present findings suggest that the polymorphism of the GSTT1 gene might be a genetic risk factor of the development of METH use disorder, and that genetic polymorphism in the GSTT1 gene may serve as a molecular marker for monitoring clinical course among patients with METH psychosis. Furthermore, it is possible that the GSH-related genes, including GSTT2, GSTA1, GSTO1, GPX1, and GCLM, have no major genetic effects on the pathogenesis of schizophrenia in a Japanese population.

ACKNOWLEDGMENTS

Funding for this study was provided by grants for Psychiatric and Neurological Disorders and Mental Health from the Ministry of Health, Labor and Welfare, Japan (to K.H.) and Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to K.H.).

REFERENCES

Achat-Mendes C, Anderson KL, Itzhak Y. 2007. Impairment in consolidation of learned place preference following dopaminergic neurotoxicity in mice is ameliorated by N-acetylcysteine but not D1 and D2 dopamine receptor agonists. Neuropsychopharmacology 32:531–541.

Ambrosone CB, Tian C, Ahn J, Kropp S, Helmbold I, von Fournier D, Haase W, Sautter-Bihl ML, Wenz F, Chang-Claude J. 2006. Genetic predictors of acute toxicities related to radiation therapy following lumpectomy for breast cancer: A case-series study. Breast Cancer Res 8:R40.

Anema SM, Walker SW, Howie AF, Arthur JR, Nicol F, Beckett GJ. 1999. Thioredoxin reductase is the major selenoprotein expressed in human umbilical-vein endothelial cells and is regulated by protein kinase C. Biochem J 342:111–117.

Asanuma M, Miyazaki I, Ogawa N. 2003. Dopamine- or L-DOPA-induced neurotoxicity: The role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res 5:165–176.

Brind AM, Hurlstone A, Edrisinghe D, Gilmore I, Fisher N, Pirmohamed M, Fryer AA. 2004. The role of polymorphisms of glutathione S-transferases GSTM1, M3, P1, T1 and A1 in susceptibility to alcoholic liver disease. Alcohol Alcohol 39:478–483.

Cadet JL, Jayanthi S, Deng X. 2003. Speed kills: Cellular and molecular bases of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. FASEB J 17:1775–1788.

Choi HJ, Yoo TM, Chung SY, Yang JS, Kim JI, Ha ES, Hwang O. 2002. Methamphetamine-induced apoptosis in a CNS-derived catecholaminergic cell line. Mol Cells 13:221–227.

Coles BF, Morel F, Rauch C, Huber WW, Yang M, Teitel CH, Green B, Lang NP, Kadlubar FF. 2001. Effect of polymorphism in the human glutathione S-transferase A1 promotor on hepatic GSTA1 and GSTA2 expression. Pharmacogenetics 11:663–669.

Cubells JF, Rayport S, Rajendran G, Sulzer D. 1994. Methamphetamine neurotoxicity involves vacuolation of endocytic organelles and dopamine-dependent intracellular oxidative stress. J Neurosci 14: 2260–2271.

Dringen R. 2000. Metabolism and functions of glutathione in brain. Prog Neurobiol 6:649–671.

Fukami G, Hashimoto K, Koike K, Okamura N, Shimizu E, Iyo M. 2004. Effect of antioxidant N-acetyl-L-cysteine on behavioral changes and neurotoxicity in rats after administration of methamphetamine. Brain Res 1016:90–95.

Goldman D, Oroszi G, Ducci F. 2005. The genetics of addictions: Uncovering the genes. Nat Rev Genet 6:521–532.

Hashimoto K. 2007. New research on methamphetamine abuse. In: Toolaney GH, editor. New research on methamphetamine abuse. New York: Nova Science Publishers, Inc. pp 1–51.

Hashimoto K, Tsukada H, Nishiyama S, Fukumoto D, Kakiuchi T, Shimizu E, Iyo M. 2004. Protective effects of N-acetyl-L-cysteine on the reduction of dopamine transporters in the striatum of monkeys treated with methamphetamine. Neuropsychopharmacol 29:2018–2023.

Hashimoto T, Hashimoto K, Matsuzawa D, Shimizu E, Sekine Y, Inada T, Ozaki N, Iwata N, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Iyo M. 2005. A functional glutathione S-transferase P1 gene polymorphism is associated with methamphetamine-induced psychosis in Japanese population. Am J Med Genet Part B 135B:5–9.

Hayes JD, Strange RC. 2000. Glutathione S-transferase polymorphisms and their biological consequences. Pharmacology 3:154–166.

Hayes JD, Flanagan JU, Jowsey IR. 2005. Glutathione transferases. Annu Rev Pharmacol Toxicol 45:51–88.

Hu YJ, Diamond AM. 2003. Role of glutathione peroxidase 1 in breast cancer: Loss of heterozygosity and allelic differences in the response to selenium. Cancer Res 12:3347–3351.

Huang CS, Chang LS, Anderson ME, Meister A. 1993. Catalytic and regulatory properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. J Biol Chem 26:19675–19680.

Kendler KS, Karkowski LM, Neale MC, Prescott CA. 2000. Illicit psychoactive substance use, heavy use, abuse, and dependence in a US population-based sample of male twins. Arch Gen Psychiatry 57:261–269.

Koizumi H, Hashimoto K, Kumakiri C, Shimizu E, Sekine Y, Ozaki N, Inada T, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Takei N, Iyo M. 2004. Association between the glutathione S-transferase M1 gene deletion and female methamphetamine abusers. Am J Med Genet Part B 126B:43–45.

Kosten TR, Markou A, Koob GF. 1998. Depression and stimulant dependence: Neurobiology and pharmacotherapy. J Nerv Ment Dis 186:737–745.

Li YJ, Scott WK, Zhang L, Lin PI, Oliveira SA, Skelly T, Doraiswamy MP, Welsh-Bohmer KA, Martin ER, Haines JL, Pericak-Vance MA, Vance JM. 2006. Revealing the role of glutathione S-transferase omega in age-at-onset of Alzheimer and Parkinson diseases. Neurobiol Aging 27:1087–1093.

Mannervik B. 1985. The isoenzymes of glutathione transferase. Adv Enzymol Relat Areas Mol Biol 57:357–417.

Matsuzawa D, Hashimoto K, Miyatake R, Shirayama Y, Shimizu E, Maeda K, Suzuki Y, Mashimo Y, Sekine Y, Inada T, Ozaki N, Iwata N, Harano M, Komiyama T, Yamada M, Sora I, Ujike H, Hata A, Sawa A, Iyo M. 2007. Identification of functional polymorphisms in the promoter region of the human PICK1 gene and their association with methamphetamine psychosis. Am J Psychiatry 164:1105–1114.

McIlwain CC, Townsend DM, Tew KD. 2006. Glutathione S-transferase polymorphisms: Cancer incidence and therapy. Oncogene 25:1639–1648.

McLellan RA, Oscarson M, Alexandrie AK, Seidegard J, Evans DA, Rannug A, Ingelman-Sundberg M. 1997. Characterization of a human glutathione S-transferase mu cluster containing a duplicated GSTM1 gene that causes ultrarapid enzyme activity. Mol Pharmacol 52:958–965.

Merikangas KR, Stolar M, Stevens DE, Goulet J, Preisig MA, Fenton B, Zhang H, O'Malley SS, Rounsaville BJ. 1998. Familial transmission of substance use disorders. Arch Gen Psychiatry 55:973–979.

Mirecki A, Fitzmaurice P, Ang L, Kalasinsky KS, Peretti FJ, Aiken SS, Wickham DJ, Sherwin A, Nobrega JN, Forman HJ, Kish SJ. 2004. Brain antioxidant systems in human methamphetamine users. J Neurochem 89:1396–1408.

Miyazaki I, Asanuma M, Diaz-Corrales FJ, Fukuda M, Kitaichi K, Miyoshi K, Ogawa N. 2006. Methamphetamine-induced dopaminergic neurotoxicity is regulated by quinone-formation-related molecules. FASEB J 20:571–573.

Nakamura S, Kugiyama K, Sugiyama S, Miyamoto S, Koide S, Fukushima H, Honda O, Yoshimura M, Ogawa H. 2002. Polymorphism in the 5′-flanking region of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. Circulation 105:2968–2973.

Nishimura M, Naito S. 2006. Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes. Drug Metab Pharmacokinet 21: 357–374.

Pemble S, Schroeder KR, Spencer SR, Meyer DJ, Hallier E, Bolt HM, Ketterer B, Taylor JB. 1994. Human glutathione S-transferase theta (GSTT1): cDNA cloning and the characterization of a genetic polymorphism. Biochem J 300:271–276.

Purcell S, Cherny SS, Sham PC. 2003. Genetic power calculator: Design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19:149–150.

Sato M, Chen CC, Akiyama K, Otsuki S. 1983. Acute exacerbation of paranoid psychotic state after long-term abstinence in patients with previous methamphetamine psychosis. Biol Psychiatry 18:429–440.

Sato M, Numachi Y, Hamamura T. 1992. Relapse of paranoid psychotic state in methamphetamine model of schizophrenia. Schizophr Bull 18: 115–122.

Schulz JB, Lindenau J, Seyfied J, Dichgans J. 2000. Glutathione, oxidative stress and neurodegeneration. Eur J Biochem 16:4904–4911.

Schweizer U, Brauer AU, Kohrle J, Nitsch R, Savaskan NE. 2004. Selenium and brain function: A poorly recognized liaison. Brain Res Brain Res Rev 3:164–178.

Smythies J, Galzigna L. 1998. The oxidative metabolism of catecholamines in the brain: A review. Biochim Biophys Acta 1380:159–162.

Sprenger R, Schlagenhaufer R, Kerb R, Bruhn C, Brockmoller J, Roots I, Brinkmann U. 2000. Characterization of the glutathione S-transferase GSTT1 deletion: Discrimination of all genotypes by polymerase chain reaction indicates a trimodular genotype-phenotype correlation. Pharmacogenetics 10:557–565.

Sreelekha TT, Ramadas K, Pandey M, Thomas G, Nalinakumari KR, Pillai MR. 2001. Genetic polymorphism of CYP1A1, GSTM1 and GSTT1 genes in Indian oral cancer. Oral Oncol 37:593–598.

Strange RC, Jones PW, Fryer AA. 2000. Glutathione S-transferase: Genetics and role in toxicology. Toxicol Lett 112–113:357–363.

Thomas JP, Maiorino M, Ursini F, Girotti AW. 1990. Protective action of phospholipid hydroperoxide glutathione peroxidase against membrane-damaging lipid peroxidation. In situ reduction of phospholipid and cholesterol hydroperoxides. J Biol Chem 265:454–461.

Tosic M, Ott J, Barral S, Bovet P, Deppen P, Gheorghita F, Matthey ML, Parnas J, Preisig M, Saraga M, Solida A, Timm S, Wang AG, Werge T, Cuénod M, Do KQ. 2006. Schizophrenia and oxidative stress: Glutamate cysteine ligase modifier as a susceptibility gene. Am J Hum Genet 79:586–592.

Uhl GR, Liu QR, Naiman D. 2002. Substance abuse vulnerability loci: Converging genome scanning data. Trends Genet 18:420–425.

Ujike H, Sato M. 2004. Clinical features of sensitization to methamphetamine observed in patients with methamphetamine dependence and psychosis. Ann NY Acad Sci 1025:279–287.

Ujike H, Harano M, Inada T, Yamada M, Komiyama T, Sekine Y, Sora I, Iyo M, Katsu T, Nomura A, Nakata K, Ozaki N. 2003. Nine- or fewer repeat alleles in VNTR polymorphism of the dopamine transporter gene is a strong risk factor for prolonged methamphetamine psychosis. Pharmacogenomics J 3:242–247.

Watson MA, Stewart RK, Smith GB, Massey TE, Bell DA. 1998. Human glutathione S-transferase P1 polymorphisms: Relationship to lung tissue enzyme activity and population frequency distribution. Carcinogenesis 19:275–280.

Whitehead RE, Ferrer JV, Javitch JA, Justice JB. 2001. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. J Neurochem 76:1242–1251.

Yoshimura K, Hanaoka T, Ohnami S, Ohnami S, Kohno T, Liu Y, Yoshida T, Sakamoto H, Tsugane S. 2003. Allele frequencies of single nucleotide polymorphisms (SNPs) in 40 candidate genes for gene-environment studies on cancer: Data from population-based Japanese random samples. J Hum Genet 48:654–658.

Phencyclidine-Induced Cognitive Deficits in Mice Are Improved by Subsequent Subchronic Administration of the Novel Selective α7 Nicotinic Receptor Agonist SSR180711

Kenji Hashimoto, Tamaki Ishima, Yuko Fujita, Masaaki Matsuo, Tatsuhiro Kobashi, Makoto Takahagi, Hideo Tsukada, and Masaomi Iyo

Background: Accumulating evidence suggests that α7 nicotinic receptor (α7 nAChR) agonists could be potential therapeutic drugs for cognitive deficits in schizophrenia. The present study was undertaken to examine the effects of the novel selective α7 nAChR agonist SSR180711 on cognitive deficits in mice after repeated administration of the N-methyl-D-aspartate receptor antagonist phencyclidine (PCP).

Methods: Saline or PCP (10 mg/kg/day for 10 days) was administered to mice. Subsequently, vehicle, SSR180711 (.3 or 3.0 mg/kg/day), SSR180711 (3.0 mg/kg/day) + the selective α7 nAChR antagonist methyllycaconitine (MLA; 3.0 mg/kg/day), or MLA (3.0 mg/kg/day) was administered IP for 2 consecutive weeks. Twenty-four hours after the final administration, a novel object recognition test was performed.

Results: The PCP-induced cognitive deficits were significantly improved by subsequent subchronic (2-week) administration of SSR180711 (3.0 mg/kg). The effects of SSR180711 (3.0 mg/kg) were significantly antagonized by co-administration of MLA (3.0 mg/kg). Furthermore, Western blot analysis and immunohistochemistry revealed that levels of α7 nAChRs in the frontal cortex and hippocampus of the PCP (10 mg/kg/day for 10 days)-treated mice were significantly lower than those of saline-treated mice.

Conclusions: These findings suggest that repeated PCP administration significantly decreased the density of α7 nAChRs in the brain and that the α7 nAChR agonist SSR180711 could ameliorate cognitive deficits in mice after repeated administration of PCP. Therefore, α7 nAChR agonists including SSR180711 are potential therapeutic drugs for treating cognitive deficits in schizophrenic patients.

Key Words: α7 Nicotinic receptors, cognition, NMDA receptor, object recognition test, schizophrenia, SSR180711

Cognitive deficits in patients with schizophrenia are a core feature of the illness, which predicts vocational and social disabilities in patients (1–5). Accumulating evidence suggests that a dysfunction in the glutamatergic neurotransmission via the N-methyl-D-aspartate (NMDA) receptors might be involved in the pathophysiology of schizophrenia (3,6–11). The NMDA receptor antagonists such as phencyclidine (PCP) and ketamine are known to induce schizophrenia-like symptoms including cognitive deficits in healthy subjects (6,12). Therefore, PCP has been used as an animal model of cognitive deficits in schizophrenia (13–16). We recently found that PCP-induced cognitive deficits in the novel object recognition test (NORT) could be significantly improved by subsequent subchronic (2-week) administration of clozapine but not haloperidol (14). These findings suggest that reversal of PCP-induced cognitive deficits as measured by the NORT might be a potential animal model of atypical antipsychotic activity related to the amelioration of cognitive deficits in schizophrenia (14–16).

Several lines of evidence suggest that α7 nicotinic receptors (α7 nAChRs) might play a role in the pathophysiology of schizophrenia and that α7 nAChR agonists might have therapeutic potential in the treatment of cognitive deficits in patients with schizophrenia (17–26). It has been reported that ARR17779, a full agonist at α7 nAChRs, significantly improved learning and memory as well as social recognition in rats (27,28) although one report did not find any effects of ARR17779 in the five-choice task (29). Furthermore, it has been reported that the administration of tropisetron or 3-(2,4)-dimethoxybenzylidine anabaseine (DMXB), partial agonists at α7 nAChRs, improved the deficient inhibitory processing of the P20-N40 auditory evoked potential in DBA/2 mice (30,31). Recently, these two drugs have been demonstrated to improve the P50 auditory gating deficits in schizophrenic patients (32,33). Thus, it is likely that α7 nAChR agonists such as tropisetron and DMXB could be therapeutic for cognitive deficits in schizophrenia.

SSR180711, 4-bromophenyl 1,4-diazabicyclo[3.2.2]nonane-4-carboxylate hydrochloride, is a novel selective partial agonist at α7 nAChRs with a high affinity (Ki = 50 nmol/L for rat α7 nAChRs, Ki = 78 nmol/L for human α7 nAChRs) (25,34,35). Furthermore, it has been reported that SSR180711 restores the selective attention deficits induced by PCP administration at the neonatal stage and the spatial working memory deficit induced by dizocilpine (35). These findings suggest that SSR180711 has the potential to improve cognitive deficits in schizophrenic patients (25,34,35).

In the present study, with the NORT, we examined the effects of subsequent acute or subchronic (2-week) treatment with SSR180711 on cognitive deficits in mice after repeated administration of PCP. Furthermore, we studied whether repeated PCP treatment alters the density of α7 nAChRs in the mouse brain.

Methods and Materials

Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The mice were housed in clear polycarbonate cages (22.5 × 33.8 × 14.0 cm) and in groups of 5 or 6 mice under a controlled 12-hour/

From the Division of Clinical Neuroscience (KH, TI, YF), Chiba University Center for Forensic Mental Health, Chiba; Department of Psychiatry (MI), Chiba University Graduate School of Medicine, Chiba; Nard Institute (MM, TK, MT), Amagasaki, Hyogo; and the PET Center (HT), Central Research Laboratory, Hamamatsu Photonics K.K., Hamamatsu, Shizuoka, Japan.

Address reprint requests to Kenji Hashimoto, Ph.D., Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, 1-8-1 Inohana, Chiba 260-8670, Japan; E-mail: hashimoto@faculty.chiba-u.jp.

Received December 5, 2006; revised April 12, 2007; accepted April 24, 2007.

0006-3223/08/$34.00
doi:10.1016/j.biopsych.2007.04.034

BIOL PSYCHIATRY 2008;63:92–97

12-hour light–dark cycle (light from 7:00 AM to 7:00 PM), with room temperature at 23 ± 1°C and humidity at 55 ± 5%. The mice were given free access to water and food pellets. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

Drugs

The PCP hydrochloride was synthesized in our laboratory at Chiba University. The SSR180711 was synthesized at Nard Institute (Amagasaki, Hyogo, Japan). Methyllycaconitine citrate (MLA) was purchased from Sigma-Aldrich Corporation (St. Louis, Missouri). Other drugs were purchased from commercial sources.

Drug Administration

Saline (10 mL/kg) or PCP (10 mg/kg expressed as a hydrochloride salt) was administered subcutaneously (SC) for 10 days (once daily on days 1–5, 8–12), with no treatment on days 6, 7, 13, and 14. In the experiment involving acute treatment, 3 days after the final administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg; saline) or SSR180711 (.3 or 3.0 mg/kg) was administered intraperitoneally (IP). In the experiment involving subchronic (2-week) treatment, 3 days after the final administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg; saline), SSR180711 (.3 or 3.0 mg/kg), SSR180711 (3.0 mg/kg) + MLA (3.0 mg/kg), or MLA (3.0 mg/kg) was administered IP for 2 consecutive weeks (once daily on days 15–28). A 3-mg/kg dose of MLA was selected, because this dose was effective in the deficient inhibitory processing of the P20-N40 auditory evoked potential in DBA/2 mice (31) or in PCP-induced cognitive deficits (15).

NORT

In the experiment involving acute treatment, 1 hour after the final administration of vehicle or SSR180711, NORT was performed as previously reported (14–16,36). In the experiment involving subchronic (2-week) treatment, a training session for NORT was performed 24 hours after the final administration of vehicle, SSR180711 (.3 or 3.0 mg/kg), SSR180711 (3.0 mg/kg) + MLA (3.0 mg/kg), or MLA (3.0 mg/kg). The apparatus for this task consisted of a black open field box (50.8 × 50.8 × 25.4 cm). Before the test, mice were habituated in the box for 3 days. During the training session, two objects (various objects differing in their shape and color but similar in size were used) were placed in the box 35.5 cm apart (symmetrically), and each animal was allowed to explore in the box for 10 min (5 min × 2). The animals were considered to be exploring the object when the head of the animal was facing the object within an inch of the object or when any part of the body, except for the tail, was touching the object. The time that the mice spent exploring each object was recorded. After the training, the mice were immediately returned to their home cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention test sessions were carried out at 1-day intervals after the initial training. During the retention test sessions, each mouse was placed back in the same box, but one of the objects in the box used during the initial training was replaced with a novel one. The mice were then allowed to freely explore for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity. 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) to the total time spent exploring both objects, was used to measure memory performance.

Western Blotting

Saline (10 mL/kg/day) or PCP (10 mg/kg/day) was administered SC for 10 days (once daily on days 1–5, 8–12). First, 3 days after the final administration of saline or PCP (10 mg/kg/day for 10 days; i.e., on day 15), the mice were killed by decapitation. Second, 3 days after the last administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg/day; saline) or SSR180711 (3.0 mg/kg/day) was administered IP into mice for 2 consecutive weeks (once daily on days 15–28). Twenty-four hours after the last administration of vehicle or SSR180711, the mice were killed by decapitation. Then, the frontal cortex and hippocampus were dissected on ice and stored at −80°C.

Briefly, brain tissue was homogenized in 10 vol of 5 mmol/L Tris/hydrochloric acid (HCl) (pH 7.4) containing .32 mol/L sucrose and centrifuged for 10 min at 1,000 × *g*. The resulting supernatant was recentrifuged for 10 min at 40,000 × *g* to obtain the crude membrane fraction. The pellet was washed twice in buffer and resuspended. Aliquots (frontal cortex: 25 μg protein, hippocampus: 40 μg protein) of the membranes were incubated for 5 min at 95°C with an equal volume of 125 mmol/L Tris/HCl, pH 6.8, 20% glycerol, .002% bromphenol blue, 10% β-mercaptoethanol, and 4% sodium dodecylsulfate (SDS), and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) with 10% mini-gels (Mini Protean II; Bio-Rad, Hercules, California). Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes with a Trans Blot Mini Cell (Bio-Rad). For immunodetection, the blots were blocked for 1–2 hours in Tris-buffered saline-Tween (TBST) (50 mmol/L Tris/HCl, pH 7.8, .13 mol/L sodium chloride, .1% Tween 20) containing 5% nonfat dry milk at room temperature, followed by incubation with rabbit anti-α7 nAChR antibody (1:2,000, Cat. No: AB5637, Chemicon International, Temecula, California) overnight at 4°C in TBST/5% blocker. The blots were washed five times with TBST. Incubation with the secondary antibody (GE Healthcare Bioscience, Buckinghamshire, United Kingdom) was performed for 1 hour at room temperature. After extensive washing, immunoreactivity was detected by enhanced chemiluminescence (ECL) plus the Western Blotting Detection system (GE Healthcare Bioscience). Images were captured with a Fuji LAS3000-mini imaging system (Fujifilm, Tokyo, Japan), and immunoreactive bands were quantified. β-Actin immunoreactivity was used to monitor equal sample loading. The levels of α7 nAChRs in the PCP-treated mice were expressed as percentages of those of saline-treated mice (control animals).

Immunohistochemistry

Three days after the final administration of saline (10 mL/kg/day for 10 days; days 1–5 and days 8–12) or PCP (10 mg/kg/day for 10 days; days 1–5 and days 8–12) (i.e., on day 15), the mice were killed with sodium pentobarbital and perfused transcardially with 10 mL of isotonic saline, followed by 40 mL of ice-cold 4% paraformaldehyde in .1 mol/L phosphate buffer (pH 7.4). The brains were removed from the skulls and postfixed overnight at 4°C in the same fixative. For the immunohistochemical analysis, 50-μm-thick serial sagittal sections of brain were cut in ice-cold .01 mol/L phosphate buffer saline (PBS; pH 7.5) with a vibrating blade microtome (VT1000S, Leica Microsystems AG, Wetzlar, Germany). Free-floating sections were treated with .3% hydrogen peroxide (H_2O_2) in .05 mol/L Tris-HCl saline (TBS) for 30 min and blocked in TBS containing .2 % Triton X-100 (TBST) and 1.5% normal serum for 1 hour at room temperature. The samples were then incubated for 36 hours at 4°C with rabbit anti-α7 nAChR antibody (1:10,000, Cat. No: AB5637, Chemicon International). The sections were washed twice in TBST and processed according to the avidin-biotin-peroxidase method (Vectastain Elite ABC, Vector Laboratories, Burlingame,

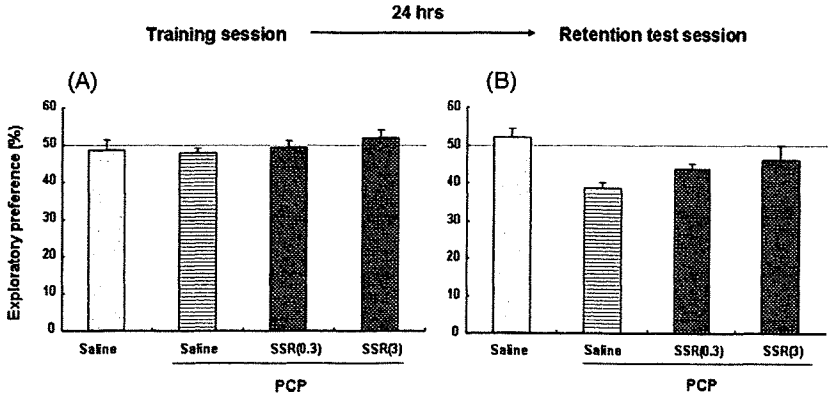

Figure 1. Effects of acute administration of 4-bromophenyl 1,4-diazabicyclo[3.2.2]nonane-4-carboxylate hydrochloride (SSR180711) on phencyclidine (PCP)-induced cognitive deficits in mice. Saline (10 mL/kg) or PCP (10 mg/kg) was administered SC for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg; saline) or SSR180711 (3 mg/kg) was administered IP to the mice. The novel object recognition test (NORT) was performed 1 hour after the administration. Values are the means ± SEM (n = 7–13). $*p < .05$ as compared with the saline-treated group (control).

California). The sections were then reacted for 5 min in a solution of .15 mg/mL diaminobenzidine containing .01% H_2O_2. Alternate sections, incubated in the absence of primary antibody as an immunohistochemical control, showed no immunostaining. The sections were mounted on gelatinized slides, dehydrated, cleared, and coverslipped under Entellan New (Merck KGaA, Darmstadt, Germany). The sections were imaged, and the intensity of α7 nAChR-immunoreactivity in the frontal cortex and hippocampus was analyzed by using a light microscope equipped with a CCD camera (Olympus IX70, Olympus Corporation, Tokyo, Japan) and the SCION IMAGE software package (Scion Corporation, Frederick, Maryland). The α7 nAChR immunoreactivity was quantified in the regions of the frontal cortex and hippocampus in a blinded manner. The levels of α7 nAChRs in the PCP-treated mice were expressed as percentages of those of saline-treated mice (control animals).

Determination of PCP and SSR180711 in the Mouse Brain

In the experiment involving an acute single treatment, mice were killed by decapitation 1 hour after a single administration of vehicle (10 mL/kg, IP) or SSR180711 (3 mg/kg, IP). In the experiment involving subchronic (2-week) treatment, saline (10 mL/kg/day, SC) or PCP (10 mg/kg/day/ SC) was administered for 10 days (once daily on days 1–5, 8–12), and no treatment was given on days 6, 7, 13, and 14. Three days after the final administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg/day; saline) or SSR180711 (3.0 mg/kg/day) was administered IP for 2 consecutive weeks (once daily on days 15–28). Twenty-four hours after the final administration of vehicle or SSR180711, the mice were killed by decapitation. Then brains were dissected on ice, and the frontal cortex and hippocampus were weighed.

Briefly, the brain tissues were homogenized in 20 volumes of methanol high-performance liquid chromatography (HPLC grade) on ice. The homogenates were centrifuged at 4,500 g for 10 min, and 20 μl of supernatant was injected into the HPLC system with UV detector (Shimadzu, Kyoto, Japan). A column (4.6 × 250 mm; TSKgel ODS-80Ts, Tosoh Corporation, Tokyo, Japan) was used. The UV detector was set at 244 nm. The mobile phases for the determination of PCP and SSR180711 were acetonitrile: 100 mmol/L sodium acetate (30: 70, vol/vol) containing .1% triethylamine (final pH 5.6) and acetonitrile: 30 mmol/L ammonium acetate: acetic acid (HPLC grade) (300: 700: 2, vol/vol), respectively. The flow rate was 1.0 mL/min.

Statistical Analysis

Data were expressed as mean ± SEM. Statistical analysis was performed by using the Student t test or one-way analysis of variance (ANOVA) and the post hoc Bonferroni test. P values < .05 were considered statistically significant.

Results

Effects of SSR180711 on PCP-Induced Cognitive Deficits in Mice

In the NORT, repeated administration of PCP (10 mg/kg/day for 10 days) caused significant cognitive deficits in the mice, a result that is consistent with previous reports (14–16). During the training session, there were no significant differences among the four groups in the total amount of time spent exploring the two objects nor in the exploratory preference (Figure 1A). In the retention session, the exploratory preference (approximately 40%) of the PCP-treated group was significantly lower than that (approximately 50%) of the saline-treated group, suggesting that the behavior of the PCP-treated mice might not have been a result of memory impairment (14). Therefore, it is likely that our model of PCP-induced cognitive deficits with NORT might show negative symptoms, such as social withdrawal, that are related to cognitive deficits (14). In the retention session, a single administration of SSR180711 (.3 or 3.0 mg/kg, 1 hour) did not alter the

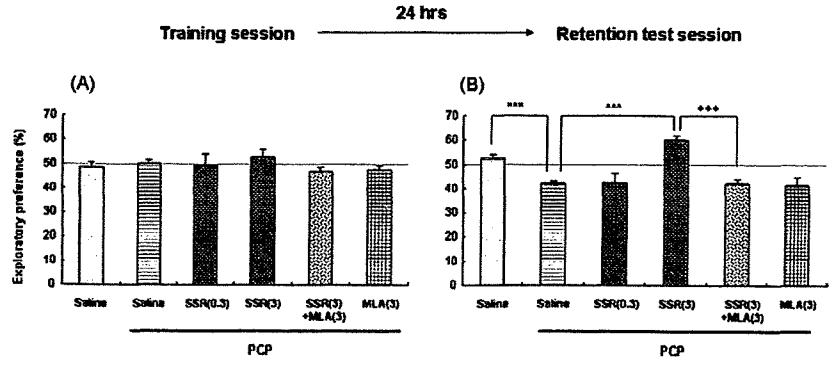

Figure 2. Effects of subchronic administration of SSR180711 on PCP-induced cognitive deficits in mice. Saline (10 mL/kg) or PCP (10 mg/kg) was administered SC for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg/day; saline), SSR180711 (.3 or 3.0 mg/kg/day), SSR180711 (3.0 mg/kg/day) + methyllycaconitine citrate (MLA) (3.0 mg/kg/day), or MLA (3.0 mg/kg/day) was administered IP to the mice for 2 consecutive weeks (once daily on days 15–28). On days 29 and 30, the NORT was performed. Values are means ± SEM (n = 7–24). $***p < .001$ as compared with the PCP + saline-treated group. $^{+++}p < .001$ as compared with the PCP + SSR180711 (3.0 mg/kg)-treated group. Other abbreviations as in Figure 1.

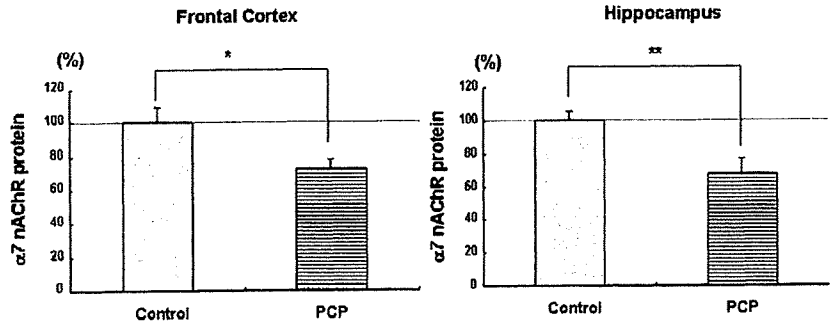

Figure 3. Effects of repeated administration of PCP on α7 nicotinic receptor (α7 nAChR) protein in the mouse brain. Saline (10 mL/kg/day) or PCP (10 mg/kg/day) was administered SC for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (i.e., on day 15), the mice were killed by decapitation. Western blot analysis with α7 nAChR antibody was performed as described in Methods. The levels of α7 nAChRs in the PCP-treated mice were expressed as percentages of those of the saline-treated mice. Values are the means ± SEM ($n = 8$). $*p < .05$, $**p < .01$ as compared with the saline-treated group (control). Other abbreviations as in Figure 1.

reduction of the exploratory preference in mice after repeated administration of PCP (Figure 1B).

In contrast, PCP-induced cognitive deficits were significantly improved after subsequent subchronic (2-week) administration of the higher dose (3.0 mg/kg/day) but not of the lower dose (.3 mg/kg/day) of SSR180711. In the training session, the exploratory preferences of the six groups were not significantly different [$F(5,74) = .762$, $p = .580$] (Figure 2A). However, in the retention session, ANOVA analysis revealed that the exploratory preferences of the six groups were significantly different [$F(5,74) = 12.11$, $p < .001$] (Figure 2B). A post hoc Bonferroni test indicated that the exploratory preference of the PCP-treated group was significantly ($p < .001$) increased after subchronic (2-week) administration of SSR180711 (3.0 mg/kg/day) but not SSR180711 (.3 mg/kg/day) (Figure 2B). Furthermore, the effect of SSR180711 (3.0 mg/kg) on the PCP-induced cognitive deficits was significantly ($p < .001$) antagonized by the co-administration of MLA (3 mg/kg/day) (Figure 2B). Moreover, subchronic (2-week) administration of MLA (3.0 mg/kg/day) alone did not alter PCP-induced cognitive deficits in mice (Figure 2B).

Effects of Repeated PCP Administration on the Levels of α7 nAChRs in the Mouse Brain

Western blot analysis revealed that the levels of α7 nAChRs in the frontal cortex ($t = 2.589$, $p = .021$) and hippocampus ($t = 3.024$, $p = .009$) of the PCP-treated (10 mg/kg/day for 10 days) mice were significantly lower than those of saline-treated mice (Figure 3). Furthermore, immunohistochemistry revealed that the immunoreactivity of α7 nAChRs in the frontal cortex ($t = 3.182$, $p = .005$) and hippocampus ($t = 5.820$, $p < .001$) of mice treated with PCP (10 mg/kg/day 10 days) was significantly lower than that of the saline-treated group (Figure 4).

Next, we examined whether subsequent subchronic (2-week) administration of SSR180711 (3 mg/kg/day) alters the reduction of α7 nAChRs in the mouse brain after repeated PCP administration. In this study, we found that subchronic administration of SSR180711 (3 mg/kg/day) did not alter the reduction of α7 nAChRs in the frontal cortex and hippocampus after repeated PCP administration (Figure 5). These findings suggest that repeated PCP administration caused a long-term reduction of α7 nAChRs in the mouse brain (more than 2 weeks after the final administration of PCP).

HPLC Determination of PCP and SSR180711 Levels in the Mouse Brain

To ascertain whether the behavioral effects of SSR180711 are due to the stimulation at α7 nAChRs in the mouse brain or due to another effect (residual PCP) in the mouse brain, we measured the PCP levels in the mouse brain at the time of the behavioral NORT test. In this study, we did not detect the compound PCP in the mouse brain 24 hours after the subsequent subchronic (2-week) administration of SSR180711 (3 mg/kg/day). Therefore, it is likely that the behavioral effect of SSR180711 on PCP-induced cognitive deficits might be due to the stimulation at α7 nAChRs in the mouse brain but not to residual PCP in the mouse brain.

Next, we measured the levels of SSR180711 in the mouse brain at the time of the behavioral NORT test. We could detect the compound SSR180711 (SSR180711-treated group: 33.04 ± 10.97 ng/mg tissue [$n = 6$]; vehicle-treated group: not detected) in the mouse brain at 1 hour after a single acute administration of SSR180711 (3 mg/kg). However, we did not detect the compound SSR180711 in the mouse brain at 24 hours after the subchronic (2-week) administration of SSR180711 (3 mg/kg/day). These findings suggest that the behavioral effect of SSR180711 on PCP-induced cognitive deficits might be due to the subchronic stimulation at α7 nAChRs in the mouse brain but not to residual SSR180711 in the mouse brain.

Discussion

The major findings of the present study are that repeated PCP administration significantly decreased the density of α7 nAChRs in the mouse brain and that PCP-induced cognitive deficits could be improved by subsequent subchronic (2-week) administration of the

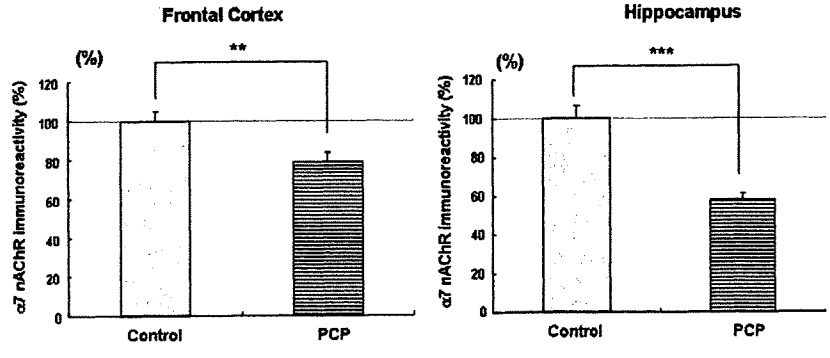

Figure 4. Effects of repeated administration of PCP on the distribution of α7 nAChR immunoreactivity in the mouse brain. Saline (10 mL/kg/day) or PCP (10 mg/kg/day) was administered SC for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (i.e., on day 15), the mice were perfused. Immunohistochemistry was performed as described in Methods. Values are the means ± SEM ($n = 11$). $**p < .01$, $***p < .001$ as compared with the saline-treated group (control). Other abbreviations as in Figure 1.

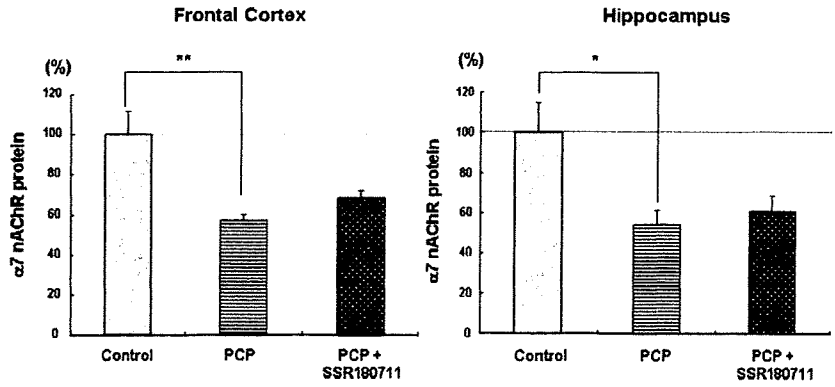

Figure 5. Effects of subsequent subchronic administration of SSR180711 on the reduction of α7 nAChR immunoreactivity in the mouse brain after the repeated administration of PCP. Saline (10 mL/kg/day) or PCP (10 mg/kg/day) was administered SC for 10 days (once daily on days 1–5, 8–12). Three days after the last administration of saline or PCP (i.e., on day 15), vehicle (10 mL/kg/day; saline) or SSR180711 (3.0 mg/kg/day) was administered IP to the mice for 2 consecutive weeks (once daily on days 15–28). Twenty-four hours after the last administration of vehicle or SSR180711, the mice were killed by decapitation. Western blot analysis with α7 nAChR antibody was performed as described in Methods. The levels of α7 nAChRs in the PCP-treated mice were expressed as percentages of those of the saline-treated mice. Values are the means ± SEM ($n = 8$). $*p < .05$, $**p < .01$ as compared with the saline-treated group (control). Other abbreviations as in Figure 1.

novel selective α7 nAChR agonist SSR180711. We reported recently that, in the NORT, PCP-induced cognitive deficits could be improved by subsequent subchronic (2-week) administration of clozapine but not of haloperidol, suggesting that the reversal of PCP-induced cognitive deficits with the NORT might be a potential animal model of atypical antipsychotic activity related to the amelioration of cognitive deficits in schizophrenia (14). Very recently, Pichat *et al.* (35) reported that SSR180711 significantly reversed dizocilpine-induced deficits in the retention of episodic memory in rats (object recognition test). SSR180711 was co-administered with dizocilpine (.1 mg/kg, IP) in that study (35), whereas SSR180711 was administered after repeated PCP administration in the present study. Taken together, these findings suggest that SSR180711 could ameliorate cognitive deficits after the administration of NMDA receptor antagonists (PCP and dizocilpine), although the treatment schedules differed between the two studies. Nonetheless, it is likely that SSR180711 can be used as a therapeutic drug in the treatment of cognitive deficits in schizophrenia.

In the present study, we found that repeated PCP administration caused the reduction of α7 nAChRs in the mouse brain. This is the first report demonstrating that the administration of PCP significantly decreased the density of α7 nAChRs in the brain. The precise mechanism(s) underlying how repeated PCP administration could modulate α7 nAChRs in the brain are currently unknown. A postmortem human brain study demonstrated decreased expression of hippocampal α7 nAChRs in schizophrenic patients (37), suggesting that schizophrenic patients have fewer α7 nAChRs in the hippocampus, a condition that might lead to the failure of cholinergic activation of the inhibitory interneurons, manifesting clinically as decreased gating of responses to sensory stimulation (18,37). Furthermore, it has been reported that the immunoreactivity of α7 nAChRs in the prefrontal cortex of schizophrenia was significantly decreased as compared with normal control subjects (38). Interestingly, α7 nAChR agonists can increase the release of glutamate from the presynaptic terminals, resulting in stimulation of the NMDA receptors on the postsynaptic neurons, suggesting that stimulation at α7 nAChRs might potentiate the NMDA receptors (11,25). Taken together, these findings suggest that α7 nAChRs might interact with the NMDA receptors in the brain (11,25), although further study on the cross-talk between α7 nAChRs and NMDA receptors in the brain is necessary (39).

Deficient inhibitory processing of the P50 auditory evoked potential is a pathophysiological feature of schizophrenia (17,18,40). Several lines of evidence suggest that α7 nAChRs play a critical role in this phenomenon (17–19,22). Similar to schizophrenic patients, DBA/2 mice (decreased levels of α7 nAChRs) spontaneously exhibit a deficit in the inhibitory processing of the P20-N40 auditory evoked potential, which is thought to be a rodent analogue of the human P50 auditory evoked potential (41). We reported that the administration of tropisetron, a partial agonist at α7 nAChRs, improved the deficient inhibitory processing of the P20-N40 auditory evoked potential in DBA/2 mice and that the co-administration of MLA (3 mg/kg) blocked the normalizing effect of tropisetron, suggesting that α7 nAChRs play a role in the mechanism of action of tropisetron (15). In addition, we also reported that tropisetron improved the deficits of P50 suppression in schizophrenic patients (32). Recently, we also reported that, via α7 nAChRs, tropisetron could improve PCP-induced cognitive deficits in mice (31). Furthermore, it has been reported that the selective α7 nAChR agonist PNU-282987 restores auditory gating deficits in rats after the administration of amphetamine (42) and that the selective α7 nAChR agonists, (R)-3'(5-chlorothiophen-2-yl)spiro-1-azabicyclo[2,2,2]octane-3,5'-[1',3']oxazolidin-2'-one (25,43) and W-56203, (R)-3'-(3-methylbenzo[b]thiophen-5-yl)spiro[1-azabicyclo[2.2.2]octane-3,5'-oxazolidin]-2'-one (25,44), significantly improved dizocilpine-induced auditory gating deficits in rats. Therefore, it might be of interest to study the P20-N40 auditory evoked potential in mice with the repeated PCP administration, because repeated PCP administration decreased the density of α7 nAChRs in the mouse brain.

In conclusion, the present findings suggest that repeated administration of PCP decreased the density of α7 nAChRs in the mouse brain and that SSR180711 could improve the cognitive deficits that occur in mice after the repeated administration of PCP. These findings suggest that α7 nAChR agonists such as SSR180711 could be used as potential therapeutic drugs in the treatment of cognitive deficits in schizophrenia.

This study was supported in part by grants from the Minister of Education, Culture, Sports, Science, and Technology of Japan (KH), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (KH), and the Research Program on Development of Innovative Technology, JST. Dr. Hashimoto reports receiving speaker fees from Solvay, Meiji Seika, Eli Lilly, Janssen, and Otsuka. Ms. Ishima, Ms. Fujita, Dr. Matsuo, Mr. Kobashi, Mr. Takahagi, and Dr. Tsukada report no competing interests. Dr. Iyo reports receiving consultant and speaker fees from Janssen, Meiji Seika., Dainippon-Sumitomo, GlaxoSmithKline, Novartis, Eli Lilly, Pfizer, and Otsuka.

1. Green M (1996): What are the functional consequences of neurocognitive deficits in schizophrenia? *Am J Psychiatry* 153:321–330.
2. Freedman R (2003): Schizophrenia. *N Engl J Med* 349:1738–1749.
3. Coyle JT, Tsai G (2004): The NMDA receptor glycine modulatory site: A therapeutic target for improving cognition and reducing negative symptoms in schizophrenia. *Psychopharmacology (Berl)* 174:32–38.

4. Green MF, Nuechterlein KH, Gold JM, Barch DM, Cohen J, Essock S, *et al.* (2004): Approaching a consensus cognitive battery for clinical trials in schizophrenia: The NIMH-MATRICS conference to select cognitive domains and test criteria. *Biol Psychiatry* 56:301–307.
5. Kurtz MM (2005): Neurocognitive impairment across the lifespan in schizophrenia: An update. *Schizophrenia Res* 74:15–26.
6. Javitt DC, Zukin SR (1991): Recent advances in the phencyclidine model of schizophrenia. *Am J Psychiatry* 148:1301–1308.
7. Olney JW, Farber NB (1995): Glutamate receptor dysfunction and schizophrenia. *Arch Gen Psychiatry* 52:998–1007.
8. Krystal JH, D'Souza DC, Petrakis IL, Belger A, Berman RM, Charney DS, *et al.* (1999): NMDA agonists and antagonists as probes of glutamatergic dysfunction and pharmacotherapies in neuropsychiatric disorders. *Harv Rev Psychiatry* 7:125–143.
9. Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N, *et al.* (2003): Decreased serum levels of D-serine in patients with schizophrenia: Evidence in support of the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch Gen Psychiatry* 60: 572–576.
10. Hashimoto K, Okamura N, Shimizu E, Iyo M (2004): Glutamate hypothesis of schizophrenia and approach for possible therapeutic drugs. *Curr Med Chem - CNS Agents* 4:147–154.
11. Hashimoto K, Shimizu E, Iyo M (2005): Dysfunction of glia-neuron communication in pathophysiology of schizophrenia. *Curr Psychiatry Rev* 1:151–163.
12. Krystal JH, Perry EB Jr, Gueorguieva R, Belger A, Madonick SH, Abi-Dargham A, *et al.* (2005): Comparative and interactive human psychopharmacologic effects of ketamine and amphetamine: Implications for glutamatergic and dopaminergic model psychoses and cognitive function. *Arch Gen Psychiatry* 62:985–994.
13. Jentsch JD, Roth RH (1999): The neuropsychopharmacology of phencyclidine: From NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. *Neuropsychopharmacology* 20:201–225.
14. Hashimoto K, Fujita Y, Shimizu E, Iyo M (2005): Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of clozapine, but not haloperidol. *Eur J Pharmacol* 519:114–117.
15. Hashimoto K, Fujita Y, Ishima T, Hagiwara H, Iyo M (2006): Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of tropisetron: Role of α7 nicotinic receptors. *Eur J Pharmacol* 553:191–195.
16. Hashimoto K, Fujita Y, Iyo M (2006): Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of fluvoxamine: role of sigma-1 receptors. *Neuropsychopharmacology* 32:514–521.
17. Freedman R, Coon H, Myles-Worsley M, Orr-Urtreger A, Olincy A, Davis A, *et al.* (1997): Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. *Proc Natl Acad Sci U S A* 94:587–592.
18. Freedman R, Adams CE, Leonard S (2000): The α7-nicotinic acetylcholine receptor and the pathology of hippocampal interneurons in schizophrenia. *J Chem Neuroanat* 20:299–306.
19. Leonard S, Gault J, Hokins J, Logel J, Vianzon R, Short M, *et al.* (2002): Association of promoter variants in the α7 nicotinic acetylcholine receptor subunit gene with an inhibitory deficit found in schizophrenia. *Arch Gen Psychiatry* 59:1085–1096.
20. Picciotto MR, Caldarone BJ, Brunzell DH, Zachariou V, Stevens TR, King SL (2001): Neuronal nicotinic acetylcholine receptor subunit knockout mice: Physiological and behavioral phenotypes and possible clinical implications. *Pharmacol Ther* 92:89–108.
21. Simosky JK, Stevens KE, Freedman R (2002): Nicotinic agonists and psychosis. *Curr Drug Targets CNS Neurol Disord* 1:149–162.
22. De Luca V, Wong AH, Muller DJ, Wong GW, Tyndale RF, Kennedy JL (2004): Evidence of association between smoking and α7 nicotinic receptor subunit gene in schizophrenia patients. *Neuropsychopharmacology* 29:1522–1526.
23. Young JW, Finlayson K, Spratt C, Marston HM, Crawford N, Kelly JS, *et al.* (2004): Nicotine improves sustained attention in mice: Evidence for involvement of the α7 nicotinic acetylcholine receptor. *Neuropsychopharmacology* 29:891–900.
24. Martin LF, Kem WR, Freedman R (2004): α7 nicotinic receptor agonists: Potential new candidates for the treatment of schizophrenia. *Psychopharmacology (Berl)* 74:54–64.
25. Hashimoto K, Koike K, Shimizu E, Iyo M (2005): α7 Nicotinic receptor agonists as potential therapeutic drugs for schizophrenia. *Curr Med Chem - CNS Agents* 5:171–184.
26. Levin ED, McClernon FJ, Rezvani AH (2006): Nicotinic effects on cognitive function: Behavioral characterization, pharmacological specification, and anatomic localization. *Psychopharmacology (Berl)* 184:523–539.
27. Levin ED, Bettegowda C, Blosser J, Gordon J (1999): AR-R17779, and α7 nicotinic agonist, improves learning and memory in rats. *Behav Pharmacol* 10:675–680.
28. Van Kampen M, Selbach K, Schneider R, Schiegel E, Boess F, Schreiber R (2004): AR-R 17779 improves social recognition in rats by activation of nicotinic α7 receptors. *Psychopharmacology (Berl)* 172:375–383.
29. Grottick AJ, Higgins GA (2000): Effect of subtype selective nicotinic compounds on attention as assessed by the five-choice serial reaction time task. *Behav Brain Res* 117:197–208.
30. Simosky JK, Stevens KE, Kem WR, Freedman R (2001): Intragastric DMXB-A, an α7 nicotinic agonist, improves deficient sensory inhibition in DBA/2 mice. *Biol Psychiatry* 50:493–500.
31. Hashimoto K, Iyo M, Freedman R, Stevens KE (2005): Tropisetron improves deficient inhibitory auditory processing in DBA/2 mice: role of α7 nicotinic acetylcholine receptors. *Psychopharmacology (Berl)* 183:13–19.
32. Koike K, Hashimoto K, Takai N, Shimizu E, Komatsu N, Watanabe H, *et al.* (2005): Tropisetron improves deficits in auditory P50 suppression in schizophrenia. *Schizophrenia Res* 76:67–72.
33. Olincy A, Harris JG, Johnson LL, Pender V, Kongs S, Allensworth D, *et al.* (2006): Proof-of-concept trial of an α7 nicotinic agonist in schizophrenia. *Arch Gen Psychiatry* 63:630–638.
34. Bilton B, Bergis OE, Galli F, Nedelec A, Lochead AW, Jegham S, *et al.* (2007): SSR180711, a novel selective α7 nicotinic receptor partial agonist: (I) binding and functional profile. *Neuropsychopharmacology* 32:1–16.
35. Pichat P, Bergis OE, Terranova JP, Urani A, Duarte C, Santucci V, *et al.* (2007): SSR180711, a novel selective α7 nicotinic receptor partial agonist: (II) efficacy in experimental models predictive of activity against cognitive symptoms of schizophrenia. *Neuropsychopharmacology* 32:17–34.
36. Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006): Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: A neurodevelopmental animal model of schizophrenia. *Biol Psychiatry* 59:546–554.
37. Freedman R, Hall M, Adler LE, Leonard S (1995): Evidence in postmortem brain tissue for decreased numbers of hippocampal nicotinic receptors in schizophrenia. *Biol Psychiatry* 38:22–33.
38. Martin-Ruiz CM, Haroutunian VH, Long P, Young AH, Davis KL, Perry EK, *et al.* (2003): Dementia rating and nicotinic receptor expression in the prefrontal cortex in schizophrenia. *Biol Psychiatry* 54:1222–1233.
39. Hashimoto K, Hattori E (2007): Candidate genes and models: Chapter 6. Neurotransmission. In: Sawa A, McInnis M. editors. Neurogenetics of Psychiatric Disorders. New York: Informa Healthcare, 81–100.
40. Braff D, Geyer MA (1990): Sensorimotor gating and schizophrenia. Human and animal model studies. *Arch Gen Psychiatry* 47:181–188.
41. Stevens KE, Freedman R, Collins AC, Hall M, Leonard S, Marks MJ, *et al.* (1996): Genetic correlation of inhibitory gating of hippocampal auditory evoked response and α-bungarotoxin-binding nicotinic cholinergic receptors in inbred mouse strains. *Neuropsychopharmacology* 15:152–162.
42. Hajos M, Hurst RS, Hoffmann WE, Krause M, Wall TM, Higdon NR, *et al.* (2005): The selective α7 nicotinic acetylcholine receptor agonist PNU-282987 [N-[(3R)-1-Azabicyclo[2.2.2]oct-3-yl]-4-chlorobenzamide hydrochloride] enhances GABAergic synaptic activity in brain slices and restores auditory gating deficits in anesthetized rats. *J Pharmacol Exp Ther* 312:1213–1222.
43. Tatsumi R, Fujio M, Satoh H, Katayama J, Takanashi S, Hashimoto K, *et al.* (2005): Discovery of the α7 nicotinic acetylcholine receptor agonists. (R)-3'-(5-Chlorothiophen-2-yl)spiro-1-azabicyclo[2.2.2]octane-3,5'-[1',3'] oxazolidin-2'-one as a novel, potent, selective, and orally bioavailable ligand. *J Med Chem* 48:2678–2686.
44. Tatsumi R, Fujio M, Takanashi S, Numata A, Katayama J, Satoh H, *et al.* (2006): (R)-3'-(3-Methylbenzo[b]thiophen-5-yl)spiro[1-azabicyclo[2,2,2] octane-3,5'-oxazolidin]-2'-one, a novel and potent α7 nicotinic acetylcholine receptor partial agonist displays cognitive enhancing properties. *J Med Chem* 49:4374–4383.

Available online at www.sciencedirect.com
ScienceDirect
Progress in Neuro-Psychopharmacology & Biological Psychiatry 32 (2008) 336–339
Progress In Neuro-Psychopharmacology & Biological Psychiatry
www.elsevier.com/locate/pnpbp

Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of the antibiotic drug minocycline

Yuko Fujita [a], Tamaki Ishima [a], Shinsui Kunitachi [a], Hiroko Hagiwara [a,b], Lin Zhang [a,b], Masaomi Iyo [b], Kenji Hashimoto [a,*]

[a] *Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, 1-8-1 Inohana, Chiba 260-8670, Japan*
[b] *Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan*

Received 14 March 2007; received in revised form 22 August 2007; accepted 22 August 2007
Available online 29 August 2007

Abstract

Background: The *N*-methyl-D-aspartate (NMDA) receptor antagonist phencyclidine (PCP)-induced cognitive deficits have been used as an animal model for schizophrenia. This study was undertaken to determine whether the antibiotic drug minocycline could improve PCP-induced cognitive deficits in mice.
Methods: Saline (10 ml/kg/day, s.c., once daily on day 1–5, 8–12) or PCP (10 mg/kg/day, s.c., once daily on day 1–5, 8–12) were administered to mice for 10 days. Subsequently, vehicle (10 ml/kg/day, i.p.) or minocycline (4.0 or 40 mg/kg/day, i.p.) was injected for 14 consecutive days. One day after the final injection, a novel object recognition test was performed.
Results: PCP-induced cognitive deficits in mice were significantly improved by subsequent subchronic (14 days) administration of minocycline (40 mg/kg), but not minocycline (4.0 mg/kg).
Conclusions: This study suggests that minocycline could be a potential therapeutic drug for cognitive deficits in schizophrenic patients.

Keywords: Cognition; Minocycline; NMDA receptor; Novel object recognition; Schizophrenia

1. Introduction

Cognitive deficits in patients with schizophrenia are a core feature of the illness, which predicts vocational and social disabilities for patients (Green, 1996). Accumulating evidence suggests that *N*-methyl-D-aspartate (NMDA) receptor plays a role in the pathophysiology of schizophrenia (Javitt and Zukin, 1991; Hashimoto et al., 2004, 2005b). The NMDA receptor antagonists such as phencyclidine (PCP) are known to induce schizophrenia-like symptoms including cognitive deficits in healthy subjects (Javitt and Zukin, 1991). In the novel object recognition test (NORT), we found that PCP-induced cognitive deficits in mice could be significantly improved by subsequent subchronic (14 days) administration of clozapine, but not haloperidol (Hashimoto et al., 2005a). These findings suggest that the reversal of PCP-induced cognitive deficits as measured by the NORT may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto et al., 2005a, 2006, 2007a).

Minocyline is a semisynthetic second-generation tetracycline which has anti-inflammatory effects that appear to be completely separate and distinct from its anti-microbial activity. Accumulating evidence suggests that minocycline has potential therapeutic effects in several animal models of neurological diseases (Domercq and Matute, 2004; Yong et al., 2004; Thomas and Le, 2004; Stirling et al., 2005). In addition, we reported that minocycline could ameliorate the behavioral changes (e.g., acute hyperlocomotion and the development of

Abbreviations: ANOVA, One-way analysis of variance; NMDA, *N*-methyl-D-aspartate; NORT, Novel object recognition test; PCP, Phencyclidine.

* Corresponding author. Tel.: +81 423 226 2147; fax: +81 423 226 2150.
E-mail address: hashimoto@faculty.chiba-u.jp (K. Hashimoto).

0278-5846/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pnpbp.2007.08.031

behavioral sensitization) and neurotoxicity that occur in mice and monkeys due to the administration of methamphetamine or 3,4-methylenedioxymethamphetamine (Zhang et al., 2006a, 2006b; Hashimoto et al., 2007b). Furthermore, we found that the hyperlocomotion and prepulse inhibition deficits in mice that occur after the administration of the NMDA receptor antagonist dizocilpine were significantly attenuated by the administration of minocycline (Zhang et al.; 2007). These findings suggest that minocycline may be a potential therapeutic drug for neuropsychiatric disorders including schizophrenia. In the present study, using the NORT, we examined the effects of subsequent subchronic (14 days) treatment with minocycline on cognitive deficits in mice after repeated administration of PCP.

2. Methods

2.1. Animals

Male ICR mice (6 weeks old) weighing 25–30 g were purchased from SLC Japan (Hamamatsu, Shizuoka, Japan). The mice were housed in clear polycarbonate cages (22.5×33.8×14.0 cm) and in groups of 5 or 6 mice under a controlled 12/12-h light–dark cycle (light from 7:00 AM to 7:00 PM) at a room temperature of 23±1 °C and humidity of 55±5%. The mice were given free access to water and to food pellets designed for mice. The experimental procedure was approved by the Animal Care and Use Committee of Chiba University Graduate School of Medicine.

2.2. Drug administration

PCP hydrochloride was synthesized by K.H. in our laboratory. Saline (10 ml/kg/day) or PCP (10 mg/kg/day expressed as a hydrochloride salt) were administered subcutaneously (s.c.) for 10 days (once daily on day 1–5, 8–12), and no treatment was given on days 6, 7, 13 and 14. In the experiment involving subchronic treatment, 3 days (day 15) after the final administration of saline or PCP, vehicle (10 ml/kg/day; physiological saline) or minocycline (4.0 or 40 mg/kg/day, Wako Pure Chemical Ltd., Tokyo, Japan) was administered intraperitoneally (i.p.) for 14 consecutive days (once daily on days 15–28). The dose (40 mg/kg) of minocycline was selected based on the fact that this dose was effective in mitigating the methamphetamine-induced hyperlocomotion (Zhang et al., 2006a) as well as prepulse inhibition deficits in mice after the administration of the NMDA receptor antagonist dizocilpine (Zhang et al., 2007). The dose (4.0 mg/kg) of minocycline was used as a low-dose, negative control dose. The experiments were conducted separately, and the individual dose groups were distributed across the duration of the experiments.

2.3. Novel object recognition test (NORT)

NORT was performed 1 day after a final administration of vehicle (10 ml/kg/day for 14 days) or minocycline (4.0 or 40 mg/kg/day for 14 days). The apparatus for this task consisted of a black open field box (50.8×50.8×25.4 cm). Before the test, mice were habituated in the box for 3 days. During a training session, two objects (various objects differing in shape and color but similar in size) were placed in the box 35.5 cm apart (symmetrically), and each animal was allowed to explore in the box for 10 min (5 min×2). The animals were considered to be exploring the object when the head of the animal was facing the object within an inch of the object or when any part of the body, except for the tail, was touching the object. The time that the mice spent exploring each object was recorded. After the training, the mice were immediately returned to their home-cages, and the box and objects were cleaned with 75% ethanol to avoid any possible instinctive odorant cues. Retention tests were carried out at 1-day intervals following the training. During the retention test, each mouse was placed back into the same box, with one of the objects used during training replaced by a novel object. The mice were then allowed to freely explore for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counter-balanced manner in terms of their physical complexity. 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 test session) over the total time spent exploring both objects, was used to measure the memory performance.

2.4. Statistical analysis

Data were expressed as means±S.E.M. Statistical analysis was performed using one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni/Dunn test. *P*-values less than 0.05 were considered statistically significant.

3. Results

During the training session, there were no significant differences (F (4,80)=1.92, p=0.115) among the five groups in the total amount of time spent exploring two objects (Table 1). In the retention session, the exploratory preference (approximately 40%) of the PCP-treated group was significantly lower than that (approximately 50%) of the saline-treated

Table 1
Total amount of time spent exploring both objects during object recognition in the training and retention sessions

Group	Training session (10 min)	Retention session (5 min)
	Time exploring objects (seconds)	
Vehicle+Vehicle	63.97±3.37	48.12±3.81
PCP+Vehicle	65.24±4.64	43.93±3.30
PCP+Minocycline (4.0 mg/kg)	63.47±6.92	42.65±5.99
PCP+Minocycline (40 mg/kg)	82.65±6.74	48.81±3.90
Vehicle+Minocycline (40 mg/kg)	71.08±13.14	32.44±6.67

Data are expressed as the mean±S.E.M (n=9–24). There were no significant differences among five groups.

group, suggesting that the behavior of the PCP-treated mice may not have been due to memory impairment (Hashimoto et al., 2005a). Therefore, it is likely that our model of PCP-induced cognitive deficits using NORT may show behavioral deficits such as reduction in motivation for a novel object, concentration, and withdrawal symptoms, which might be related to cognitive deficits.

We examined the effects of subsequent subchronic (14 day) administration of minocycline on PCP-induced cognitive deficits in mice. As shown in Fig. 1, PCP-induced deficits were significantly improved after subsequent subchronic (14 days) administration of minocycline (40 mg/kg/day), but not minocycline (4.0 mg/kg/day). In the training session, the exploratory preference was not different among the five groups (F (4,80)=1.011, p=0.407) (Fig. 1). However, in the retention test session, ANOVA analysis revealed that the exploratory preferences of mice in the five groups were significantly different (F (4,80)=14.30, p<0.001) (Fig. 1). A *post hoc* Bonferroni test indicated that the exploratory preference of the PCP-treated group was significantly (p<0.001) increased after subchronic (14 days) administration of minocycline (40 mg/kg/day) (Fig. 1). Furthermore, subchronic (14 days) administration of minocycline (40mg/kg/day) alone did not alter the exploratory preference in either the training session or the retention session (Fig. 1).

4. Discussion

In this study, we found that PCP-induced cognitive deficits could be improved by subsequent subchronic (14 days) administration of minocycline. In the NORT, no significant differences in the total amount of time spent exploring two objects or in exploratory preference were found among all of the groups during the training session, suggesting that the levels of motivation, curiosity, and interest in exploring novel objects were the same in all groups (Hashimoto et al., 2005a, 2006, 2007a). In the NORT, we reported that PCP-induced cognitive deficits in mice could be improved by subsequent subchronic (14 days) administration of the atypical antipsychotic drug clozapine, but not the typical antipsychotic drug haloperidol (Hashimoto et al., 2005a). Therefore, the reversal of PCP-induced cognitive deficits using the NORT may be a potential animal model of atypical antipsychotic activity in relation to the amelioration of cognitive deficits in schizophrenia (Hashimoto et al., 2005a).

It has been reported that minocycline enhanced the discriminative stimulus effects of PCP in rats, suggesting that minocycline may interact either directly or indirectly with the NMDA receptors (Munzar et al., 2002). These data seem to be in conflict with the present data. The precise mechanisms underlying this discrepancy are currently unclear. The discrepancy may be due to the difference in the treatment schedule. In the paper by

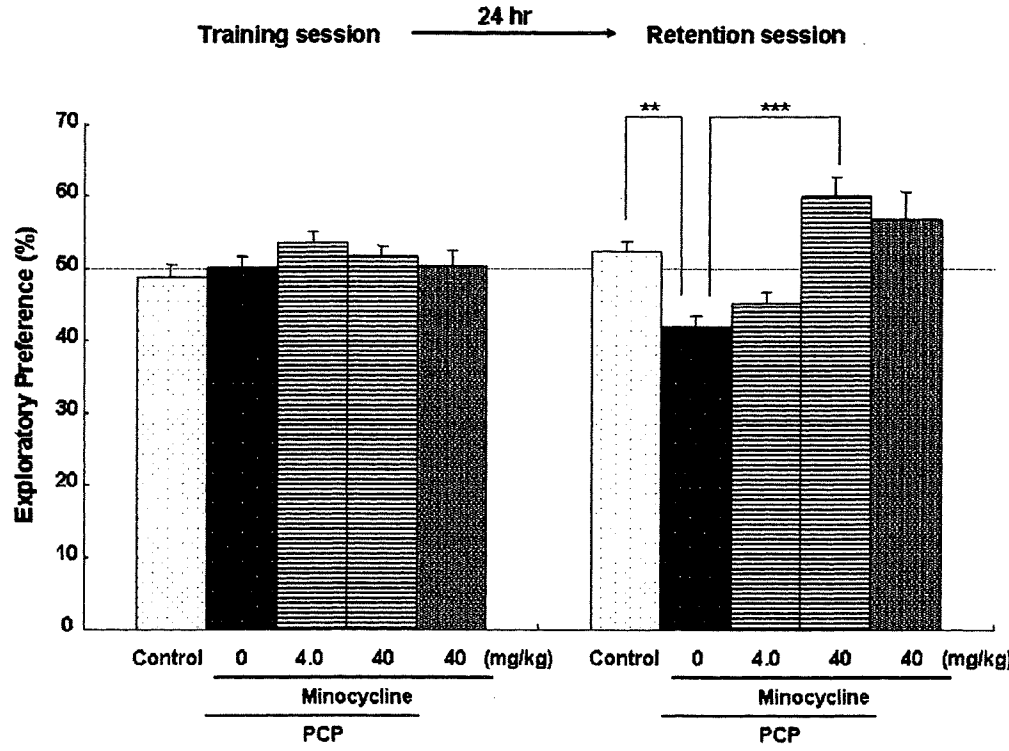

Fig. 1. Effects of minocycline on PCP-induced cognitive deficits in mice Saline (10 ml/kg/day) or PCP (10 mg/kg/day) were administered s.c. for 10 days (once daily on day 1–5, 8–12). (A) Three days (day 15) after the last administration of saline or PCP, vehicle (10 ml/kg/day; saline), or minocycline (4.0 or 40 mg/kg/day) were administered i.p. into mice for consecutive 14 days (once daily on day 15–28). Twenty four hours (day 29) after the last administration of vehicle or minocycline, the training session of NORT was performed. Then the retention test session was performed 24 h after the training session. Values are the mean±S.E.M. (n=9–24). **p<0.01, ***p<0.001 as compared with PCP plus saline-treated group.

Munzar et al. (2002), minocycline was administered 30 min before PCP administration. It is, therefore, possible that minocycline may increase PCP levels in the rat brain by pharmacokinetic interaction. In contrast, in the present study, minocycline was administered 24 h after the final administration of PCP.

Recently, we reported that minocycline significantly attenuated hyperlocomotion in mice after the administration of methamphetamine (Zhang et al., 2006a), and that hyperlocomotion and prepulse inhibition deficits in mice after the administration of the NMDA receptor antagonist dizocilpine were significantly attenuated by the administration of minocycline (Zhang et al., 2007). Taken together, these results suggest that minocycline is likely to have antipsychotic activity in animal models of schizophrenia. Although the molecular and cellular mechanisms underlying the efficacy of minocycline on PCP-induced cognitive deficits are currently unclear, it seems that at least two mechanisms (the attenuation of innate and adaptive immunity and the blockade of apoptotic cascades) (Domercq and Matute, 2004) may be implicated in the mechanism of minocycline. Further studies of the mechanism of minocycline are necessary.

Recently, Miyaoka et al. (2007) reported two cases of schizophrenic patients treated with minocycline. In the two patients, minocycline was effective in the treatment of acute schizophrenia with predominantly catatonic symptoms (Miyaoka et al., 2007). Ahuja and Carroll (2007) have hypothesized that minocycline acts as a functional NMDA receptor antagonist and helps improve the catatonic symptoms when used as an adjunct to antipsychotic medication. However, the precise mechanisms underlying the improvement induced by minocycline are currently unknown. Further double-blind placebo control studies of minocycline in schizophrenic patients as well as molecular and cellular studies of the mechanisms of minocycline are necessary.

5. Conclusion

This study shows that PCP-induced cognitive deficits in mice could be attenuated by subsequent subchronic administration of minocycline, although the precise mechanisms underlying the mechanism of action for minocycline remain unresolved. Therefore, minocycline may be a potential therapeutic drug for schizophrenia.

Acknowledgements

This study was supported in part by grants from the Minister of Education, Culture, Sports, Science, and Technology of Japan (to K.H.), the Ministry of Health, Labor and Welfare of Japan (to K.H.), and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (to K.H.).

References

Ahuja N, Carroll BT. Possible anti-catatonic effects of minocycline in patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2007;31:968–9.

Domercq M, Matute C. Neuroprotection by tetracyclines. Trends Pharmacol Sci 2004;25:609–12.

Green M. What are the functional consequences of neurocognitive deficits in schizophrenia? Am J Psychiatry 1996;153:321–30.

Hashimoto K, Fujita Y, Ishima T, Hagiwara H, Iyo M. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of tropisetron: role of α7 nicotinic receptors. Eur J Pharmacol 2006;553:191–5.

Hashimoto K, Fujita Y, Iyo M. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of fluvoxamine: role of sigma-1 receptors. Neuropsychopharmacology 2007a;32:514–21.

Hashimoto K, Fujita Y, Shimizu E, Iyo M. Phencyclidine-induced cognitive deficits in mice are improved by subsequent subchronic administration of clozapine, but not haloperidol. Eur J Pharmacol 2005a;519:14-117.

Hashimoto K, Okamura N, Shimizu E, Iyo M. Glutamate hypothesis of schizophrenia and approach for possible therapeutic drugs. Curr Med Chem-CNS Agents 2004;4:147–54.

Hashimoto K, Shimizu E, Iyo M. Dysfunction of glia-neuron communication in pathophysiology of schizophrenia. Curr Psychiatry Rev 2005b;1:151–63.

Hashimoto K, Tsukada H, Nishiyama S, Fukumoto D, Kakiuchi T, Iyo M. Protective effects of minocycline on the reduction of dopamine transporters in the striatum after administration of methamphetamine: a PET study in conscious monkeys. Biol Psychiatry 2007b;61:577–81.

Javitt DC, Zukin SR. Recent advances in the phencyclidine model of schizophrenia. Am J Psychiatry 1991;148:1301–8.

Miyaoka T, Yasukawa R, Yasuda H, Hayashida M, Inagaki T, Horiguchi J. Possible antipsychotic effects of minocycline in patients with schizophrenia. Prog Neuropsychopharmacol Biol Psychiatry 2007;31:304–7.

Munzar P, Li H, Nicholson KL, Wiley JL, Balster RL. Enhancement of the discriminative stimulus effects of phencyclidine by the tetracycline antibiotics doxycycline and minocycline in rats. Psychopharmacology 2002;160:331–6.

Stirling DP, Koochesfahani KM, Steeves JD, Tetzlaff W. Minocycline as a neuroprotective agent. Neuroscientist 2005;11:308–22.

Thomas M, Le WD. Minocycline: neuroprotective mechanisms in Parkinson's disease. Curr Pharm Des 2004;10:679–86.

Yong VW, Wells J, Giuliani F, Casha S, Power C, Metz LM. The promise of minocycline in neurology. Lancet Neurol 2004;3:744–51.

Zhang L, Kitaichi K, Fujimoto Y, Nakayama H, Shimizu E, Iyo M, et al. Protective effects of minocycline on behavioral changes and neurotoxicity in mice after administration of methamphetamine. Prog Neuropharmacol Biol Psychiatry 2006a;30:1381–93.

Zhang L, Shirayama Y, Shimizu E, Iyo M, Hashimoto K. Protective effects of minocycline on 3,4-methylenedioxymethamphetamine-induced neurotoxicity in serotonergic and dopaminergic neurons of mouse brain. Eur J Pharmacol 2006b;544:1–9.

Zhang L, Shirayama Y, Iyo M, Hashimoto K. Minocycline attenuates hyperlocomotion and prepulse inhibition deficits in mice after administration of the NMDA receptor antagonist dizocilpine. Neuropsychopharmacology 2007;32:2004–10.

厚生労働科学研究費補助金（医薬品・医療器機等レギュラトリーサイエンス総合研究事業）
分担研究報告書

乱用薬物による易再発性精神病様状態および依存症の予防・治療法開発に関する研究

分担研究者：西川　徹
研究協力者：竹林裕直、海野麻未、山本直樹、車地暁生
東京医科歯科大学大学院精神行動医科学分野

[研究要旨]
乱用薬物による精神障害の分子病態を明らかにする目的で、(1) PCP、ケタミン等のNMDA型グルタミン酸受容体遮断薬による依存や精神病状態が小児期には生じにくいことや、(2)実験動物において一定の発達期（臨界期）以降に成熟期タイプの異常行動が生ずるようになることに注目し、PCP精神病関連候補分子として、ラットの脳において臨界期前後でPCPへの応答が異なる遺伝子を探索した。DNAアレイ、RT-PCR、In situ hybridization 等により、leiomodin 2遺伝子（*Lmod2*）が、視床前核群を中心とした視床の限局した部位で、生後32日以降に発現が増加する発達依存的な発現増加を示すことが明らかになった。これらの所見は、*Lmod2* mRNAを発現する視床核群がPCP精神病の病態に関与する神経回路を構成することを示唆している。また、*Lmod2*遺伝子や蛋白を含む分子カスケードが薬物性精神病状態に対する新しい診断・予防・治療法開発の分子標的になる可能性がある。

A. 研究目的

本研究は、乱用が問題となっている覚醒剤（アンフェタミン、メトアンフェタミン(MAP)など)、コカインなどのドーパミン作動薬や、フェンサイクリジン（PCP)、ケタミンなどのNMDA型グルタミン酸受容体遮断薬による易再発性の統合失調症様精神病状態の分子機構を解明し、新しい治療法開発の標的となる神経機構や、臨床診断、経過、予後などのマーカー物質を見いだすことを目的としている。このため、1) ドーパミン作動薬やNMDA 受容体遮断薬の精神機能に及ぼす影響が思春期前後では異なり、小児期までは依存形成や精神症状が生じ難いことが知られている[12,16]、2) 齧歯類では、上記の薬物が引き起こす行動変化およびその易再発性状態（逆耐性現象）がヒトの統合失調症型精神病状態のモデルと考えられているが、こうした行動異常は一定の生後発達期（臨界期）以降に見られるようになる[13]、などの点に着目して分子生物学的研究を進めた。以上の事実は、これらの乱用薬物が惹起する依存あるいは精神病状態に特異的に関与する脳内情報処理系は、特定の発達段階に成熟して薬物の影響を受けるようになることを示唆している。実際、MAP やPCP 投与ラットの脳の活動異常のパターンも生後発達に伴って変化し、上述した行動異常の臨界期頃から成熟期のパターンとなる[12,13,18,27]。したがって、こうした変化が著しい脳部位が、これらの薬物による精神障害に特異的に関与する情報処理系に含まれる可能性が高い[8,9,12,13,22]。

そこで、このような情報処理系を構築する分子群を明らかにするため、臨界期以後に、MAP やPCP に対する応答性を獲得する遺伝子群を検索

している。本年度は、PCP 投与後の活動性異常の生後発達期における変化が最も顕著な部位のひとつである視床から、PCP に発達依存的応答示す遺伝子として、*Lmod2* (leimodin 2) を検出し薬理学的・組織化学的な解析を行った。

B. 研究方法

今回報告した研究は、東京医科歯科大学の実験動物委員会の承認を得た上、倫理ガイドラインを遵守して行った。

1. 対象および薬物

動物実験には、生後 8ー56 日令の Wistar 系雄性ラットを用いた。動物は 25.0±0.5℃、湿度 55%、8 時より 20 時を明期とする明暗条件下で飼育した。

PCP は山之内製薬（現、アステラス製薬）のご好意で合成・分与していただいた。そのほかの試薬は、すべて市販のものを用いた。薬物投与は、皮下（s.c.）への注射により行った。対照群の動物には注射溶媒を投与した。薬物の投与量は、常に free base で計算した。

2. DNA アレイ

PCP または生理食塩水を投与した、生後 8 日齢および 56 日齢のラット視床から total RNA を抽出した。このうち、0.4 ug を用いて、random hexamer priming による逆転写反応によって cDNA を合成し、約 28,000 遺伝子の 30,000 転写産物以上に対する DNA チップ（Affymetrix GeneChip^{R} Rat Genome 230 2.0 Array）を使って、生後 8 日と 56 日の間で薬物応答に差のある遺伝子のスクリーニンを行った。さらに、この結果を定量的 RT-PCR により確認した。

3. 遺伝子発現の定量的解析 [8,9,22]

上記のように調整した、ラット大脳新皮質 cDNA を，10 倍量の TE buffer で希釈した。この cDNA 溶液 5ul を以後の定量的 PCR のテンプレートとして用いた。標的遺伝子の発現量補正のための内因性コントロールとしては GAPDH を用いた。標的遺伝子 mRNA および GAPDH mRNA に特異的なプライマーペアーにより，LightCycler（Roche）を用いてリアルタイム PCR（LightCycler-FastStart DNA master SYBR Green 1 Kit) を行った(Kajii Ito Takebayashi)。PCR 増幅産物の量は Syber Green の蛍光強度として，各サイクルの伸長反応の終わりの時点で測定した。RT-PCR には、次のプライマーセットを用いた：*Lmod2*, 5'-AATGGAAGAAAGCTCCCGG-3" および 5'-CCACCCTCCGTAGCTGTCTTAT-3'; *Tmod1*, 5'-GACAGTGATGGCTCAAAAGCTG-3" および 5'-CGATGCCACTCTAGTTACCCTG-3'; *Tmod2*, 5'-CGTTTCCGTTAACCATATCCG-3" および 5'-TGTACAAGCCACCAAGCGTGT-3'; GAPDH, 5'-ACATCATCCCTGCATCCACT-3' および 5'-GGGAGTTGCTGTTGAACTCA-3"。得られた PCR 増幅曲線から LightCycker Software を用いて，GAPDH および標的遺伝子の mRNA 量の相対値を算出した。標的遺伝子の mRNA 発現量は GAPDH mRNA 量に対する比として補正した後，統計解析を行った。なお，PCR 産物の特異性の検討は，PCR 後の融解曲線解析およびアガロースゲル電気泳動による単一バンドの確認により行った。mRNA 発現の定量は、mRNA の濃度に比例した測定値が得られる条件下における RT-PCR によっても検討した。

4. ノーザンブロット分析 [9,22]

各ラット視床より poly(A)+RNA を、oligo(dT)-cellulose column (Amersham, UK)を用いて抽出しした。それぞれのサンプルをアガロース-ホルムアルデヒドゲル(6.3% formaldehyde, 1% agarose, 1x 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA: pH 7.0)) を使った電気泳動によって分離した。これをナイロンメンブレイン (Hybond-XL, Amersham Bioscience, Freiburg,

Germany) に転写し、紫外線照射により固定した。プレハイブリダイゼイションを68°Cに保ったバッファー(DIG-Easy Hyb buffer, Roche)中で2時間行った後、ハイブリダイゼイションとして、digoxigenin (DIG)で標識した RNAプローブ(ラット *Lmod2* cDNA , 335 bases (nucleotides 1549-1883, GenBank Accession No. AB331240) ; ラットGAPDH cDNA, 609 bases (nucleotides 331-939, GenBank Accession No. X02231)) を、同じバッファー中において一晩反応させた。0.1%(w/v) SDS (sodium dodecyl sulfate) を含む、2× 標準生理食塩水-クエン酸ナトリウムバッファー(SSC)により室温で1時間、さらに0.1×SSC/0.1%SDSにより68°Cで1時間洗浄した。ハイブリダイゼションはアルカリフォスファターゼに結合させた抗DIG抗体によって免疫反応的に検出し、化学発光物質CDP-Star (Roche)で可視化した。ラット各臓器間の発現を比較する実験では、各臓器から抽出されたmRNAを吸着させたメンブレインのRat Multiple Tissue Northern (MTN) Blot (Takara Bio/Clontech)をハイブリダイゼイションに用いた。

5. In situハイブリダイゼーション

Northernで用いたのと同じcRNAプローブをDIG [11,14]または^{35}Sで標識[23,24]し、木山らの方法に従い生後50日齢の脳切片に対してin situハイブリダイゼーションを行った。いずれも、スライドグラスに凍結脳切片（厚さ16μm）を貼り付け、次の過程の順に処理した後、ハイブリダイゼイションを行った：1) 自然乾燥、2) 4% パラフォルムアルデヒドを含むリン酸バッファー生理的食塩水（PBS）中で20分間固定、3) PBS で2分間リンスを2回、4) 5rSSC で15分間処置。

6.統計解析

データの統計的解析においては、2 群間の平均値の比較は Student's t-test または Cokran-Cox t-test を使って行った。多群間（3 群以上）の比較には、一元分散分析（one-way ANOVA）にもとづく、多重比較（Scheffe's test）を用いた。一部の実験結果は、二元分散分析（two-way ANOVA）により比較検討した。

C. 研究結果

1．PCP に対し発達依存的応答を示す遺伝子 *Lmod2* のスクリーニング

成体ラットの視床において、PCP 投与により、その発現が 2 倍以上増加する遺伝子として 5 個の既知遺伝子と 3 個の未確立の遺伝子転写産物が検出された。未確立の 3 個の遺伝子に注目したところ、この中の一つがクローニングにより既知遺伝子 *Lmod2*[2] のラット ortholog であることが明らかになった。本研究で得られたラット *Lmod2* 遺伝子 cDNA から予測されるアミノ酸配列はマウスと 97%の相同性が見られた。*Lmod2* 遺伝子は、主として心筋に発現し、アクチンキャッピングドメイン（Fig.1）で細胞骨格蛋白と相互作用する蛋白をコードすることが知られている [2]が、他に検出された 5 つの既知遺伝子への PCP の作用は報告があるのに対し、これまで脳における研究報告がなく、その機能も十分に解明されていないため、本研究の解析対象とした。

ノーザンブロット解析において、成熟ラット *Lmod2* mRNA は、主として心筋に発現し骨格筋でも認められたが、脳全体では検出できなかった。RT-PCR を使って脳の部位別発現を検討した

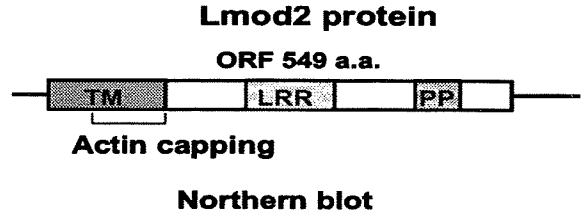

Fig.1 Schematic representation of the structure of rat Lmod2 cDNA and the nucleotidesequences encoding the open reading frame (ORF)

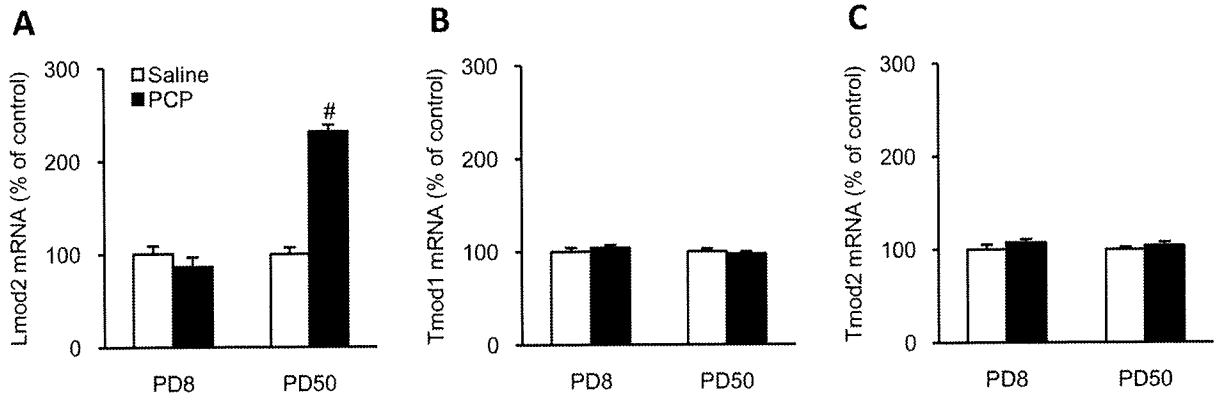

Fig. 2. Differential effects of PCP on Lmod2 (A), Tmod1 (B) and Tmod2 (C) expression in thethalamus of the infant (PD8) and adult (PD50) rats. The data were obtained by the real-time RT-PCR method and are expressed as percentage of the values of the respective saline-treated controls. ** P<0.01 vs. respective saline-treated controls (Scheffee test). n=5. (a) Lmod2: two-way ANOVA, postnatal days effect; F1,16=76.878, p<0.0001 ; PCP treatment effect : F1,16=51.066, p<0.0001 ; postnatal daysrPCP treatment effect: F1,16=77.456, p<0.0001).

ところ、ほぼ視床に限局していることがわかった。

2．*Lmod2* の PCP に対する応答

Lmod2 遺伝子の発現が、生後 50 日齢の視床では PCP の急性投与により増加し、8 日齢の視床では増加しないという DNA アレイの結果は、RT-PCR によっても確認された（Fig.2)。*Lmod2* 遺伝子は tropomodulin (Tmod)遺伝子ファミリーに属しているが、脳で同定されている他の Tmod アイソフォームの *Tmod1*、*Tmod2* [2,6] は、*Lmod2* とは対照的に、脳全体に広く発現しており、視床において、成熟期（50 日齢)、新生仔期（8 日齢）ともに PCP による影響は受けなかった（Fig.2)。また、心筋の *Lmod2* 遺伝子は、視床と同一の塩基配列をもっていたが、その反応性は視床とは異なり、PCP 投与後も有意な変化を示さなかった（データ省略)。生後発達の観察では、*Lmod2* 遺伝子の基礎発現は生後 26 日まで増加し、PCP への応答性は生後 32 日以降に出現することが明らかになった（Fig. 3)。これらの結果から、*Lmod2* 遺伝子は発達依存的で視床選択的な PCP による発現誘導を受けることが示唆された。

さらに、成熟期 において、PCP 投与後の *Lmod2* mRNA 発現は、1-3 時間でピークを迎え 6 時間後

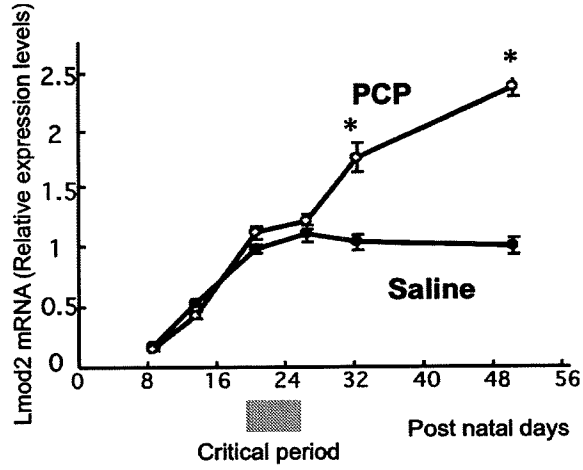

Fig. 3. Postnatal changes in the basal and PCP-induced expression of the thalamic Lmod2 mRNA. Rats at PD 8, 13, 20, 26, 32 and 50 were treated with 7.5 mg/kg PCP subcutaneously and the relative expression levels of Lmod2 mRNA (Lmod2:GAPDH mRNA ratio) were determined by the real-time RT–PCR method 60 min after administration of PCP or saline. Results are the means with S.E.M. of data obtained from five or six rats per group and are expressed as a percentage of the values of the adult (PD 50) saline-treated animals (# p<0.01 vs. respective saline-treated controls ; # p<0.01 vs. saline-treated rats at PD 8)

には saline 投与と同じレベルにまで低下した。これに対して、新生仔期（生後 8 日）では、投与 1、2、3、6 時間後のいずれでも、有意な変化は観察