Table 1. Genetic and Biochemical Analyses in Schizophrenic Patients and Control Subjects

Characteristic	No. (%) Schizophrenic Patients (n=1761)	Control Subjects (n=1921)	Main Application
Institutions where DNA was collected, No.			
Tokyo Institute of Psychiatry	261	302	Resequence
Tokyo Metropolitan Matsuzawa Hospital (postmortem brain tissue)	70	1	Resequence plasmid construction
RIKEN Brain Science Institute	1156	1502	Resequence
Okayama University	274	116	Resequence
Pentosidine level[a]			
Very high, >130 ng/mL	3 (6.7)[b]	0	HPLC
High, >55.2 ng/mL	18 (40.0)	2 (3.3)	
Normal, <55.2 ng/mL	24 (53.3)	59 (96.7)	
Vitamin B_6, pyridoxal level[a]			
Normal: male, 6-27 ng/mL; female, 4-42 ng/mL	19 (42.2)	54 (88.5)	HPLC
Low: male, <6 ng/mL; female, <4 ng/mL	15 (33.3)	7 (11.5)	
Very low, <3 ng/mL	11 (24.4)[b]	0	

Abbreviation: HPLC, high-performance liquid chromatography.
[a] Forty-five schizophrenic patients; 61 healthy control subjects.
[b] For detailed information, see Table 3.

cation system is ubiquitous in human tissues, including the brain. The GLO1 detoxification system interacts with several metabolizing cascades, and some compounds in these cascades have been reported as candidates for involvement in the etiology of schizophrenia, such as glutathione, homocysteine, and folic acid metabolites (eFigure 1, available at http://www.prit.go.jp/En/PSchizo/TSchizo/archives.html).[12-19]

Recent studies have revealed that dysfunction of GLO1 is involved not only in systemic diseases such as diabetes mellitus[20] and vascular injury,[21] but also in neuropsychiatric disorders such as mood disorder,[22] autism,[23,24] anxiety disorders,[25] alcoholism,[26] and Alzheimer disease.[7] In mice, levels of *Glo1* expression have been associated with anxiety-like behavioral phenotypes.[27-29] *GLO1* has been mapped to chromosome 6p21, a linkage region for schizophrenia.[30-32] A missense polymorphism, Glu111/Ala111, has been reported in 2 multiplex Caucasian pedigrees with schizophrenia spectrum disorders.[33] However, the functional significance of this polymorphism has not been addressed.

The present study examined whether plasma levels of pentosidine and serum vitamin B_6 are altered in patients with schizophrenia. If so, *GLO1* polymorphisms associated with functional deficits could be an underlying substrate of schizophrenia. To the best of our knowledge, this is the first study to suggest enhanced carbonyl stress as an underlying mechanism of schizophrenia.

METHODS

SUBJECTS

Materials for resequencing of the *GLO1* gene were obtained from 1761 schizophrenic patients (mean age, 50.1 years [SD, 13.9 years]) and 1921 healthy control subjects (mean age, 42.5 years [SD, 14.4 years]) (**Table 1**). For genetic study, the affected individuals were randomly recruited from among both inpatients and outpatients. Cases were composed of 961 men (mean age, 49.0 years [SD, 13.4 years]) and 800 women (mean age, 51.4 years [SD, 14.3 years]). Control subjects were composed of 779 men (mean age, 41.2 years [SD, 13.6 years]) and 1142 women (mean age, 43.0 years [SD, 14.8 years]). DNA extracted from 71 postmortem brain tissue specimens was used for resequencing. We did not assess associations between common variants and schizophrenia, as the aim of this study was to focus on rare variations to reveal large biological effects, thus enabling clarification of pathophysiology in rare cases of schizophrenia. These samples were therefore not matched by age or sex. Schizophrenia was diagnosed according to the *DSM-IV* to obtain a best-estimate lifetime diagnosis, with consensus of at least 2 experienced psychiatrists. No structured interviews were performed. Ten percent of patients exhibited discordant subtypes. The available medical records and family informant reports were also taken into consideration. Control subjects were recruited from among hospital staff and company employees documented to be free from mental illness based on brief interviews by experienced psychiatrists. The companies that provided employees as control subjects for our study were biochemical, pharmaceutical, and medical device manufacturers. We personally announced recruitment of volunteers for our research at annual meetings such as those of the Japanese Society of Biological Psychiatry and the Japanese Society of Schizophrenia Research.

Fresh plasma and serum samples were obtained from 45 available schizophrenic patients and 61 healthy controls among the subjects included in the genetic study (Table 1). Diabetes mellitus and renal dysfunction were criteria for exclusion in selecting patients and healthy control subjects, as these diseases may potentially increase pentosidine levels.

All participants provided written informed consent, and the study protocols were approved by the ethics committees of all participating institutions (Tokyo Institute of Psychiatry,[34] Tokai University, RIKEN Brain Science Institute,[35-38] Okayama University,[39] Tokyo Metropolitan Matsuzawa Hospital, Hamamatsu University, Chiba University, and Tohoku University).

RESEQUENCING ANALYSIS OF *GLO1*

All the coding regions and exon-intron boundaries as well as the 5′ upstream region of *GLO1* were examined by direct sequencing of the polymerase chain reaction (PCR) products. Polymerase chain reaction amplification was performed using the sets of

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primers listed in eTable 1 and Blend Taq polymerase (Toyobo, Osaka, Japan). Detailed information on the PCR amplification conditions is available from the authors upon request. Sequencing of PCR products was performed using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, California) and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). We read both strands when an inserted or deleted nucleotide yielded dual signals derived from wild-type and mutant-type strands. Moreover, to confirm a single base insertion or deletion, PCR fragments were subcloned into a pTA2 plasmid vector (Toyobo) and sequenced.

GLO1 ENZYMATIC ASSAY

Fresh blood samples were obtained from 45 schizophrenic patients and 61 healthy control subjects (Table 1). Red blood cells (RBC), plasma, and serum were separated by centrifugation and used in subsequent studies. Glyoxalase I enzymatic activity in RBC was determined using the spectrophotometric method described by McLellan and Thornalley.[40] Briefly, washed RBC were lysed with 4 volumes of ice-cold distilled water and kept on ice for more than 30 minutes to complete hemolysis. Debris was removed by centrifugation and the supernatant was assayed for enzymatic activity. Activity of the GLO1 enzyme is given in units/10^6 RBC, where 1 unit is the amount of enzyme required to catalyze the formation of 1 μmol of S-D-lactoylglutathione per minute from hemithioacetal. Hemithioacetal was prepared by preincubation of 2mM methylglyoxal with 2mM glutathione in a 50mM sodium phosphate buffer (pH 6.6) at 37°C for 10 minutes. The increase in absorbance at 240 nm owing to the formation of S-D-lactoylglutathione was measured by spectrophotometry. Prominently low enzymatic activities were confirmed by at least 3 measurements.

MEASUREMENT OF PENTOSIDINE AND VITAMIN B_6

Pentosidine, an AGE, was determined by high-performance liquid chromatography assay as described previously.[41] In brief, the plasma sample was lyophilized, hydrolyzed in 100 μL of 6N of hydrochloric acid for 16 hours at 110°C under nitrogen, neutralized with 100 μL of 5N of sodium hydroxide and 200 μL of a 0.5M sodium phosphate buffer (pH 7.4), filtered through a 0.5-μm filter, and diluted with phosphate-buffered saline (PBS). A sample (corresponding to 25 μg of protein) was injected into a high-performance liquid chromatography system and fractionated on a C18 reverse-phase column. Effluent was monitored at excitation-emission wavelengths of 335/385 nm using a fluorescence detector (RF-10A; Shimadzu, Kyoto, Japan). Synthetic pentosidine was used to obtain a standard curve. We measured pentosidine at least twice, and additional measurements were performed 3 times to confirm 3 outliers. Three forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) were measured in serum samples by high-performance liquid chromatography according to a previously described method.[42] Other parameters (glucose, glycohemoglobin A_{1C}, total cholesterol, triglyceride, aspartate aminotransferase, alanine aminotransferase, creatinine, urea nitrogen, total protein, and albumin) were measured in blood samples. Glomerular filtration rate was estimated using the abbreviated Modification of Diet in Renal Diseases study equation.[43]

WESTERN BLOTTING

The GLO1 protein expression in RBC lysate was assessed by Western blotting analysis after sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 5% to 20% polyacrylamide gradient gel. Polyclonal anti-GLO1 sera, designated NT2, were raised in rabbits by immunization with a human GLO1 peptide MAEPQPPSGGLTDEAALSC (corresponding to amino acids 1-19) conjugated to keyhole limpet hemocyanin. Equal volumes of RBC lysates were treated with Laemmli buffer, boiled at 100°C for 5 minutes, applied to the gel, and transferred to polyvinylidene fluoride membranes. Blots were treated with 100% BlockingOne (Nacalai, Kyoto, Japan) to block any nonspecific binding sites at 4°C overnight. The membrane was washed with PBS containing 0.05% Tween 20 (PBS-T) and then incubated with 1-μg/mL rabbit anti-GLO1 antibody (NT2) and mouse anti–glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, California) as an internal control in PBS-T containing 5% BlockingOne for 1 hour at room temperature. Anti-GLO1 antibody was affinity-purified using beads coupled with the antigen peptide. The membrane was washed again 3 times with PBS-T and then incubated with peroxidase-conjugated anti-mouse Ig (1:1000) and peroxidase-conjugated anti-rabbit Ig (1:1000) (Vector, Burlingame, California) for 1 hour at room temperature, followed again by a wash and eventual development with 3,3′-diaminobenzidine tetrahydrochloride solution (Sigma, St Louis, Missouri). The GLO1 signals that were normalized to GAPDH were quantified using National Institutes of Health image software (http://rsb.info.nih.gov/nih-image/). Researchers were blind to *GLO1* genotypes during experiments with Western blotting. We performed at least 2 determinations for each sample.

CELL CULTURE

Epstein-Barr virus–transformed lymphoblastoid cell lines derived from patients and normal subjects were established at SRL Inc (Tokyo, Japan). Lymphoblastoid cell lines were grown in RPMI 1640 medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, California) and antibiotic liquid (Nacalai, Kyoto, Japan). Cell lines were cultured at 37°C in a humidified atmosphere incubator under 5% carbon dioxide.

STATISTICAL ANALYSIS

Data were analyzed using PRISM software (GraphPad Software, San Diego, California). Simple comparisons of means and standard errors of data were performed using an unpaired *t* test or the Mann-Whitney test (both 2-tailed). The χ^2 and Pearson correlation tests were used to assess the significance of association between the data. For comparison of more than 2 groups, 1-way analysis of variance was used. If the results of analysis of variance were significant, the Bonferroni procedure was used as a post hoc test. Significance was defined as $P < .05$.

RESULTS

PENTOSIDINE ACCUMULATION AND PYRIDOXAL DEPLETION

We measured plasma pentosidine and serum pyridoxal (vitamin B_6) levels using samples from 45 patients with schizophrenia and 61 mentally healthy subjects (**Figure 1**). Neither schizophrenic patients nor healthy subjects had diabetes mellitus or chronic kidney disease (estimated glomerular filtration rate >60 mL/min), which are 2 major causes of elevated AGEs. An increase in plasma pentosidine (to above the mean plus 2 SDs of control sub-

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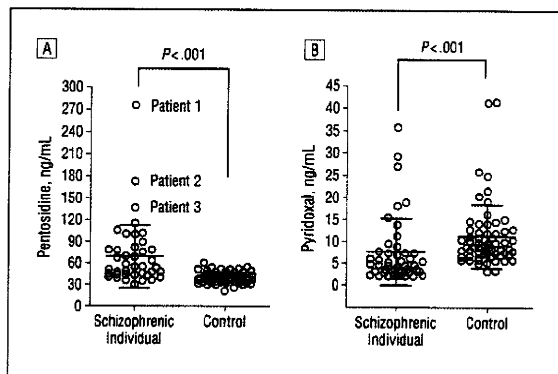

Figure 1. Plasma pentosidine accumulation and serum pyridoxal (vitamin B_6) depletion. Levels of plasma pentosidine (A) and serum pyridoxal (B) were analyzed using high-performance liquid chromatography techniques. Values were compared using the Mann-Whitney *U* test (2-tailed). Error bars indicate standard deviations.

jects, >55.2 ng/mL) was observed in 21 schizophrenic individuals (approximately 47%), as shown in Table 1. Three patients (patients 1, 2, and 3 in Figure 1A) exhibited extremely high pentosidine levels. The mean pentosidine level was 1.73-fold higher in schizophrenic individuals than in control subjects ($P<.001$) (Figure 1A and **Table 2**).

A concomitant marked decrease in pyridoxal levels was found in 11 schizophrenic patients (Table 1), most of whom were hospitalized and had been treated with well-controlled daily nutrition by a registered dietitian approved by the Japanese Ministry of Health, Labour, and Welfare based on the National Dietitian Law. Significant reduction of pyridoxal level was observed in schizophrenic patients compared with healthy control subjects ($P<.001$) (Figure 1B).

Mean values of pentosidine and vitamin B_6 in control samples were 39.6 ng/mL (SD, 7.8 ng/mL) and 11.1 ng/mL (SD, 7.3 ng/mL), respectively. These values do not deviate markedly from the standard levels in adult subjects without diabetes mellitus or renal dysfunction reported in previous studies.[44-46]

GENETIC ANALYSES OF *GLO1*

We next attempted to determine the mechanism underlying the alterations in pentosidine/pyridoxal levels observed in schizophrenia by resequencing analysis (all exons and flanking introns) of *GLO1* using 1761 patients with schizophrenia and 1921 control subjects (Table 1). These subjects included not only those for whom pentosidine/pyridoxal levels were examined, but also many other schizophrenic individuals and controls to ensure thorough genetic scrutiny. This analysis detected 2 heterozygous frameshift mutations. The first was an adenine insertion at nt 79 in exon 1, causing a frameshift starting from codon 27 and introducing a premature termination codon after aberrant translation of 15 amino acid residues (T27NfsX15) in 1 patient with schizophrenia (**Figure 2**A and eTable 2). The second heterozygous frameshift mutation, c.365delC, generated a frameshift from codon 122 in exon 4 and a premature termination after an aberrant 27–amino acid addition (P122LfsX27) (Figure 2B). This mutation was detected in 4 schizophrenic individuals and 10 control subjects (eTable 2). No relatives of subjects exhibiting c.365delC were available for analysis.

Furthermore, we identified 36 nucleotide changes, including 8 common polymorphisms (minor allele frequency >0.03) and 28 rare variants (eTable 2 and eTable 3). We also identified 13 homozygous Ala111 carriers: 9 schizophrenic patients and 4 controls (9 of 1586 schizophrenic patients [0.6%]; 4 of 1685 control subjects [0.2%]) (Figure 2C and eTable 3).

Seven heterozygous frameshift carriers (3 schizophrenic individuals and 4 controls), 10 homozygotes for Ala111 (7 schizophrenic individuals and 3 controls), 22 subjects with Glu111/Ala111 genotype (12 schizophrenic individuals and 10 controls), and 67 subjects with Glu111/Glu111 genotype (23 schizophrenic individuals and 44 controls) were available for biochemical assays (Figure 1 and Table 2).

BIOCHEMICAL ANALYSES OF GLO1

We focused on the heterozygous frameshift mutations and Glu111/Ala111 variation of *GLO1* in an attempt to assess the functional significance of these changes. We first quantified the levels of expression of GLO1 protein in RBC by Western blotting in 45 schizophrenic patients and 61 control subjects. Marked reductions (40%-50%) to full-length GLO1 protein expression were found in 10 subjects carrying heterozygous frameshift mutations ($P<.001$) (Table 2 and eFigure 2A). Significantly reduced (approximately 15%) GLO1 expression was observed in 7 homozygous Ala111 carriers compared with homozygous Glu111 or heterozygous Glu111/Ala111carriers in the schizophrenia group (both $P<.05$) (Table 2). In control subjects, levels of GLO1 protein expression in 3 homozygous Ala111 carriers did not differ significantly from those carrying other genotypes (Table 2).

The GLO1 enzymatic activity in RBC was measured by spectrophotometric assay (Table 2). Marked reductions (40%-50%) in enzymatic activity were found in all individuals carrying heterozygous frameshift mutations ($P<.01$). The 7 homozygous Ala111 carriers also exhibited significantly decreased enzymatic activity (an approximately 20% reduction) compared with homozygous Glu111 carriers in the schizophrenic group ($P<.001$) but not in control subjects.

In addition, we established a cell line from lymphocytes of a heterozygous frameshift carrier and performed functional analysis of these cell lysates (eFigure 2B). They exhibited the same functional abnormalities as identified in RBC, ie, decrease in GLO1 activity and its protein expression.

CONFOUNDING FACTORS AND BIOCHEMICAL DATA

Three patients (patients 1, 2, and 3 in Figure 1A) exhibiting extremely high pentosidine levels had especially severe schizophrenia, though they were free of systemic disease. These 3 schizophrenic individuals had chronic and

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Table 2. Samples Used in the Biochemical Analyses

	Mean (SD)									
	Schizophrenic Patients					Control Subjects				
Characteristic	All (n=45)	Glu/Glu (n=23)	Glu/Ala (n=12)	Ala/Ala (n=7)	Frameshift (n=3)	All (n=61)	Glu/Glu (n=44)	Glu/Ala (n=10)	Ala/Ala (n=3)	Frameshift (n=4)
Sex, No., M/F	29/16	13/10	9/3	5/2	2/1	23/38	17/27	3/7	0/3	3/1
Age, y	51.0 (12.2)[a]	47.6 (12.5)[a]	51.5 (12.7)	59.0 (8.6)[a]	57.3 (4.6)[a]	36.0 (9.4)	35.1 (9.4)	41.9 (8.2)	24.3 (1.5)	40.5 (5.7)
Age at onset, y	25.0 (8.7)	24.4 (5.8)	25.8 (11.9)	28.0 (12.7)	20.0 (2.6)					
Relative protein expression	0.95 (0.15)[b]	0.99 (0.11)	1.01 (0.08)	0.86 (0.06)[c]	0.55 (0.09)[d]	0.88 (0.12)	0.91 (0.10)	0.87 (0.09)	0.86 (0.05)	0.60 (0.06)[e]
Enzymatic activity, mU/10^6 RBC	5.43 (1.00)[f]	6.00 (0.75)	5.47 (0.35)	4.70 (0.65)[g]	3.00 (0.20)[h]	5.94 (1.00)	6.18 (0.61)	6.11 (0.69)	5.83 (0.29)	2.90 (0.08)[i]
Pentosidine, ng/mL	68.37 (43.42)[j]	64.73 (32.8)[k]	54.96 (17.83)[l]	97.95 (82.67)[m]	80.91 (53.26)	39.59 (7.82)	39.17 (8.41)	39.27 (6.25)	39.08 (3.24)	45.34 (6.12)
Pyridoxal, ng/mL[n]	7.46 (7.56)[o]	8.20 (8.70)[p]	7.36 (7.66)	6.82 (4.69)	3.60 (2.12)	11.14 (7.31)	11.91 (8.02)	8.45 (2.76)	14.63 (8.95)	6.88 (1.56)

Abbreviation: RBC, red blood cell.
[a]Unpaired *t* test, $P<.05$ (vs controls).
[b]Mann-Whitney test, $P<.01$ (vs controls).
[c]Analysis of variance, $F_{3,41}=21.76$, $P<.001$; Bonferroni multiple comparison test, $P<.05$ in schizophrenic patients (vs Glu/Glu and Glu/Ala).
[d]Analysis of variance, $F_{3,41}=21.76$, $P<001$; Bonferroni multiple comparison test, $P<.001$ in schizophrenic patients (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[e]Analysis of variance, $F_{3,57}=13.71$, $P<.001$; Bonferroni multiple comparison test, $P<.01$ in controls (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[f]Mann-Whitney test, $P<.001$ (vs controls).
[g]Analysis of variance, $F_{3,41}=23.44$, $P<.001$; Bonferroni multiple comparison test, $P<.001$ in schizophrenic patients (vs Glu/Glu).
[h]Analysis of variance, $F_{3,41}=23.44$, $P<.001$; Bonferroni multiple comparison test, $P<.01$ in schizophrenic patients (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[i]Analysis of variance, $F_{3,57}=37.41$, $P<.001$; Bonferroni multiple comparison test, $P<.001$ in controls (vs Glu/Glu, Glu/Ala, and Ala/Ala).
[j]Mann-Whitney test, $P<.001$ (vs controls).
[k]Mann-Whitney test, $P<.001$ (vs controls).
[l]Mann-Whitney test, $P<.01$ (vs controls).
[m]Mann-Whitney test, $P<.05$ (vs controls).
[n]Pyridoxal levels less than 2.0 were calculated as 2.0.
[o]Mann-Whitney test, $P<.001$ (vs controls).
[p]Mann-Whitney test, $P<.001$ (vs controls).

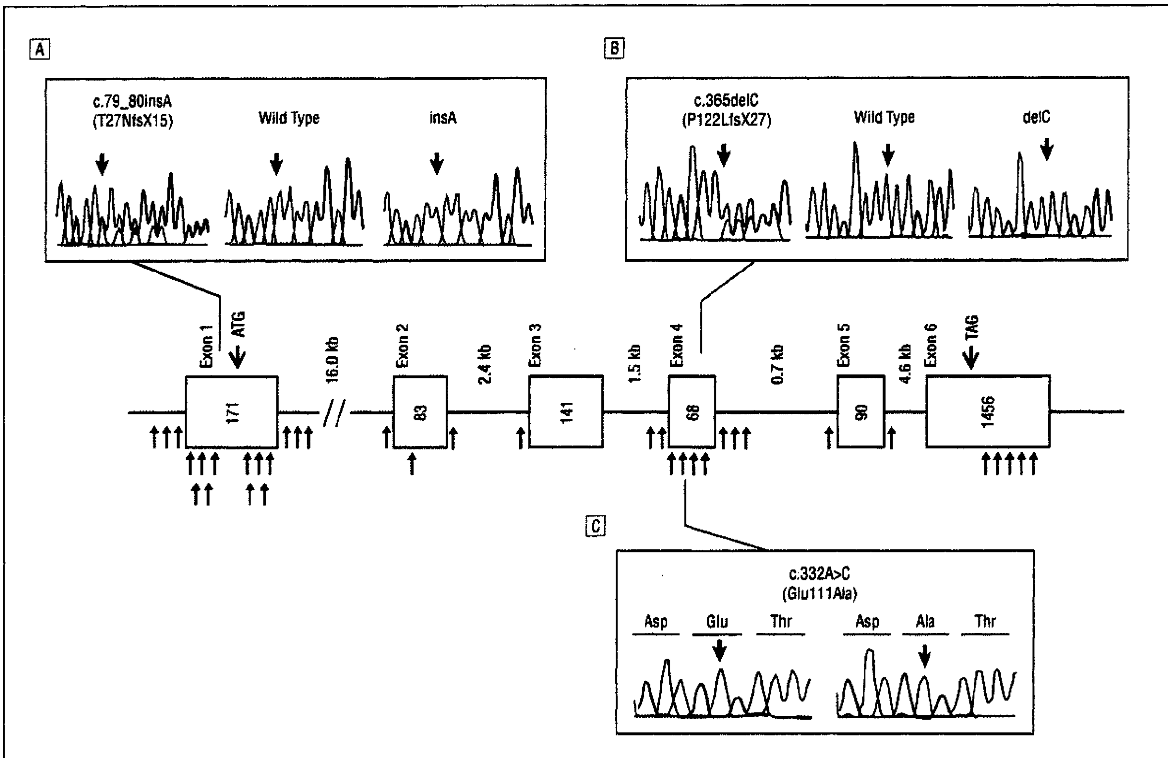

Figure 2. DNA sequence chromatograms showing frameshift and missense variants. Heterozygous sequence traces derived from individuals carrying an adenine insertion within exon 1 (A) and a cytosine deletion within exon 4 (B). TA cloning and subsequent sequencing analyses revealed normal (denoted "wild type") and mutant (denoted insA or delC) sequences. C, Chromatogram showing a Glu111/Ala111 missense variant located within exon 4. Positions of common and rare variants of *GLO1* are indicated by arrows (see also eTable 2 and eTable 3). kb indicates kilobase pairs.

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Table 3. Summary of Demographic Data of Patients With High Pentosidine and/or Low Pyridoxal Levels

	Patient No.												
Characteristic	**MZ65**	**TZ5**	**MZ70**	**TZ77**	**NP50**	**TZ72**	**MZ192**	**TZ40**	**TZ16**	**TZ41**	**SF114**	**SF136**	**TZ20**
Sex	M	F	M	F	F	F	M	M	M	M	M	M	M
Age, y	66	53	60	46	60	59	57	41	60	41	63	60	41
Age at onset, y	17	18	17	16	21	17	18	25	22	19	48	20	19
High pentosidine level	Yes[a]	Yes[b]	Yes[c]										
Very low pyridoxal level, <3.0 ng/mL			Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
GLO1 genotype	Ala/Ala	Glu/Glu	T27NfsX15	Glu/Glu	P122LfsX27	Glu/Glu	Glu/Ala	Glu/Glu	Glu/Ala	Glu/Glu	Ala/Ala	Ala/Ala	Glu/Ala
Enzymatic activity, mU/10^6 RBC	4	5.5	2.8	5.7	3	6.6	5.9	6.6	5.5	6.1	5.5	4.9	5.5
Pentosidine, ng/mL	276.6	172.6	137	106.6	74.7	55.8	49	47.8	46.7	43.3	42.9	40.6	40.6
Pyridoxal, ng/mL	7.3	3.4	2.8	2.4	<2.0	2.3	2.4	<2.0	2.4	2.1	2.8	<2.0	<2.0
Antipsychotics, haloperidol equivalent, mg/d	34.6	54	38	18	7	8	16	20.5	13	9	8	12.3	10.1
Minor tranquilizer, diazepam equivalent, mg/d				10				6.7		6.3			18.8
Benzodiazepine hypnotics, nitrazepam equivalent, mg/d	5	25	20	10	10		10	10	10	10	10	20	7.5
Other medications		CBZ		PB, CBZ, GBP	VPA, CLN	CBZ	VPA	CBZ	Li_2CO_3, CBZ		CLN		
Smoking	No	No	Yes	Yes	No	Yes	Yes	Yes	No	Yes	Past smoker	Yes	Yes
Duration of hospitalization, y	33	10.6	21.4	25.3	14.2	2.7	3.4	0.8	35.8	0	12	35	15
Educational background	HS	College, 2 y	HS dropout	HS	JHS	JHS	U, 8 y	JHS	JHS	JHS	JHS	JHS	College dropout
Case type			Familial	Familial				Familial	Familial	Familial			
Criminal record			Yes			Yes							

Abbreviations: CBZ, carbamazepine; CLN, clonazepam; GBP, gabapentin; HS, high school; JHS, junior high school; Li_2CO_3, lithium carbonate; PB, phenobarbital; RBC, red blood cell; U, university; VPA, sodium valproate.

[a] Patient 1 in Figure 1A.
[b] Patient 2 in Figure 1A.
[c] Patient 3 in Figure 1A.

treatment-resistant schizophrenia (with doses of antipsychotics in haloperidol equivalents of 34.8-54.0 mg/d), with more than a 20-year disease history and more than 10 years of hospitalization each (range, 10.6-33 years) (**Table 3**). Patient 3 (Figure 1A) has an elder brother who committed suicide and 2 maternal uncles, all of whom had schizophrenia; patient 3 killed his mother and exhibited violent behavior against hospital staff.

Most of the patients had been taking multiple medications; we did not control for smoking by subjects. The daily dose of medication in haloperidol equivalents was significantly correlated with plasma pentosidine level (r=0.513, P=.001) but not with serum vitamin B_6 level (r=−0.087, P=.61). The significance of correlation between pentosidine and medication dose disappeared when the data for patients 1, 2, and 3 were excluded (r=0.186, P=.29). The mean value of medication dose in the high-pentosidine group was not significantly different from that in the normal pentosidine group (17.0 mg/day [SD, 12.4 mg/day] vs 12.4 mg/day [SD, 9.1 mg/day], respectively; P=.495). No significant correlation was found between pentosidine and dose of medication (high-pentosidine group, r=0.027, P=.93; normal group, r=−0.067, P=.78). Pentosidine level in smokers was not significantly different from that in nonsmokers (smokers, 65.6 ng/mL [SD, 29.7 ng/mL]; nonsmokers, 80.3 ng/mL [SD, 60.9 ng/mL]; P=.69), nor did vitamin B_6 level differ between these groups (smokers, 5.5 ng/mL [SD, 6.4 ng/mL]; nonsmokers, 7.3 ng/mL [SD, 5.4 ng/mL]; P=.08). Plasma pentosidine and vitamin B_6 levels did not appear to be affected by confounding factors such as duration of hospitalization, since there were no correlations between biochemical data and duration of hospitalization (pentosidine, r=0.295, P=.07; vitamin B_6, r=−0.072, P=.67).

COMMENT

This study revealed that some patients with schizophrenia are predisposed to enhanced carbonyl stress. Pyridoxal is 1 of the 3 forms of vitamin B_6, ie, pyridoxine, pyridoxal, and pyridoxamine. In vivo, pyridoxamine is biosynthesized from both pyridoxal and pyridoxine. Marked decreases in serum pyridoxal levels were found in 11 schizophrenic patients, but not in the control subjects (Table 1 and Table 3). Two schizophrenic patients with heterozygous frameshift mutations displayed markedly lowered pyridoxal levels (Table 3). Depletion of pyridoxal might thus reflect elevated carbonyl stress induced by *GLO1* defects and other unknown factors in these patients. Carbonyl stress and AGEs are known to interfere with cellular functions in various fashions. First, carbonyl compounds are biologically active and initiate a variety of cellular responses.[47] Second, AGEs induce not only structural alterations in proteins, but also influence cellular functions on interaction with receptors for

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AGEs.[48] Agents able to inhibit AGE formation or entrap carbonyl compounds may also prove to be of therapeutic value, if carbonyl stress is directly linked to schizophrenic signs and symptoms. Some AGE inhibitory compounds are already clinically available (eg, angiotensin receptor blockers).[49] Others, including pyridoxamine[50] and TM2002,[51] have potent abilities to entrap toxic carbonyl compounds and prevent toxicity. In particular, the markedly lower vitamin B_6 levels in schizophrenic patients with high pentosidine levels suggest that pyridoxamine, a nontoxic, water-soluble vitamin B_6, may prove clinically useful.

To examine the molecular mechanisms underlying the carbonyl stress we observed and determine whether elevated carbonyl stress plays a causative role in schizophrenia, we performed a deep resequencing analysis of one of the target genes, *GLO1*. We focused on *GLO1*, because it is ubiquitous and because a highly active defense against glycation appears to be associated with the risk of development of various disorders,[8] though several enzymes are capable of reduction of α-dicarbonyls, eg, aldose reductase, betaine-aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase.[52] We identified rare but drastic genetic variants, 2 different heterozygous frameshift mutations, and a functional Glu111Ala polymorphism. Biochemical analyses revealed that all of these resulted in a 10% to 50% reduction in GLO1 activity in RBC and were linked to attendant biochemical abnormalities, ie, increased plasma pentosidine and decreased serum vitamin B_6. These *GLO1* genetic defects/alterations were also identified in a fraction of control subjects; though in contrast to schizophrenic patients, these controls exhibited normal pentosidine and vitamin B_6 levels, implying the existence of compensatory mechanisms, such as upregulation of other relevant enzymes. Such compensatory mechanisms might not function in schizophrenia owing to additional unknown defects. The mechanisms through which healthy subjects with *GLO1* genetic defects/alterations escape carbonyl stress are of special interest. Elucidation of such mechanisms might clarify not only the sequential events involved in the development of schizophrenia, but also provide clues to novel therapeutic approaches in patients with carbonyl stress. Collectively, our findings suggest a cross-sectional link, albeit incomplete, between *GLO1* defect–elicited carbonyl stress and a subgroup of patients with schizophrenia.

We detected 13 Ala111/Ala111 genotype carriers among 3271 Japanese subjects. The frequency of the Ala111 allele exhibits high population diversity: 0.354 to 0.475 in Europeans, 0.239 to 0.395 in African Americans, 0.267 in sub-Saharan Africans, and 0.033 to 0.125 in Asian populations. The allelic frequency of Ala111 determined in the present study is identical to that described by Thornally.[11] The high prevalence of the Ala111 allele in European and African American populations suggests the existence of a mechanism maintaining normal plasma pentosidine and serum vitamin B_6 levels, despite diminished GLO1 activity, in individuals from these populations.

We estimate that approximately 20% of patients exhibited enhanced carbonyl stress–related schizophrenia based on our biochemical analyses using as criteria both high accumulation of pentosidine (>55.2 ng/mL) and depletion of vitamin B_6 (male, <6 ng/mL; female, <4 ng/mL), as shown in eTable 4. The frequency of such individuals was estimated to be approximately 1% when the criterion was carriage of a heterozygous frameshift mutation or homozygote for Ala111.

There are possible limitations of our study. First, all patients in our study had taken medication. We could not exclude the possibility of an increase of carbonyl stress through antipsychotic medicines. We hope to clarify whether carbonyl stress is involved in psychiatric illnesses using drug-naive patients in the near future. Second, the sample size of biochemical analyses was modest. Further investigations of reciprocal relationships between pentosidine accumulation/vitamin B_6 depletion and genetic defects using large Japanese samples and individuals from different ancestral populations are needed. Third, for biochemical analyses, we arbitrarily selected molecules and cofactors affecting glyoxalase detoxification systems in vivo, as shown in eFigure 1. We thus may have missed important molecules involved in the metabolic cascades maintaining homeostasis by compensating for *GLO1* genetic defects. Fourth, we could not exclude effects of exercise on our biochemical findings, as we were unable to quantify the physical activity of patients in a systematic fashion. In future work, we plan to focus on profiling the metabolomics, genomics, and clinical manifestations of carbonyl stress–related schizophrenia with or without *GLO1* defects. Fifth, the reason why low GLO1 protein expression was observed only in patients with the Ala111/Ala111 genotype in vivo remains unclear.

In summary, our study revealed the pivotal role of carbonyl stress in some patients with schizophrenia, and subsequent intensive resequencing analysis of *GLO1* detected 2 novel frameshift mutations with loss of function and moderate-effect Glu111/Ala111 polymorphism in Japanese cohorts. Additional studies of carbonyl stress in schizophrenia may well pave the way toward novel therapeutic/preventive measures for this devastating disease.

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Author Affiliations: Project for Schizophrenia Research, Tokyo Institute of Psychiatry, Tokyo, Japan (Drs Makoto Arai, Haga, Ichikawa, Nishida, Tanaka, Furukawa, and Itokawa; and Mss Nohara, Obata, and Mayumi Arai); Institute of Medical Sciences, Tokai University, Bohseidai, Isehara, Kanagawa, Japan (Ms Yuzawa and Dr Miyata); Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan (Drs Ohnishi, Toyota, Yoshikawa, and Itokawa; and Ms Iwayama); Department of Neuropsychiatry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan (Dr Ujike); Department of Psychiatry, Tokyo Metropolitan Matsuzawa Hospital, Tokyo (Drs Aikawa, Kuroda, Niizato, Izawa, Matsushita, Okazaki, and Itokawa); Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan (Drs Nakamura and Mori); Department of Integrative Neurophysiology, Chiba University Graduate School of Medicine (Dr Matsuzawa), Division of Clinical Neuroscience, Chiba University Center for Forensic

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Mental Health (Dr Hashimoto), and Department of Psychiatry, Chiba University Graduate School of Medicine (Dr Iyo), Chiba, Japan; Department of Neuroscience, Division of Psychobiology, Tohoku University Graduate School of Medicine, Miyagi, Japan (Dr Sora); Core Research of Evolutional Science & Technology, Japan Science and Technology Agency, Tokyo (Drs Yoshikawa and Itokawa); and Center for Translational and Advanced Research on Human Disease, Tohoku University Graduate School of Medicine, Miyagi (Dr Miyata).
Correspondence: Masanari Itokawa, MD, PhD, Project for Schizophrenia Research, Tokyo Institute of Psychiatry, 2-1-8 Kamikitazawa, Setagaya, Tokyo 156-8585, Japan (itokawa-ms@igakuken.or.jp); Toshio Miyata, MD, PhD, Center for Translational and Advanced Research on Human Disease, Tohoku University Graduate School of Medicine, Miyagi 980-8575, Japan (t-miyata@mail.tains.tohoku.ac.jp).
Author Contributions: Drs Arai Makato, Miyata, and Itokawa had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
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Infusions of Allopregnanolone Into the Hippocampus and Amygdala, but not Into the Nucleus Accumbens and Medial Prefrontal Cortex, Produce Antidepressant Effects on the Learned Helplessness Rats

Yukihiko Shirayama,[1,2*] Katsumasa Muneoka,[1,3] Makoto Fukumoto,[1] Shigenori Tadokoro,[1] Goro Fukami,[1] Kenji Hashimoto,[4] and Masaomi Iyo[1]

ABSTRACT: Patients with depression showed a decrease in plasma and cerebrospinal fluid allopregnanolone (ALLO). But antidepressants increased the contents of ALLO in the rat brain. We examined the antidepressant-like effects of infusion of ALLO into the cerebral ventricle, hippocampus, amygdala, nucleus accumbens, or prefrontal cortex of learned helplessness (LH) rats (an animal model of depression). Of these regions, infusions of ALLO into the cerebral ventricle, the CA3 region of hippocampus, or the central region of amygdala exerted antidepressant-like effects. Infusion of ALLO into the hippocampal CA3 region or the central amygdala did not produce memory deficits or locomotor activation in the passive avoidance and open field tests. It is well documented that ALLO exerts its effects through GABA receptors. Therefore, we examined the antagonistic effects of flumazenil (a GABA receptor antagonist) on the antidepressant-like effects of ALLO. Coinfusion of flumazenil with ALLO into the hippocampal CA3 region, but not into the central amygdala, blocked the antidepressant-like effects of ALLO. However, coinfusion of (+)MK801 (an NMDA receptor antagonist), but not cycloheximide (a protein synthesis inhibitor), blocked the antidepressant-like effects of ALLO in the central amygdala. These results suggest that ALLO exerts antidepressant-like effects in the CA3 region of hippocampus through the GABA system and in the central region of amygdala, dependently on the activation of the glutamatergic mechanisms. © 2010 Wiley-Liss, Inc.

KEY WORDS: learned helplessness (LH); allopregnanolone (ALLO); depression; hippocampus; amygdala

[1] **Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan; [2] Department of Psychiatry, Teikyo University Chiba Medical Center, Ichihara, Japan; [3] Department of Anatomy I, Showa University School of Medicine, Tokyo, Japan; [4] Division of Clinical Neuroscience, Chiba University Center for Forensic Mental Health, Chiba, Japan**

Abbreviations used: ALLO, allopregnanolone; ANOVA, one-way analysis of variance; AP, anteroposterior; BST, bed nucleus of the stria terminalis; CSF, cerebrospinal fluid; DG, dentate gyrus; DMSO, dimethyl sulfoxide; DV, dorsoventral; HPA, hypothalamo–pituitary–adrenal; ICV, intracerebroventricular; LH, learned helplessness; MRI, magnetic resonance imaging; NAcc, nucleus accumbens core; PF, prefrontal; PVN, paraventricular nucleus.

*Correspondence to: Yukihiko Shirayama, M.D., Ph.D., Department of Psychiatry, Teikyo University Chiba Medical Center, 3426-3 Anesaki, Ichihara, Chiba 290-0111, Japan. E-mail: shirayama@rapid.ocn.ne.jp

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INTRODUCTION

Depression has multiple biological markers. Recent studies have been directed at allopregnanolone (3α5α-tetrahydroprogesterone, ALLO), a marker of depression that is one of the neurosteroids. In clinical studies, depressed patients showed lower ALLO concentrations in the plasma and cerebrospinal fluid (CSF) than controls (Uzunova et al., 1998; Ströhle et al., 1999). Furthermore, antidepressant treatments increased ALLO levels in plasma and CSF of depressed patients (Romeo et al., 1998; Uzunova et al., 1998; Ströhle et al., 1999; Schüle et al., 2006). These studies demonstrate a possible involvement of ALLO in the pathophysiology of depression.

In animal studies, the possibility that ALLO is involved in the pathophysiology of depression is supported by a previous report that ALLO levels were decreased in the amygdala, frontal cortex and hippocampus of the olfactory bulbectomized rat (an animal model of depression, Uzunova et al., 2003). Furthermore, subchronic antidepressant treatment elevated ALLO levels in the rat and mouse brain (Uzunov et al., 1996; Griffin and Mellon, 1999; Nechmad et al., 2003). These studies demonstrate the possibility that ALLO contributes to the amelioration of depression. A previous study has reported that intracerebroventricular (ICV) administration of ALLO exerted antidepressant-like effect in the forced swim test (an antidepressants screening model) (Khisti et al., 2000).

The current study examines the effects of infusion of ALLO into the hippocampus, amygdala, nucleus accumbens, and medial prefrontal cortex of learned helplessness (LH) rats (an animal model of depression) on the conditional active avoidance test. Many clinical studies using functional magnetic resonance imaging (MRI) or postpartum victims indicated that there are several limbic brain regions that have been implicated in mood disorders, including the frontal cortex, nucleus accumbens, amygdala, and hippocampus (reviewed by Sheline, 2003; McCabe et al., 2009; Sibille et al., 2009). We chose the sites for infusion

for the purpose of screening the sites where ALLO works for the treatment of stress-related depression.

MATERIALS AND METHODS

Animal and Treatments

The procedures for animal use were in accordance with the Chiba University Graduate School of Medicine Guide for the Care and Use of Laboratory Animals and were approved by the Chiba University Graduate School of Medicine Animal Care and Use Committee. Male Sprague-Dawley rats (190–220 g) were used. The animals were housed under 12-h light/dark cycle with free access to food and water.

Surgery was performed using a stereotaxic apparatus (Kopf, Tujunga, CA) under anesthesia with pentobarbital sodium solution (50 mg/kg, intraperitoneal injection, Abbott Laboratories, Abbott Park, IL), one day after the acquisition of LH. ALLO was suspended in 20% hydroxypropyl-β-cyclodextrin (CDX; Sigma, St. Louis, MO) and dissolved in 0.9% saline to yield a working ALLO solution in 2% CDX. This solvent (2% CDX) is used in controls. Rats received bilateral microinjection of different amounts of ALLO (0.5 or 0.05 μg/side), ALLO and flumazenil (a GABA receptor antagonist, 0.05 ng in 0.8% Tween 80/side), ALLO and (+)MK801 (an NMDA receptor antagonist, 2.5 μg/side), or 0.9% saline (control) into various regions of the brain. A protein synthesis inhibitor, cycloheximide (10 μg in 5% DMSO/side) was infused bilaterally 20 min before the infusion of ALLO. The solvent (5% DMSO) is used as controls. A total volume of 1.0 μl was infused into each side over 15 min, and the injection syringe was left in place for an additional 5 min to allow for diffusion. The coordinates for the cerebral ventricle, dentate gyrus and CA3 region of the hippocampus (HIPdg, HIPca3), central and basolateral regions of the amygdala (CeA, BLA), nucleus accumbens core (NAcc), and medial prefrontal cortex (mPF) relative to bregma, according to the atlas of Paxinos and Watson (1997) were as follows: −0.3 anteroposterior (AP), ±1.2 lateral, −3.4 dorsoventral (DV) from dura (cerebral ventricle); −3.8 AP, ±2.0 lateral, −3.2 DV from dura (HIPdg); −3.6AP, ±3.6 lateral, −2.8 DV from dura (HIPca3); −2.3AP, ±4.0 lateral, −7.7 DV from dura (CeA); −2.8AP, ±4.8 lateral, −7.4 DV from dura (BLA); +2.2AP, ±1.6 lateral, −6.7 DV from dura (NAcc); +3.2AP, ±0.6 lateral, −2.8 DV from dura (mPF). The placements of injection cannula in the brain are shown in Figure 1.

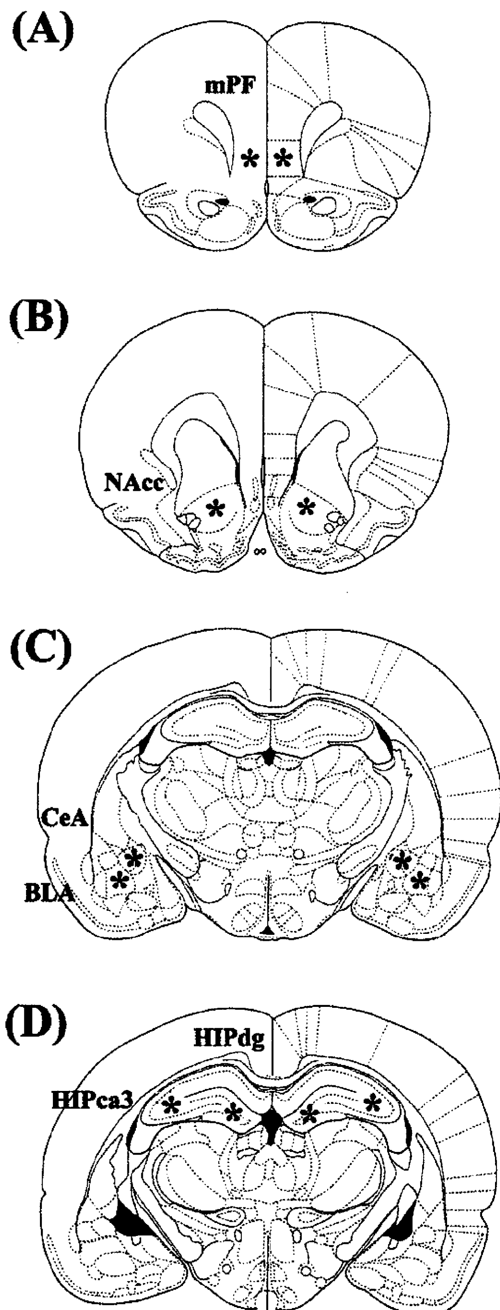

FIGURE 1. A schematic representation of microinjection sites within the medial prefrontal cortex (A), the core region of nucleus accumbens (B), the dentate gyrus and CA3 region of hippocampus (C), and the central and basolateral regions of amygdala (D). Abbreviations: mPF, medial prefrontal cortex; NAcc, nucleus accumbens core; HIPdg, dentate gyrus of hippocampus; HIPca3, CA3 region of hippocampus; BLA, basolateral amygdala; CeA, central amygdala.

LH Paradigm

To create the LH paradigm, animals are initially exposed to uncontrollable stress. When the animal is later placed in a situation in which shock is controllable (escapable), it not only fails to acquire the escape responses, but also often makes no efforts to escape the shock at all (Overmier and Seligman, 1967). This escape deficit is reversed by subchronic antidepressant treatment (Shirayama et al., 2002; Iwata et al., 2006).

Learned helplessness behavioral tests were performed using the Gemini Avoidance System (San Diego Instruments, San Diego, CA). This apparatus was divided into two compartments by a retractable door. On days 1 and 2, rats were subjected to 30 inescap-

able electric footshock [0.65 mA, 30-s duration at random intervals (mean 30 s, average 18–42 s)]. On day 3, a two-way conditioned avoidance test was performed as a postshock test to determine if the rats would show the predicted escape deficits. This screening session consisted of 30 trials in which electric foot shocks [0.65 mA, 6-s duration at random intervals (mean 30 s, average 18–42 s)] were preceded by a 3 s conditioned stimulus tone that remained on until the shock was terminated. Rats with more than 25 escape failures in the 30 trials were regarded as having reached criterion and were used for further experiments. Approximately 65% of the rats met this criterion. On day 4, rats received bilateral microinjections of ALLO and/or other chemicals (flumazenil, (+)MK-801, cycloheximide) as described above. On day 8 (4 days after surgery), a two-way conditioned avoidance test was performed. This test session consisted of 30 trials in which electric foot shocks [0.65 mA, 30-s duration at random intervals (mean 30 s, average 18–42 s)] were preceded by a 3 s conditioned stimulus tone that remained on until the shock was terminated. The numbers of escape failures and the latency to escape in each 30 trial were recorded by the Gemini Avoidance System.

For antidepressant treatment, imipramine (20 mg/kg, once per day) or saline (0.9%) was administered i.p. for 12 days after the postshock screening test until 1 day before the conditioned avoidance test.

Open Field Test

Four days after surgery, an open field test was performed in a square area (76.5 × 76.5 × 49 cm^3) using a standard procedure (Lacroix et al., 1998). The open field was divided into two areas, a peripheral area and a square center (40 × 40 cm^2). The test room was dimly illuminated (60 W light, indirect). Rats were allowed to explore for 30 min. A computer software program (Be Trace: Behavioral and Medical Sciences Research Consortium, Hyogo, Japan) calculated the velocity of movement, the distance of travel, and the time spent in the center of the open field. These parameters are thought to reflect locomotor activity and fear or anxiety, respectively.

Passive Avoidance Test

The passive avoidance test was conducted according to standard procedures with the following modifications (Ferry et al., 1999). The apparatus was divided into two compartments by a retractable door: a lighted safe compartment and a darkened shock compartment (Gemini Avoidance System). Four days after surgery, the test animal received a single inescapable foot shock (0.80 mA; 4 s duration). Twenty-four hours later, each rat was placed in the lighted safe compartment and the latency until reentry into the darkened shock compartment was recorded as the measure of retention.

Statistical Analysis

Statistical differences among more than three groups were estimated by a one-way analysis of variance (ANOVA), followed by Tukey's test. For comparison of the mean values between the two groups, statistical evaluation was done using the two-tailed Student's t-test. The criterion of significance was $P < 0.05$.

RESULTS

Effects of ALLO Infusion Into the Cerebral Ventricle or Other Regions of LH Rat Brain

Subchronic treatment of LH rats with the tricyclic antidepressant imipramine produced a significant improvement in the conditioned avoidance test (Fig. 2A). This demonstrates that the LH paradigm is responsive to antidepressant treatment, as reported previously (Shirayama et al., 2002; Iwata et al., 2006).

LH rats that received bilateral microinjections of ALLO into the cerebral ventricle demonstrated a significant improvement in the conditioned avoidance test relative to saline-treated controls (Fig. 2B). Infusion of ALLO into the infralimbic prefrontal cortex or nucleus accumbens core failed to produce the antidepressant-like effects (Figs. 2C,D).

LH rats that received bilateral microinjection of ALLO into the CA3 region, but not the dentate gyrus, of the hippocampus demonstrated a significant improvement in the conditioned avoidance test relative to saline-treated controls (Figs. 2E,F). Infusion of ALLO into the central, but not the basolateral, region of the amygdala of LH rats significantly decreased escape failure in the conditioned avoidance test (Figs. 2G,H).

Effect of Infusion of ALLO Into CA3 Region of Hippocampus or Central Region of Amygdala on Locomotor Activity or Passive Avoidance

Infusions of ALLO into the CA3 region of the hippocampus or the central region of the amygdala failed to affect the time spent in the center, distance traveled, or velocity in the open field test (Figs. 3A,B). This would not be the result expected if a general increase in locomotor activity contributed to the effect of ALLO on conditioned avoidance in the LH models of depression.

Infusions of ALLO into the CA3 region of the hippocampus or the central region of the amygdala did not alter the length of time spent in the darkened compartment in consecutive retention tests (Figs. 3C,D). These results suggest that ALLO infusions do not cause a deficit in learning, which could result in the effects observed in the LH paradigms.

Effects of Coadministration of GABA Antagonist Flumazenil With Infusion of ALLO Into CA3 Region of Hippocampus or Central Region of Amygdala of LH Rats

It is well documented that ALLO exerts its effects through GABA receptors. Coadministration of flumazenil (a GABA receptor antagonist) and ALLO into the CA3 region of the hippocampus blocked the antidepressant-like effects of ALLO (Fig. 4A), whereas coadministration of flumazenil and ALLO

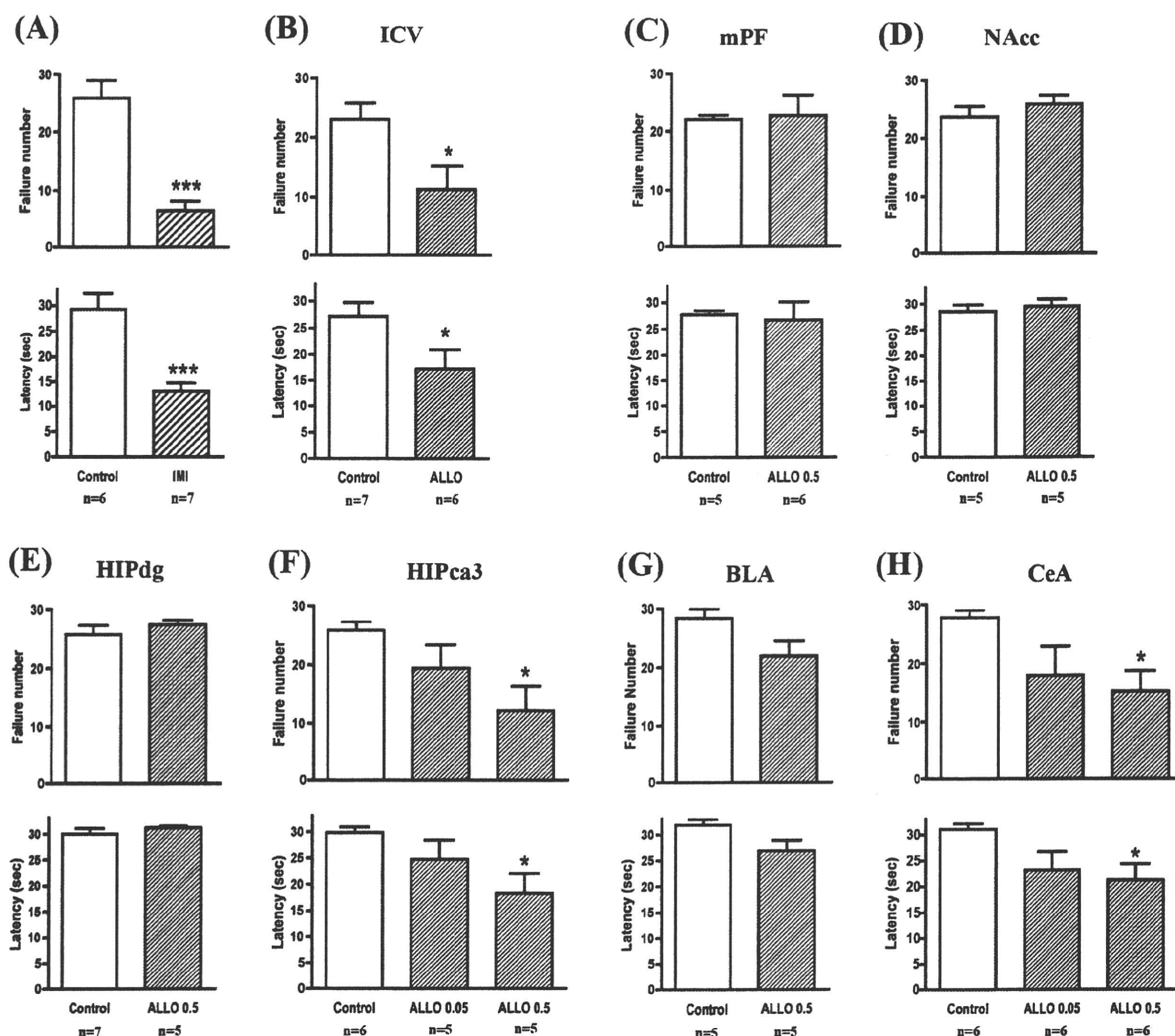

FIGURE 2. Influence of ALLO infusion into the brain on the LH paradigm. Imipramine administration (12 days) for comparison (A). ALLO or saline was administered via bilateral infusion into the cerebral ventricle (B), the medial prefrontal cortex (C), the nucleus accumbens core (D), the dentate gyrus of hippocampus (E), the CA3 region of hippocampus (F), the basolateral amygdala (G), or the central amygdala (H). Animals were subjected to conditioned avoidance test 4 days later. Escape failure and latency to escape were determined. The results are expressed as mean ± standard error of mean (SEM). The number of animals is listed under each column. (A) Top, t = 5.668, P = 0.0001; bottom, t = 4.830, P = 0.0005; (B) top, t = 2.517, P = 0.0286; bottom, t = 2.288, P = 0.0429; (C) top, t = 0.168, P = 0.8698; bottom, t = 0.258, P = 0.8020; (D) top, t = 0.905, P = 0.3915; bottom, t = 0.535, P = 0.6067; (E) top, t = 1.206, P = 0.2585; bottom, t = 0.8648, P = 0.4074; (F) top, F (2,13) = 4.560, P = 0.0316; bottom, F (2,13) = 4.076, P = 0.0422; (G) top, t = 2.150, P = 0.0638; bottom, t = 2.163, P = 0.0625; (H) top, F (2,14) = 4.028, P = 0.0415; bottom, F (2,14) = 3.862, P = 0.0462. *$P < 0.05$; ***$P < 0.001$ when compared with saline-injected controls (Student's t-test or ANOVA followed by Tukey's test). Abbreviations: ICV, cerebral ventricle; mPF, medial prefrontal cortex; NAcc, nucleus accumbens core; HIPdg, dentate gyrus of hippocampus; HIPca3, CA3 region of hippocampus; BLA, basolateral amygdala; CeA, central amygdala.

into the central region of the amygdala failed to block the antidepressant-like effects of ALLO (Fig. 4B). Infusion of flumazenil alone into the hippocampal CA3 region failed to exert any effects on the conditioned avoidance test (Fig. 4A). This indicates that ALLO exerts antidepressant-like effects through GABA receptors in the CA3 region of the hippocampus, but independent of the GABA system in the central region of the amygdala.

Effects of Coadministration of NMDA Antagonist (+)MK-801 With Infusion of ALLO Into Central Region of Amygdala of LH Rats

Coadministration of (+)MK-801 (an NMDA receptor antagonist) and ALLO into the central region of the amygdala blocked the antidepressant-like effects of ALLO (Fig. 4C). Infusion of (+)MK-801 alone into the central amygdala failed to exert any effects on the conditioned avoidance test (Fig. 4C).

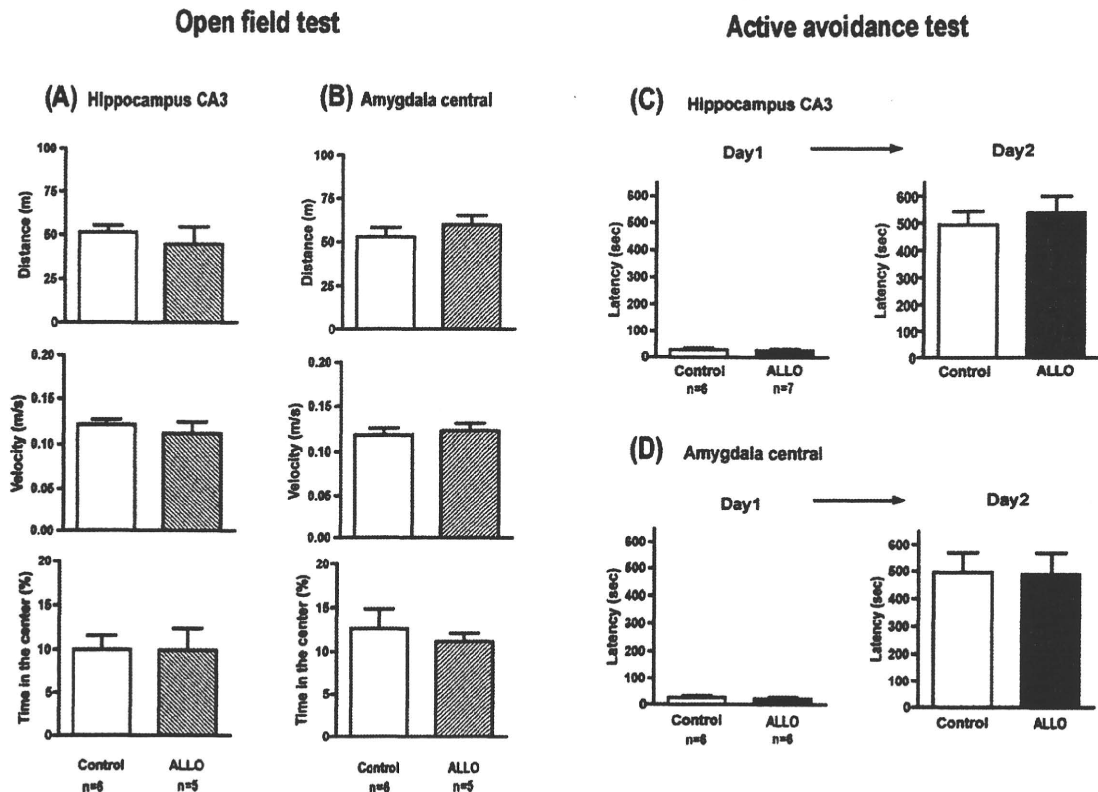

FIGURE 3. Influences of ALLO infusion into the hippocampus or amygdala on locomotor activity or passive avoidance. ALLO or saline was infused into the CA3 of hippocampus (A, C) or central amygdala (B, D), and 4 days later, the distance traveled, velocity, and time spent in center in an open field were determined or passive avoidance test was conducted. The results are the mean ± SEM of the number of animals indicated under each column. (A) Top, distance, $t = 0.6821$, $P = 0.5124$; middle, velocity, $t = 0.7700$, $P = 0.4610$; bottom, time in the center, $t = 0.0238$, $P = 0.9815$. (B) Top, distance, $t = 0.9464$, $P = 0.3687$; middle, velocity, $t = 0.4033$, $P = 0.6961$; bottom, time in the center, $t = 0.5686$, $P = 0.5835$. (C) Day 1, $t = 0.2849$, $P = 0.7810$; Day 2, $t = 0.5328$, $P = 0.6047$. (D) Day 1, $t = 0.4395$, $P = 0.6696$; Day 2, $t = 0.0753$, $P = 0.9414$.

This indicates that ALLO exerts antidepressant-like effects through the activation of glutamatergic mechanisms in the central region of the amygdala.

Effects of Preinfusion of Protein Synthesis Inhibitor Cycloheximide on Antidepressant-Like Effects of ALLO Infusion Into Central Amygdala

Preadministration of cycloheximide (a protein synthesis inhibitor) failed to block the antidepressant-like effects of ALLO in the central region of the amygdala (Fig. 4D). This indicates that ALLO does not produce antidepressant-like effects through a genomic-based mechanism.

DISCUSSION

The primary finding of the present study is that infusions of ALLO into the cerebral ventricle, CA3 region of hippocampus, or the central region of amygdala, but not into the dentate gyrus of hippocampus, basolateral region of amygdala, nucleus accumbens, and medial prefrontal cortex, produced antidepressant-like effects in LH rats, an animal model of depression. Thus, the antidepressant action of ALLO on brain is the region-specific.

The open field test did not demonstrate any significant differences in distance traveled, velocity, or time spent in the center, suggesting that the antidepressant-like effects of ALLO did not contribute to enhanced locomotion or modulation of anxiety. Furthermore, the results of the passive avoidance test did not show any differences between the ALLO and control groups, indicating that the antidepressant-like effects of ALLO did not contribute to the memory deficits. Thus, the antidepressant-like effects of ALLO were not because of nonspecific actions.

It is noteworthy that infusions of ALLO into the central amygdala as well as into the CA3 region of hippocampus exerted antidepressant effects. Previous studies showed that chronic administration of antidepressant drugs increased the number of Fos-positive neurons in the central nucleus of the amygdala (Duncan et al., 1996; Veening et al., 1998; Morelli et al., 1999). Other studies demonstrated that uncontrollable

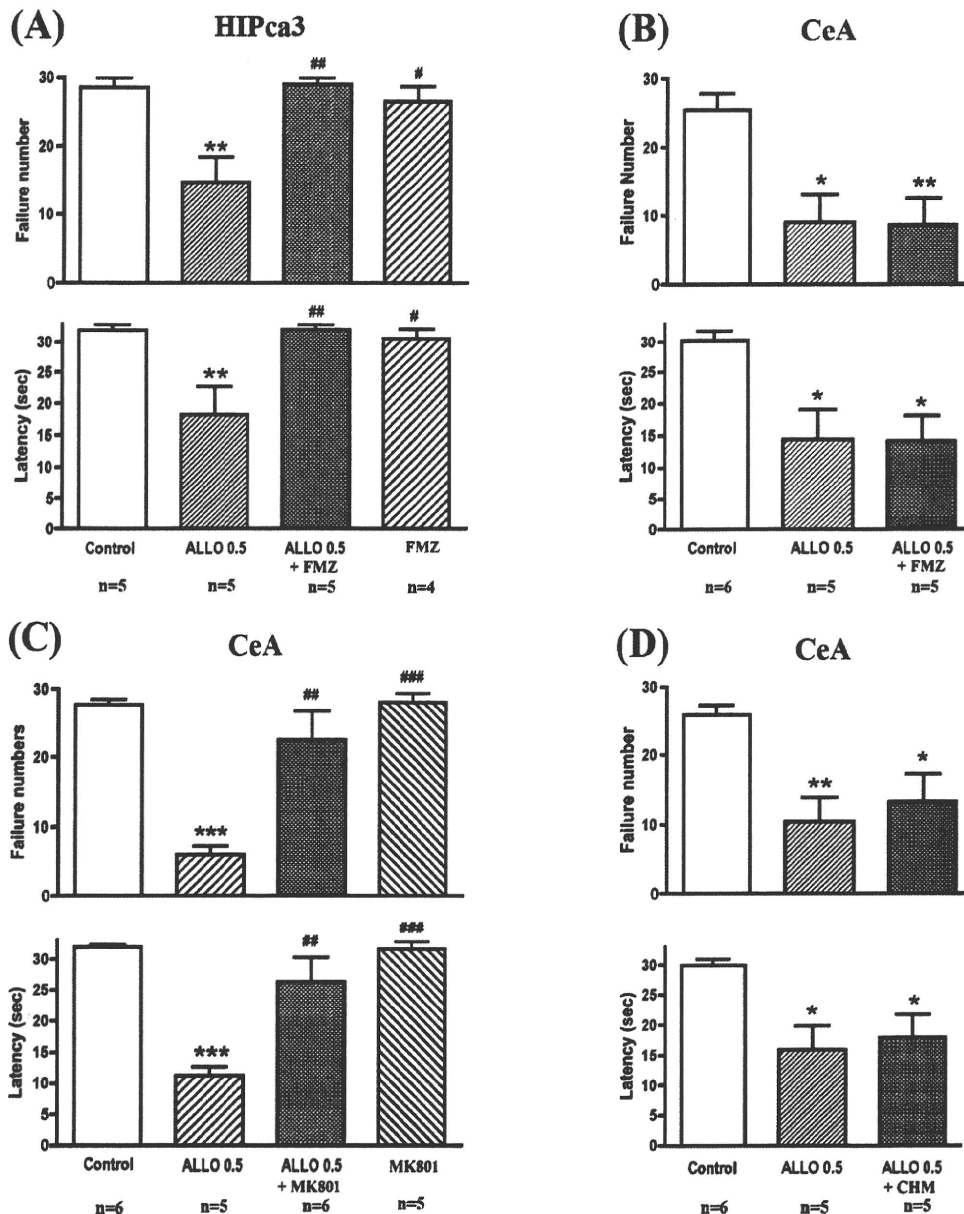

FIGURE 4. Influence of flumazenil (GABA receptor antagonist), (+)MK-801 (NMDA receptor antagonist), or cycloheximide (protein synthesis inhibitor) on the antidepressant-like effects of ALLO infusions into the hippocampus or amygdala in the LH paradigm. Coinfusion of flumazenil blocked the antidepressant-like effects of ALLO in the CA3 region of hippocampus (A), but not in the central amygdala (B). Coinfusion of (+)MK-801 (C), but not cycloheximide (D), blocked the antidepressant-like effects of ALLO infusions into the central amygdala in the LH paradigm. Animals were subjected to conditioned avoidance test 4 days later. Escape failure and latency to escape were determined. The results are expressed as mean ± SEM. The number of animals is listed under each column. (A) Top, F (3,15) = 8.653, P = 0.0014; bottom, F (3,15) = 7.066, P = 0.0035. (B) Top, F (2,13) = 8.579, P = 0.0042; bottom, F (2,13) = 7.591, P = 0.0065. (C) Top, F (3,18) = 16.07, P < 0.0001; bottom, F (3,18) = 15.61, P < 0.0001. (D) Top, F (2,13) = 9.053, P = 0.0040; bottom, F (2,13) = 7.254, P = 0.0086. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ when compared with saline-injected animals; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ when compared with the ALLO-injected group (ANOVA followed by Tukey's test). Abbreviations: HIPca3, CA3 region of hippocampus; CeA, central amygdala; FMZ, flumazenil; CHM, cycloheximide.

stress (a kind of learned helplessness) caused extracellular signal-regulated kinase phosphorylation in the hippocampus, basolateral, and central amygdala (Yang et al., 2008), and that the central amygdala of Flinders sensitive line rats (a genetic animal model of depression) did not respond to acute restraint stress without increased mRNA expression of corticotropin-releasing hormone (Zambello et al., 2008). ALLO levels were decreased in the amygdala, frontal cortex, and hippocampus of olfactory

bulbectomized rat (an animal model of depression) (Uzunova et al., 2003). Taken together, the findings indicate that the central amygdala and hippocampus could be involved in the pathophysiology of depression and the mechanism of antidepressant effects of ALLO.

The second finding is that coinfusion of flumazenil (a GABA receptor antagonist) with ALLO into the hippocampal CA3 region blocked the antidepressant-like effects of ALLO. ALLO is a positive allosteric modulator of the $GABA_A$ receptor, and thus this finding indicates that ALLO exerts antidepressant-like effects through $GABA_A$ receptors in the CA3 region of the hippocampus. Forced swim stress increased ALLO expression in the brain and plasma (Purdy et al., 1991) and decreased function of $GABA_A$ receptors in the rat brain (Drugan et al., 1989). This increase in ALLO levels has been postulated as a homeostatic mechanism to restore decreased GABA system functions. In contrast to acute stress, social isolation induced decreases in both ALLO levels and $GABA_A$ receptor function in the cortex and hippocampus of rats (Serra et al., 2000). Chronic stress altered mRNA expression of glutamate decarboxylase, a GABA synthesizing enzyme, in the hippocampus (Bowers et al., 1998) and chronic exposure to stress levels of corticosterone altered GABAergic function in the hippocampus of rats (Orchinik et al., 1995). Social isolation decreased 5α-reductase, an enzyme necessary for ALLO synthesis, in the hippocampus, amygdala, and prefrontal cortex of mouse (Agís-Balboa et al., 2007). It is likely that long-term mild stress finally decreases ALLO levels, which follow the decreased $GABA_A$ receptor function. Therefore, it may be that ALLO exerted antidepressant effects by compensating for the reduction of ALLO and restoring GABA function in the hippocampus of the LH rats. An alternative hypothetic mechanism might be that ALLO attenuates GABAergic neurotransmission through $GABA_A$ receptors on the GABAergic interneurons, in turn enhancing the glutamatergic transmission in the CA3 region of the hippocampus. Further study will be needed to elucidate the involvement of GABAergic interneurons in the antidepressant-like effects of ALLO in the hippocampus.

The third finding is that coinfusion of flumazenil (a $GABA_A$ receptor antagonist) with ALLO into the central amygdala failed to block the antidepressant-like effects of ALLO. This suggests that the $GABA_A$ receptors involved in the antidepressant-like effects of ALLO infusions into the central amygdala are insensitive to benzodiazepine modulation because flumazenil works as a benzodiazepine-sensitive $GABA_A$ receptor antagonist. It was reported that chronic administration of ALLO increases expression of the α4 subunit of the $GABA_A$ receptor, whereas α4 subunit-containing $GABA_A$ receptors are insensitive to benzodiazepine modulation (Smith et al., 2007). Hence, the antidepressant-like effects of ALLO in the central amygdala might be mediated by benzodiazepine-insensitive α4 subunit-containing $GABA_A$ receptor. Otherwise, the benzodiazepine-insensitive $GABA_A$ receptor might be exprasynaptic δ subunit-containing $GABA_A$ receptor (Lambert et al., 2009). Therefore, it remains possible that ALLO exerts its effects through benzodiazepine-insensitive $GABA_A$ receptors in the central amygdala.

Furthermore, coinfusion of (+)MK-801 (an NMDA receptor antagonist) with ALLO into the central amygdala blocked the antidepressant-like effects of ALLO. Since the anatomical fact showed that the central region of amygdala receives glutamatergic projections from the basolateral region of amygdala and sends the outputs to the brainstem and hypothalamus (Pitkanen et al., 1997), the antidepressant-like effects of ALLO could be dependent on the activation of glutamatergic mechanisms. It was reported that NMDA receptors could mediate regular synaptic transmission in GABAergic interneurons in the lateral amygdala (Szinyei et al., 2003). An electrophysiological study has demonstrated that ALLO reduces $GABA_A$ receptor-mediated inhibitory postsynaptic currents in the central nucleus of the amygdala via an NMDA receptor-mediated mechanism (Wang et al., 2007). Although direct effects of ALLO on glutamatergic receptors have never been reported, other sulfated neurosteroids have modulatory effects on the NMDA receptors (Elfverson et al., 2008; Sedlacek et al., 2008). These support the assumption that $GABA_A$ receptor function is modulated by the NMDA receptor in the central amygdala.

The present result cannot be simply attributed to an amnesiac effect because of an enhancement of GABAergic function by ALLO because memory function was intact in the passive avoidance test (Fig. 3). Moreover, ALLO exerted not only memory-inhibitory action (Ladurelle et al., 2000; Johansson et al., 2002) but also memory-facilitatory action, probably with relation to glutamatergic systems (Cheney et al., 1995). Further study will be needed to elucidate the relevance of memory modulation by ALLO to the antidepressant-like effects of ALLO in the LH rat.

LH has a kind of matter of learning and extinction, which may be related to the function of the hippocampus and amygdala. It could be that LH rats have a difficulty in starting new reasonable behaviors because of obsession by past experience (Overmier and Seligman, 1967). The matter of memory in the LH rats may be consistent with the memory deficits in human depression. ALLO was found to inhibit the glucocorticoid receptor-mediated gene transcription *in vitro* (Basta-Kaim et al., 2007). Therefore, the gene transcription in the central amygdala induced by increased glucocorticoid during the process attaining LH might be the cause of depression. Learning requires long-term memory, which needs gene transcription. However, the present study showed that preadministration of cycloheximide (a protein synthesis inhibitor) into the central amygdala failed to block the antidepressant-like effects of ALLO infusion into the central amygdala of LH rats, indicating that the antidepressant-like effects of ALLO were not exerted through the gene transcription in the central amygdala.

The preferable mechanism is that bed nucleus of the stria terminalis (BST) is a switching relay point because BST has the outputs from both stress-inhibitory structure hippocampus and stress-excitatory region CeA (reviewed by Ulrich-Lai and Herman, 2009). In accordance with this, the effects of ALLO were blocked by antagonism of GABAergic system in the CA3 of hippocampus and antagonism of glutamatergic system in the CeA. The paraventricular nucleus of the hypothalamus (PVN)

receives projections from brainstem, BST, and CeA and participates in hypothalamo–pituitary–adrenal (HPA) activation. Thus, BST and PVN could be key modulators for HPA activation to stress conditions driven by LH, although this is speculation. Otherwise, the central nucleus of the amygdala sends projections to the VTA, locus coeruleus, and dorsal raphe (Wallace et al., 1992; Gonzales and Chesselet, 1990). The precise mechanism remains to be elucidated.

In summary, infusion of ALLO into the CA3 region of hippocampus or the central region of amygdala produced antidepressant-like effects in LH rats. Coadministration of flumazenil (a GABA receptor antagonist) with ALLO into the CA3 region of the hippocampus, but not into the central amygdala, blocked the effect of ALLO. However, coinfusion of (+)MK801 (an NMDA receptor antagonist) with ALLO into the central amygdala blocked the antidepressant-like effects. Finally, infusion of ALLO into the amygdala or hippocampus did not alter memory ability or locomotion. These results demonstrate that the antidepressant-like effects of ALLO in the CA3 region of hippocampus are produced through the GABA system, and in the central amygdala the effects are contingent on activation of the glutamatergic mechanisms.

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Effects of etizolam and ethyl loflazepate on the P300 event-related potential in healthy subjects

Goro Fukami[1*], Tasuku Hashimoto[1], Yukihiko Shirayama[1], Tadashi Hasegawa[1], Hiroyuki Watanabe[1], Mihisa Fujisaki[1], Kenji Hashimoto[2], Masaomi Iyo[1]

Abstract

Background: Benzodiazepines carry the risk of inducing cognitive impairments, which may go unnoticed while profoundly disturbing social activity. Furthermore, these impairments are partly associated with the elimination half-life (EH) of the substance from the body. The object of the present study was to examine the effects of etizolam and ethyl loflazepate, with EHs of 6 h and 122 h, respectively, on information processing in healthy subjects.

Methods: Healthy people were administered etizolam and ethyl loflazepate acutely and subchronically (14 days). The auditory P300 event-related potential and the neuropsychological batteries described below were employed to assess the effects of drugs on cognition. The P300 event-related potential was recorded before and after drug treatments. The digit symbol test, trail making test, digit span test and verbal paired associates test were administered to examine mental slowing and memory functioning.

Results: Acute administration of drugs caused prolongation in P300 latency and reduction in P300 amplitude. Etizolam caused a statistically significant prolongation in P300 latency compared to ethyl loflazepate. Furthermore, subchronic administration of etizolam, but not ethyl loflazepate, still caused a weak prolongation in P300 latency. In contrast, neuropsychological tests showed no difference.

Conclusions: The results indicate that acute administration of ethyl loflazepate induces less effect on P300 latency than etizolam.

Background

Benzodiazepines have anxiolytic, sedative, anticonvulsant and myorelaxant properties, and have been widely prescribed in various clinical settings. These compounds, however, also induce adverse effects such as oversedation, cognitive impairment, motor impairment and withdrawal. These adverse effects may be partly associated with the elimination half-life (EH) of the compounds from the body; that is, long-term use of the compounds with a short elimination rate may induce withdrawal syndromes, whereas accumulation-related effects of a long elimination rate may include oversedation, cognitive dysfunction and motor impairment [1-4].

It has been observed previously that cognitive impairment induced by benzodiazepines may go unnoticed while profoundly disturbing social activity [5]. Therefore, it is clinically very important to take note of the cognitive effects of benzodiazepines. In order to assess the effects of benzodiazepines on cognition, the event-related potential (ERP), P300, may be useful [6], as well as neuropsychological tests. The P300 components of ERP are elicited by an auditory oddball paradigm in which a subject detects infrequent task-relevant stimuli randomly presented among frequent stimuli. P300 reflects stimulus context and stimulus meaning [7]. P300 components are associated with cognitive processes such as attention, memory, orientation and evaluation. Relationships between P300 and neuropsychological function have been reported [8-14]. Benzodiazepine anxiolytic drugs, as well as benzodiazepine hypnotic drugs, have been reported to induce reductions in P300 amplitude and prolongation in P300 latency [6,15-20].

As far as is known, however, there are no reports on the effects of chronic or subchronic administration of benzodiazepines on cognition and P300 from the viewpoint of elimination rates. Here, we studied the effects

* Correspondence: fukami@faculty.chiba-u.jp
[1]Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan
Full list of author information is available at the end of the article

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of anxiolytic benzodiazepines on neuropsychological functions and P300 components of auditory ERP under acute and subchronic administration of ethyl loflazepate and etizolam. Ethyl loflazepate is a potent, non-sedative, anxiolytic drug with a long EH of 122 h [21], whereas etizolam is characteristic of a potent antianxiety and sedative drug with a short EH of 6 h [22]. Therefore, etizolam is often used as a sleep inducer. However, it is well known that benzodiazepine drugs including etizolam and ethyl loflazepate have the effects of reducing the deep sleep stage 3, resulting in loss of good sleep. The aim of the present study is to examine whether sedative or anxiolytic actions of benzodiazepines have some effects on ERP and neuropsychological tests.

Methods

Study design and subjects

All subjects had normal acoustic function and were right handed. The ethics committee of Chiba University Graduate School of Medicine approved the experiments. Subjects were free from treatment for past psychiatric illness. Written informed consent was obtained after the procedure had been fully explained.

In the acute experiment, 10 healthy men (n = 5) and women (n = 5) ranging in age from 16 to 38 (average age 28.6 (SD 6.5)) participated in the study. First, all subjects were measured for the P300 components of ERP and received neuropsychological tests. Then they took etizolam (1 or 2 mg, orally). Then, 2 h later, the same ERP and neuropsychological tests were performed, since the blood concentration of the drugs reaches a maximum 1-2 h after consumption. After a 2-week washout period, the same experimental procedures were repeated, but subjects took ethyl loflazepate (1 or 2 mg, orally).

In the subchronic experiment, 17 healthy men (n = 8) and women (n = 9) ranging in age from 22 to 34 (average age 27.4 (SD 4.1)) participated in the study. The 17 subjects were divided into 2 groups: the first group was given etizolam (1 mg, orally, for 14 days), and the second given ethyl loflazepate (1 mg, orally for 14 days). Subjects were asked to take drugs in the evening every day, and performed ERP recording and neuropsychological tests 14-20 h after taking the last drug. Subjects performed ERP recording and neuropsychological tests twice before and after subchronic treatment with etizolam or ethyl loflazepate.

Doses examined in the present study were chosen based on the equivalent conversion table for anxiolytic drugs (5 mg of diazepam, 1.5 mg of etizolam, and 1.67 mg of ethyl loflazepate) [23,24].

ERP procedure

Electroencephalogram electrodes were attached at Fz, Cz and Pz according to the international 10-20 system. Earlobe electrodes were linked for reference. Electrooculography was also recorded from vertical and lateral derivations to check ocular artefacts. Subjects sat on a semi-reclined chair in a sound-attenuated and electrically shielded room during recordings. Subjects were instructed to press a button as quickly as possible upon hearing the infrequent high-pitched tones. Event-related potentials were recorded under an oddball paradigm. The stimuli consisted of a 1,000 Hz tone burst (frequent non-target stimulus) and a 2,000 Hz tone burst (rare target stimulus). In each paradigm, 200 stimuli were presented through bilateral earphones by using a Neuropack 10 (MEB-2200, Nihon Kohden, Tokyo, Japan). The ratio of the rare versus frequent stimuli was 0.25. Stimuli were presented in a random order, the duration of each stimulus being 120 ms, with rise and fall times of 10 ms. The intensity was 40 dB for all stimuli. The interstimulus interval was 1.5 s.

Neuropsychological tests

The trail making test consists of two parts [25]. In part A, subjects are asked to draw lines connecting 25 consecutively numbered circles on a worksheet. In part B, they draw lines connecting 25 consecutively numbered and lettered circles, alternating between the sequences (for example, 1-A-2-B-3 and so on). Part A examines psychomotor speed and attention. Part B examines set alternation or divided attention.

The digit symbol modalities test is a measure of switching attention [26]. Subjects are asked to identify nine different symbols corresponding to the numbers 1 through 9, and write the correct number under the corresponding symbol. Thus, visual shifting and pairing of specific digits is directed, with a set of prespecified symbols.

The forward digit span test is a measure of simple attention, immediate memory and attentional control processing. In contrast, backward digit span is not only a test of attentional control processing but also working memory test.

The verbal paired associates test from the Wechsler Memory Scale-Revised (WMS-R) is a cued recall test of verbal memory [27]. Subjects learned a list of eight verbal paired associates. Then, either immediately or after a delay, the examiner says one word of each pair and the subjects recall the other word. Three sets of immediate memory testing and one set of delayed recall testing were administered.

Statistical analysis

Two-way repeated measures analysis of variance (ANOVA) was performed to assess the overall differences between variables. Where a significant interaction in the within-subject variables was found, subsequent one-way

ANOVA was carried out among more than three groups by a *post hoc* comparison using Fisher's protected least significant difference test. For comparison of the mean values between the two groups, statistical evaluation was performed using the two-tailed Student's t test. The significance level was set at $P < 0.05$.

Results

Effects of acute treatments with etizolam and ethyl loflazepate on P300

For acute drug treatment on the P300, two-way repeated ANOVA indicated significant effects of treatment (Fz, F (1,16) = 49.397, $P < 0.0001$; Cz, F (1,16) = 59.022, $P < 0.0001$; Pz, F (1,16) = 45.623, $P < 0.0001$), but not effects of group, on latency, with a significant interaction (Fz, treatment × group, F (3,16) = 3.846, $P = 0.0301$; Cz, treatment × group, F (3,16) = 3.436, $P = 0.0423$; Pz, treatment × group, F (3,16) = 3.278, $P = 0.0483$) (Figure 1a-c). The subsequent one-way ANOVA on the changes of P300 latency indicated significant differences (Fz, F (3,16) = 3.431, $P = 0.0425$; Cz, F (3,16) = 3.436, $P = 0.0423$; Pz, F (3,16) = 3.387, $P = 0.0441$), and the *post hoc* comparison using Fisher's protected least significant difference test indicated the following: ethyl loflazepate 1 mg has less effects than etizolam 1 mg and etizolam 2 mg in Fz (Figure 1g), Cz (Figure 1h), and Pz (Figure 1i). For amplitude, two-way repeated ANOVA indicated significant effects of treatment (Cz, F (1,16) = 7.967, $P = 0.0123$; Pz, F (1,16) = 8.807, $P = 0.0091$; but see Fz, F (1,16) = 4.032, $P = 0.0618$), but not effects of group, without a significant interaction (Figure 1d-f). This seems to reflect that benzodiazepine reduced P300 amplitude. Although the subsequent one-way ANOVA on the changes of P300 amplitude revealed no significant difference among drug groups (Figure 1j-l), the magnitude of changes showed that etizolam (2 mg) produced a trend in reduction of amplitude.

Effects of subchronic treatments with etizolam and ethyl loflazepate on P300

For subchronic drug treatment on the P300, two-way repeated ANOVA indicated significant effects of treatment region specifically (Fz, F (1,15) = 7.734, $P = 0.0140$; but see Cz, F (1,15) = 2.391, $P = 0.1491$; Pz, F (1,15) =

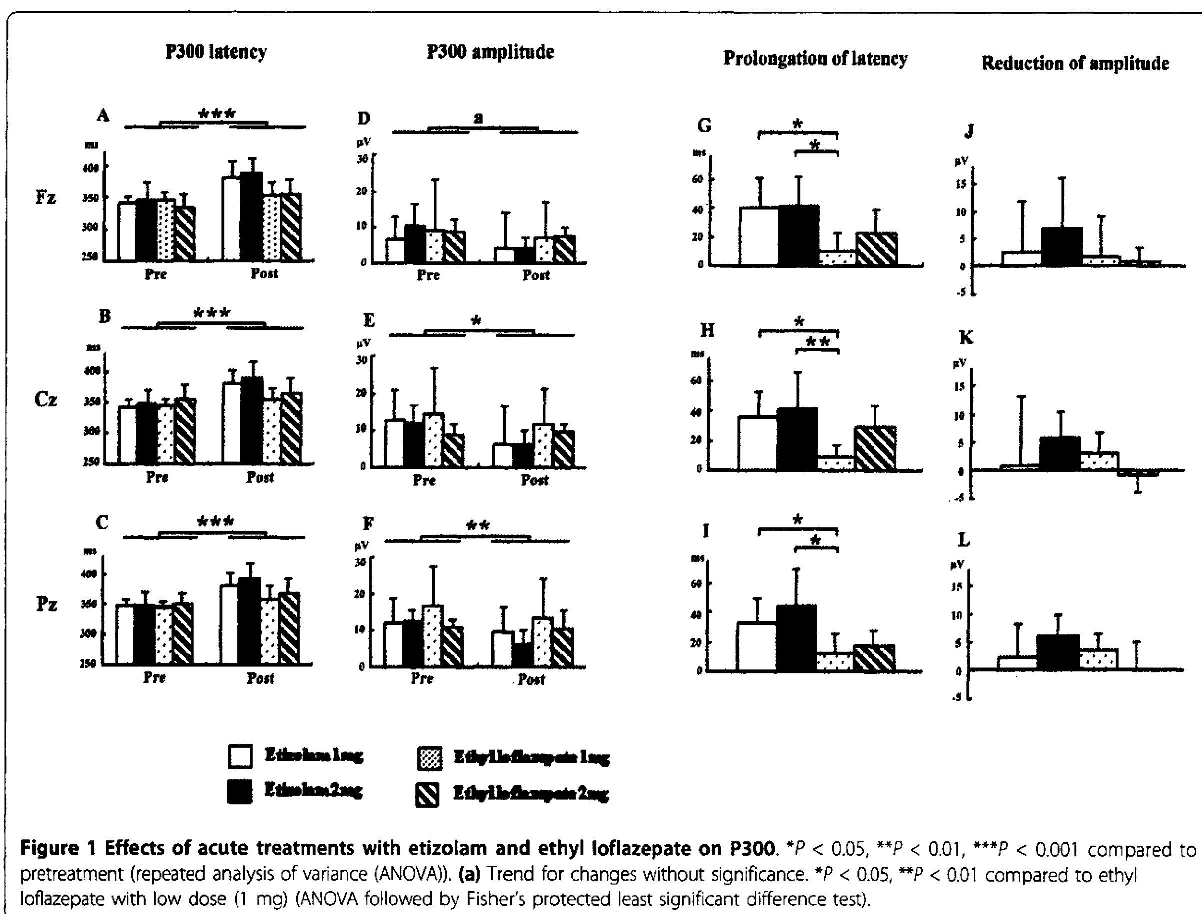

Figure 1 Effects of acute treatments with etizolam and ethyl loflazepate on P300. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to pretreatment (repeated analysis of variance (ANOVA)). **(a)** Trend for changes without significance. *$P < 0.05$, **$P < 0.01$ compared to ethyl loflazepate with low dose (1 mg) (ANOVA followed by Fisher's protected least significant difference test).