Possible Association between a Haplotype of the GABA-A Receptor Alpha 1 Subunit Gene (*GABRA1*) and Mood Disorders

Yasue Horiuchi, Junko Nakayama, Hiroki Ishiguro, Tsuyuka Ohtsuki, Sevilla D. Detera-Wadleigh, Tomoko Toyota, Kazuo Yamada, Masahiro Nankai, Haruo Shibuya, Takeo Yoshikawa, and Tadao Arinami

Background: *The γ-aminobutyric acid (GABA) neurotransmitter system has been implicated in the pathogenesis of mood disorders. The* GABRA1 *gene encodes one of the subunits of GABA-A receptor and is located on human chromosome 5q34-q35, which is a region reportedly linked to mood disorders. We examined the* GABRA1 *gene as a candidate for mood disorders.*

Methods: *We performed mutation screening of* GABRA1 *in 24 Japanese bipolar patients and evaluated associations in Japanese case-control subjects consisting of 125 patients with bipolar disorder, 147 patients with depressive disorders, and 191 healthy control subjects. Associations were confirmed in the National Institute of Mental Health (NIMH) Initiative Bipolar Pedigrees, which consists of 88 multiplex pedigrees with 480 informative persons.*

Results: *We identified 13 polymorphisms in the* GABRA1 *gene. Nonsynonymous mutations were not found. Association of a specific haplotype with affective disorders was suggested in the Japanese case-control population (corrected* p = *.0008). This haplotype association was confirmed in the NIMH pedigrees (*p = *.007).*

Conclusions: *These results indicate that the* GABRA1 *gene may play a role in the etiology of bipolar disorders.* Biol Psychiatry 2004; 55:40–45 © *2004 Society of Biological Psychiatry*

Key Words: Mood disorder, association, transmission disequilibrium test, *GABRA1*, genetics

The hypothesis that heredity is a major etiologic factor in the pathogenesis of mood disorders is supported by findings from family, twin, and adoption studies; however, the nature of the predisposing gene(s) and the exact mode of transmission remain unclear. Gamma-aminobutyric acid (GABA) is one of the most important inhibitory neurotransmitters in the vertebrate brain, and GABAergic inhibitory interneurons are widely distributed throughout the central nervous system. Over the past 20 years, several lines of evidence from preclinical and clinical studies have suggested that GABA deficit may be involved in mood disorders (Shiah and Yatham 1998) and that increasing GABAergic neurotransmission may have an antidepressant effect and perhaps a mood stabilizing effect similar to that of lithium (Petty et al 1995).

The GABA-A receptors are ligand-gated chloride channels that mediate fast synaptic inhibition in the brain. Each is a heteropentameric protein complex assembled from different classes of subunits (α1–6, β1–4, γ1–3, δ, ε, π, θ; Moss and Smart 2001). Four of the GABA-A receptor subunit genes (*GABRA1*, *GABRA6*, *GABRB2*, and *GABRG2*) form a cluster on chromosome 5q34-q35 (Johnson et al 1992), which is a region reported to be linked to mood disorders (Rice et al 1997). In addition, the α1β2γ2 combination is the most abundant form of the receptor in the brain (Sieghart et al 1999).

During our systematic association screening of the *GABRA1*, *GABRA6*, *GABRAB2*, and *GABRAG2* genes, we found preliminary evidence for association of *GABRA1* polymorphisms with mood disorders. The *GABRA1* gene contains a highly polymorphic (dC-dA)n repeat that varies from 15–28 dinucleotides in intron 5 (Johnson et al 1992). Association studies of this polymorphism with mood disorders (Coon et al 1994), symptoms (Serretti et al 1998), and response to lithium prophylaxis in mood disorders (Serretti et al 1999) have been reported; however, the results of these studies were not statistically significant. Significant results in unipolar disorder and as a trend in bipolar disorder were reported by a collaborative European study (Massat et al, unpublished data). No systematic mutation analysis of the *GABRA1* gene for association with mood disorders has been reported.

Methods and Materials

Samples

The case-control subjects were 125 unrelated Japanese patients with bipolar disorders (72 bipolar I and 53 bipolar II; 70 men, 55 women; mean age 50.5 ± 12.1 years), 147 patients with depressive disorders (recurrent major depression; 63 men, 84 women; mean age 55.5 ± 14.1 years), and 191 control subjects (104 men, 87 women; mean age 53.6 ± 9.1 years). All subjects examined in this study were recruited from a geographic area located in central Japan. All patients met DSM-IV criteria for mood disorders. The subjects, both outpatients and inpatients, were followed for at least 6 months from the time of diagnosis. Data on DSM Axis I and II comorbidities were not available. Control subjects had not been evaluated for psychiatric disorders by a psychiatrist.

The National Institute of Mental Health (NIMH) Genetics Initiative Bipolar Pedigrees are a panel of 88 multiplex pedigrees

From the Department of Medical Genetics, Institute of Basic Medical Sciences (YH, JN, HI, TO, TA), University of Tsukuba, Tsukuba, Ibaraki, Japan; Intramural Research Program (SDD-W), National Institute of Mental Health, Bethesda, Maryland; Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute (TT, KY, TY), Wako, Saitama; Tokyo Metropolitan Police Hospital, Department of Neuropsychiatry (MN), Tokyo; National Sanatorium Minami-Hanamaki Hospital (HS), Hanamaki, Iwate, Japan.

Address reprint requests to Tadao Arinami, M.D., Department of Medical Genetics, Institute of Basic Medical Science, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan.

Received March 13, 2003; revised June 16, 2003; accepted June 23, 2003.

0006-3223/04/$30.00
doi:10.1016/S0006-3223(03)00689-9

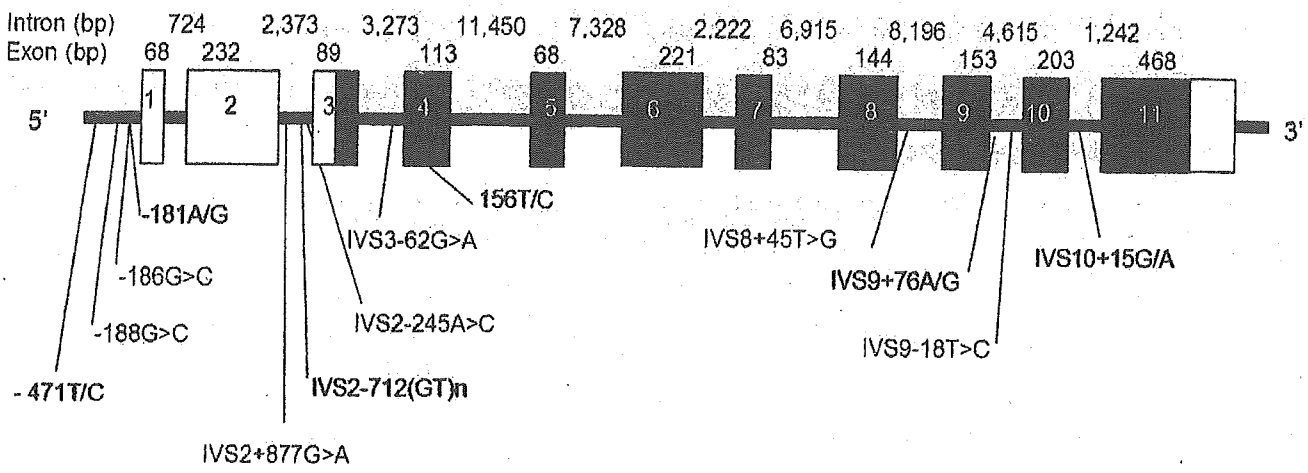

Figure 1. Schematic of the *GABRA1* gene. The 11 exons and 10 introns of the *GABRA1* gene and the approximate location of each polymorphism identified in the present study are shown. Polymorphisms in bold letters were genotyped for association.

that include 480 informative persons. Diagnosis and ascertainment methods are described in detail elsewhere (NIMH Genetics Initiative Bipolar Group 1997). In addition to bipolar I disorder, three hierarchical diagnoses were used in this study. Model I comprised affected individuals diagnosed with schizoaffective disorder, bipolar type (SA/BP), or bipolar I disorder (BPI). Affected individuals under model II included those diagnosed under model I as well as those with bipolar II disorder (BPII). Model III included all individuals classified as affected under model II as well as those with unipolar recurrent depression.

Written informed consent was obtained from all subjects. The study was approved by the Ethics Committees of University of Tsukuba, RIKEN, Tokyo Medical and Dental University, and NIMH.

DNA Analysis

The genomic structure of the *GABRA1* gene was determined from the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgGateway?org = human) and the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). To screen for nucleotide variants, we used a denaturing high-performance liquid chromatography (dHPLC) method with the WAVE DNA fragment analysis system (Transgenomic, San Jose, California) and subsequent direct sequencing with a Big Dye Terminator Cycle Sequencing kit and ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Norwalk, Connecticut) in 24 randomly selected patients with bipolar disorders.

Six polymorphisms were genotyped: -471T/C, -181A/G, IVS2–712(GT)n, 156T/C, IVS9+76A/G, and IVS10+15G/A. The -471T/C and -181A/G mutations were genotyped by direct sequencing after amplification with the forward PCR primer 5′-GGA GAA AAT GAC CAG TGA GCT T and the reverse PCR primer 5′-CTG AAT TGT GCT GGG TTC CT. IVS2–712(GT)n was amplified with the forward polymerase chain reaction (PCR) primer 5′-TCC AGC TTC CAT CTG TTT GA and the reverse PCR primer 5′-CCG GAG TCG TGC TTT TAT TC and genotyped with an ABI 3100 Genetic Analyzer and Genescan software (Applied Biosystems). The 156T/C and IVS10+15G/A polymorphisms were genotyped by primer extension method (AcycloPrime-FP SNP Detection Kit, PerkinElmer, Wellesley, Massachusetts). The IVS9+76A/G polymorphism was genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis with *Mbo*II (New England BioLabs, Beverly, Massachusetts; A allele, 194 bp and 21 bp; G allele, 215 bp).

Statistical Analysis

Deviations of the genotype distributions from Hardy–Weinberg equilibrium were assessed by chi-square analysis for single nucleotide polymorphisms (SNPs) and the MEGA2 program (Mukhopadhyay et al 1999) for multiallelic polymorphisms. For the reasons discussed by Devlin and Roeder (1999), we chose the additive model and used Armitage's Trend Test (Armitage 1955) to examine genotypic associations. Allelic and haplotype associations with disorders were examined with the COCAPHASE software (Dudbridge et al 2000). A family-based linkage and association analysis was conducted with TDTPHASE (Dudbridge et al 2000). Options of "droprare," "missing," "EM," and "tsu" in TDTPHASE were used. Because we examined associations for six polymorphisms, we corrected the results of comparisons of each polymorphism between the Japanese total case and control groups for multiple testing by six and those between Japanese bipolar or depressive and control groups by 12. When we tested multiple allele and haplotype distributions, we calculated empirical probabilities on 1000, or more if necessary, replicates. For haplotype associations, we analyzed the full set of markers; therefore, we did not correct results for haplotype distribution and corrected results for each haplotype by four because of only four common haplotypes in our Japanese population. In a replication sample, results were not corrected. A corrected p value $< .05$ was considered statistically significant and $< .1$ was considered to indicate a trend.

Results

Japanese Population

We identified 13 variants and polymorphisms in the *GABRA1* gene through mutation analysis of 24 Japanese bipolar patients. Four SNPs were located in the promoter region, seven SNPs and one short tandem repeat polymorphism (STRP) were located in introns, and one exonic synonymous SNP (156T/C) was located in exon 4 (Figure 1). Among these polymorphisms, only two

Table 1. The −471T/C, −181A/G, 156T/C, IVS9 + 76A/G, and IVS10 + 15G/A Allele and Genotype Frequencies in the Japanese Population

Polymorphism Population	*n*	Genotype Count (frequency)			p^a	Allele Count (frequency)		p^b
−471T/C		TT	TC	CC		T	C	
Control	178	64 (.36)	91 (.51)	23 (.13)		219 (.62)	137 (.39)	
Mood disorder	252	100 (.40)	107 (.42)	45 (.18)	.86	307 (.61)	197 (.39)	.89
Bipolar	114	45 (.40)	46 (.40)	23 (.20)	.65	136 (.60)	92 (.40)	.67
Depressive	138	55 (.40)	61 (.44)	22 (.16)	.99	171 (.62)	105 (.38)	.93
−181A/G		AA	AG	GG		A	G	
Control	185	81 (.44)	75 (.41)	29 (.16)		237 (.64)	133 (.36)	
Mood disorder	241	96 (.40)	111 (.46)	34 (.14)	.73	303 (.63)	179 (.37)	.77
Bipolar	107	44 (.41)	50 (.47)	13 (.12)	.99	138 (.65)	76 (.36)	.93
Depressive	134	52 (.39)	61 (.46)	21 (.16)	.53	165 (.62)	103 (.38)	.56
156T/C(rs1129647)		TT	TC	CC		T	C	
Control	191	100 (.52)	75 (.39)	16 (.08)		275 (.72)	107 (.28)	
Mood disorder	271	171 (.63)	88 (.33)	12 (.04)	.01	430 (.79)	112 (.21)	.01
Bipolar	125	83 (.66)	36 (.29)	6 (.05)	.01	202 (.81)	48 (.19)	.01
Depressive	146	88 (.60)	52 (.36)	6 (.04)	.07	228 (.78)	64 (.22)	.08
IVS9 + 76A/G		AA	AG	GG		A	G	
Control	191	110 (.58)	75 (.39)	6 (.03)		295 (.77)	87 (.22)	
Mood disorder	268	150 (.56)	98 (.37)	20 (.08)	.30	398 (.74)	138 (.26)	.13
Bipolar	123	68 (.55)	48 (.39)	7 (.06)	.47	184 (.75)	62 (.25)	.50
Depressive	145	82 (.57)	50 (.35)	13 (.09)	.30	214 (.74)	76 (.26)	.32
IVS10+15G/A(rs2279020)		GG	GA	AA		G	A	
Control	191	37 (.19)	100 (.52)	54 (.28)		174 (.46)	208 (.55)	
Mood disorder	270	53 (.20)	122 (.45)	95 (.35)	.32	228 (.42)	312 (.58)	.35
Bipolar	123	19 (.16)	63 (.51)	41 (.33)	.25	101 (.41)	145 (.59)	.29
Depressive	147	34 (.20)	59 (.45)	54 (.37)	.55	127 (.43)	167 (.57)	.58

[a]Armitage trend test (uncorrected).
[b]Fisher's Exact Test (uncorrected).

SNPs, 156T/C (rs1129647) and IVS10+15G/A (rs2279020), had been deposited previously in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/).

Among the 13 variants and SNPs, five had minor alleles with greater than 10%. We genotyped these five SNPs and the IVS2–712(GT)n polymorphism for associations with mood disorders. With the exception of the -471T/C and -181A/G polymorphisms, the other SNPs were in significant linkage disequilibrium with each other (data not shown).

The genotype and allele distributions of the five SNPs in the patient and control groups are shown in Table 1. The genotype distributions of the SNPs and IVS2–712(GT)n polymorphism did not deviate significantly from Hardy–Weinberg equilibrium in the patient or control group. The C allele of 156T/C occurred less frequently in the patient group (p = .01), particularly in the bipolar group (p = .01), than in the control group. The genotype distributions of this SNP also differed between the patient and control groups (p = .01). The differences between the patient and control groups were only marginally significant after the results were corrected for multiple testing (corrected, p = .06).

The allele frequencies of the IVS2–712(GT)n polymorphism in the patient and control groups are shown in Table 2. The distribution differed between the total patient group (empirical, p = .03) or bipolar group (empirical, p = .04) and the control groups; however, the difference in distribution was not significant after correction for multiple testing. The frequency of the 17-repeat allele was lower in the bipolar patients than in the control group and that of the 16-repeat allele was higher in the bipolar patients than in the control group.

We estimated the haplotype distributions of the full set of the markers in the patient and control groups (Table 3). The haplotype distributions differed significantly between the patient

Table 2. The IVS2-712(GT)n Allele and Genotype Frequencies in the Japanese Population

	n	(GT)10	(GT)12	(GT)13	(GT)14	(GT)15	(GT)16	(GT)17	(GT)18	(GT)19	p^a
Control Subjects	378	7	123	0	0	7	54	171	16	0	
Frequency		.02	.33	.00	.00	.02	.14	.45	.04	.00	
Patients Total	526	8	184	3	1	5	87	206	27	5	.03
Frequency		.02	.35	.01	.002	.01	.17	.39	.05	.01	
Bipolar	242	3	84	3	1	4	47	85	14	1	.04
Frequency		.01	.35	.01	.004	.02	.19	.35	.06	.004	
Depression	284	5	100	0	0	1	40	121	13	4	.29
Frequency		.02	.35	.00	.00	.004	.14	.43	.05	.01	

n indicates chromosome numbers.
[a]Compared with control subjects by COCAPHASE (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/unphased/) permutation analysis.

Table 3. Estimated Haplotype Frequencies and Association Significance in Japanese Case-Control Population

	Control (n = 374)	Mood Disorders (n = 522)		Bipolar (n = 282)		Depressive (n = 240)	
	Frequency	Frequency	Haplotype p	Frequency	Haplotype p	Frequency	Haplotype p
Haplotype[a]							
T-A-12-T-A-A	.32	.29	.44	.28	.36	.30	.66
C-G-16-T-A-A	.13	.11	.47	.13	.87	.09	.19
C-A-17-C-A-G	.21	.13	.0002	.11	.002	.14	.02
T-G-17-T-G-G	.17	.17	.87	.14	.43	.19	.67
Specific Haplotype							
C-A-17-C-A-/	.24	.14	.0002	.11	.0001	.16	.02
/-A-17-C-A-G	.22	.13	.001	.12	.004	.14	.01
C-A-17-C-/-/	.24	.14	.0009	.11	.0002	.17	.05
/-A-17-C-A-/	.24	.14	.0003	.12	.0003	.16	.01
/-/-17-C-A-G	.22	.13	.0003	.11	.001	.14	.007
C-A-17-/-/-/	.24	.17	.02	.14	.006	.20	.23
/-A-17-C-/-/	.24	.15	.001	.12	.0008	.17	.04
/-/-17-C-A-/	.26	.14	.00004	.12	.00005	.16	.004
/-/-/-C-A-G	.23	.15	.002	.14	.004	.16	.02
C-A-/-/-/-/	.22	.26	.28	.22	.48	.22	.28
/-A-17-/-/-/	.26	.20	.04	.17	.02	.22	.25
/-/-17-C-/-/	.26	.16	.0004	.15	.002	.16	.01
/-/-/-C-A-/	.28	.18	.002	.16	.003	.19	.02
/-/-/-/-A-G	.23	.20	.32	.20	.38	.21	.45

Haplotypes with frequencies < .05 are not listed.
Global p values are .04 in total patients, .11 in bipolar patients, and .27 in depressive patients versus control subjects comparisons (permutation test).
[a]Markers are shown from 5′ to 3′ order (-471T/C–-181A/G–IVS2-712(GT)n–156T/C–IVS9 + 76A/G–IVS10 + G/A) as indicated in Figure 1.

and control groups (p = .04) but not between the bipolar and control groups (p = .11) or between depressive and control groups (p = .27). Because of linkage disequilibrium, only four haplotypes were common, and the C-A-17-C-A-G (polymorphism order from the 5′ to 3′ direction, -471T/C – -181A/G – IVS2–712(GT)n – 156T/C – IVS9+76A/G – IVS10+15G/A) haplotype was significantly less frequent in the total patient (p = .0002, corrected p = .0008) and bipolar (p = .002, corrected p = .008) groups than in the control group (Table 3). Although the haplotype association was stronger in bipolar disorder than in depressive disorder, there was no statistical evidence of heterogeneity between bipolar and depressive disorders (χ^2 = .63, p = .43). The four common haplotypes can be distinguished by genotyping the repeat polymorphism IVS2+712(GT)n and one SNP (excluding for IVS10+15G/A), and the power to detect haplotype association was not decreased by reducing the number of markers typed to the repeat polymorphism and 156T/C SNP (Table 3). For further analyses, we selected IVS2–712(GT)n and 156T/C as haplotype tagging polymorphisms.

Table 4. Transmission Disequilibrium Test (TDT) of the IVS2-712(GT)n and 156T/C Polymorphisms in NIMH Initiative Bipolar Pedigrees

			Model I			Model II			Model III		
	Allele	Frequency	Trans	No Trans	Allelic p	Trans	No Trans	Allelic p	Trans	No Trans	Allelic p
156T/C	C	.30	44	63	.07	55	78	.04	78	90	.04
	12	.51	121	98	.02	154	127	.02	170	149	.08
	13	.02	4	4	1.00	5	5	1.00	6	5	.76
	14	.05	10	10	1.00	14	12	.69	14	15	.85
	15	.01	2	2	1.00	2	3	.65	2	3	.65
	16	.02	3	4	.70	5	6	.76	5	6	.76
IVS2-712(GT)n	17	.20	24	39	.04	37	50	.12	48	55	.45
	18	.07	9	13	.38	11	22	.05	13	22	.11
	19	.01	1	2	.56	1	3	.30	1	4	.16
	22	.01	2	1	.56	2	1	.56	2	1	.56
	23	.06	4	12	.04	7	15	.08	9	15	.21
	24	.04	9	7	.61	11	8	.48	11	9	.65
	25	.00	3	0	.04	3	0	.04	3	0	.04

Trans, transmission.
Model I: bipolar I disorder + schizoaffective disorder, bipolar type; model II: model I + bipolar II disorder; model III: model II + unipolar recurrent depression.
Global TDT p values for the IVS2-712(GT)n polymorphism are .047 in Model I, .09 in Model II, and .25 in Model III.

Table 5. Transmission of the IVS2-712(GT)n and 156T/C Haplotypes in NIMH Initiative Bipolar Pedigrees

		Model I			Model II			Model III		
Haplotype[a]	Frequency	Trans	No Trans	Haplotype *p*	Trans	No Trans	Haplotype *p*	Trans	No Trans	Haplotype *p*
/-/-12-T-/-/	.58	108	84	.007	130	103	.007	145	121	.02
/-/-17-C-/-/	.09	8	22	.007	12	27	.01	17	31	.04
/-/-17-T-/-/	.06	8	6	.61	11	17	.21	15	18	.54

Trans, transmission.

Model I: bipolar I disorder + schizoaffective disorder, bipolar type; model II: model I + bipolar II disorder; model III: model II + unipolar recurrent depression.

Global *p* values are .04 in model I, .03 in model II, and .19 in model III.

[a]Markers are shown from 5′ to 3′ order as indicated in Table 3. Haplotypes with frequencies < .05 are not listed.

NIMH Pedigrees

Replication analysis was conducted by genotyping the selected haplotype tagging polymorphisms in the NIMH Genetics Initiative Bipolar Pedigrees. Transmission disequilibrium test (TDT) analysis showed that transmission of the C allele of 156T/C to patients occurred significantly less frequently than expected in models II ($p = .04$) and III ($p = .04$; Table 4).

As for the IVS2–712(GT)n polymorphism, the repeat range was larger in the NIMH pedigrees than in the unrelated Japanese population. In the NIMH pedigrees, 12–25-repeat alleles were found, compared with 12–19-repeat alleles in our Japanese population. The 17-repeat allele was the most common and the 12-repeat allele the second most common allele in the Japanese, whereas the 12-repeat allele was the most common and the 17-repeat allele the second most common allele in the NIMH pedigrees. The 16-repeat allele was frequent in the Japanese but rare in the NIMH pedigrees. The 12-repeat allele was transmitted more frequently to patients ($p = .02$), and the 17-repeat allele was transmitted less frequently to patients ($p = .04$) in model I. Global TDT was significant for the IVS2–712(GT)n polymorphism in model I ($p = .047$) but not in model II ($p = .09$) or model III ($p = .25$).

The IVS2–712(GT)n and 156T/C polymorphisms are in linkage disequilibrium ($D' = .81$). Three haplotypes had greater than 5% haplotype frequencies. TDT revealed less frequent transmission of the 17-C haplotype to patients ($p = .007$ in model I, $p = .01$ in model II, and $p = .04$ in model III) and more frequent transmission of haplotype 12-T ($p = .007$ in model I, $p = .007$ in model II, and $p = .02$ in model III) (Table 5). Global haplotype TDT was significant in model I ($p = .04$) and model II ($p = .03$).

Discussion

We found a significant association between the haplotype of the *GABRA1* gene and mood disorders in a Japanese case-control population. The association was confirmed in the NIMH pedigrees. Thus, the findings of this study indicate that the 17-C haplotype of the IVS2–712(GT)n and 156T/C polymorphisms, which is the third most common in our Japanese sample and the second most common in the NIMH pedigrees, is likely to be associated with a protective role in mood disorders; however, no definitively functional polymorphisms were detected. The haplotype may be associated with altered expression of *GABRA1* or may be in linkage disequilibrium with unidentified functional mutation(s). The tissue-specific expression of *GABRA1* hampers analysis of the association of the haplotypes with gene expression; however, the 156T/C polymorphism is transcribed into mRNA; therefore, this polymorphism may be a tool for expression analysis in subjects heterozygous for the polymorphism.

There are some limitations of our study. First, control subjects in our Japanese population were not screened for psychiatric disorders. Second, mutation screening was carried out only in Japanese patients, and it is likely that more informative variants would have been identified in an ethnically diverse population. Third, we examined multiple affection phenotypes. The potential for type I errors remains, given the multiple disease phenotypes tested and the number of individual SNPs genotyped initially. Fourth, the definition of the affection status differed between the Japanese and NIMH samples because bipolar type schizoaffective patients were included as affected in the NIMH sample but not in the Japanese sample. In addition, different test procedures (case-control comparison vs. TDT) were used. Therefore, the use of different affection status and test procedures is problematic for the purpose of testing a specific hypothesis in a replication sample. Finally, we did not examine *GABRA6*, which is 120 kb centromeric to *GABRA1*, or *GABRG2*, which is 150 kb telomeric to *GABRA1*. Yamada et al (in press) found a possible association between a SNP in *GABRA1* and a SNP in *GABRA6* and mood disorders in a Japanese population, which partially overlapped our Japanese patient subjects. Thus, the possible involvement of *GABRA6* and *GABRG2* in mood disorders cannot be excluded.

To date, a functional mutation in the *GABRA1* gene has been detected only in a pedigree with an autosomal-dominant form of juvenile myoclonic epilepsy (Cossette et al 2002). This mutation showed a reduced amplitude of GABA-activated currents in vitro, suggesting that seizures may result from loss of GABA-A receptor function. We speculate that mild dysfunction or dysregulation of GABA-A receptors may be associated with mood disorders. A shared mechanism between epilepsy and mood disorders associated with GABA-A receptors has been proposed (Weiss and Post 1998). GABA deficiency may be involved in epilepsy and mood disorders. Sodium valproate, an anticonvulsant agent, is a structural analog of GABA that enters various metabolic pathways, has multiple clinical effects (Barrueto et al 2002), and enhances GABA activity. Mania is associated with depletion of inhibitory transmitters in the central nervous system, and GABA is one of the most important inhibitory transmitters. The GABAergic effects of valproate provide a theoretical basis for its use in mood disorders. European studies, which have been both open and controlled, showed beneficial effects of valproate in acute and prophylactic treatment of bipolar illness, with particularly good results in mania (Fawcett 1989). The *GABRA1* gene haplotypes found in our study may be useful in pharmacogenetic studies to evaluate the response of bipolar patients to drugs with primary actions on the GABA system. In conclusion, the consistent findings between the Japanese and NIMH samples may indicate the existence of common mutation(s) or haplotype(s) associated with susceptibility to mood disorder across ethnicities.

Data and biomaterials were collected in four projects that participated in the National Institute of Mental Health (NIMH) Bipolar Disorder Genetics Initiative. From 1991–98, the principal investigators and coinvestigators were as follows: Indiana University, Indianapolis, Indiana, Grant No. U01 MH46282, John Nurnberger, M.D., Ph.D., Marvin Miller, M.D., and Elizabeth Bowman, M.D.; Washington University, St. Louis, Missouri, Grant No. U01 MH46280, Theodore Reich, M.D., Allison Goate, Ph.D., and John Rice, Ph.D.; Johns Hopkins University, Baltimore, Maryland, Grant No. U01 MH46274, J. Raymond DePaulo, Jr., M.D., Sylvia Simpson, M.D., M.P.H., and Colin Stine, Ph.D.; NIMH Intramural Research Program, Clinical Neurogenetics Branch, Bethesda, Maryland, Elliot Gershon, M.D., Diane Kazuba, B.A., and Elizabeth Maxwell, M.S.W. This study was supported by a grant from Research on Brain Science; a grant from the Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare; and Grant-in-Aid for Scientific Research on Priority Areas "Medical Genome Science" from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Human Molecular Genetics, 2004, Vol. 13, No. 5 **551–561**
DOI: 10.1093/hmg/ddh047
Advance Access published on January 6, 2004

Association between schizophrenia with ocular misalignment and polyalanine length variation in *PMX2B*

Tomoko Toyota[1,2], Kiyoshi Yoshitsugu[1,2], Mitsuru Ebihara[1], Kazuo Yamada[1], Hisako Ohba[1], Masayuki Fukasawa[1,2], Yoshio Minabe[1,3], Kazuhiko Nakamura[1,3], Yoshimoto Sekine[3], Noriyoshi Takei[3], Katsuaki Suzuki[3], Masanari Itokawa[1], Joanne M.A. Meerabux[1], Yoshimi Iwayama-Shigeno[1], Yoshiro Tomaru[4], Hiromitsu Shimizu[5], Eiji Hattori[1], Norio Mori[3] and Takeo Yoshikawa[1,*]

[1]Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Wako, Saitama 351-0198, Japan, [2]Department of Neuropsychiatry, Tokyo Medical and Dental University, Tokyo 113-8519, Japan, [3]Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan, [4]Yamada Hospital, Chofu, Tokyo 182-0005, Japan and [5]Hokushin General Hospital, Nakano, Nagano 383-8505, Japan

Received November 10, 2003; Revised and Accepted December 17, 2003

The increased incidence of minor physical anomalies (MPAs) in schizophrenia is the fundamental basis for the neurodevelopmental hypothesis of schizophrenia etiology. Ocular misalignment, or strabismus, falls into the category of MPAs, but this phenotype has not been assessed in schizophrenia. This study reveals that a subtype of strabismus, constant exotropia, displays marked association with schizophrenia ($P=0.00000000906$). To assess the genetic mechanisms, we examined the transcription factor genes *ARIX* (recently identified as a causative gene for syndromic strabismus) and its paralogue, *PMX2B*. We identified frequent deletion/insertion polymorphisms in the 20-alanine homopolymer stretch of *PMX2B*, with a modest association between these functional polymorphisms and constant exotropia in schizophrenia ($P=0.029$). The polymorphisms were also associated with overall schizophrenia ($P=0.012$) and more specifically with schizophrenia manifesting strabismus ($P=0.004$). These results suggest a possible interaction between *PMX2B* and other schizophrenia-precipitating factors, increasing the risk of the combined phenotypes. This study also highlights the unique nature of the polyalanine length variations found in PMX2B. In contrast with other transcription factor genes, the variations in *PMX2B* show a high prevalence, with deletions being more common than insertions. Additionally, the polymorphisms are of ancient origin and stably transmitted, with mild phenotypic effects. In summary, our study lends further support to the disruption of neurodevelopment in the etiology of schizophrenia, by demonstrating the association of a specific MPA, in this case, constant exotropia with schizophrenia, along with molecular variations in a possible causative gene.

INTRODUCTION

The view that neurodevelopmental abnormalities are involved in, at least partially, the etiologies of schizophrenia has become prevalent, as can be seen from a range of epidemiological, clinical and neurobiological evidence (1). One facet of such supporting evidence is the observation of a significantly higher prevalence of minor physical anomalies (MPAs) in schizophrenic patients than in healthy controls (reviewed in 2). MPAs involve slight dysmorphic features representing subtle alterations in the development of various ectoderm-derived bodily structures in the mouth, eye, ear, global head, hand and foot areas. MPAs are believed to develop during the first and/or early second trimesters of gestation (3). As the bodily structures involved in

*To whom correspondence should be addressed at: Laboratory for Molecular Psychiatry, Brain Science Institute, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Tel: +8 1484675968; Fax: +8 1484675916; Email: takeo@brain.riken.go.jp

the expression of MPAs typically share an embryonic origin with that of the brain (4), an organ of ectodermal origin, MPAs represent potentially valuable indices of disturbances in early neuronal development. MPAs may result from both genetic factors and environmental influences, such as complications during pregnancy (2). The findings that MPAs are particularly common among schizophrenic patients with a family history of the disorder (5), and that siblings display significantly more MPAs than normal subjects (6) represent evidence of a genetic effect.

MPAs in schizophrenia have been scored using the Waldrop scale (7), including modified versions (8) and additional or different items (9). The Waldrop scale was originally devised for use in children with Down's syndrome, and is a standardized tool for assessing 18 features of the head, eyes, ears, mouth, hands and feet. However, the study of MPAs in schizophrenia is still in the exploratory phase, and the instruments used to measure MPAs have been criticized for their inherent limitations, including content. For eye assessments, the items that have been addressed thus far include telecanthus, epicanthus (7), heterochromia and ptosis (6). Abnormalities in eye position, such as the presence of strabismus, have not been addressed in previous studies. Isolated non-syndromic strabismus affects 1–5% of the general population (10). Most forms of strabismus are multifactorial in origin, with possible inherited components. Strabismus can result from errors in developmental co-ordination of cranial nerves innervating the extraocular muscles, namely the oculomotor (nIII), trochlear (nIV) and abducens nerves (nVI). These cranial nerves differentiate from neural crest cells in the embryonic stage (11). Once formed in the developmental stage, strabismus persists into adult life and is readily detected on simple visual examination, as with other MPAs. We therefore set out to compare the prevalence of strabismus in schizophrenia and mentally normal cohorts in this study, to determine whether this developmentally minor anomaly is associated with schizophrenia.

The genes responsible for strabismus have long remained quite unknown. However, Nakano *et al.* (12) recently reported homozygous mutations in *ARIX* (*PHOX2A*) in congenital fibrosis of the extraocular muscles type 2 (CFEOM2), which accompanies strabismus. The *ARIX* paralogue, *PMX2B* (*PHOX2B*) (12), is 100% identical to ARIX within the homeodomain and 71% identical over the whole gene. The two proteins show an overlapping pattern of expression, including co-expression in the nIII and nIV cranial nerve nuclei (13) that control eye alignment. Both genes are also known to be involved in the development of catecholaminergic neurons (13). We therefore screened for polymorphisms in these two candidate genes and evaluated the contribution of detected variants to risk of strabismus and schizophrenia.

RESULTS

Strabismus in schizophrenia and controls

Much of the difficulty in studying strabismus lies in the use of varying definitions and measures of strabismus (14). For the purpose of the present study, strabismus was defined as ocular misalignment in which both eyes are not directed to the object of regard (for details see Materials and Methods). This includes both misalignment in the primary position (straight ahead gaze) and eccentric gaze. All subjects defined as displaying strabismus in this study manifested concomitant strabismus without any systemic abnormalities: angle of misalignment was approximately the same for all directions of gaze (i.e., none of the subjects demonstrated incomitant/paralytic strabismus). Concomitant strabismus is the most common form of ocular motility defect, and has been the target of numerous epidemiological studies (14). The observed concomitant strabismus was divided into subtypes according to the direction of squint and based on whether the condition was constant or intermittent, as different mechanisms have been suggested for different forms of strabismus. The complication of strabismus as a whole displayed a highly significant association with schizophrenic cohorts compared to control subjects ($P = 0.0000161$; Table 1). When subtypes of strabismus were inspected, this marked association was attributable to the over-representation of constant exotropia in schizophrenia ($P = 0.00000000906$; Table 1, Fig. 1). The odds ratio for constant exotropia in schizophrenia was 20.6 (95% confidence interval, 5.03–56.2). Age of onset of schizophrenia in cohorts with (mean ± SD, 24.4 ± 6.5 years) and without (mean ± SD, 26.1 ± 9.5 years) strabismus did not differ significantly ($P = 0.625$ using the Mann–Whitney test).

Analyses of candidate genes

Strabismus is widely acknowledged as displaying genetic components, although the etiology is multifactorial (14). Confirming this notion, disruption of ARIX protein, a homeodomain transcription factor, has recently been reported to cause strabismus as one symptom in CFEOM2 (12). We therefore first examined *ARIX* in the 24 schizophrenic patients manifesting constant exotropia (Table 1). However, no polymorphisms were detected.

As *ARIX* has a close paralogue, *PMX2B*, this gene was the next to be screened in the same samples. Human *PMX2B* (paired mesoderm homeobox 2b, also known as *NBPhox*) encodes a transcription factor with a paired-like homeodomain (15). The chromosomal assignment of the gene was first reported to be 5p12–p13 (15), but later amended to 4p12–13 by GenBank (accession no. AB015671). We further refined the location to 4p13, by fluorescence *in situ* hybridization using the BAC clone RP11-227F1910, which spans the *PMX2B* and by radiation hybrid mapping using the Stanford G3 panel (http://shgc-www.stanford.edu/RH/index.html) (linked to SHGC4-435, LOD 13.6). The PMX2B protein contains two polyalanine regions, comprising nine (Ala9) and 20 alanines (Ala20), both located downstream of a homeobox domain, and none of which are present in ARIX (Fig. 2). Interestingly, mutation screening detected variations in length of the Ala20 tract, with variant alleles derived from an in-frame deletion of alanine residues and an insertion. Examination of all schizophrenia ($n = 346$) and control samples ($n = 542$) revealed three different mutated alleles: −15 bp (−5 Ala), −21 bp (−7 Ala) and +6 bp (+2 Ala) (mutated alleles: 5.9% in schizophrenia, 4.8% in controls) (Figs 3 and 4). Initial analysis using ordinal denaturing acrylamide gels detected bands migrating to a −3 base position (Fig. 5). Use of c^7dGTP in the PCR reaction mixtures to

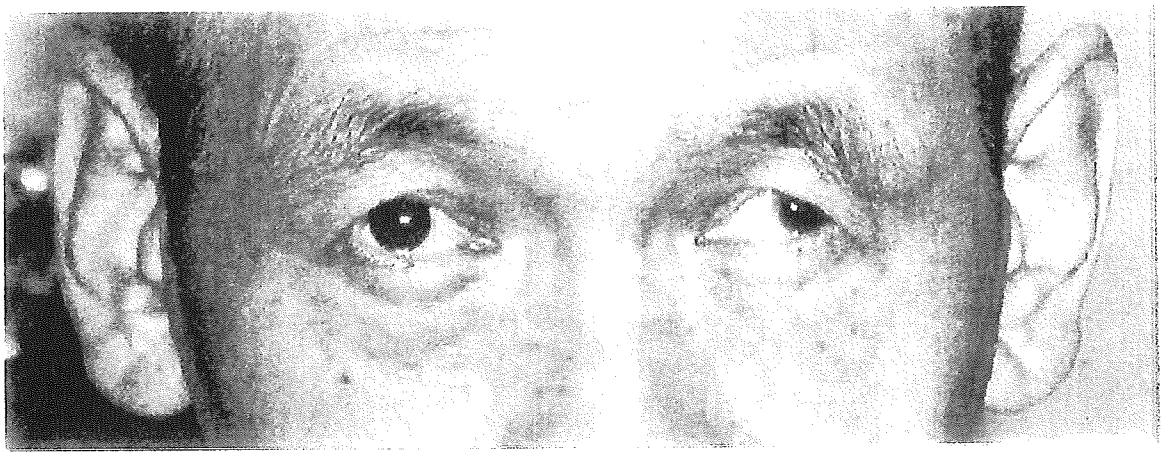


Figure 1. An example of constant exotropia, a subtype of strabismus.

Table 1. Prevalence of strabismus in schizophrenia and control groups

Sample	*n*	No strabismus (%)	Total strabismus (%)	Constant strabismus			Intermittent strabismus	
				Exotropia (%)	Esotropia (%)	Hypertropia (%)	Exotropia (%)	Esotropia (%)
Schizophrenia	346	300 (87)	46 (13)	24 (6.9)	0 (0)	1 (0.3)	20 (5.9)	1 (0.3)
Control	542	515 (95)	27 (5)	2 (0.4)	1 (0.2)	0 (0)	17 (3.1)	7 (1.3)
P-value[a]			0.0000161	0.00000000906	1.00	0.369	0.038	0.27

[a]Statistical significance was calculated between no strabismus and strabismus groups, using Fisher's exact test.

breakdown hydrogen bonds in the highly GC template revealed that the 3-base shift was induced by a c.762A-to-C substitution (Ala254Ala) (Figs 2 and 5). We also observed that the alleles having both 15 bp deletion and c.762C migrated at a −18 base position. All the alleles with 21 bp deletion and 6 bp insertion displayed c.762A.

Deletion/insertion polymorphisms of *PMX2B* were in Hardy–Weinberg equilibrium in all sample groups, and displayed a modest association with constant exotropia in the schizophrenic group [nominal $P=0.029$ by 2×4 Fisher's exact test; *P*-value after Bonferroni correction is not significant, when multiple tests for three-way comparisons (Table 2) plus three-way comparisons (Table 3) are considered] and in the combined samples (schizophrenia + controls) (nominal $P=0.017$), but not in controls ($P=1.000$) (Table 2). In schizophrenia, the deleted allele (−15 bp) was over-represented in subjects with constant exotropia. The polymorphisms were weakly associated with overall schizophrenia (nominal $P=0.012$), and more specifically with the subset of schizophrenia that carried constant exotropia (nominal $P=0.004$, corrected $P=0.024$) (Table 3). Power analysis showed that the present sample size had powers of 0.757 and 0.912 ($\alpha<0.05$) in an additive model with a genotype relative risk of 1.5 and allele frequencies of 0.1 and 0.2, respectively.

The polyalanines affect the protein function

To understand the functional role of alanine repeats in PMX2B, we generated constructs with total deletion or varying length of the Ala20 sequence and total deletion of the Ala9 sequence (Fig. 6A). Activities as transcription factors under the dopamine β-hydroxylase gene (*DBH*) promoter were then examined. Deletion of Ala20 or Ala9 sequences reduced luciferase activity to approximately half the normal PMX2B value (Fig. 6B). Deletion of Ala20 produced a larger reduction than deletion of Ala9. Increasing [+5 alanine residues (16)] or decreasing (−1, −5, −6, −7, or −13 alanine residues: we found one schizophrenic patient with a −13 alanine deletion who was unavailable for ophthalmologic examination) the Ala20 stretch also reduced promoter activity, with the greatest change seen in the +5 alanine insertion (16) (Fig. 6C).

During the preparation of our manuscript, Amiel *et al.* (16) reported an association between CCHS and variants with +5 to +9 alanine expansions within the Ala20 tract of PMX2B. In some of their patients, expansions resulted from *de novo* mutations, prompting Amiel *et al.* to suggest unequal crossing-over during meiosis as a mechanism for the mutations (16). In the separate panel of family samples, we analyzed allele transmission, but found no evidence of *de novo* mutations (transmitted alleles included 259 wild-type and 15 deleted variants). If the polymorphisms detected in this study were attributable to unequal crossing-over, the area surrounding the Ala20 stretch should represent a recombination hot spot, giving rise to an LD gap in this region. Mutation screening and a database search identified nine SNPs in the genomic region surrounding the Ala20 stretch (Fig. 1). LD analysis between these SNPs excluded the possibility of an LD gap (Table 4). Furthermore, analysis of the evolutionary history of haplotypes

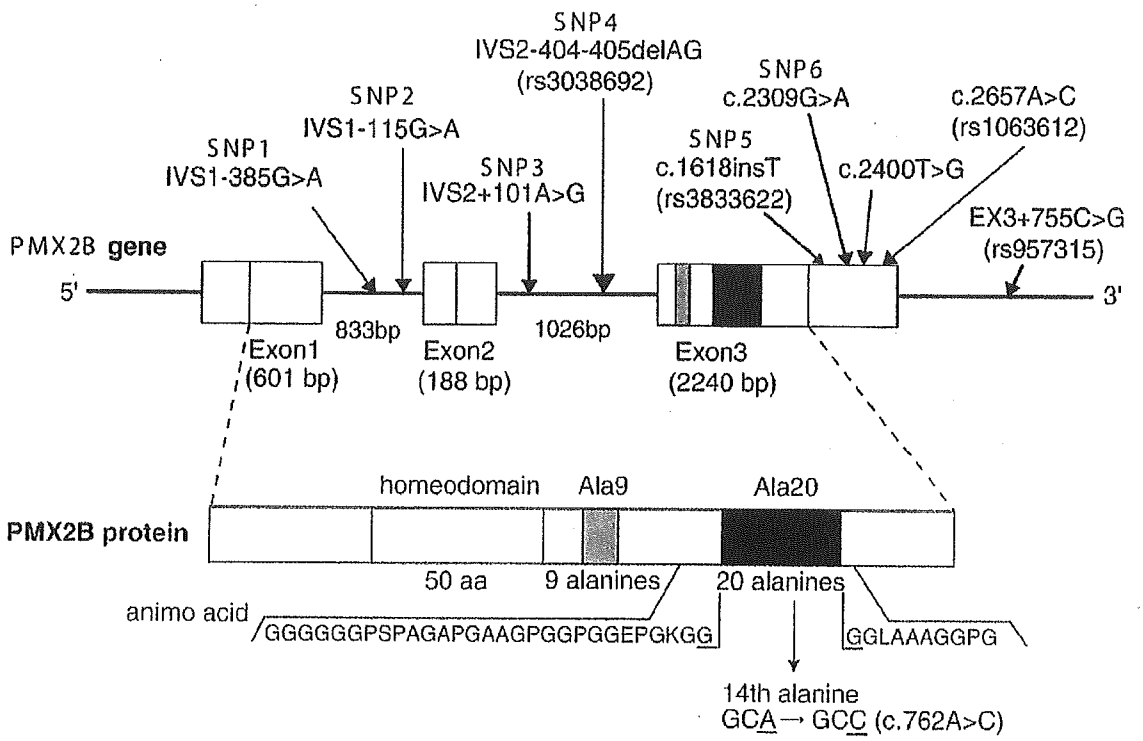


Figure 2. Schematic representation of *PMX2B* and the associated protein structure. Nine single nucleotide polymorphisms (SNPs) (upper panel) and A-to-C transversion at the 14th alanine codon in the Ala20 region (c.762A>C, Ala254Ala) (lower panel) are shown. Polymorphisms assigned SNP numbers were used for linkage disequilibrium analysis, based on genetic informativeness (Table 4). Amino acid residues flanking Ala20 are also denoted.

defined by these SNPs and alanine length variations suggested a relatively ancient origin for polymorphisms of the Ala20 stretch (Fig. 7). The amino acid sequence of human PMX2B differs from that seen in mouse (17) by one residue located outside either polyalanine stretch. Orthologous genes in other species have not been reported. We examined the Ala20 homopolymer in mouse *Pmx2b* from each of the parental lines C57BL/6 and C3H/He, in addition to F1 intercrosses ($n = 120$ each), and found no polymorphisms. These results imply that the Ala20 stretch (and the genetic variations in humans) is stably transmitted in both species. However, this stability of transmission differs from the duplications seen in the rare CCHS (16).

DISCUSSION

This is the first study to report the frequency of strabismus, an ocular misalignment, in schizophrenia. Constant exotropia, a subtype of strabismus, was found to be extremely prevalent in schizophrenia compared to normal controls. In addition, the rate of overall strabismus (13%) or constant exotropia (6.9%) in our schizophrenic cases was higher than that of ptosis (2%) (6), another MPA of the eye. We therefore propose that strabismus (more specifically, constant exotropia) should be considered in the item list for MPAs that are assessed in schizophrenia from the perspective of neurodevelopmental etiology. Some researchers have reported rare cases of schizophrenia exhibiting temporal strabismus affected by psychotic state (18,19). None of our cases displayed this type of 'fluctuating' strabismus. All current schizophrenic patients in this study were maintained on the appropriate medication. Neuroleptics are known to sometimes cause acute dystonic reactions, and involvement of the extraocular muscles may result in oculogyric crisis, wherein the eyes are elevated and 'locked' in this position (20). However, this symptom is easily differentiated from strabismus in the clinical situation.

The reason for accumulation of this specific subtype of strabismus (constant exotropia) in schizophrenia is unknown. Comitant strabismus is likely to display an etiologically heterogenous, complex and multifactroial phenotype, possibly with genetically distinct backgrounds according to subtype (reviewed in 14). For instance, Schlossman and Priestley (21) reported the presence of a family history in 50% of esotropes and 37% of exotropes. Direction of squint in each family was concordant with that of the affected proband. Waardenburg (22) described families in which exotropia was transmitted through generations, implying dominant transmission. Maumenee and Alston (23) described inheritance of congenital esotropia. Our results suggest an overlap of genetic etiology and developmental trigger for constant exotropia and schizophrenia.

Prevalences of the various forms of concomitant strabismus vary widely among populations. Gover and Yankey (24) found prevalences of 2.5 and 0.6% for strabismus among Caucasians and African Americans, respectively; most of the latter were exotropic. Nordlow (25) examined a Caucasian population, finding prevalences of 2.59% constant esotropes, 0.93%

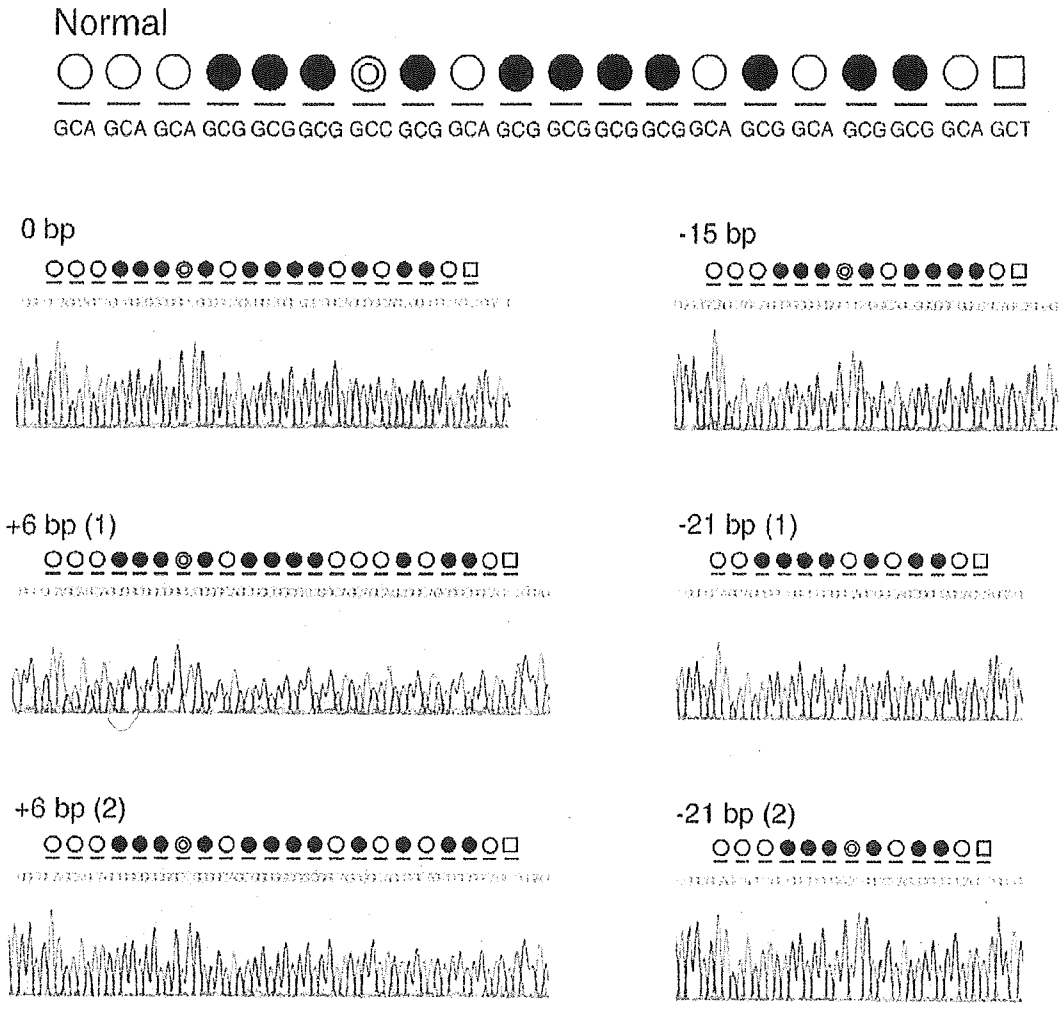


Figure 3. Sequences of Ala20 deletions/insertion mutants. Two different sequences were detected for each of the 6 bp insertion and 21 bp deletion mutants.

intermittent esotropes, 0.13% constant exotropes, 0.3% intermittent exotropes and 0.05% hypertropes, for a total of 4% strabismics. Laatikainen and Erkkila (26) reported 2.9% esotropes and 1.7% exotropes in a study of 411 Finnish schoolchildren. Ing and Pang (27) reported the frequency in Asian populations, as 33% esotropia and 67% exotropia. Concordant with their Asian data, occurrence of exotropia (3.5%) is about 2-fold higher than that of esotropia in our Japanese controls (1.5%). Differences between races in the frequencies and types of strabismus may again be attributable to genetic factors, providing further evidence of a genetic contribution. We could not examine the heritability of strabismus in schizophrenia (and controls) in the present case–control study, because of a lack of information on the phenotypic status of parents and siblings, but this would be an important issue to be solved in future studies.

Human *PMX2B* and its mouse ortholog are expressed in neural crest cell derivatives and play a primary role in the generation and survival of adrenergic neurons and a subpopulation of brainstem motor neurons (13). PMX2B protein is expressed in the nIII and nIV cranial nerve nuclei (13) that control eye alignment. This expression pattern may explain the contribution of functional polymorphisms to the risk of strabismus in schizophrenia. However, *PMX2B* mutations alone may not be sufficient to induce strabismus, it may require interaction with an additional causative gene(s) and/or environmental factors relevant to schizophrenia, since the mutations were not associated with strabismus in normal subjects. PMX2B regulates the expression of tyrosine hydroxylase and DBH, which are required for the biosynthesis of dopamine and noradrenaline, respectively, in catecholaminergic cells (13). Perturbations in the expression of these enzymes have been linked to the pathophysiology of schizophrenia (28). In line with the pivotal roles of *PMX2B* in catecholaminergic neurons, variation of the Ala20 length in *PMX2B* sequence exerted a genetic effect, albeit modest, on the development of overall schizophrenia. A stronger association of *PMX2B* variation with the subset of schizophrenics who manifested constant exotropia suggests that

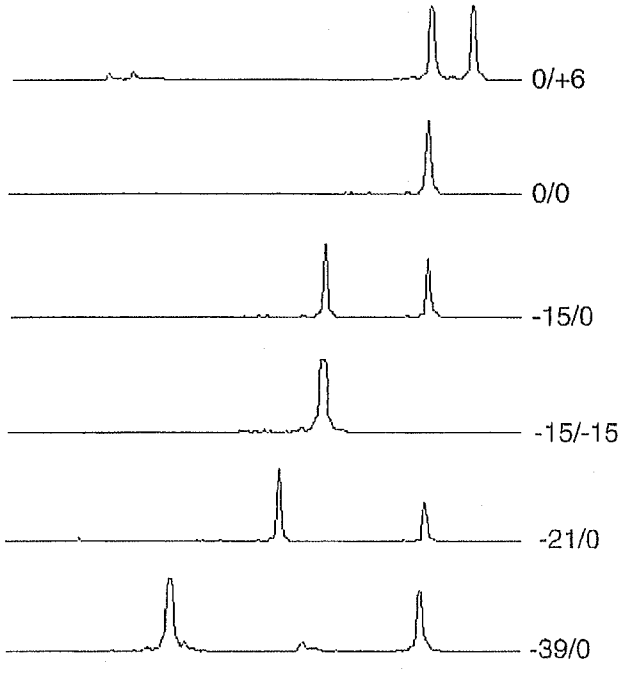


Figure 4. Fragment analysis of polymorphic Ala20 length genotypes. Four different Ala20 variants were detected: three in-frame deletions (−15, −21 and −39 bp) and one in-frame insertion (+6 bp) within the homopolymeric stretch. The study identified one schizophrenic with a 13 alanine deletion, but the patient was unavailable for ophthalmologic examination.

schizophrenia with and without the MPA may have an etiologically distinct predisposition.

Data on transcriptional activity suggest that even homozygote carriers of variant alanine homopolymer stretches may possess residual PMX2B function. This is consistent with the fact that *Pmx2b* heterozygous knockout mice are viable, with no reported abnormalities (29). This may also account for the lack of robust association between schizophrenia and *PMX2B* Ala20 variations. In-frame expansions but not deletions of polyalanine stretches in transcription factors and homeodomain genes have been well documented, along with associated clinical traits, all of which are inherited in an autosomal dominant manner, as with *CBFA1* (30), *HOXA13* (31), *HOXD13* (32), *ZIC2* (33) and, as recently reported, *PMX2B* (16). All these mutations are rare, possibly resulting from *de novo* generation (and a mechanism of unequal crossing-over during meiosis), and result in profound phenotypic defects. The uniqueness of polyalanine length variations in PMX2B is thus interesting to consider in terms of commonness, more frequent deletion than insertion, all in the context of a historically ancient human-specific origin with stable transmission and mild phenotypic effect. The relevance of polymorphisms in Ala20 in contrast to Ala9 of human *PMX2B* gene is unknown. Intriguingly, Ala20 in PMX2B is flanked by sequences rich in serine, glycine, proline (residues that could be generated by substitution of the first or second nucleotides within a progenitor alanine codon, GCX), and additional alanine residues (Fig. 1), whereas Ala9 is not. The suggestion is that the current Ala20 stretch might have undergone dynamic evolutional change (34).

In summary, MPAs have been found to display increased prevalence in a range of neurodevelopmental disorders other than schizophrenia, including learning disabilities, congenital speech, hearing impairments, attention deficit hyperactivity disorder (35) and autism (36). Investigation of the prevalence of strabismus in those neuropsychiatric disorders, and assessment of the role of *PMX2B* variations in strabismus conveyed by these illnesses and in the development of the illnesses themselves may therefore prove worthwhile.

MATERIALS AND METHODS

Subjects

For ophthalmologic examinations, 346 schizophrenia (mean age 42.8 ± 8.3 years) and 542 mentally healthy controls (mean age 42.5 ± 11.0 years) were recruited. All subjects were collected from a single geographic area in central Japan. Diagnosis of schizophrenia was achieved by direct interview, based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (37), with consensus from at least three experienced psychiatrists. All available medical records were taken into consideration. All patients underwent computer tomography examination of the brain to exclude organic abnormalities. None of the current subjects displayed mental retardation or congenital central hypoventilation syndrome (CCHS) (16). Control subjects were recruited from among volunteers documented as free of psychoses. A total of 124 families were recruited separately to test for transmission stability of *PMX2B* variants. These comprised 80 families with one offspring plus parents, 18 with two offspring plus parents and five with three offspring plus parents. Biological parentage in all families was confirmed by examining 444 highly polymorphic microsatellite markers from Japanese cohorts (mean heterozygosity = 0.73) (38). The present study was approved by the Ethics Committees of RIKEN and Hamamatsu Medical University, and all participants provided written informed consent.

Ophthalmologic examination

Clinical assessments were made by trained medical doctors (Tomoko Toyota, Yoshio Minabe and Kiyoshi Yoshitugu) in a blind study, and confirmed by an independent and experienced ophthalmologist (Hajime Fujikura). The Hirschberg test and two kinds of cover tests (a cover–uncover test and an alternating cover test) were conducted to detect and classify strabismus and to exclude heterophoria. None of the strabismus sufferers displayed incomitant strabismus, accommodation esotropia, or systemic abnormalities including developmental and metabolic defects, brain damage or mental retardation.

Mutation screening of *ARIX* and *PMX2B*

ARIX comprises three exons with the initiation codon in exon 1 and a stop codon in exon 3 (39). We screened the coding region, flanking introns and promoter sequences (1210 bp upstream from the reported 5′ end of exon 1) (39), using PCR amplification and sequencing of genomic DNA from 24 schizophrenia samples who showed constant exotropia (Table 1).

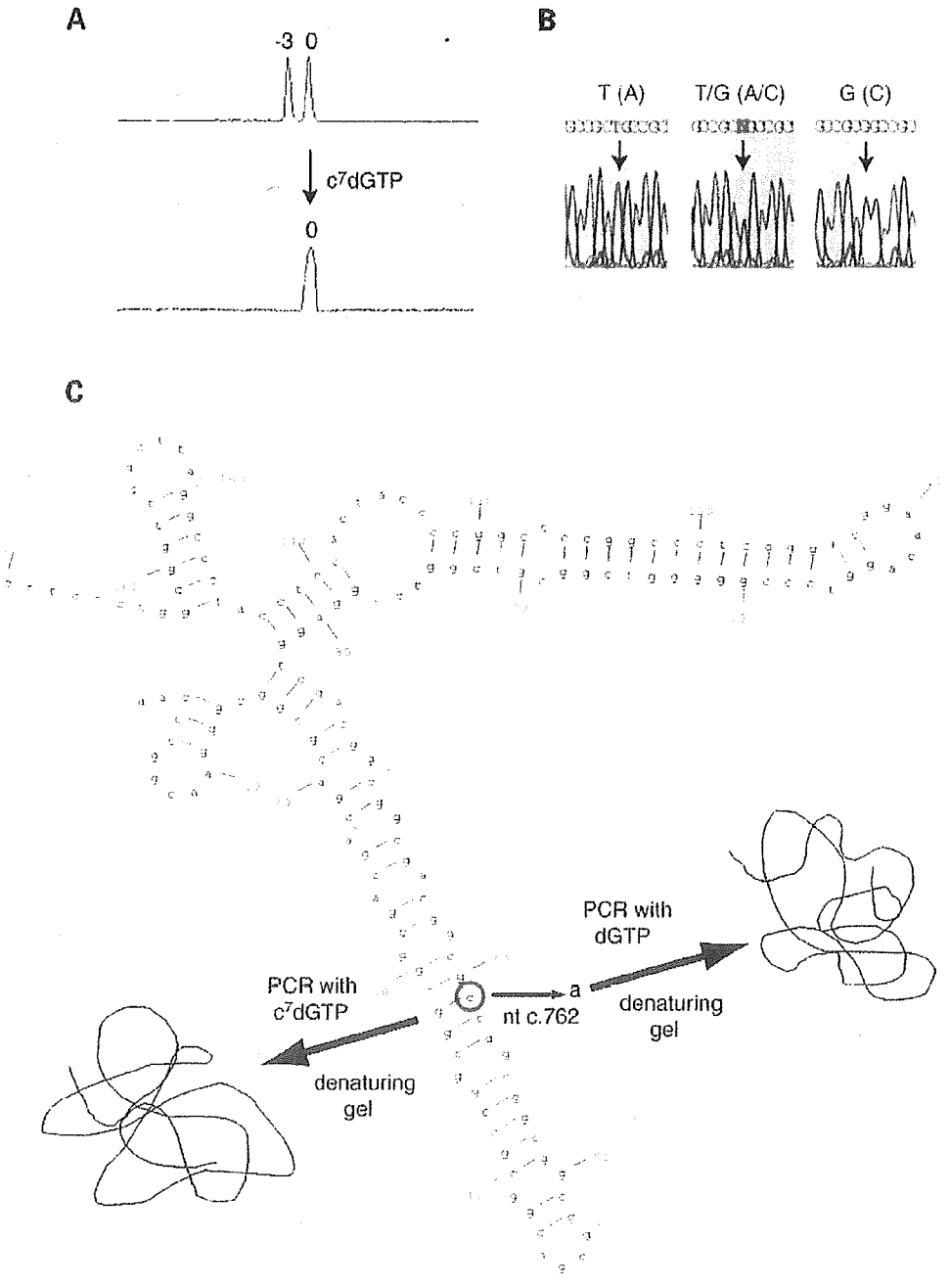


Figure 5. Effect of c.762A>C polymorphism on aberrant mobility shift in a denaturing gel. Ala20 templates with a c.762C migrate at a −3 base position in the absence of 7-deaza-2′-deoxyguanosine triphosphate (c^7dGTP). This aberrant −3 base allele peak was removed on the addition of c^7dGTP to the PCR reaction mixture (**A**). Without c^7dGTP, 17% of total non-deletion/insertion alleles displayed the −3 base migration. Diagram of sequences (anti-sense strands) shows the c.762A>C polymorphism (**B**). Secondary structure of the DNA fragment spanning the Ala20 portion was predicted using GENETYX-MAC version 11 software (GENETYX Corporation, Tokyo, Japan) (**C**). Minimum free energy generated by the secondary structure was −78.3 kcal/mol for the fragment with c.762A and −83.5 kcal/mol for the fragment with c.762C. The fragment with c.762C was deemed to retain at least partial secondary structure even under denaturing conditions. This remnant secondary structure is apparently destroyed when the fragment is PCR-amplified in a reaction mixture containing c^7dGTP.

For detecting mutations in *PMX2B*, an initial screen was performed on the coding region, flanking introns and promoter sequences (130 bp upstream from the reported 5′ end of exon 1) (15), using the same samples as in *ARIX*. To identify single nucleotide polymorphisms (SNPs) for use in linkage disequilibrium analysis, we analyzed a region up to 2204 bp upstream from the 5′ start of the gene, introns 1 and 2 and 1913 bp downstream from the 3′ end, by examining 30 additional

Table 2. Strabismus and the Ala20 polymorphism of *PMX2B* gene

Sample	Strabismus	*n*	Genotype counts[a]					*P*-value[b]
			0/0	0/−15	−15/−15	0/−21	0/+6	
Control	Constant exotropia	2	2	0	0	0	0	1.000
	No strabismus	515	472	33	3	6	1	
Schizophrenia	Constant exotropia	24	17	6	0	0	1	0.029
	No strabismus	300	266	32	0	1	1	
Total	Constant exotropia	41	19	6	0	0	1	0.017
	No strabismus	815	738	65	3	7	2	

[a]0 indicates the wild type allele, and − or + denotes the variant alleles with the indicated number of deletions (−) or insertions (+) of Ala20-coding nucleotides.
[b]*P*-value for association was calculated by Fisher's exact test.

Table 3. Schizophrenia and the Ala20 polymorphism of *PMX2B* gene

Subjects	*n*	Genotype counts (%)[a]					*P*-value[b]
		0/0	0/−15	−15/−15	0/−21	0/+6	
Total schizophrenia	324	283 (87)	38 (12)	0 (0)	1 (0.3)	2 (0.6)	0.012
Without strabismus	300	266 (87)	32 (11)	0 (0)	1 (0.3)	1 (0.3)	0.076
With constant exotropia	24	17 (71)	6 (25)	0 (0)	0 (0)	1 (4.2)	0.004
Controls[c]	515	472 (92)	33 (6.4)	3 (0.6)	6 (1.2)	1 (0.2)	

[a]0 indicates the normal allele, and − or + denotes the variant alleles with the indicated number of deletions (−) or insertions (+) of the Ala20-coding nucleotides.
[b]*P*-value for association was calculated by Fisher's exact test.
[c]Subjects who had strabismus were excluded.

samples. Information on primer sequences used in this study is available on request.

Genotyping of *PMX2B* polymorphisms

The genomic region encoding the 20 alanine (Ala20) tract was amplified using fluorescently labeled forward primer (5′-AACCCGGCAAGGGCGGCGCAGCA, 3′ end at nt c.726) and reverse primer (5′-GAAGGGACCCCCAAGCGAAT, 3′ end at nt c.854), rTaq polymerase (Takara, Tokyo, Japan) and MasterAmp K buffer (Epicentre Technologies, Madison, WI). To avoid artefactual −3 base shifts seen in templates with c.762C, we added 0.2 mM of 7-deaza-2′-deoxyguanosine triphosphate (c^7dGTP) to PCR reaction mixtures (Fig. 5). PCR products were run on an ABI 3700 genetic analyzer and the resulting data analyzed using GeneScan and Genotyper software (Applied Biosystems, Foster City, CA). Genotypes of mutants were verified using both direct sequencing and subcloning of amplicons into a TA vector (Invitrogen, Carlsbad, CA) and sequencing. Sequencing was performed using a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences, Piscataway, NJ). TaqMan assay was used to type SNP markers (Applied Biosystems). The mouse Ala20 tract of *Pmx2b* was examined in 120 C57BL/6 mice, 120 C3H/He mice and 120 of the F1 progeny, using the forward primer (5′-AGGCGAACCCGGCAAGGGCGGT, 3′ end at nt c.657) and reverse primer (5′-GAAGGGCCCCCCAAGA GAATCT, 3′ end at c.789).

Constructs for luciferase assay

The coding region of *PMX2B* (accession no. NM_003924) was amplified using Human Brain Marathon-Ready cDNA as a template (Clontech, Palo Alto, CA), then cloned into pIRES-neo2 expression vector (Clontech). Altered Ala20 length constructs were prepared by swapping the Ala20 region with those amplified from mutant genomic DNA or using PCR-based techniques (40). The gene promoter for dopamine β-hydroxylase (*DBH*) (41) was amplified using a primer set designed from the genomic sequence (accession no. AC001227), then cloned into the pGL3-basic reporter vector (Promega, Madison, WI). Constructs lacking the entire Ala20 and Ala9 regions were generated using PCR-based techniques (40).

Transfection and luciferase assay

HepG2 cells were purchased from the Riken Cell Bank (Tsukuba, Japan). The plasmid mixture was prepared by combining 1.3 μg of construct DNA (pIRES-neo2-*PMX2B*:pGL3-basic-*DBH* promoter = 400 μg : 900 μg), 100 μg of pRL-TK as an internal control and 2.5 μl of LipofectAMINE2000 in 100 μl of OPTI-MEM (Invitrogen). Transfections were performed using Lipofect AMINE2000 (Invitrogen) according to the instructions of the manufacturer. Transcriptional assay was performed using the PicaGene Dual SeaPansy kit in accordance with the manufacturer's instructions (Toyo Ink, Tokyo, Japan). Luciferase activity was measured using a luminometer Lumat LB 9507 (EG&G Berthold, Bad Wildbad, Germany).

Statistical analyses

Phenotype–genotype association tests were assessed using the χ^2 test, or Fisher's exact test where appropriate. Linkage disequilibrium (LD) statistics were calculated using COCAPHASE (42) (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/), and estimation

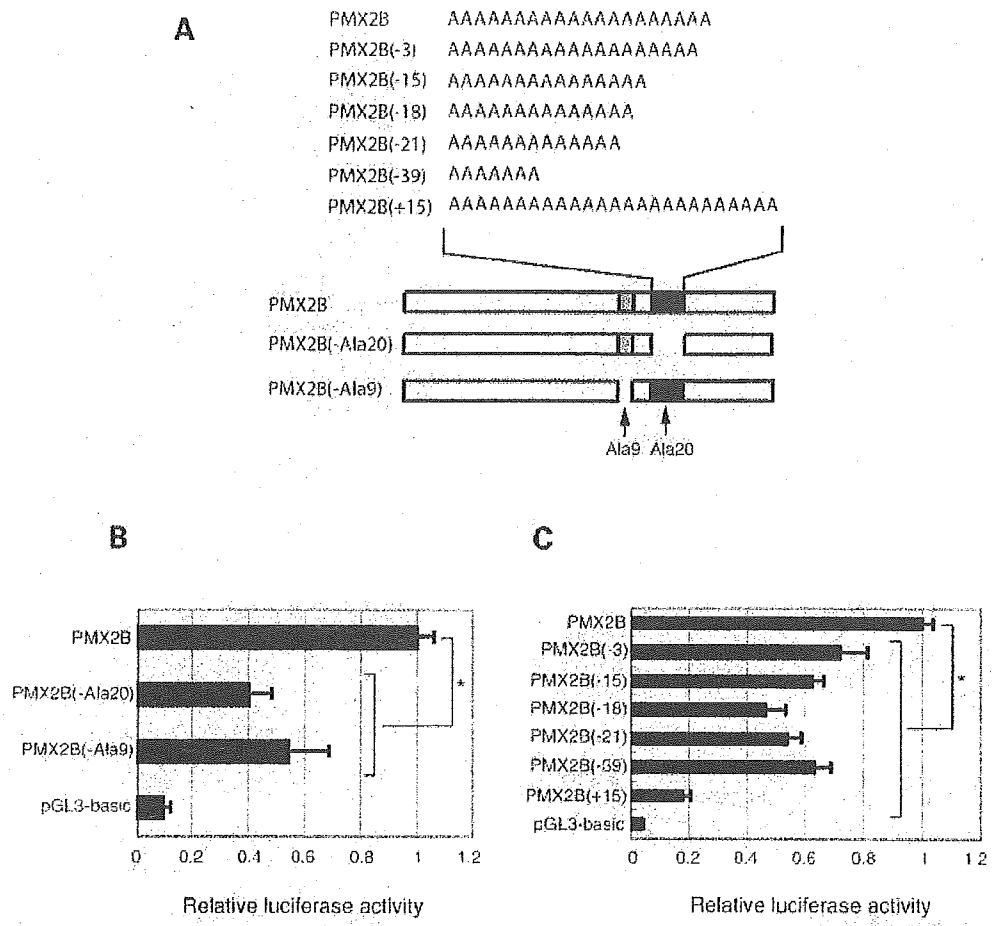


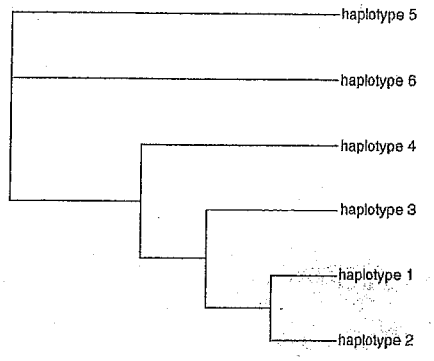
Figure 6. Functional consequences of *PMX2B* mutant alleles. Preparation of *PMX2B* deletion/insertion mutants is shown (**A**). A, alanine. Effects of Ala20 and Ala9 stretch deletions were examined under luciferase assay. Reporter constructs containing the *DBH* promoter were co-transfected into HepG2 cells with either normal *PMX2B* or deletion mutants. Luciferase activity of each construct was normalized by the internal control, pRL-TK. Activity of the normal *PMX2B* was defined as 1. Results shown represent means ± SEM for at least three separate transfections, each run in triplicate. pGL3-basic is a promotorless negative control vector. $^*P < 0.01$ by Tukey–Kramer multiple comparison test (**B**). Assay of Ala20 mutants was performed under the same conditions as in B (**C**). $^*P < 0.01$ by Tukey–Kramer test.

Table 4. Pairwise linkage disequlibrium estimations between polymorphisms in the *PMX2B* gene

Polymorphism	IVS1-385G>A (SNP1)	IVS1-115G>A (SNP2)	IVS2+101A>G (SNP3)	IVS2-404-405delAG (SNP4)	c.1618insT (SNP5)	c.2309G>A (SNP6)
SNP1	—	1.000	1.000	1.000	1.000	1.000
SNP2	1.000	—	1.000	1.000	1.000	1.000
SNP3	0.984	0.984	—	0.984	1.000	1.000
SNP4	0.984	0.984	0.968	—	1.000	1.000
SNP5	0.969	0.969	0.954	0.954	—	0.967
SNP6	0.86	0.858	0.846	0.846	0.831	—

Values above the diagonal show standardized D' in 200 unrelated subjects, calculated by using the COCAPHASE program. Values below the diagonal show r^2 (squared correlation coefficient).
The polymorphisms used in this linkage disequlibrium analysis (SNPs1–6) were those whose minor allele frequencies were more than 3% (also see Fig. 1).
The Ala20 stretch is located between SNP4 and SNP5.

of haplotype frequencies and assessment of Hardy–Weinberg equilibrium were performed using Arlequin software (http://lgb.unige.ch/arlequin/). Genotype data from 100 males and 100 females were used for LD and haplotype analyses. CLUSTALW (program for multiple alignments and tree-making; http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) (43) and TreeView ver.1.6.6 (program for displaying phylogenies; http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (44) software



	SNP1	SNP2	SNP3	SNP4	Ala20	SNP5	SNP6	frequency
haplotype 1	A	A	G	D	1	I	A	0.0784
haplotype 2	A	A	G	D	2	I	A	0.1303
haplotype 3	G	G	A	I	1	D	A	0.0262
haplotype 4	G	G	A	I	1	D	G	0.6453
haplotype 5	G	G	A	I	2	D	G	0.0379
haplotype 6	G	G	A	I	3	D	G	0.0608

Figure 7. Phylogram of haplotypes in *PMX2B*. For nomenclature of SNPs 1–6, see Figure 1. For SNP4, 'D' indicates deletion and 'I' denotes insertion. For Ala20, allele 1 = c.762A, allele 2 = c.762C, allele 3 = 15-bp deletion. Six different haplotypes covered 97.9% of the total number of haplotypes.

was used to depict the evolutionary history of haplotypes in a phylogram. Power analysis was performed using the Genetic Power Calculator (http://stagen.iop.kcl.ac.uk/gpc/) (45).

ACKNOWLEDGEMENTS

We would like to express our gratitude to all participants in this study. We greatly appreciate the assistance of ophthalmologist H. Fujikura for his expert examinations. We wish to thank Y. Ishituka for his technical assistance and the following psychiatrists for their help in collecting psychiatric patients: T. Kumakura and T. Ichimiya. We would like to thank nurse C. Tachibana for her assistance with physical examinations. This work was supported by RIKEN BSI Funds and Research on Brain Science Funds from the Ministry of Health Labor and Welfare, Japan.

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The Pharmacogenomics Journal (2004) 4, 114–126

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ORIGINAL ARTICLE

Comprehensive expression analysis of a rat depression model

N Nakatani[1]
H Aburatani[1,2]
K Nishimura[3]
J Semba[1,4]
T Yoshikawa[1]

[1]*Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Wako, Saitama, Japan;* [2]*Department of Cancer Systems Biology, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan;* [3]*Department of Information Systems, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan;* [4]*The University of the Air, Chiba, Japan*

Correspondence:
Dr T Yoshikawa, Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
Tel: +81 48 467 5968
Fax: +81 48 467 7462
E-mail: takeo@brain.riken.go.jp

Received: 20 May 2003
Revised: 03 November 2003
Accepted: 10 December 2003

ABSTRACT

Herein we report on a large-scale analysis of gene expression in the 'learned helplessness' (LH) rat model of human depression, using DNA microarrays. We compared gene expression in the frontal cortex (FC) and hippocampus (HPC) of untreated controls, and LH rats treated with saline (LH-S), imipramine or fluoxetine. A total of 34 and 48 transcripts were differentially expressed in the FC and HPC, respectively, between control and LH-S groups. Unexpectedly, only genes for NADH dehydrogenase and zinc transporter were altered in both the FC and HPC, suggesting limited overlap in the molecular processes from specific areas of the brain. Principal component analysis revealed that sets of upregulated metabolic enzyme genes in the FC and downregulated genes for signal transduction in the HPC can distinguish clearly between depressed and control animals, as well as explain the responsiveness to antidepressants. This comprehensive data could help to unravel the complex genetic predispositions involved in human depression. *The Pharmacogenomics Journal* (2004) 4, 114–126. doi:10.1038/sj.tpj.6500234

Keywords: learned helplessness; DNA microarray; frontal cortex; hippocampus; antidepressant

INTRODUCTION

Depression is a complex psychiatric disease with specific symptoms that include depressed mood, loss of interest, diminished appetite, sleep disturbances and psychomotor retardation. Depression is common, with lifetime prevalence estimated to be up to 20%,[1] and the condition exacts high personal and social costs on sufferers. The illness is also a major cause of suicide. Epidemiological studies suggest a genetic component to affective disorder,[1,2] and efforts to identify susceptibility genes by linkage and other genetic analyses are being conducted.[3] However, the precise etiologies remain elusive, as does the development of new therapies against depression, particularly for cases that are refractory to conventional therapy. In the case of complex trait diseases, isolating genetic mechanisms using human disease material is often difficult because of sample heterogeneity and other confounding factors. Analysis of suitable animal models under strictly controlled conditions would therefore be beneficial.

To investigate the molecular basis of depression, we have applied DNA microarray technology to analyze gene expressions in learned helplessness (LH) rats, an animal model of depression. After pretreatment with repeated inescapable shocks, animals with LH display decreased ability to escape adverse situations. This behavioral model was originally described in dogs,[4] and later analogous behavior was induced in rats.[5] LH animals display behavioral phenotypes resembling human depressive symptoms, and LH can be ameliorated

using antidepressant drugs.[6,7] LH therefore fulfills the parameters of construct validity, face validity and predictive validity,[8] confirming the suitability of the model for studying the neurobiology of depressive illness and the actions of antidepressants.[9–11] It is also important to note that the 'depressive state' in LH animals lasts for over 3 weeks,[12] making this model particularly useful for studying the chronic changes in brain physiology that accompany depression. We examined the frontal cortex (FC) and hippocampus (HPC) of LH rats, because positron emission tomography scanning and functional magnetic resonance imaging studies have recently indicated a potential abnormality in the frontal cortex of both familial bipolar and unipolar depressives.[13] In addition, recent evidence has suggested that neurogenesis in the HPC may be disturbed in depressive patients.[14–16]

In this study, we have analyzed brain transcripts altered during LH and followed their responsiveness to a classical tricyclic antidepressant (TCA), imipramine, and a new generation selective serotonin reuptake inhibitor (SSRI), fluoxetine. In addition, we performed principal component analysis (PCA) to extract essential gene sets from complex expression data sets that can best explain the different pathophysiological conditions. This was achieved by considering genes as variables in PCA. When genes are variables, the analysis creates a set of principal gene components indicating the features of genes that best explain the experimental responses. Using these comprehensive pharmaco-behavioral genetic approaches, we have attempted to generate data that would eventually allow for the formulation of hypotheses to help understand the molecular and genetic pathophysiology of depression. This in turn could lead to the development of novel antidepressants with greater efficacy.

RESULTS AND DISCUSSION

Effectiveness of Antidepressants in Learned Helplessness

The LH model is difficult to generate, requiring meticulous refinement of multiple experimental parameters. In our experimental setting, after inescapable shock pretreatment, animals were subjected to 15 avoidance trials at 30 s intervals. In each trial, a current was applied via the floor grid during the first 3 s. If an animal moved to a neighboring compartment within this period (escape response), the shock was terminated. Failures in escape response were counted as a measure of LH. We defined animals as being in a state of LH when escape failures were demonstrated in more than half of the trials in the session. Using this system, we reproducibly induced LH in rats with a success rate of ~40%. LH rats were subsequently treated with repeated injections of saline (LH-S), fluoxetine (LH-F) or imipramine (LH-I), then re-evaluated for escape responses in the test session. Figure 1 shows a schematic of these procedures. During the escapable shock of the test session, all animals in the LH-S group ($n=10$) showed more than eight escape failures, and the mean failure was significantly higher than that of control rats (those that were not given inescapable shock, $n=15$) (Figure 2). Antidepressant administration significantly reduced the number of escape failures for both LH-F ($P<0.05$) and LH-I ($P<0.01$). Imipramine recovered all

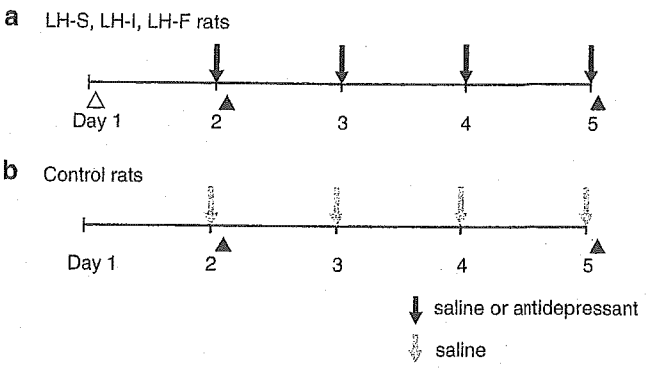

Figure 1 Schematic representation of behavioral procedures. (a) To induce the LH state, animals were given inescapable shock (△) on day 1. On day 2, they received escapable shock (▲), and were selected as 'LH rats' if they showed greater than 50% failure in escape responses. LH animals were then administered saline (LH-S) or antidepressants (LH-F, LH-I) for 4 consecutive days. These animals received escapable shock (▲) again on day 5 to determine whether they were still in the LH state. (b) Control rats were not given inescapable shock on day 1, but treated in the same way thereafter as the LH rats.

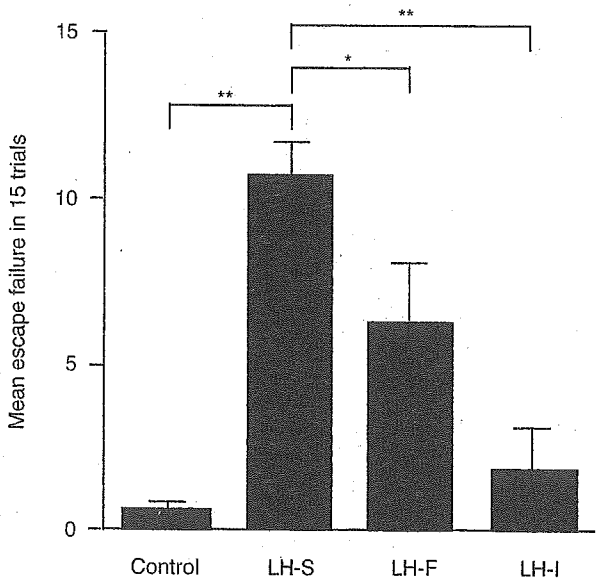

Figure 2 Mean number of escape failures (±SE) during the 15 avoidance trials. Controls ($n=15$) were not given inescapable shocks. Rats exposed to prior inescapable shocks were treated with saline (LH-S) ($n=10$), fluoxetine (LH-F) ($n=7$) or imipramine (LH-I) ($n=9$) once a day for consecutive days (days 2–5). Escape failure refers to the failure of animals to move into the safe compartment during electric footshock (0.5 mA, 3 s duration). The mean numbers of escape failures among groups were evaluated by ANOVA, $F(3,37)=23.69$. $^{*}P<0.01$ and $^{**}P<0.001$ by *post hoc* Tukey–Kramer test.

rats from the LH state ($n=9$), and fluoxetine reinstated five out of seven. These results confirm the persistency of LH in our animals and the effectiveness of antidepressants in this model.[5,17–20] Fluoxetine produced a weaker response compared to imipramine in alleviating the LH phenotype (Figure 2). We also tested larger doses of each drug, 10 mg/kg i.p. of fluoxetine and 50 mg/kg i.p. of imipramine, but did not observe any significant change in the number of escape failures (10 mg/kg of fluoxetine, 5.0 ± 1.0 ($n=3$); 50 mg/kg of imipramine, 2.0 ± 1.3 ($n=3$)) between the two doses. In addition, Anthony *et al*[21] showed that 5 mg/kg of fluoxetine, the same dose as used in the present study, was enough to elicit monoaminergic perturbation such as a reduced 5-hydroxytryptamine receptor 1B expression in the dorsal raphe of rats. Bristow *et al*[22] also demonstrated the validity and efficacy of 5 mg/kg of fluoxetine in ameliorating depressive behavior in rats. We attempted to minimize the nonspecific effects of the drugs by using the lowest possible dose that maintained antidepressive efficacy.

The present results may reflect the different clinical potencies of the individual agents. TCAs such as imipramine inhibit the reuptake of both serotonin and norepinephrine at nerve terminals by acting on monoamine transporters. In contrast, SSRIs including fluoxetine specifically block the reuptake of serotonin.[2] These differences in pharmacological profiles underlie the distinct antidepressive competences exerted by TCA and SSRI. Although human patients require over 2 weeks of medication before antidepressive effects are observed, we administered the drugs for 4 days in our rodent experiments, in keeping with the protocols of Geoffroy *et al*[6] (5-day treatment) and Tordera *et al*[23] (4-day treatment), and could replicate distinct behavioral responses to therapy.

We also measured weight gain during the 5 days of experiments. Weight increase in the LH-S group was significantly lower than that in controls (LH-S, 18.8 ± 4.4 g; control, 31.7 ± 2.2 g; $P<0.05$).

General Profiles of Gene Expressions Associated with LH and Antidepressant Treatments

We selected six animals each from the control (rats showing no escape failure in the escapable shock session) and LH-S groups, and five each from the LH-F and LH-I groups (these showed $\leqslant 7$ failures in the 15 trials), to perform microarray analyses. Patterns of gene expression in the two brain regions from the four rat groups were examined using the Affymetrix GeneChip U34A, which represents 8799 probe sets and codes over 8000 transcripts including known genes (>5000) and expressed sequence tags (ESTs). Transcript expression from extracted RNA displayed good linearity in both FC and HPC samples (Figure 3). Our stringent criteria identified 34 and 48 transcripts as differentially expressed between control ($n=6$) and LH-S ($n=6$) groups in the FC and HPC, respectively (henceforth referred to as 'LH-associated transcripts') (Figures 3a and b). However, none of these transcripts survived after the Benjamin and Hochbery False Discovery Rate analysis. This observation may confirm the statements of Mirnics *et al*[24] that true gene-expression changes in psychiatric traits are small and

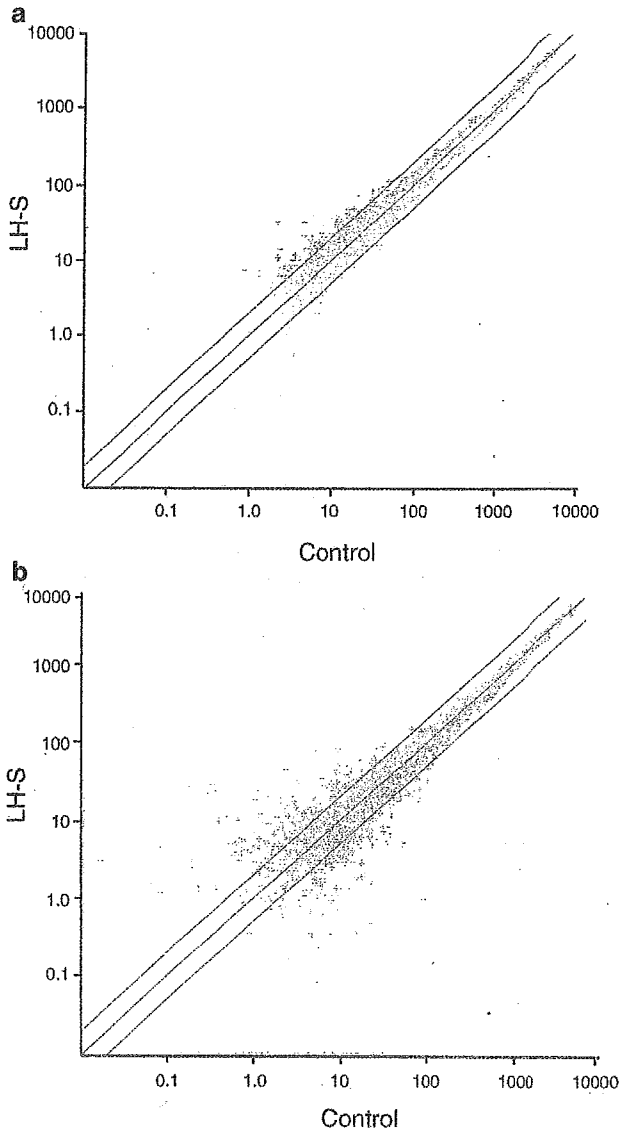

Figure 3 Scatter plot of log-intensity values for the over 8000 genes assayed with the RG-U34A chip in the frontal cortex (a) and hippocampus (b). Each point represents the log value from an average of six control or six LH-S animals.

psychiatric diseases may result from cumulative subtle changes.

Among LH-associated transcripts, five transcripts and one gene showed significant recovery to control levels from the LH state under both imipramine ($n=5$) and fluoxetine ($n=5$) administration in the FC and HPC, respectively (white portions in Figures 4a and b). Transcripts in the pink and yellow areas of Figure 4 represented expression levels that returned to normal after administration of imipramine and fluoxetine, respectively. Interestingly, no LH-associated transcripts demonstrated significant deviation from control levels after drug treatment. That is, none of the LH-associated genes were further decreased or excessively