is located in intron 3, the physiologic functions of which are unclear. To date, it has been reported that introns and their removal by the spliceosome can influence many stages of mRNA metabolism, including initial transcription of the gene, editing and polyadenylation of the pre-mRNA, nuclear export, translation, and decay of the mRNA product [11]. Therefore, the IVS3 + 258T > C polymorphism may have direct and/or indirect effects on the regulation of mRNA metabolism, although the functional roles for this SNP in the *FZD3* gene remain to be clarified. It is also possible that there is an unknown functional mutation in an exon that is in linkage disequilibrium with the IVS3 + 258T − 435G haplotype and influences the gene-product functions.

In conclusion, our data show significant association between schizophrenia and IVS3 + 258T > C polymorphism or IVS3 + 258T − 435G haplotype in the *FZD3* gene, suggesting that dysregulation of the Wnt-signaling pathway may be involved in the predisposition to schizophrenia. Our findings may need to be confirmed by replication in other populations or methods like the transmission disequilibrium test to avoid population stratification. Functional analyses of the IVS3 + 258T > C polymorphism, precise mapping, and mutation screening in the entire *FZD3* gene are necessary to further understand the role of FZD3 in the etiology of schizophrenia.

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Relationship Between Catechol-*O*-Methyltransferase Polymorphism and Treatment-Resistant Schizophrenia

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Catechol-*O*-methyltransferase (COMT) plays a crucial role in the regulation of central dopaminergic systems. We examined the allelic association of a functional polymorphism of the COMT gene with the clinical manifestations and the response to antipsychotics of 100 schizophrenic patients and 201 healthy controls from the general Japanese population. No statistically significant difference was observed in the allele and genotype frequencies between the schizophrenic patients and the healthy controls. The daily neuroleptic dosage that patients received during their maintenance therapy was significantly higher in patients with the L/L genotype than in the other patients ($P < 0.05$). The present results suggest that the presence of the COMT genotype does not help in evaluating the susceptibility to the development of schizophrenia, but that it may help in the estimation of treatment-resistant features of schizophrenia.

KEY WORDS: COMT; dopamine; antipsychotic drug; extrapyramidal symptom; gene

INTRODUCTION

Polymorphisms at gene loci related to central dopaminergic systems have been extensively examined as candidate loci for schizophrenia because antipsychotic drugs, which act as dopamine antagonists, are effective in the treatment of psychiatric symptoms seen in schizophrenic patients.

Although the results are still controversial, a number of studies have shown a positive association between schizophrenia as a whole or subgroups of this disease with some specific polymorphic sites of the gene loci related to central dopaminergic transmission. However, whether specific mutations in the dopamine-related genes can contribute significantly to the clinical features of schizophrenia still remain to be demonstrated.

Catechol-*O*-methyltransferase (COMT) metabolizes catecholamines such as dopamine, and catechol drugs such as L-DOPA. The *COMT* gene, which lies on the cytogenetic band of 22q11.2 on chromosome 22, has been considered to be one of the candidate genes for schizophrenia because it inactivates dopamine. There is an amino acid alteration that determines the activity of the COMT enzyme [Lotta et al., 1995; Lachman et al., 1996; Sander et al., 1997]. It has been shown that a G–A transition exists at codon 158 of the *COMT* gene that results in a valine-to-methionine substitution. The association with this functional polymorphism has been examined extensively in the general population of schizophrenic patients or in subgroups with various clinical features. For example, relationships with this polymorphism have been reported in schizophrenia [Ohmori et al., 1998], violence in patients with schizophrenia or schizoaffective disorders [Lachman et al., 1998], homicidal schizophrenia [Kotler et al., 1999], suicidal behavior in patients with schizophrenia [Nolan et al., 2000], and aggressive behavior in patients with schizophrenia [Jones et al., 2001]. This polymorphism is reportedly associated with specific subgroups of various psychiatric diseases such as type-I alcoholism [Tiihonen et al., 1999], higher alcohol consumption [Kauhanen et al., 2000], delirium tremens in alcoholics [Nakamura et al., 2001], polysubstance abuse [Vandenbergh et al., 1997], obsessive-compulsive disorder [Karayiorgou et al., 1997], rapid-cycling mood disorder [Papolos et al., 1998], female bipolar disorder [Mynett-Johnson et al., 1998], and depressive disorder

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[Ohara et al., 1998]. However, there are also some reports of a lack of significant association with schizophrenia [Daniels et al., 1996; Strous et al., 1997; Wei and Hemmings, 1999; de Chaldee et al., 2001; Liou et al., 2001], affective disorders [Kunugi et al., 1997], and alcoholics [Ishiguro et al., 1999], suggesting that the exact role of COMT polymorphism in determining the phenotype in terms of clinical psychiatry and human behavior is not yet fully elucidated.

The present study examined the relationship between COMT and schizophrenia in the Japanese population, focusing on the allelic association of COMT polymorphism with the clinical manifestations and antipsychotic features of schizophrenia.

MATERIALS AND METHODS

Ethical Considerations

This study had the approval of the ethics committee of the Kohnodai Area, National Center of Neurology and Psychiatry, Chiba, Japan. Written informed consent was obtained from all subjects who participated.

Subjects

The subjects for the present study were chronic inpatients who met the criteria of the DSM-IIIR diagnosis [American Psychiatric Association, 1987] for schizophrenia, who had been hospitalized and had been receiving antipsychotic therapy for at least 1 year during their current hospitalization. Volunteer control subjects were mostly medical staff with no history of psychosis or substance abuse, or of receiving antipsychotic medication. All subjects were Japanese and had Japanese parents.

Status of Extrapyramidal Symptoms

Extrapyramidal symptoms (EPS) induced by antipsychotic medication were basically evaluated using DIEPSS [Inada et al., 2002]. We assumed that acute EPS had been present within 3 months of the initial neuroleptic therapy if the clinical records clearly described EPS and a subsequent reduction in the neuroleptic dosage or a subsequent addition of antiparkinsonian drugs. Patients who showed no signs of acute EPS in spite of receiving neuroleptic therapy for more than 6 months were regarded as not having acute EPS. Tardive dyskinesia (TD) was assessed with the Japanese version of the Abnormal Involuntary Movement Scale (AIMS). The diagnosis of TD was made according to the criteria of Schooler and Kane [1982]. The inclusion criteria used to subclassify patients with or without TD were those reported by Inada et al. [1997]. Briefly, patients who had been suffering from TD with at least one item persistently rated 3 on the AIMS for more than 1 year or patients who had been suffering from persistent TD for more than 1 year that had developed within 5 years after the first neuroleptic exposure were classified as having TD. Patients who had never developed TD despite receiving neuroleptics for more than 10 years were regarded as not having TD. In rating EPS and TD, suspected cases were videotaped for later evaluation. When information about the presence of acute EPS in the initial stage of neuroleptic therapy for the first episode of schizophrenia was incomplete, the patient was excluded from the acute EPS study. When the TD status could not be obtained, the patient was omitted from the TD study.

Neuroleptic Dosage

Information about the neuroleptic therapy that schizophrenic patients had been receiving was obtained from their clinical records. The daily neuroleptic dosage was calculated from the most recent 1-year neuroleptic prescription history. The chlorpromazine-equivalent daily dose administered to each patient was calculated from a table developed specifically for Japanese patients [Inagaki et al., 1998].

Definition of Treatment-Resistant Schizophrenia

Schizophrenic patients were diagnosed as having treatment-resistant schizophrenia (TRS) when they had been hospitalized for more than 1 year and had been receiving antipsychotic therapy at dosages of at least 1,000 mg/day chlorpromazine equivalents for more than 1 year.

Experimental Procedure

Genomic DNA was extracted from samples of total blood obtained from the subjects. The COMT polymorphism is generated by the presence of a G or A encoding a valine or methionine at codon 158. The 210-bp target segment was amplified by the polymerase chain reaction (PCR) method according to the standard protocol [Daniels et al., 1996], using the primers 5′-ACT GTG GCT ACT CAG CTG TG and 5′-CCT TTT TCC AGG TCT GAC AA. The PCR products were digested with 5 units of Nla III for 2 hr at 37°C, and electrophoresed using automated and manual staining of DNA separated on polyacrylamide gels with a DNA silver staining kit (Pharmacia Biotech, Tokyo, Japan).

Statistics

Allele and genotype frequencies were compared by using the chi-squared test for 2×2 and 2×3 contingency tables. The association between neuroleptic-induced EPS (the status of TD and acute EPS) and the polymorphism was also assessed by the chi-squared test. Comparison of the daily neuroleptic dosage between the L/L and the other genotypic subgroups was performed using the Mann–Whitney U test. The probability values are listed in the tables. The computer package SPSS for Windows (release 11.0J, SPSS Japan, Tokyo, Japan) was used for the statistical analyses.

RESULTS

The COMT genotype (H/H, H/L, L/L) and allele frequency in schizophrenic subjects are shown in Table I. No significant differences in allele frequencies were

TABLE I. Genotype and Allele Frequencies of Catechol-*O*-Methyltransferase H/L Polymorphism in Schizophrenic Patients and Controls

Group	Genotype distribution (%)			*P* value (vs. controls)	Allele frequency (%)		*P* value (vs. controls)
	H/H	H/L	L/L		H	L	
Controls (n = 201)	43.8	49.8	6.5	—	68.7	31.3	—
Schizophrenia (n = 100)	41.0	51.0	8.0	0.88	66.5	33.5	0.59
Delusion and hallucination (n = 64)	51.6	43.8	4.7	0.53	73.4	26.6	0.31
Bizarre behavior (n = 70)	50.0	44.3	5.7	0.67	72.1	27.9	0.44
Disorganization (n = 72)	44.4	51.4	4.2	0.77	70.1	29.9	0.74
Negative symptoms (n = 54)	55.6	38.9	5.6	0.30	75.0	25.0	0.20
Positive first-degree family history (n = 42)	47.6	45.2	7.1	0.87	70.2	29.8	0.78
Onset at 21 years or younger (n = 45)	44.4	48.9	6.7	0.99	68.9	31.1	0.97

For comparison between controls and schizophrenic patients as a whole or their subgroups listed in this table, two-tailed chi-squared tests for 2 × 2 and 2 × 3 contingency tables were performed.

observed between the healthy controls and schizophrenic patients as a whole or any of the subgroups classified according to the psychiatric symptoms seen at the patient's first episode.

Table II shows the genotype and allele frequencies of the COMT H/L polymorphism and the status of TD and acute EPS in schizophrenic patients. No significant differences were observed in the susceptibility to neuroleptic-induced extrapyramidal side effects among three subgroups of the COMT genotype.

The characteristics of neuroleptic treatment among the three subgroups showing COMT H/L polymorphism are shown in Table III. The daily neuroleptic dosage received during maintenance therapy was significantly higher in patients with the L/L genotype than in the other patients (Mann–Whitney U test, $Z = -2.248$, $P = 0.0246$). The rate of TRS tended to be higher in patients with the COMT L/L genotype than in the other patients, although this is a marginal difference statistically expressed as a significant trend level (chi-squared = 3.782, df = 1, $P = 0.052$). The odds ratio of L/L for TRS was 4.392 (95% confidence interval: 0.894–21.588).

DISCUSSION

In the present study, no significant association was observed between COMT polymorphism and schizophrenic patients as a whole or any of the subgroups classified according to the psychiatric symptoms seen at the patient's first episode, suggesting that this polymorphism is unlikely to play an essential role in the development of schizophrenia. The lack of association with the general population of schizophrenic subjects is consistent with previous reports of French [de Chaldee et al., 2001], Taiwanese [Liou et al., 2001], Turkish [Herken and Erdal, 2001], and Caucasian [Wei and Hemmings, 1999] schizophrenic populations, but contradictory to the report of Ohmori et al. [1998]. Although most of the studies failed to detect significant relationships between this polymorphism and schizophrenic subjects as a whole [Chen et al., 1999; Semwal et al., 2001], relationships with this polymorphism have been reported in certain subgroups of schizophrenia [Herken and Erdal, 2001; Liou et al., 2001]. Recently, Egan et al. [2001] reported that the COMT low-activity genotype slightly increases the risk for schizophrenia by its effect of increasing prefrontal dopamine catabolism.

Here we have used a definition of TRS that is modified from that proposed by Kane et al. [1988], which was defined based on observations of monotherapy antipsychotic treatment. The main reason for modifying the definition of TRS is that Japanese psychiatrists generally prefer polypharmacy in routine clinical practice. If patients relapse during their antipsychotic maintenance therapy, Japanese psychiatrists often prescribe another antipsychotic agent in addition to the original one, instead of switching to it, so that it is quite rare for patients in Japanese routine psychiatric practice to be diagnosed as having TRS as proposed by Kane et al.

TABLE II. Genotype and Allele Frequencies of Catechol-*O*-Methyltransferase H/L Polymorphism and the Status of TD and Acute EPS in Schizophrenic Patients

Group	Genotype (%)			*P* value	Allele frequency (%)		*P* value
	H/H	H/L	L/L		H	L	
Status of TD							
Patients with TD (n = 33)	42.4	51.5	6.1	0.79	68.2	31.8	0.60
Patients without TD (n = 46)	34.8	58.7	6.5		64.1	35.9	
Status of acute EPS							
Acute EPS present (n = 30)	43.3	50.0	6.7	0.67	68.3	31.7	0.44
Acute EPS absent (n = 45)	33.3	57.8	8.9		62.2	37.8	

For comparison of the status of TD and acute EPS, two-tailed chi-squared tests for 2 × 2 and 2 × 3 contingency tables were performed. TD, tardive dyskinesia; EPS, extrapyramidal symptom.

TABLE III. Characteristics of Neuroleptic Treatment Among Three Subgroups Showing Catechol-*O*-Methyltransferase H/L Polymorphism

	Catechol-*O*-methyltransferase H/L polymorphism		
	H/H	H/L	L/L
Daily neuroleptic dosage (mg/day) (n = 78)	728 ± 655 (n = 32)	685 ± 621 (n = 40)	1,226 ± 1,069* (n = 6)
Rate of treatment-resistant cases	25.0%	22.0%	57.1%

Data are expressed as mean ± SD. Daily neuroleptic dosage was calculated from the most recent 1-year neuroleptic therapy as chlorpromazine equivalent dose using the table of Inagaki et al. [1998].
*$P < 0.05$ when compared to the (H/H + H/L) subgroups (Mann–Whitney U tests).

[1988], even when the schizophrenia is considered treatment-resistant. In the present study, a significantly higher daily neuroleptic dosage was observed in patients with the L/L genotype (average: 1,226 mg/day) than in those with the other genotypes. In addition, the rate of TRS tended to be higher in patients with the COMT L/L genotype than in the other patients. These findings suggest that the low-activity COMT genotype is more common in TRS. This is consistent with the results of Herken and Erdal [2001], who reported that Turkish schizophrenics with the L/L genotype may have clinical signs that are much more severe. Here we assumed that the patients with persistent and severe condition of psychoses usually receive high doses of antipsychotics for the long period. Base on this assumption, we did not examine the direct observation of 'current and persistent positive symptoms of psychosis and at least moderate overall severity of current illness' in the present study, although they are included in the Kane's criteria of TRS. Therefore, this is a limitation of this study; some of the patients who were identified as TRS in this study might contain the mere chronic schizophrenic patients with poor positive symptoms [Conley and Buchanan, 1997], although they had been hospitalized for more than 1 year and had been receiving high doses of antipsychotic therapy for more than a year.

As for the susceptibility to neuroleptic-induced EPS, no significant differences were observed among three subgroups of the COMT genotype. If the low-activity COMT genotype contributed to the high dopamine concentration in the synaptic cleft due to the decrease in COMT activity, then EPS would be less likely to develop in patients with the COMT L/L genotype. On the other hand, the patients with the COMT L/L genotype are observed to receive significantly higher doses of antipsychotics in the present study, which suggests that EPS would be more likely to develop in these patients. Our present results of no differential characteristics of neuroleptic-induced EPS among three subgroups of the COMT genotype may be the consequence of offset of these two factors discussed above. To clarify the exact role of the COMT H/L polymorphism for the susceptibility to neuroleptic-induced EPS, the comparison should be done under the condition that the neuroleptic dosage patients receive is strictly controlled.

Since the COMT H/L polymorphism examined in the present study has been demonstrated to alter enzyme activity [Lotta et al., 1995; Lachman et al., 1996; Sander et al., 1997], the polymorphism is considered to affect the clinical manifestations related to central dopaminergic systems. However, it should be noted that the present conclusion was drawn mainly from the findings in only eight patients with the COMT L/L genotype, which represents a small sample size. Further research using a larger sample set is required to clarify the exact role of this polymorphism; that is, whether alteration of enzyme activity could modify the phenotype of various psychiatric diseases and human behavior, including the characteristics of TRS suggested in this study.

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ORIGINAL RESEARCH ARTICLE

Modification of human 5-HT$_{2C}$ receptor function by Cys23Ser, an abundant, naturally occurring amino-acid substitution

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A human serotonin (5-HT)$_{2C}$ receptor gene polymorphism leads to the substitution of cysteine for serine at codon 23 (Cys23Ser); the frequency of the Ser23 allele in unrelated Caucasians is approximately 0.13. In the present study, we assessed whether Cys23Ser could affect receptor function. The two alleles were functionally compared following expression in COS-7 cells. The constitutive activity of the receptor in an *in situ* reconstitution system was also evaluated following expression of each allele in Sf9 cells. Using radioligands, Ser23-expressed membranes showed reduced high-affinity binding to meta-chlorophenylpiperazine (*m*-CPP) and 5-HT. Although the amplitude of the 5-HT-induced intracellular Ca^{2+} peak did not differ between the alleles, Ser23 required higher 5-HT concentrations to elicit the same response. These differences might be due to more extensive desensitization in the Ser23 form. In the *in situ* reconstitution system, the 5-HT$_{2C}$ receptor displayed considerable constitutive activity, with the Ser23 allele being significantly higher in this regard than the Cys23 form. After prolonged serum deprivation in order to resensitize the receptor, four of the 15 cells expressing Ser23 showed abnormally higher *m*-CPP-induced sensitivity of the Ca^{2+} response. These results indicate that the Ser23 allele may be constitutively more active than Cys23. Thus, Ser23 appears to be an abundant candidate allele capable of directly influencing inter-individual variation in behavior, susceptibility to mental disorder, and response to drugs including atypical antipsychotic and some antidepressant drugs that are potent 5-HT$_{2C}$ inverse agonists or antagonists.
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Keywords: serotonin receptor; polymorphism; 5-HT; m-CPP

The serotonin (5-HT)$_{2C}$ receptor is distributed widely throughout the brain, but is densely expressed in regions implicated in anxiety, mood, drug-induced hallucinogenesis, reward, neuroendocrine regulation, and appetite.[1] 5-HT$_{2C}$ receptor knockout mice become overweight as a result of abnormal control of feeding behavior and are prone to spontaneous death from seizure,[2] while 5-HT$_{2C}$ receptor mutant mice exhibit abnormal spatial learning and a reduced aversion to a novel environment[3] and consume more food.[4] Mutant mice also showed a decreased satiety response to D-fenfluramine, a releaser of 5-HT.[5] In studies that compared healthy human subjects with patients afflicted with alcoholism, anxiety disorders, or affective disorders, differences in hormonal and psychological responses were observed following administration of m-chlorophenylpiperazine (*m*-CPP), a nonselective 5-HT$_{2C}$ agonist.[5–9] Furthermore, various antipsychotic drugs and antidepressants have been shown to be direct acting inverse agonists or antagonists of 5-HT$_{2C}$ receptors.[10–12] Therefore, 5-HT$_{2C}$ receptors have been hypothesized to be a target of drugs used for the treatment of schizophrenia, affective disorders, anxiety disorders, and dyskinesias.[13,14] Thus, diverse lines of evidence implicate genetic perturbation of 5-HT$_{2C}$ receptors as a potential origin of differences in behavior and drug response.

We identified a human 5-HT$_{2C}$ receptor gene (HTR2C) polymorphism that leads to substitution of cysteine for serine at position 23 (Cys23Ser).[15] The

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amino-acid residue at this position is located in the putative extracellular N-terminus of the 5-HT_{2C} receptor, a seven-transmembrane-domain G protein-coupled receptor (GPCR). Cysteine residues play a key role in the formation of a disulfide bridge found within GPCRs [16] and in the formation of disulfide-linked GPCR dimers.[17] Since Cys23 is the only cysteine residue in the N-terminus of the receptor, the naturally occurring Ser23 substitution could disrupt a disulfide bridge within or between 5-HT_{2C} receptors. In addition, it has been noted[18] that 5-HT_{2C} receptors possess an extra hydrophobic domain within the N-terminal region, and modification of this domain may affect the receptor's function by changing the hydrophobicity of a putative membrane-docking domain.

The frequencies of the Cys23 and Ser23 alleles in unrelated Caucasians are 0.87 and 0.13, respectively.[15] As HTR_{2C} is located on the X chromosome, about 13% of male Caucasians are hemizygous for Ser23, expressing only this form of the 5-HT_{2C} receptor. Due to X-inactivation, there is also the possibility for chimeric expression in the brains of females. Direct evidence that Cys23Ser is functional has not been presented. However, because this amino-acid substitution is abundant and nonconservative, it has been frequently investigated for involvement in psychiatric disease and drug response. We have reported that Cys23Ser is related to CSF monoamine metabolite concentrations and DSM-III-R psychiatric diagnosis.[19] Cys23Ser has also been reported to significantly associate with clozapine response in schizophrenia,[20] bipolar disorder [21], tardive dyskinesia in chronic schizophrenia[14] and the personality trait of reward dependence,[22] although the relations of Cys23Ser to behavior and drug response are still controversial.

The difficulties just outlined in evaluating the phenotypic role of Cys23Ser are typical of the problems faced in the analysis of the role of genes in complex behavioral phenotypes. However, the search for a link between phenotype and candidate genes can be guided by prior demonstration of *in vitro* functional significance of their variants. Such functionally significant alleles have a higher prior probability of genuine involvement in phenotype. Also, the effects of allelic variants on phenotype may be better anticipated if their cellular function is understood. Comparison of the expression of allelic variants *in vitro* can reveal a variety of potential functional effects under carefully controlled experimental conditions.

5-HT_{2C} receptors couple to the G_q family of G proteins, activating phospholipase C to hydrolyze phosphoinositide and produce two second messengers, diacylglycerol and inositol triphosphate.[1] Inositol triphosphate mobilizes calcium from intracellular stores, increasing the intracellular calcium concentration ($[Ca^{2+}]_i$), whereas diacylglycerol activates protein kinase C. Human 5-HT_{2C} receptors are silenced for otherwise high constitutive activity by RNA editing.[23,24] Altered editing would likely influence either the baseline state of behavior or drug response. Recently, altered editing of 5-HT_{2C} receptors was detected in the prefrontal cortex of depressed suicide victims.[25,26] A point mutation in the third intracellular loop causes constitutive activation of the 5-HT_{2C} receptor,[27] and mutations in several protein domains can lead to constitutive activation of GPCRs.[28,29]

We therefore investigated whether Cys23Ser affects the ligand affinity, transduction, and constitutive activity of the 5-HT_{2C} receptor. To detect functional differences between the two alleles, we compared their receptor-binding profiles and ability to mobilize calcium in COS-7 cells. We also explored the mechanism for the difference between the constitutive activities of the two alleles using a novel procedure for *in situ* reconstitution.

Materials and methods

Materials

The Sf9 cell line and COS-7 kidney cell line were obtained from GIBCO/BRL (Grand Island, NY, USA) and the American Type Culture Collection (Rockville, MD, USA), respectively. Tissue culture media, transfection media, and lipofectamine were purchased from GIBCO/BRL. The expression vector, pcDNA3, was purchased from Invitrogen (San Diego, CA, USA). [^{125}I](±)-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane ([^{125}I]DOI; 2200 Ci/mmol) and [^{35}S] guanosine 5′-*o*-(3-thio)-triphosphate (GTPγS; 1000 Ci/mmol) were from New England Nuclear (Boston, MA, USA), and [^{3}H]mesulergine (82 Ci/mmol) was from Amersham Corporation (Arlington Heights, IL, USA). 5-HT, *m*-CPP, (±) DOI, mesulergine, ritanserin, and methysergide were from Research Biochemical Incorporated (Natick, MA, USA). All chemical reagents were of the highest commercially available grade.

Cell culture

COS-7 cells were grown in Dulbecco's modified Eagle medium (D-MEM) supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin sulfate, at 37°C, in a humidified atmosphere containing 5% CO_2. Cells were passaged every 3–4 days.

Overexpression of human 5-HT_{2C} receptor in COS-7 cells

A human 5-HT_{2C} receptor cDNA was obtained from Dr Alan Saltzman (Rhone-Poulenc Rorer Central Research, King of Prussia, PA, USA). For transient expression of the 5-HT_{2C} receptor, COS-7 cells were transfected with pcDNA3 using lipofectamine. Briefly, 4×10^6 COS-7 cells were seeded in 150-mm dishes (NUNC, Naperville, IL, USA) in D-MEM with 10% fetal bovine serum and 50 μg/ml gentamycin sulfate. This medium was replaced with OptiMEM medium 24 h after seeding. Lipofectamine (15 μg/ml) and the transfection vector (1.5 μg/ml) were then

added, and the cells were incubated for 12–14 h. Afterwards, they were again incubated in D-MEM with 10% fetal bovine serum and 50 μg/ml gentamycin sulfate. Cys23 and Ser23 allele-expressing COS-7 cells were prepared under conditions as similar as possible. Only the plates of COS-7 cells, whose cellular condition had been verified as good and largely similar, were divided into two pools and transfected with vectors containing either Cys23 or Ser23 alleles. Membranes were then prepared as described below. Consequently, we obtained several pairs of membrane preparations expressing either Cys23 or Ser23 5-HT_{2C} receptors with nearly identical protein amounts.

Radioligand-binding assays

For analysis of ligand-binding, cells were harvested 48–60 h after transfection, frozen in PBS buffer, and stored at −70°C. The transfected cells were homogenized and centrifuged at 30 000 *g* for 20 min. Crude membranes were washed twice and resuspended in binding buffer containing 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 7.4) at a protein concentration of about 100–200 μg/ml. For competition experiments, 100 μl of membrane suspension was incubated in a final volume of 500 μl with 0.8 nM [^{125}I]DOI or 1.6 nM [^{3}H]mesulergine and various concentrations of test agents at 37°C for 20 min. In the displacement assay, we measured a pair of Cys23 and Ser23 samples at the same time, each in triplicate. Six tubes containing 0.8 nM [^{125}I]DOI and the displacing concentrations of agonist (5-HT or *m*-CPP) were prepared; Cys23 membranes were then added to 3 of these tubes, and Ser23 membranes to three other three tubes. This design eliminates any systematic differences in the free tracer concentrations between samples of Cys23 and Ser23. For Scatchard analyses, [^{125}I]DOI or [^{3}H]mesulergine was isotopically diluted with unlabeled ligand to vary the concentration. Con-centrations of the tracer ligands were confirmed by measurement in an aliquot of the binding reactions. Binding assays were terminated by rapid filtration through Whatman GF/C filters that were pretreated with 0.1% polyethyleneimine. Filters were then washed three times with 5 ml of 50 mM Tris-HCl (pH 7.4) at 4°C. Radioactivity was detected by liquid scintillation counting. Nonspecific binding was estimated in parallel incubations containing 10 μM mianserin. Protein concentrations were determined using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Melville, NY, USA). All comparisons between the Cys23 and Ser23 5-HT_{2C} receptors were carried out on the same day and with the same drug solutions. In Scatchard analyses, [^{125}I]DOI binding could not be measured at con-centrations higher than 10 nM, since a dose-dependent decrease in specific binding of [^{125}I]DOI was observed.

Measurements of $[Ca^{2+}]_i$

Transfected COS-7 cells were placed in supplemented D-MEM in the absence of fetal bovine serum 15–18 h before the experiment. Observations on the transfected cells were made with the cells in a standard perfusion medium containing 150 mM NaCl, 4.5 mM KCl, 1.2 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 10 mM glucose, 2 mg/ml BSA, and 10 mM HEPES (pH 7.4). Prior to addition of 5-HT, the cells were loaded with 10 μM Fura-2 AM for 20 min according to the method of Fatatis *et al.*[30] 5-HT or *m*-CPP was administered for 30 s, and the procedure was repeated five times at intervals of 20 min.

Fura-2 fluorescence in cells on coverslips was imaged with an inverted fluorescent microscope on a vertical optical bench using a Nikon × 20 objective (1.3 numerical aperture). Fluorescence was measured at 1-s intervals with a CCD camera. Approximately 10% of the 50–70 cells in each field responded to 10 μM 5-HT or *m*-CPP with at least a three times the resting level.

GDP/GTPγS exchange assay

The coding region of the human 5-HT_{2C} cDNA (INI form) was subcloned into the BacPAK9 transfer plasmid (Clontech, Palo Alto, CA, USA) in the *Eco*RI and *Xba*I position of the multiple cloning site. Sf9 cells were cotransfected with BSU361-digested BacPAK6 baculovirus DNA (Clontech) and with the 5-HT_{2C}-containing recombinant BacPAK9 plasmid. Cells were harvested 60 h postinfection, and the postnuclear fraction (P2) was collected as described previously.[31] To remove endogenous G proteins as well as other extrinsic membrane proteins, 5-HT_{2C} receptor-expressing membranes were immediately extracted from this fraction with 7 M urea, using a minor modification of the previous procedure.[31] The final pellet was resuspended in solution A with 12% sucrose, and aliquots were frozen and stored at −80°C. Urea-extracted 5-HT_{2C}-receptor–ligand binding sites were quantitated by analysis of binding with [^{3}H]mesulergine and the amount of Cys23 or Ser23 5-HT_{2C} receptor-containing membrane used in the reaction was adjusted based upon this value (77 ± 6 pmol/mg of protein for Cys23 ($n = 3$) and 59 ± 2 pmol/mg of protein for Ser23 ($n = 3$), respectively.) Squid retina $G\alpha_q$ and bovine retina $G\beta\gamma$ were purified as described previously.[31] The receptor-catalyzed GDP/GTPγS exchange on G_α was determined essentially by the method of Hartman and Northup,[31] with the addition of 10 μM GDP to compete for uncatalyzed GTPγS binding. Receptor-containing membranes were mixed with G protein subunits with or without agonist on ice in a total volume of 30 μl. The addition of 20 μl of reaction solution was used to initiate the reactions. The combined solution contained 50 mM MOPS (pH 7.5), 100 mM NaCl, 1 mM EDTA, 3 mM $MgSO_4$, 1 mM DTT, 3 mg/ml BSA, 10 μM GDP, and ^{35}S-GTPγS. Reactions were conducted at 30°C, terminated by adding 2 ml of ice-cold solution B (20 mM Tris/HCl, 25 mM $MgCl_2$, 100 mM NaCl, pH 8.0), and filtered over nitrocellulose membranes on a vacuum manifold. The filters were washed four times with 2 ml of ice-cold solution B and dried. The radioactivity on

the filters was determined in a Wallac 1219 liquid scintillation spectrometer (Wallac, Inc., Gaithersburg, MD, USA).

Statistical analysis

In binding assays, B_{max} and apparent K_d were computed by Scatchard analysis using Grafit4 (Erithacus Software, London, UK). In competition experiments, K_{fH} and K_{fL} values were computed by the Pka Double program using Grafit4. The statistical significance of differences between Cys23 and Ser23 receptor alleles was determined by one-way ANOVA. Paired *t*-test was performed using the SAS statistical computer package (version 8.0).

Results

Ligand-binding properties of 5-HT$_{2C}$ receptor allelic variants overexpressed in COS-7 cells without serum deprivation

[^{3}H]mesulergine binds to 5-HT$_{2C}$ receptors in the G protein-coupled and uncoupled states.[32] The ligand-binding properties of Cys23 and Ser23 expressed in COS-7 cells revealed a homogeneous class of binding site for [^{3}H]mesulergine that was absent in untransfected cells (Cys23, $B_{max} = 5.93 \pm 0.62$ pmol/mg of protein, $K_d = 3.12 \pm 0.62$ nM, $n = 11$; Ser23, $B_{max} = 6.19 \pm 0.59$ pmol/mg of protein, $K_d = 3.33 \pm 0.63$ nM, $n = 11$). Low-affinity sites of [^{125}I]DOI binding, which were assumed to represent the G protein-uncoupled form of the receptor, could not be detected since at concentrations greater than 20nM the specific activity of [^{125}I]DOI gradually decreased, probably due to desensitization. Therefore, [^{125}I] DOI binding fits a one-site model, and the binding properties of Cys23 and Ser23 were nearly identical (Cys23, $B_{max} = 0.58 \pm 0.10$ pmol/mg of protein, $K_d = 0.93 \pm 0.1$ nM, $n = 6$; Ser23, $B_{max} = 0.68 \pm 0.18$ pmol/mg of protein, $K_d = 1.09 \pm 0.08$ nM, $n = 6$).

Figure 1 represents the displacement of [^{125}I]DOI by two agonists of the 5-HT$_{2C}$ receptor, 5-HT and *m*-CPP, for the two alleles using the Grafit4 program of a two-site model. The allelic variants differed in that the high-affinity components (K_{fH}) of 5-HT and *m*-CPP binding for Ser23 were significantly greater than those for Cys23. The low-affinity components (K_{fL}) were not significantly different (Table 1). The displacement of [^{125}I]DOI by DOI also showed high- and low-affinity components, but no significant difference was observed between the two alleles (data not shown). The K_{fH} values in Table 1, while statistically different for Cys23 *vs* Ser23, are relatively small in magnitude. A difference in the amount of membrane protein in the assays might produce such a difference. In the present study, since we added 100 μl of membranes without correction for protein concentration, the added protein amounts were not identical for each experiment. However, the membrane protein was not systematically different, the mean values were indistinguishable (247 ± 39 μg/ml for Cys23, $n = 6$; 247 ± 37 μg/ml for Ser23, $n = 6$), and the paired *t*-test revealed no significant difference ($P = 0.99$). Since the difference in added membrane amounts is subtle and not systematic, the membrane protein amount cannot explain the difference in binding profiles between Cys23 and Ser23.

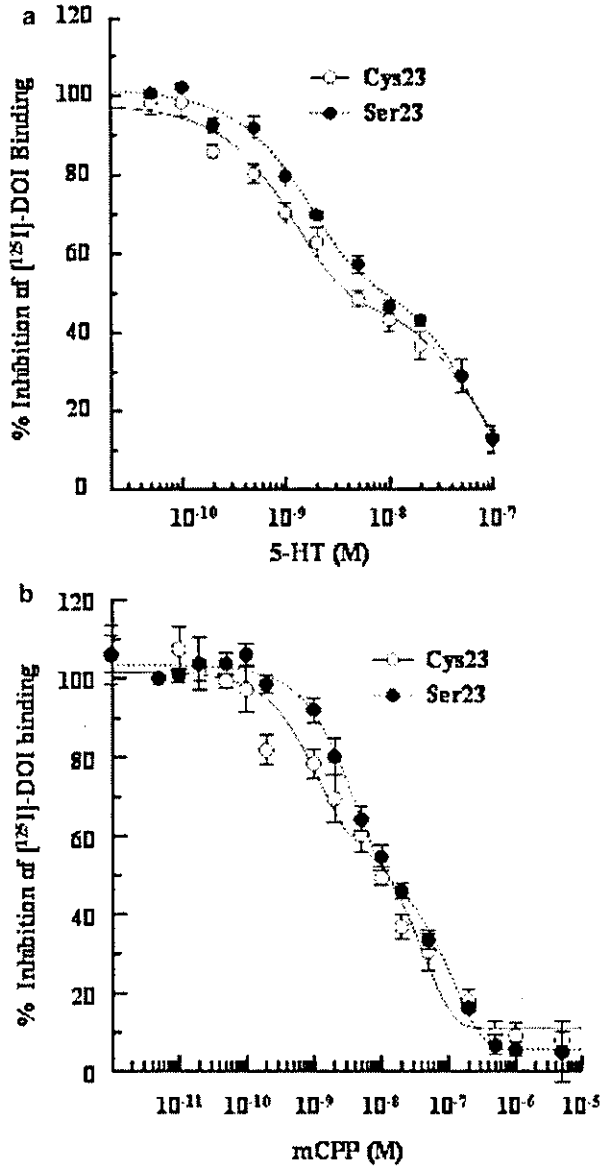

Figure 1 Competition for [^{125}I]DOI binding by 5-HT (a) and *m*-CPP (b) for 5-HT$_{2C\text{-}INI}$ Cys23 and Ser23 allelic receptors expressed in COS-7 cells. Radioligand binding was performed as described in Materials and methods using 0.8 nM [^{125}I]DOI in the absence and presence of the indicated concentrations of test ligands. The data are the mean $\pm$ SE for values obtained by triplicate determinations made in six independent experiments.

Table 1 Agonist-binding profiles of allelic $5HT_{2C}$ receptors (K_i values for [^{125}I]DOI)

	5-HT (n=6)	*m-CPP (n=6)*
Cys23	0.77±0.13	1.18±0.36
K_{iH} (nM)	57.5±17.4	64.5±16.1
K_{iL} (nM)		
Ser23	1.35±0.18*	2.46±0.26*
K_{iH} (nM)		
K_{iL} (nM)	74.0±15.4	126.8±26.1

DOI binding was performed as described in Materials and methods in the presence of 14 concentrations of the indicated ligands from 0.1 to 2000 nM. Results are shown as means±SEM for the binding parameters obtained from the indicated number of experiments.
*$P<0.05$; Cys23 *vs* Ser23.

5-HT-stimulated intracellular Ca^{2+} mobilization by Cys23 and Ser23 receptors overexpressed in COS-7 cells after overnight serum deprivation

We also evaluated the Cys23 and Ser23 alleles for differences in transduction, measured as intracellular Ca^{2+} mobilization following stimulation with ligand in COS-7 cells. The resting $[Ca^{2+}]_i$ was similar in cells transfected with either allele (Cys23, 89.9±7.1 nM, $n=11$; Ser23, 98.7±4.9 nM, $n=11$). The addition of 10 μM 5-HT increased $[Ca^{2+}]_i$ to 833±201 nM ($n=11$) with the Cys23 allele and 615±60 nM ($n=11$) with the Ser23 allele. These values were not significantly different. As shown in Figure 2, 5-HT saturation of the intracellular Ca^{2+} response for Cys23 was best fit by a single-site model with an EC_{50} of 227±67 nM ($n=11$). 5-HT saturation for Ser23 did not fit a single-site model. The maximal responses elicited by 5-HT were identical for the two alleles. However, at subsaturating concentrations of 5-HT, the Cys23 allele produced greater responses than the Ser23 allele. The reasons for the unusually steep dose–response curve of Ser23 are not clear, but the steepness could indicate increased desensitization.

Cys23 and Ser23 receptor-catalyzed activation of G_q

To evaluate the effect of the alleles on the functional interaction between the receptor and G protein subunits, we analyzed receptor-catalyzed GTPγS binding to G_q by *in situ* reconstitution. Sf9 cells infected with baculovirus constructs encoding the Cys23 and Ser23 alleles expressed these receptors in high abundance. Scatchard analysis of [^{3}H]mesulergine binding to urea-extracted membranes again revealed a single high-affinity binding site with a K_d of 5.5±1.1 nM ($n=3$) for Cys23 and 5.2±0.3 nM ($n=3$) for Ser23. The binding capacities for the two alleles were 77±6 pmol/mg of protein for Cys23 ($n=3$) and 59±2 pmol/mg of protein for Ser23 ($n=3$). Little [^{125}I]DOI binding could be observed in these two membrane fractions (about 1.8% of mesulergine binding). These results suggest that in Sf9 membranes, $5\text{-}HT_{2C}$ receptors are expressed abundantly, and that urea extraction produces a homogeneous population of the uncoupled form. The results also indicate that the intrinsic antagonist-binding properties of the two allelic variants are essentially identical.

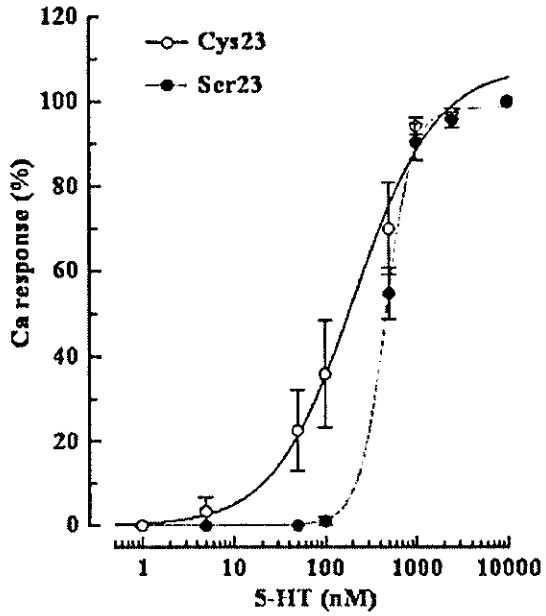

Figure 2 Dose–response relation with 5-HT. 5-HT-induced increases in $[Ca^{2+}]_i$ with $5\text{-}HT_{2C\text{-}INI}$ Cys23 (open circles, $n=11$) and Ser23 (closed circles, $n=11$) alleles were measured after overnight serum deprivation showing the % increase in $[Ca^{2+}]_i$ from the resting levels at a 5-HT concentration of 10 μM, which yields a maximal response. Data are means ± SE. The *0* values are for the different batches of cells from the same transfection. The B_{max} values of [^{3}H]mesulergine binding to membranes expressing Cys23 and Ser23 were 5.1 pmol/mg protein for Cys23 and 6.4 pmol/mg protein for Ser23, respectively, which are estimates of average receptor expression.

Coupling between the $5\text{-}HT_{2C}$ receptor and heterotrimeric G proteins is a fundamental step in the signal transduction cascade initiated by the receptor–ligand interaction. Quantitative analysis of the catalytic activation of squid retina $G\alpha_q$ and bovine brain $G\beta\gamma$ enabled us to evaluate the direct interaction between each variant of the $5\text{-}HT_{2C}$ receptor and G protein subunits under almost uniform experimental conditions. Figure 3 documents the suitability of the 7 M urea-extracted membrane preparation for reconstitution of $5\text{-}HT_{2C}$ receptors with G proteins. In the absence of added G proteins, these membranes display a low capacity for GTPγS binding, which is not stimulated by 5-HT. Upon reconstitution with both $G\alpha_q$ and $G\beta\gamma$, 5-HT-stimulated binding of GTPγS was restored.

In the presence of an excess amount of 5-HT, there was no significant difference between the two alleles in affinity or catalytic activity for $G\alpha_q$, suggesting that

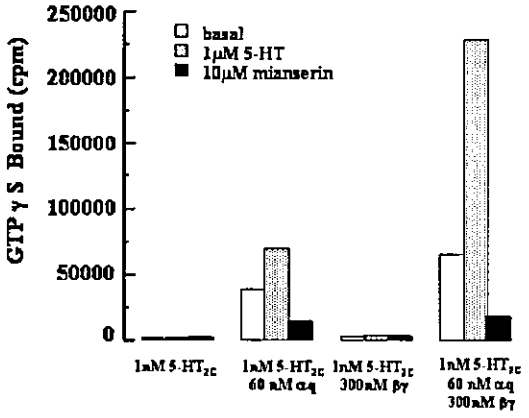

Figure 3 *In situ* reconstitution of 5-$HT_{2C\text{-}INI}$ receptor-catalyzed GTPγS binding. Urea-washed Sf9 membranes providing 1 nM 5-HT_{2C} receptor (Cys23) were tested either alone or mixed with 60 nM squid α_q, 300 nM bovine retina $\beta\gamma$ or both 60 nM α_q and 300 nM $\beta\gamma$. Each condition of protein composition was assayed in the presence of no added ligand, 1 μM 5-HT or 10 μM mianserin. For all conditions, the GTPγS-binding reactions proceeded for 10 min at 30°C and bound GTPγS was determined as described in Materials and methods.

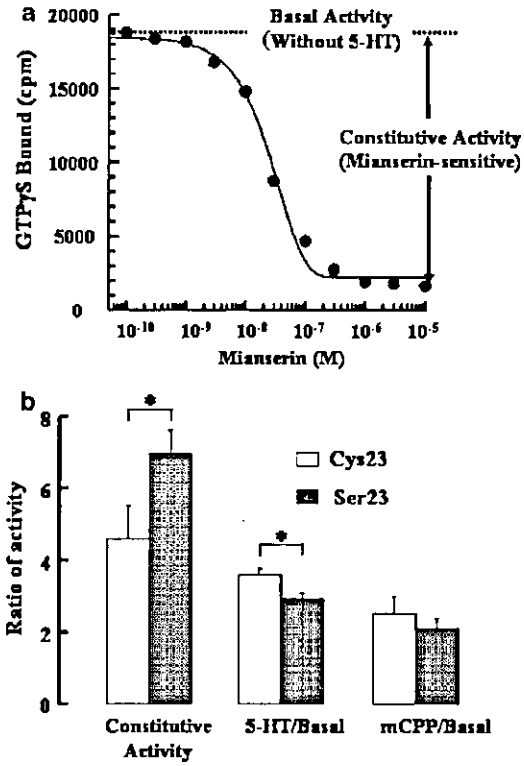

Figure 4 Basal inhibition by mianserin. (a) Representative mianserin inhibition of 5-$HT_{2C\text{-}INI}$ receptors (Cys23). 5-HT_{2C} receptor-catalyzed GTPγS binding to α_q was assessed in the absence of 5-HT. The exchange catalyzed by 4 nM 5-HT_{2C} receptor was determined with 200 nM α_q and 500 nM $\beta\gamma$. The GTPγS-binding reactions proceeded for 30 min at 30°C and bound GTPγS was determined as described in 'Materials and methods'. Under our experimental condition, the IC_{50} value for inhibition of basal activity by mianserin was 44 nM, which was consistent with the range of K_i value for competition binding assays or the IC_{50} value for inhibition of basal inositol phosphate production by mianserin as reported previously.[34] (b) Allelic differences in the constitutive activity of 5-HT_{2C} receptors. The exchange catalyzed by 4 nM 5-Ht_{2C} Cys23 or Ser23 receptors was determined with 200 nM α_q and 500 nM $\beta\gamma$. The GTPγS-binding reactions proceeded for 10 min at 30°C and bound GTPγS was determined as described in 'Materials and methods'. Each condition of protein composition was assayed in the presence of no added ligand (Basal), 10 μM 5-HT or 10 μM mianserin. Data are means ± SEM for the calculated ratios of these activities. Values of each allele are based on five urea-extracted membrane fractions infected separately.

the maximum response caused by 5-HT did not differ between the alleles (data not shown).

The 5-HT_{2C} receptor exhibits constitutive activity in transfected cell lines and the choroid plexus.[33] As previously noted for the rat 5-HT_{2C} receptor,[31] the human receptor also displays an appreciable activation of $G\alpha_q$ following *in situ* reconstitution, even in the absence of added 5-HT.

Figure 4a shows the basal activity for Cys23 (INI form), defined as the catalytic activity of the 5-HT_{2C} receptor/G protein complex in the absence of any agonist. The constitutive activity was assessed by the ratio of the basal activity to the activity in the presence of 10 μM mianserin. When there is a high constitutive activity of the receptor, the inverse agonist mianserin will inhibit the basal activity and this ratio will be greater. Figure 4b shows that the Ser23 allele had a higher ratio than the Cys23 allele (ANOVA, $P<0.05$), suggesting that the Ser23 receptor is constitutively more active. Concomitant with an increase in constitutive activity, there was a decrease in the activation ratio by 5-HT above basal activity for the Ser23 allele as compared with the Cys23 allele. This appears to be an intrinsic difference between the two alleles, since in this experiment receptor concentration was titrated so that the ratio of receptors to reconstituted $G\alpha_q$ and $G\beta\gamma$ was the same for both Ser23 and Cys23.

m-CPP-stimulated intracellular Ca^{2+} mobilization by Cys23 and Ser23 receptors overexpressed in COS-7 cells after 48 h of serum deprivation

To evaluate whether the Ser23 allele could be resensitized to reveal a sensitivity for an agonist more closely resembling that of Cys23, we extended the period of serum deprivation from overnight to 48 h and measured the increase in $[Ca^{2+}]_i$ stimulated by varying concentrations of *m*-CPP (0.1,. 0.2, 1, 2, and 10 μM). Representative traces from cells expressing either Cys23 or Ser23 are shown in Figure 5a–c. At 0.1 μM, five of the six cells evaluated did not show any response, and the remaining cell produced a faint response (14.6% of the maximal response) in the Cys23-expressed cells. As shown in Figure 5d, *m*-CPP

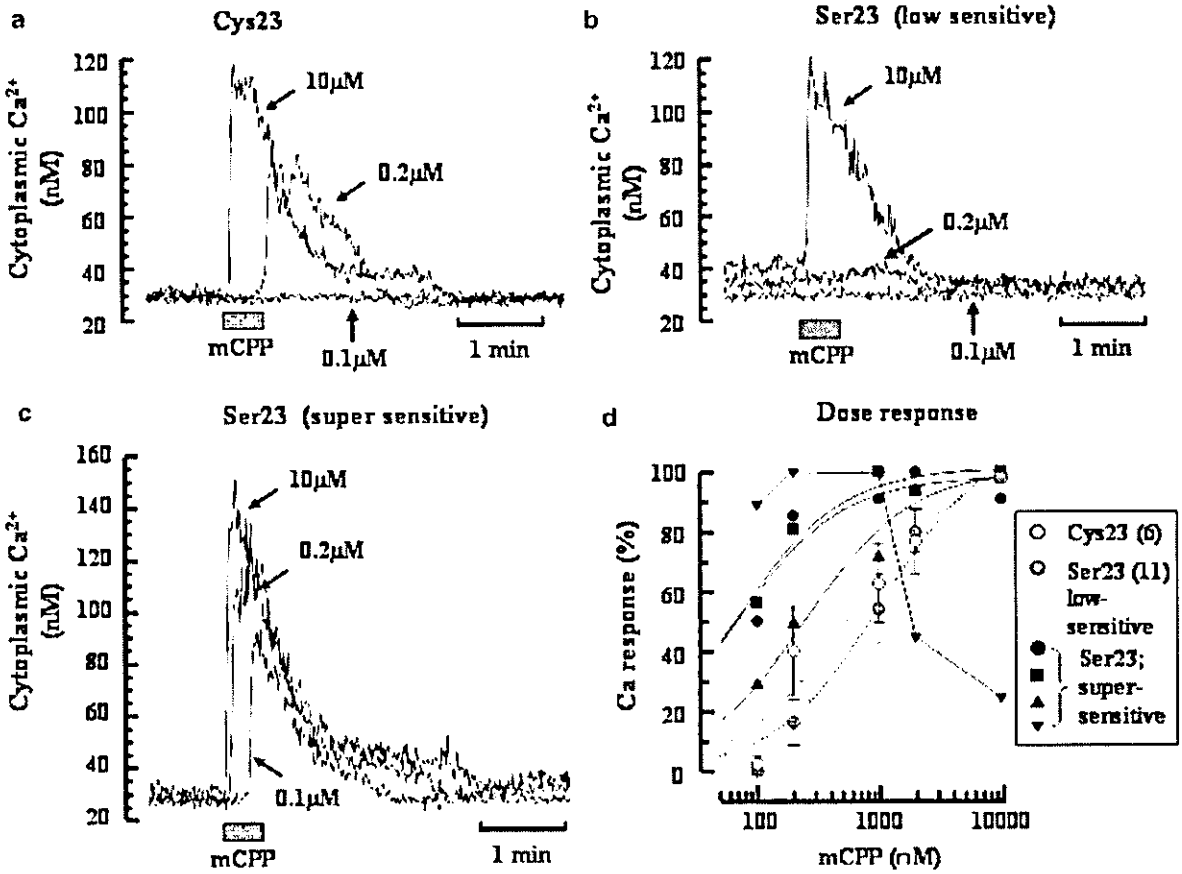

Figure 5 Dose–response relation for *m*-CPP after 48 h serum deprivation. (a–c) the representative traces showing $[Ca^{2+}]_i$ increases induced by 0.1, 0.2, and 10 μM *m*-CPP in 5-$HT_{2C\text{-}INI}$ Cys23, Ser23 (low sensitive) and Ser23 (supersensitive). (d) Dose–response curves are shown for *m*-CPP showing the % increase in $[Ca^{2+}]_i$ from the resting levels at an *m*-CPP concentration, which yields a maximal response. Data are means $\pm$ SE. The B_{max} values of [^{3}H]mesulergine binding to Cys23- and Ser23-expressing membranes were 6.5 pmol/mg protein for Cys23 and 7.4 pmol/mg protein for Ser23, respectively, these values being estimates of average receptor expression.

saturation of the intracellular Ca^{2+} response for Cys23 was best fit by a single-site model with an EC_{50} of 555 ± 244 nM ($n = 6$). In contrast, responses were more varied in the 15 cells expressing Ser23. Four cells showed a high sensitivity for *m*-CPP. At 0.1 μM, three of these four cells produced more than 50% of the maximal response, and one produced a response that was 28.6% of maximal. In addition, one of the four cells showed potent desensitization in the presence of 1–10 μM *m*-CPP. In the 11 remaining Ser23 cells, the *m*-CPP saturation of $[Ca^{2+}]_i$ was best fit by a single-site model with an EC_{50} of 1038 ± 299 nM. Consistent with the data following overnight serum deprivation, *m*-CPP displayed a lower potency in these 11 Ser23 cells than for the Cys23 cells.

Discussion

The present data demonstrate that Cys23Ser, a human 5-HT_{2C} receptor polymorphism, is functional. We observed that the rarer Ser23 allele was constitutively more active when reconstituted with G protein subunits and was more markedly desensitized when overexpressed in mammalian cells. Therefore, Cys23-Ser may be capable of influencing interindividual variations in behavior, susceptibility to mental disorders, and drug response.

The reconstitution analysis of 5-HT_{2C} receptors enabled us to evaluate the constitutive activity of the Cys23 and Ser23 alleles under almost identical conditions. We found that the binding profiles of the two alleles to mesulergine were almost identical and the K_d values were consistent with those of putative uncoupled 5-HT_{2C} receptors expressed in COS-7 cells. These results indicate that there may be no difference between the two alleles in binding when the receptor is uncoupled and inactive. There also is no significant difference between the two alleles in either affinity for G proteins or in G protein catalytic activity when 5-HT is present in excess, which indicates that the amplitude of maximal receptor activation (due to the fully active form of the receptor) does not differ

between Cys23 and Ser23. On the other hand, Ser23 can activate $G\alpha_q$ to a higher degree than can Cys23 in the absence of 5-HT, suggesting that Ser23 is constitutively more active.

Expressed in mammalian cells, the more highly constitutively active Ser23 allele was functionally downregulated, showing a lower affinity for both 5-HT and *m*-CPP. We hypothesize that this difference is due to a higher proportion of partially desensitized Ser23 receptors than Cys23 receptors. This hypothesis is consistent with the proposal by Fathy *et al* [35] that receptor desensitization occurs in proportion to constitutive activity. Increased desensitization has been seen in constitutively active mutant α_{1b}-adrenergic and β_2-adrenergic receptors.[28,29] Constitutively active 5-HT_{2C} receptors are thought to be constitutively desensitized.[36] As the fetal calf serum we used to grow cells contains 5-HT, the HTR2C-transfected COS-7 cells tend to undergo agonist-induced desensitization. The constitutively more active Ser23 may be more desensitized under conditions where 5-HT is present. In the present study, Cys23Ser did not affect the affinity for DOI. The ligand-binding pocket of the receptor contributes to the specificity of ligand recognition and binding.[37] The differential effect of Cys23Ser on the affinity of *m*-CPP, 5-HT and DOI suggests that these ligands may have different points of interaction within the ligand-binding pocket. Since m-CPP and DOI are the relatively selective agonist of 5-$HT_{1B/2C}$ receptors and the selective agonist of 5-$HT_{2A/2B/2C}$ receptors, respectively,[38,39] it is likely that the favored positions of the 5-HT_{2C} receptor to interact with these two ligands are not identical. Cys23Ser might influence the points in the binding pocket that interact with 5-HT and *m*-CPP when the receptors are partially desensitized.

The level of expression of GPCRs can also influence constitutive activity. Gurdal *et al.*[40] showed that marked myocardial overexpression of β_2-adrenergic receptors leads to induction of a maximal response without agonist. Similarly, in our reconstituted system, higher concentrations of 5-HT_{2C} receptors led to higher levels of receptor activation without agonist, as long as the concentration of G protein subunits was sufficient (data not shown). This receptor-density-dependent increase in 5-HT_{2C} receptor efficacy may be a result of receptor–receptor interactions, such as an increase in the stability of the dimeric state.[40] With the transient transfection approach we used in COS-7 cells, 5-HT_{2C} receptors were expressed at very high concentrations, about 50 times those in the cortex reported by Pandey *et al.*[41] We suggest that both Ser23 and Cys23 5-HT_{2C} receptors may have been shifted towards the constitutively active state due to overexpression. This shift may have enhanced the tendency of the Ser23 receptor to desensitize in the presence of agonist. In the present study, the dose–response curve of 5-HT for Cys23 after overnight serum deprivation fits a one-site model, while the dose–response curve for Ser23 fits a two-site model. These results indicate that overnight serum deprivation was insufficient to resensitize all Ser23 5-HT_{2C} receptors and not a few Ser23 receptors seemed to remain partially desensitized. The dose–response curve of 5-HT for Ser23 appears to shift to the right without any change in the maximal response when receptors are partially desensitized. As Dunwiddie *et al*[42] reported concerning agonist-induced desensitization of the adenosine A_3 receptor, partial desensitization of the Ser23 5-HT_{2C} receptor may reduce the affinity of 5-HT for the receptor rather than uncouple it.

It is intriguing that when serum deprivation was maintained for 48 h, four of the 15 cells expressing Ser23 exhibited abnormally high sensitivity to *m*-CPP, while the remaining 11 cells still showed low affinity. These results indicate that when completely resensitized, Ser23 may exhibit higher affinity for agonist than Cys23. Alternatively, prolonged serum deprivation may cause cell damage, and the appearance of abnormally high sensitivity for agonist may be a response of these Ser23 cells to pathological insult rather than full resensitization.

The 5-HT_{2A} and 5-HT_{2C} receptors are closely related structurally and evolutionarily, and both activate phospholipase C via the G_q family of proteins.[43,33] Interestingly, when both receptors are overexpressed in the same cellular environment, they exhibit a similar maximal response to 5-HT. The 5-HT_{2A} receptor, however, has a much lower constitutive activity than does the 5-HT_{2C} receptor [33] and is less sensitive to 5-HT-mediated desensitization.[43] These results parallel the differences in constitutive activity and desensitization that we have proposed based on our data on the Cys23 and Ser23 alleles.

The discovery of the involvement of constitutively active receptors in human diseases [44,45] has increased interest in treatment with blockers of constitutive activity, known as inverse agonists. 5-HT_{2C} receptors, except those that are fully RNA edited, exhibit potent constitutive activity.[31,33,24] Significantly, several antipsychotic and antidepressant drugs display inverse agonist activity for the 5-HT_{2A} and/or 5-HT_{2C} receptors.[11,12] Can the Cys23Ser polymorphism alter clinical sensitivity to inverse agonists? Sodhi *et al.* [20] reported that 90% of subjects (19/21) who had one or more Ser23 alleles were classified as clozapine responders, compared with 59% of subjects (84/141) who lacked this allele ($\chi^2 = 7.7$, $P = 0.005$), suggesting that Ser23 may be a predictor of good response to clozapine. Since clozapine has high affinity for the human 5-HT_{2C} receptor and acts as an inverse agonist,[12] higher constitutive activity of Ser23 might mechanistically explain this association.

A well-known clinical effect of clozapine is weight gain, and Westberg *et al.* [46] have recently reported an association between the Ser23 allele and weight loss in teenage girls never treated with clozapine. Since 5-HT_{2C} receptor knockout mice become overweight as a result of abnormal control of feeding behavior,[2] it is possible that Ser23 could lead to higher food intake

via enhanced constitutive activity, and that clozapine treatment could also lead to weight gain through its action to block 5-HT_{2C} receptors. Ertugrul *et al*[47] have found that genotype 5-HT_{2C} ser, ser/ser or cys/ser is associated with basal metabolic index in patients with schizophrenia than is genotype 5-HT_{2C} cys or cys/cys at baseline and after 6 weeks treatment with clozapine.

RNA editing is an important mechanism to silence the remarkable constitutive activity of 5-HT_{2C} receptors.[24] The editing status of this receptor in the brain differs for subjects with psychiatric disorders and controls.[25,26] In particular, D-site editing is decreased significantly in depressed suicide victims.[26] D-site editing is essential for generating the fully edited forms of human 5-HT_{2C} receptors (VSV and VGV). These two forms of the edited receptor would contribute to silencing constitutive activity because their activities are markedly lower than that of the nonedited form (INI). It is conceivable that a decrease in D-site editing in the prefrontal cortex of suicide victims may result in an increase in constitutive activity of 5-HT_{2C} receptors in that area. If increased constitutive activity arising from altered RNA editing contributes to or complicates therapy in certain psychiatric disorders, Ser23 would promote this effect by further enhancing constitutive receptor activity.

The Ser23 allele is hemizygous in 13% of Caucasian males and is found in more than one in four females. Year by year, there is growing interest in the constitutive activity of GPCRs since such activity appears to be implicated in psychiatric disorders and because elaborate mechanisms have evolved to regulate it. The more constitutively active Ser23 allele is, therefore, a potentially significant allele in human behavior. In concert with RNA editing, the Cys23Ser polymorphism may contribute to a variety of behavioral traits and psychiatric disorders or influence the therapeutic efficacy of drugs that act at the 5-HT_{2C} receptor.

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SCIENTIFIC CORRESPONDENCE

No association with the neuregulin 1 haplotype to Japanese schizophrenia

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SIR—Schizophrenia is a complex genetic disorder and has heritability of around 80%. The pathogenesis of the disease is hypothesized to be neurodevelopmental in nature based on reports of an excess of adverse events during the pre- and perinatal periods, the presence of cognitive and behavioral signs during childhood and adolescence, and the lack of evidence of a neurodegenerative process in most individuals with schizophrenia.[1]

To date, studies of the association between schizophrenia and genes coding for neurodevelopmental role has been published.[2] Recently, it has been reported that genetic variants around the gene neuregulin 1 (*NRG1*) are associated with schizophrenia in an Icelandic sample.[3] The replications of this finding have been reported independently from Scottish population[4] and Caucasians born in UK and Ireland.[5] The at-risk haplotype was found to be over-represented in schizophrenics compared to controls.

Here, we attempt to perform a replication of these results in our collection of Japanese schizophrenic patients and controls. Subjects consisted of 607 patients with schizophrenia and 515 controls. All subjects were unrelated Japanese. Most patients (93%) had more than 1-year duration of illness and 77% patients had history of hospitalization. Consensus diagnosis according to DSM-IV was made. After description of the study, written informed consent was obtained from each subject. This study was approved by the Fujita Health University and the National Center of Neurology and Psychiatry Hospital Ethics Committees.

SNP8NRG221132, SNP8NRG221533, SNP8NRG24 3177, and SNP8NRG433E1006 were genotyped using primer extension method by dHPLC. SNP8NRG24 1930 was genotyped using PCR-RFLP method. The microsatellites 478B14-848 and 420M9-1395 were genotyped following analysis on an ABI3100 capillary sequencer. Allele 0 for markers 478B14-848 and 420M9-1395 refers to PCR-product sizes of 219 and 274 bp, respectively.

Deviation from the genotype counts predicted by Hardy–Weinberg equilibrium expectations was tested using an exact test. D, D', and Δ^2 for pair-wise linkage disequilibrium (LD) were calculated. Estimation of the haplotype frequencies was performed by the expectation–maximization algorithm. Test for single-marker allelic association was performed by χ^2 analysis. Test for haplotypic association was performed using the software SAS/Genetics (Release 8.2 TS2M0, SAS Institute Japan) with a permutation test to obtain the empirical significance.[6]

We genotyped the five SNPs (SNP8NRG221132, SNP8NRG221533, SNP8NRG241930, SNP8NRG2431 77, SNP8NRG433E1006) and two microsatellite markers (478B14-848, 420M9-1395) that constitute the core haplotype in 607 Japanese patients and 515 Japanese control individuals (Table 1). The genotypic distribution of each locus was not significantly different from the distribution expected according to Hardy–Weinberg equilibrium in this ethnic population. We found no association between each marker and schizophrenia, including the C allele of SNP8NRG221533 ($P=0.42$) that was significantly in excess in both Icelandic[3] and Scottish[4] schizophrenia ($P=0.0028$ and 0.000064, respectively). The estimated frequency of the core at-risk haplotype, which was enriched in the Scottish and Icelandic patients (10.2%, 14.4%) than in control individuals (5.9%, 7.6%) by Stefansson *et al*, had no difference between Japanese patients (4.5%) and Japanese controls (4.3%). The estimated Odds Ratio was 1.13 and the 95% confidence interval was (0.60–2.11). In order to be consistent with the literature by Williams and his colleagues, we performed three-marker 'at-risk' haplotype analysis with and without a family history of schizophrenia. However, there are no significant differences among each patent group and control.

Our results do not confirm an involvement of the NRG1 in conferring susceptibility to schizophrenia. One possible explanation for this result could stem from the clinical heterogeneity of schizophrenia. Although the disease is discussed as if it is a single disease, it probably comprises a group of disorders with heterogeneous etiologies. Indeed, the original study was based on individuals taken from large multiply affected families from Iceland.[3] Subsequently, Williams *et al*[5] discussed sample stratification in which the genetic component might be different in their samples. Further investigations are needed by taking the effect of genetic load into consideration.

Another explanation is that population stratification may exist, since the initial findings started from linkage studies that point out initial evidence for schizophrenia to 8p22-21, where *NRG1* is located.[3] In contrast, from Japanese linkage study, no loci on chromosome 8 fulfiled the criteria for significant or suggestive evidence for linkage.[7] According to the common disease/common variants (CD/CV) hypothesis, if the at-risk haplotype is linked to real susceptibility variants, haplotype association test should detect the linkage disequilibrium to the

Table 1 Replication study showing allele frequencies for markers and haplotypes of the NRG1 gene

Marker	Allele	Frequency (%) Control (%)	(Number)	Schizophrenia (%)	(Number)	P (empirical)	OR (95% CI)
a. Test of the seven- and five-marker haplotypes and each SNP							
Seven-marker haplotype		4.3	488	4.5	597	0.96	1.05 (0.59–1.89)
Five-marker SNP		45.1	514	45.2	603	0.98	1.01 (0.79–1.27)
SNP8NRG221132	G	100.0	515	100.0	607	NA[a]	NA[a]
SNP8NRG221533	C	51.5	515	53.1	606	0.42	1.07 (0.85–1.35)
SNP8NRG241930	G	87.9	515	89.4	607	0.26	1.16 (0.80–1.68)
SNP8NRG243177	T	52.7	515	54.4	607	0.43	1.07 (0.55–1.36)
SNP8NRG433E1006	G	98.9	514	98.9	604	0.99	1.01 (0.34–3.02)
478B14-848	0	42.2	507	49.1	603	0.50	1.32 (1.04–1.67)
420M9-1395	0	39.3	494	48.3	605	0.21	1.44 (1.13–1.84)

Test	Frequency (%)	χ	P-value (one tailed)	OR (95% CI)
b. Test of the 'at-risk' haplotype of markers SNP8NRG221533, 478B14-848, and 420M9-1395				
Case (n–600)	5.7	0.51	0.66	1.13 (0.67–1.92)
Control ($n = 490$)	5.1			
Familial cases ($n = 69$)	7.7	0.19	0.36	1.46 (0.54–3.95)
Nonfamilial cases ($n = 531$)	5.0	0.86	0.93	0.98 (0.45–2.17)

[a]NA = not analysis.

disease variants in different ethnic populations.[8] However, the sample size was not enough such that the power of analysis was reached at 0.18 (when $\alpha = 0.05$). Additional studies of *NRG1* to evaluate across larger numbers of individuals and other ethnic populations should be needed to confirm the hypothesis.

However, it is unclear that single common variants will be the only relevant variants.[9] It is possible that the risk for some common diseases is due to a very large number of loci, with each having a low frequency of disease-predisposing alleles. Allelic heterogeneity may also be contributing to the association of the *NRG1* locus with schizophrenia.[10] In the study, we did not search for genetic variants at *NRG1* locus in our Japanese samples. Much greater depth of DNA resequencing should be conducted to confirm that unknown rare variants in linkage disequibrium might be a real disease gene.

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ORIGINAL RESEARCH ARTICLE

Serotonin transporter missense mutation associated with a complex neuropsychiatric phenotype

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Two common serotonin transporter (SERT) untranslated region gene variants have been intensively studied, but remain inconclusively linked to depression and other neuropsychiatric disorders. We now report an uncommon coding region SERT mutation, Ile425Val, in two unrelated families with OCD and other serotonin-related disorders. Six of the seven family members with this mutation had OCD ($n=5$) or obsessive-compulsive personality disorder ($n=1$) and some also met diagnostic criteria for multiple other disorders (Asperger's syndrome, social phobia, anorexia nervosa, tic disorder and alcohol and other substance abuse/dependence). The four most clinically affected individuals—the two probands and their two sibs—had the I425V SERT gene gain-of-function mutation and were also homozygous for 5′-UTR SERT gene variant with greater transcriptional efficacy.

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Obsessive-compulsive disorder (OCD) occurs in 1–3% of the US population and is among the ten leading causes of disability worldwide.[1] Its etiology is unknown, although family members are at a sevenfold higher risk than control.[2] As OCD differs from other neuropsychiatric disorders that are treated effectively with multiple drugs in responding only to serotonin transporter (SERT) antagonists, we and others have been examining SERT gene variants in this disorder.[3]

The human SERT gene, SCL6A4, has been cloned and sequenced; it maps to chromosome 17 q11.1–q12.[4–6] Like other Na^+- and Cl^--coupled monoamine transporters, it is organized in 14 exons that yield a protein with 12 membrane spanning domains.[7] Mice with a deletion of one or two SERT gene alleles exhibit profound alterations in neurochemistry, behavior, body weight, drug responses and thalamocortical circuitry.[8]

Two candidate gene investigations of the one previously described functional SERT gene 5′-UTR variant, designated 5-HTTLPR, found preferential transmission of the L allele and an excess of the LL genotype in OCD.[3,9] However, other studies of 5-HTTLPR in OCD reported no differences.[10] One view of this conflicting literature follows from data indicating that OCD clinical heterogeneity may likely be obscuring possible associations.[10,11]

We sought to further investigate the SERT gene in OCD and two other serotonergic neuropsychiatric disorders by scanning its coding sequence using single strand conformational polymorphism (SSCP) analysis. We analyzed DNA samples from 170 unrelated individuals, including patients with OCD ($n=30$), eating disorder ($n=30$), and seasonal affective disorder ($n=30$) plus healthy controls ($n=80$).

Two uncommon variants were detected. An A to G transversion at nucleotide 1273 of the coding sequence, substituting Val425 for Ile425, was observed in two patients with OCD. Ile425 is conserved across all five mammalian species investigated, as well as the fly.[12] The other variant, G1317A, was a synonymous substitution seen in one control. Neither of these uncommon SERT gene variants was observed in 213 additional unrelated individuals consisting of 143 OCD, eating disorder and seasonal affective disorder patients including 82 with OCD or a dual diagnosis of OCD and eating disorder plus 70 Centre d'Etude du Polymorphism Humain (CEPH) caucasian population controls.

We psychiatrically interviewed available relatives (A2 to 4, B2 to 10, Figure 1) of both OCD probands (A1 and B1) with Val425, and genotyped them for both I425V and 5-HTTLPR.[13] Including the probands, seven of 14 members of these two pedigrees were heterozygous for 425V (Figure 1). Six of these seven individuals had OCD ($n=5$) or OC personality disorder ($n=1$). None of the other members of these families whom we studied had OCD or OC personality disorder. As summarized (Figure 1), the OCD probands and their sibs in the two families had an unusually severe clinical course with multiple and somewhat unusual coexistent neuropsychiatric disorders.

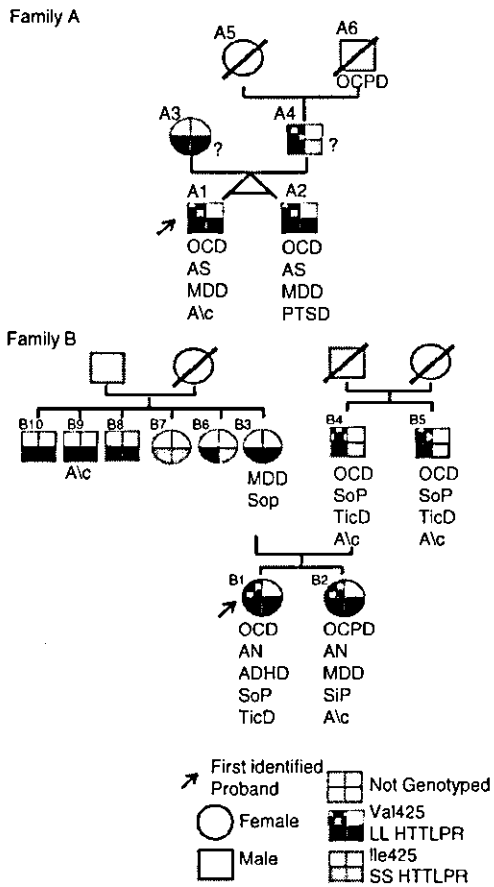

Figure 1 Families with OCD and the I425V mutation. Two families with OCD probands with the I425V mutation and with other disorders: AS, Asperger's syndrome; MDD, major depressive disorder, Alc, alcohol abuse/dependence; AN, anorexia nervosa; OCPD, obsessive-compulsive personality disorder; SoP, social phobia; SiP, simple phobia; TicD, tic disorder; PTSD, Post-Traumatic Stress Disorder; ADHD, attention-deficit hyperactivity disorder. ?—Declined assessment interviews. Thus, six out of the seven individuals with I425V had OCD ($n=5$) or OCPD ($n=6$), while one individual who was diagnostically indeterminate was considered as non-OCD (Fisher's exact test two-sided $P=0.005$; relative risk = 8.00; odds ratio = 65.00).

Thus, this SERT gene mutation, which leads to defective regulation of SERT,[14] may contribute to an uncommon familial form of OCD that is associated with Asperger's syndrome (AS)/autism, anorexia and a dense constellation of other serotonergic neuropsychiatric disorders proposed to be related to OCD. Owing of its rarity, I425V is not implicable in most individuals with these disorders. However, we have already observed the variant in two unrelated families, and I425V was also reported once in a SNP survey of 450 psychiatrically uncharacterized individuals.[15] Therefore, it is likely that I425V, while uncommon, will be found in other families with OCD and related disorders.

Overall, OCD is a moderately heritable disorder which is cotransmitted in certain families with other disorders including OC personality disorder, depression, tic disorder and grooming disorders.[7,16,17] Similar to the proposed action of 5-HTTLPR, I425V may confer an overall greater vulnerability to multiple disorders related to serotonin. However, the striking number, additional variety, severity and frequent treatment resistance of other disorders in the A and B families suggest that the actual phenotype defined by I425V is an atypical 'OCD plus': a specific syndrome incorporating OCD and an AS/autism/social phobia and eating disorder complex.

In fact, specific social difficulties were present in five of seven individuals with Val425, with two meeting criteria for AS and three for social phobia (SP). The AS found in two brothers is an unusual comorbidity since AS and autism are uncommon disorders with a population frequency of 2–15 in 10 000, *vs* 1–3% for OCD.[18] Major studies of OCD comorbidity in adults do not mention AS/autism, while social phobia was found in only 1% of 1078 individuals with epidemiologically surveyed OCD,[19] although some small studies found a higher incidence.[16] AS is considered a pervasive developmental disorder related to autism but distinguished by normal language development and later onset with higher overall functioning.[19] While repetitive behaviors are a superficial commonality between OCD and AS/autism, the interpersonal, cognitive, language and articulation deficits that are not seen in OCD, render these very distinct diagnoses.[9,19,20]

Family members of autistic probands have significantly higher rates of autism/AS (four- to nine-fold) plus SP, depression and alcohol/substance abuse and some particular personality traits, but not 15 other disorders, including OCD,[21,22]—except in one report.[23] SP has 10-fold higher frequency (20%) in family members of autistic probands *vs* controls, and is observed most often in families of higher functioning autistic individuals without mental retardation (38% incidence), who are part of the broader autism spectrum.[21,22] SP is characterized by fear and avoidance of interpersonal situations. Twin and family studies show SP to be hereditable,[22,24] but, as with autism and OCD, contributory genes have not been found and verified. It is thus possible that these I425V families are linked, in addition to OCD, by AS/autism and SP, possibly representing a dimensional social dysfunction phenotype.

With regard to the multiple other disorders in these families, anorexia nervosa, found in two siblings with I425V, occurs in 5–13% of individuals with OCD and in 1% of the general population.[25,26] Patients with anorexia nervosa frequently have OCD characteristics including perfectionism and compulsions related to both food and exercise. These observations might indicate that several disorders partially overlap in clinical comorbidity and in causality, including genetic transmission. I425V may operate as an aggravating factor for severity of these disorders.

These two probands and their families constituted the only individuals among >380 sampled who