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## Mutation screening and association study of the beta-adrenergic receptor kinase 2 gene in schizophrenia families

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### Abstract

Chromosome 22q12 is one of the most promising regions for harboring a risk gene for schizophrenia. We have reported significant linkage of intermediate phenotypes for schizophrenia with markers within or near the beta-adrenergic receptor kinase 2 (ADRBK2, or GRK3) gene, which is highly expressed in dopaminergic pathways in the central nervous system, and mediates homologous desensitization for a variety of neurotransmitters and hormones through phosphorylation of G protein-coupled receptors (GPCRs). A polymorphism in the promoter region of the ADRBK2 was reported to be associated with bipolar disorder. We screened the putative promoter region, and all 21 exonic and flanking intronic regions of the ADRBK2 gene for mutations in 48 schizophrenia probands (including 16 Japanese and 32 Chinese patients), and evaluated the detected polymorphisms and those reported in the JSNP database for associations with schizophrenia in 113 family trios of schizophrenia probands. Four single nucleotide variants in the 5'-UTR/promoter region, and 16 rare variants in exonic and flanking regions, were identified. Among them, the Cys208Ser variant was the only non-synonymous mutation. Cys208Ser was found in one family without cosegregation between the variant and schizophrenia. Moreover, allelic, genotypic and haplotypic analyses provided no evidence for association between alleles at these polymorphisms and schizophrenia. The present study indicates that the ADRBK2 gene is unlikely to contribute strongly to schizophrenia susceptibility in this set of families.

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**Keywords:** Beta-adrenergic receptor kinase 2 (ADRBK2); G protein-coupled receptor kinase 3 (GRK3); Schizophrenia; Mutation; Single-nucleotide polymorphism; Transmission disequilibrium test

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## 1. Introduction

The evidence from family, twin, adoption, and molecular studies has demonstrated that genetic factors play an important role in the etiology of schizophrenia (Kety, 1988; Gottesman and Bertelsen, 1989; Kendler and Diehl, 1993; McGuffin et al., 1995; Baron, 2001). Efforts have been made to map the schizophrenia susceptibility genes by using direct candidate gene analysis, systematic scans for linkage, and positional candidate gene approaches by combining the linkage information and candidate gene analyses. Although the corresponding results are inconsistent, some chromosomal regions have shown potential promise, including 6p, 8p, 10p, 13q, 22q (Pulver, 2000; Riley and McGuffin, 2000; Liu et al., 2002).

We previously developed a method called the exploratory eye movement (EEM) test, and found that the number of eye fixations in responsive search (NEFRS) appears to be an indicator of schizophrenia (Kojima et al., 1992; Matsushima et al., 1998; Kojima et al., 2001). We performed a genome-wide linkage analysis utilizing the NEFRS deficit as an intermediate phenotype to identify susceptibility gene(s) for schizophrenia, and found linkage between NEFRS and markers between D22S429 and D22S310 on chromosome 22q12 (LOD=4.63) (Takahashi et al., 2003). A gene coding for the beta-adrenergic receptor kinase 2 (ADRBK2), alternatively known as G protein-coupled receptor kinase 3 (GRK3), is mapped to human chromosome 22q12 and located near D22S429. In a linkage study using a quantitative composite inhibitory phenotype consisting of a P50 sensory gating measure and antisaccade ocular motor performance in schizophrenia, a marker, D22S315, in intron 2 of the ADRBK2 gene yielded a high LOD score (LOD score=3.55,  $\theta=0$ ) (Myles-Worsley et al., 1999).

The G protein-coupled receptor kinases (GRKs), which are widely distributed in the brain and periphery, play an important role in the regulation of responsiveness of various G protein-coupled receptors (GPCRs). These protein kinases have the unique ability to recognize and phosphorylate their GPCR substrates only in their active conformations, after which phosphorylated receptors may

bind accessory proteins, known as arrestins, which further uncouple the receptor from the G protein (Pitcher et al., 1998). ADRBK2 is one of the beta-adrenergic receptor kinases (beta-ARK) in the GRK family. Arriza et al. (1992) showed that beta-adrenergic receptor kinase 1 [ADRBK1, or G protein-coupled receptor kinase 2 (GRK2)] and ADRBK2 are found in presynaptic and postsynaptic localizations in various brain regions, consistent with a general role for these kinases in the desensitization of neuronal GPCRs and their putative role in the regulation of neuronal activity. Through phosphorylation of GPCRs, ADRBK2 mediates homologous desensitization for a variety of neurotransmitters and hormones. For example, dopamine D1 receptors can be phosphorylated and desensitized via an ADRBK2 mechanism (Tiberi et al., 1996), and ADRBK2 expression is particularly high in dopaminergic pathways in the central nervous system (Arriza et al., 1992). ADRBK2 has also been shown to play a role in the desensitization of beta-adrenergic receptors and corticotrophin-releasing factor receptors, and may mediate desensitization for a variety of different neurotransmitter receptors (Hauger et al., 2000). These findings suggest that a major physiological role for ADRBK2 in neurons may be to act as a brake for signal transduction by some GPCRs, and a defect in ADRBK2 function may lead to an inability to desensitize receptors, and a heightened responsiveness to dopamine and other neurotransmitter signals in the brain (Niculescu et al., 2000). Niculescu et al. (2000) reported that the ADRBK2 gene was up-regulated in an animal model of psychotic mania using a GeneChip microarray. This was further supported by decreased protein levels of the ADRBK2 gene in lymphoblastoid cell lines from a subset of bipolar disorder patients that correlated with disease severity through Western blot analysis. In addition, Garcia-Sevilla et al. (1999) also found increased levels of ADRBK1/2 protein in postmortem tissue from the prefrontal cortex of depressed patients who committed suicide. Furthermore, Barrett et al. (2003) reported that a single nucleotide polymorphism (SNP) in the promoter region of ADRBK2 gene was associated with bipolar disorder.

Table 1  
PCR primers used to search for nucleotide variants in the ADRBK2 gene

Region	Primers	3' end of primer position	PCR fragment length	Annealing temperature (°C)
Promoter 1	Forward	GAC GAA GCT CCT TCA ACT GC	588	60
	Reverse	ATA CAG CCC AAA CAC CGA AG		
Promoter 2	Forward	CGA GAC CGA AGA TCA GGA AG	592	55
	Reverse	TCT GCT CCC TGG ACA CAG TA		
Promoter 3	Forward	GGA CCA GAG CAG GAG TTT CA	700	55
	Reverse	CTC CAT GGC CAT CAG GTA AC		
Exon 1	Forward	AGC GGG GAG GCG TGC TGC GA	392	65
	Reverse	CCG ACC CTC CAG GAA GCA GG		
Exon 2	Forward	TTG TGG CAA GAA GGG TCT CT	295	60
	Reverse	TAA AGC ACT AGG GGG TGC AT		
Exon 3	Forward	TTG CTT TGA AAA CAC CCT TG	309	60
	Reverse	CCT GTT AGC ATC TCA AAC TTG ATA CTA		
Exon 4	Forward	CAT GTG CAA GTG GTT TCT GC	270	60
	Reverse	CAT TCT GGA AAC GCT GAA CA		
Exon 5	Forward	CGT AGG TTA CTG TTT AGT AAT TGC TGT	327	60
	Reverse	GTC CCA GAC AAA GCT CCA GA		
Exon 6	Forward	GGG CAG GTA CCA GAC ACA AT	325	60
	Reverse	GGC ACA TCG TAC ATG GTC AG		
Exon 7	Forward	TAC GGG TGA ATG CTC TGT GA	314	60
	Reverse	CAG TGG ACC CTC TGG GAG TA		
Exon 8	Forward	TCA GTC ATG AAA AGG GTA GAT CC	371	60
	Reverse	CCC GGC CTA CAG CTT ATT TC		
Exon 9	Forward	CAT GGC GCT TGG GTA TTA CT	351	60
	Reverse	ACC CAC CCA AGA ACT CAC TG		
Exon 10	Forward	TGT AGC ATG GTG TTT GGT GG	257	60
	Reverse	AAA GAC CCA CAT TGC CAG TC		
Exon 11	Forward	TCC CTT TTT CTG CTG AGT CC	344	60
	Reverse	AGG GCT GAG ATG AGG GCT AT		
Exon 12	Forward	ATG CCC CAC TTT GAT GTG AT	330	60
	Reverse	AGA AAT GGG GTG ACA AAT GG		
Exon 13	Forward	CAG TCG CTG CCA TCT AAT GA	339	60
	Reverse	GCA CAT GGC TTA AAG ATC ACT G		
Exon 14	Forward	TAC GGC TTT TGC ATT TCT CC	259	60
	Reverse	TTG ACG GAA TGA AGC ATG AC		
Exon 15	Forward	CTG GTT GAG TGT TAG GAT GCT G	284	60
	Reverse	CCC TTT CAA GTC AAA ATG GAA		
Exon 16	Forward	TGG AAA TAC TTG CTC CCC TG	273	60
	Reverse	GCC AGC AGT GCA TAG AGA GA		
Exon 17	Forward	TCG CAC TGT GCT GTC TTT GT	279	60
	Reverse	CAG CCC AAT ATT AAA ACA ACC A		

Table 1 (Continued)

Region	Primers	3' end of primer position	PCR fragment length	Annealing temperature (°C)
Exon 18	Forward	CGA AAT GAT TAC TGC TGC CA	396	60
	Reverse	TGC AAA ATA CCT TAC GAC CCA		
Exon 19	Forward	TGC TGA CTT CTG TAA TGC GTT C	387	60
	Reverse	GAA TCA CAG AGT TTC GGA GGA		
Exon 20	Forward	CCA TCA AAG AAA GAA AAC CGA	377	60
	Reverse	AAA CCT TGT ACC TTG GGG CT		
Exon 21a	Forward	CAG GGA CGC TGG TAA TCA AT	421	60
	Reverse	AAA TGT GAC AAG GCA GGG AG		
Exon 21b	Forward	AGG ACA CAC CAG GGT CTC AG	456	60
	Reverse	AAC CCC AGG ACA AGA AGT CA		
Exon 21c	Forward	CTT TGC TCC ATA CTG CTG GG	485	60
	Reverse	GGC AGA AGG AAC CAG AAT GA		
Exon 21d	Forward	AGG CCG TGG AGT TTT AGG TT	476	60
	Reverse	CCA GCA TCT ATT TGG GGG TA		
Exon 21e	Forward	AGC ATC TGC CCT GTG AAG TT	378	60
	Reverse	TGC AAA GTA ACT CTT ATG AAA TAA TGG		

Table 2

Identified variants in the putative promoter region, exonic, and adjacent intronic region of ADRBK2 gene in patients with schizophrenia

Position	Variant/polymorphism	Flanking sequence of the variant	ID in dbSNP database
Promoter	–845G/A	gctcttccatcag a/g gggctccctaaggccat	
Promoter	–689A/G	gagcggaggtgggg a/g cccggcccctgggcgc	
Promoter	–465T/C	cttctctggatgag t/c tggcgggcatgagaa	
Promoter	–317T/C	agggagggtlac t/c gtagagacttgg	
Exon 6	459C/T (I153I)	atacatagaagaaat c/t tgtgaaagccttcca	
Exon 8	623C/G (C208S)	gggaagtattatggt c/g caggaaagcagacac	
Intron 9	IVS9–72T/A	agaaacttgcccag t/a gtgacatatattgt	rs2877289
Intron 9	IVS9–58C/T	gagtacatatattg c/t ttaaattagtctaga	
Intron 12	IVS12–4G/T	ttattcttttctgt g/t tagccagcaaatatt	
Exon 15	1296T/C (D432D)	cttgctcagcgaga t/c gtagcaagcgctg	rs3730316
Intron 15	IVS15–39G/A	ttaggatgctgttic g/a tgaacggatatttga	rs.3730315
Intron 15	IVS15+13G/C	ggtaggccattgtt g/c ctgctttcggtac	
Intron 16	IVS16–18C/T	tactcagcaactgta c/t gactctttctctcc	
Intron 19	IVS19–21C/T	agcctatttaactcc c/t agtgattttgtatc	rs3730312
Exon 21	2174A/G	ccgggactctccag a/t ctcccagaggagtc	rs.6519622
Exon 21	2331G/T	gaagtgactctact g/t atcacgtaaat	
Exon 21	2626T/C	ctctctgggagccg t/c acccacatgactgcc	rs6519623
Exon 21	2754C/T	ttcatccgtccatca c/t tggaaagatttacag	
Exon 21	3270A/C	gctgggttatgagaa a/c cagcgaatccccca	
Exon 21	3431C/A	gtccttgatatttt c/a gcagttccaaatct	

The position of each of the SNPs is indicated relative to the first bp of the translation start site.

Because of the known physiological role of ADRBK2 in the desensitization of receptors and its map location, we hypothesized that the ADRBK2 gene was a candidate gene for schizophrenia. In order to test this hypothesis, we systematically sequenced the ADRBK2 gene to search for mutations and genotyped SNPs in a sample of schizophrenia-segregating families of Japanese and Chinese origin.

## 2. Materials and methods

### 2.1. Subjects

Sixteen Japanese families and 97 Chinese families were analyzed in this study. Each pedigree included the proband and both parents. The Japanese pedigrees were recruited through schizophrenic probands visiting the Nihon University Hospital in Tokyo and five other affiliated hospitals. The Han Chinese pedigrees were recruited through the Mental Health Institute of Beijing University in Beijing and other affiliated hospitals.

All individuals provided written informed consent for participation in the study. The study was approved by the Ethics Committee of Nihon University.

Of 16 Japanese families ascertained, five had one affected parent, and one had both affected parents. Of 97 Chinese families, five had one affected parent. Each family member was diagnosed according to DSM-IV criteria (American Psychiatric Association, 1994) by two senior psychiatrists with clinical and research experience for their respective diagnoses. The diagnosis was made based on the content of the interview, information provided by the relatives, and a complete review of the medical chart.

### 2.2. DNA Analysis

Genomic DNA was extracted from peripheral blood cells.

#### 2.2.1. Mutation screening

The mutation-screening sample consisted of 16 Japanese probands and 32 Chinese probands ran-

Table 3  
Transmission disequilibrium test results for polymorphisms in the ADRBK2 gene

SNP	ID in JSNP	Position	Allele	Freq.	Transmitted	Not transmitted	TDT	P-value	Genomic position from translation starting site	Cofliguous position in NT011520.9
-845G/A		Promoter	A	0.93	14	7	2.3	0.1892	-845	5350670
-689A/G		Promoter	A	0.08	10	14	0.7	0.5413	-689	5350826
-465T/C		Promoter	C	0.74	32	28	0.3	0.6989	-465	5351050
-317T/C		Promoter	C	0.74	32	28	0.3	0.6989	-317	5351198
rs2267080 (C/T)	IMS-JST020398	Intron 1	C	0.87	20	19	0.0	1.00	18038	5369553
rs2283812 (G/C)	IMS-JST033446	Intron 1	G	0.47	65	65	0.0	1.00	19642	5371157
rs752186 (C/T)	IMS-JST033447	Intron 1	C	0.97	7	5	0.3	0.77	19878	5371393
rs2283814 (C/G)	IMS-JST033448	Intron 1	C	0.6	67	70	0.1	0.86	19995	5371510
rs2257229 (G/T)	IMS-JST033449	Intron 1	G	0.38	69	63	0.3	0.66	20106	5371621
623C/G		Exon 8	G	0.04	1	0	1.0	1.00	109525	5461040
rs3747127 (C/T)	IMS-JST098743	Exon 21	C	0.06	5	7	0.3	0.77	157554	5509069
2626T/C		Exon 21	T	0.94	11	7	0.9	0.48	158030	5509545
2754C/T		Exon 21	C	0.04	9	9	0.0	1.00	158158	5509673
rs2235357 (A/G)	IMS-JST015353	3' flanking region	A	0.99	4	4	0.0	1.00	164288	5515803

Table 4  
Pair-wise linkage disequilibrium in the CHGB

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	-845G/A	-689A/G	-465T/C	-317T/C	rs2267080	rs2283812	rs5752186	rs2283814	rs2257229	623C/G	2626T/C	2754C/T	rs3747127	rs2235357
1	-845G/A	1.00	0.15	0.15	1.00	0.79	1.00	1.00	1.00	1.00	0.24	0.50	0.61	1.00
2	-689A/G	0.25	0.43	0.43	0.14	0.61	1.00	0.71	0.82	1.00	1.00	0.51	0.12	0.46
3	-465T/C	0.00	1.00	1.00	0.23	0.14	1.00	0.47	0.49	1.00	0.71	0.10	0.04	1.00
4	-317T/C	0.00	0.00	1.00	0.23	0.14	1.00	0.47	0.49	1.00	0.71	0.10	0.04	1.00
5	rs2267080	0.15	0.00	0.00	0.00	0.16	0.64	0.28	0.09	1.00	1.00	0.68	0.31	0.81
6	rs2283812	0.05	0.04	0.01	0.01	0.02	0.02	0.81	0.85	1.00	0.70	0.81	0.22	0.02
7	rs5752186	0.14	0.14	0.10	0.15	0.02	0.05	1.00	0.80	1.00	1.00	1.00	0.14	1.00
8	rs2283814	0.05	0.05	0.13	0.01	0.50	0.03	0.87	0.95	1.00	0.60	1.00	0.01	0.00
9	rs2257229	0.05	0.06	0.13	0.00	0.55	0.03	0.87	0.37	0.62	0.69	1.00	0.12	1.00
10	623C/G	0.16	0.16	0.18	0.83	0.00	0.15	0.36	0.37	1.00	1.00	1.00	0.50	1.00
11	2626T/C	0.04	0.15	0.01	0.15	0.03	0.14	0.03	0.03	0.25	1.00	0.57	0.27	1.00
12	2754C/T	0.21	0.00	0.00	0.00	0.04	0.14	0.04	0.04	0.00	0.01	0.57	0.48	1.00
13	rs3747127	0.04	0.00	0.00	0.01	0.02	0.00	0.00	0.01	0.00	0.00	0.01	0.48	1.00
14	rs2235357	0.14	0.05	0.15	0.02	0.00	0.14	0.00	0.01	0.14	0.14	0.14	0.01	0.56

Upper diagonal figures are  $D'$  and lower diagonal figures are  $r^2$ .  
Pairs in LD ( $D'/0.8$  or  $r^2/0.7$ ) are shown as bold values.



domly selected from our subjects. Primers were designed to amplify the putative promoter region, all 21 exons, and flanking intronic splice sites from genomic DNA using the Primer3 program based on the Whitehead Institute Center for Genome Research (<http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>) (Table 1). In the putative promoter region and exon 21, the sequences were split into several pieces with sufficient overlap. The genomic nucleotide sequence was based on GenBank NT\_011520.9.

PCRs were performed in a final volume of 20  $\mu$ l containing the following: 3 ng genomic DNA; 4 mM each of dATP, dGTP, dTTP, and dCTP; 4 mM  $MgCl_2$ ; 5 pmol primers for each; 2  $\mu$ l 10XPCR Gold Buffer II [150 mM Tris-HCl (pH 8.0), 500 mM KCl], 0.8 U Gold Taq polymerase (ABI) or 1 U native Pfu (Stratagene) by a GeneAmp PCR System 9700 (Perkin-Elmer, Foster City, CA). The PCR reaction began with an initial denaturation step at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 or 55 °C or 65 °C for 30 s, and extension at 72 °C for 1 min. The reaction was completed with a final synthesis step consisting of 7 min at 72 °C.

PCR products were then purified by a Multi-screen-PCR 96-well Filtration System (Millipore) and sequenced in both directions, using the same primers with the ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit Mix v2 (Applied Biosystems, Foster City, CA). Sequencing fragments were filtered by Multiscreen-HV Filter Plates for High Throughput Separations (Millipore) using Sephadex™ G-50 Fine (Amersham Pharmacia Biotech AB), separated by capillary electrophoresis, and detected via laser-induced fluorescence on an ABI PRISM 3700 DNA Analyzer (Perkin-Elmer) using POP6 polymer (Applied Biosystems, Foster City, CA). Sequence data were compared with the published sequence for ADRBK2 using the Sequencher 3.1 gene analysis computer program (Gene Codes Corporation).

### 2.2.2. SNP typing

The Cys208Ser variant, detected in this study, was genotyped by the restriction fragment length

polymorphism (RFLP) method after PCR amplification using *EcoRII* in all available subjects. The Cys allele is 371 bp, and the Ser allele is 200 bp + 171 bp. The fragments were separated on 2% agarose gel, and the bands were visualized by ethidium bromide staining and ultraviolet transillumination.

The other six variants detected in the mutation screening, and all SNPs in or near the ADRBK2 gene reported in the JSNP database (<http://SNP.ims.u-tokyo.ac.jp/cgi-bin/>), were genotyped by direct sequencing in all our subjects. The primers and PCR conditions were the same as the mutation screening or chosen according to the JSNP Database.

### 2.3. Statistical analysis

The transmission disequilibrium test (TDT), and  $D'$  and  $r^2$  of linkage disequilibrium were conducted with TDTPHASE (Dudbridge et al., 2000). Options of 'EM' in TDTPHASE were not used.

## 3. Results

We detected sixteen single nucleotide variants in the exonic and flanking intronic region in 48 probands (Table 2). Three were in the coding region, six were in the 3' untranslated region, and seven were in the intronic regions. Six of them, IVS9–72T/A, 1296T/C, IVS15–39G/A, IVS19–21C/T, 2174A/G and 2626T/C have been reported in the dbSNP database. Among the variants, only 623C/G in exon 8 was a non-synonymous mutation with a substitution of Cys with Ser in codon 208. The IVS9–72T/A, IVS15–39G/A, IVS19–21C/T, 2174A/G, 2626T/C and 2754C/T polymorphisms were found in the same five Chinese probands. The other variants were found in only one or two probands. All variants were observed to be heterozygous.

We genotyped the Cys208Ser variant in all family members, and found that it was only in the one Chinese family that was initially identified. The mother and proband were heterozygous for the Cys208Ser polymorphism; however, only the proband had schizophrenia.

The ADRBK2 gene promoter has yet to be functionally identified, but the putative promoter region was considered to lie in the genomic region immediately 5' to the first coding sequence (Barrett et al., 2003). We screened 1500-bp upstream of exon 1, and identified four single nucleotide variants in 48 probands (Table 2). Except the  $-845G > A$  reported as P-4 previously (Barrett et al., 2003), all are novel.

We genotyped the  $-845G/A$ ,  $-689A/G$ ,  $-465T/C$ ,  $-317T/C$ ,  $2626T/C$  and  $2754C/T$  polymorphisms detected in the mutation screening and seven other SNPs in the JSNP database in all family members. The results of the TDT analysis are summarized in Table 3. No significantly deviated transmission patterns were observed for any polymorphisms. No significant association was observed for three-marker sliding window haplotypes (data not shown). Linkage disequilibrium between polymorphisms is shown in Table 4.

#### 4. Discussion

In this study, we hypothesized that the ADRBK2 gene was an important candidate for schizophrenia; however, we found only one rare non-synonymous variant, and noticed that even synonymous variants were rarer than expected. We calculated the nucleotide diversity ( $\pi$ ) defined as the average heterozygosity per nucleotide site based on the variants that we found in 3627 bp of 21 exons of the ADRBK2 gene. The  $\pi$  was  $1.1 \times 10^{-4}$  in our population, which is lower than the nucleotide diversity found in most other human autosomal genes (Halushka et al., 1999). The  $\pi$  was  $0.9 \times 10^{-4}$  across 4601 bp in the intronic region that we screened in our population. Although five SNPs in ADRBK2 exons were reported in the dbSNP database, they were in unconfirmed validation status at the time of manuscript preparation. Only one validated SNP, which we did not find in our mutation screening sample, was reported in exon 21 in the JSNP database (IMS-JST098743). The minor allele frequency was 0.06. These SNP databases also suggest low nucleotide diversity in the ADRBK2 gene.

We found the Cys208Ser variant in one family. Cys208 localizes in the protein kinase domain and

the serine/threonine protein kinase region of ADRBK2, and is conserved in the mouse, rat, bovine, Didelphidae, Homarus, and *Drosophila* genomes; it is also conserved in ADRBK1. Therefore, the Cys208Ser variant may alter kinase function; however, since the variant was also found in a non-schizophrenic mother, no hint of association of this variant with schizophrenia was obtained.

Barrett et al. (2003) reported that the polymorphism that the authors named as P-5 in the promoter region was associated with bipolar disorder. However, we did not find the polymorphism in our samples. It is likely that the P-5 is not present in Asian populations. We genotyped four SNPs identified in the promoter region, and one of them,  $-317T/C$ , is 10 bp downstream to the P-5, and  $-465T/C$  and  $-317T/C$  are in complete linkage disequilibrium (Table 4). We did not find an association between these polymorphisms and schizophrenia.

In conclusion, the data in this study indicate that the ADRBK2 gene is not largely associated with schizophrenia in our population.

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## Functional MRI mapping of brain activation during visually guided saccades and antisaccades: cortical and subcortical networks

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### Abstract

Antisaccade tasks require a subject to inhibit a saccade toward a briefly appearing peripheral target and instead to immediately generate a saccade to an equivalent point in the opposite hemifield. Using functional magnetic resonance imaging (fMRI), we investigated the neural networks required to inhibit reflexive saccades and to voluntarily generate saccades. The results demonstrated that saccade and antisaccade tasks often bilaterally activate frontal, parietal and supplementary eye fields, lenticular nuclei and occipital cortex. Additional activation of bilateral dorsolateral prefrontal cortices, supramarginal gyri, anterior cingulate cortices and thalamus was observed during antisaccade tasks. These results indicate that fronto-parietal and fronto-striato-thalamo-cortical circuits are involved in antisaccade tasks. The fronto-parietal circuit is thought to be related to the planning of saccadic eye movements that involve attentional control, while the fronto-striato-thalamo-cortical circuits connect to cortical region as a feedback network. We speculate that the abnormalities in spatial attention and eye movement control observed in schizophrenia stem from dysfunctions in the fronto-parietal and fronto-striato-thalamo-cortical circuits.

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**Keywords:** Saccade; Antisaccade; Functional MRI; Eye movements; Fronto-parietal network; Fronto-striato-thalamo-cortical network

### 1. Introduction

Saccadic eye movements represent the primary mechanism used by primates to visually explore their environment. Saccades serve to focus points of visual interest on the fovea or parafoveal region of the retina. Several processing steps are usually performed before the neural command signals are sent to the oculomotor

nuclei in the brainstem for saccade execution. These processes include disengagement of attention from a fixated target, saccade target selection, reallocation of spatial attention to the saccade target, calculation of spatial information for the saccade target, and the decision of when to give the signal to execute saccade.

Single unit recording studies in animals have revealed contributions from several cortical and subcortical regions to the generation of saccades. Knowledge of the human cortical control of saccades has been accumulated from observations of cerebral lesions (Guitton et al., 1985; Evdokimidis et al., 1996; Crevits

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et al., 2000), transcranial magnetic stimulation (TMS) (Kapoula et al., 2001; Leff et al., 2001), positron emission tomography (PET) (Anderson et al., 1994; O'Driscoll et al., 2000), and functional magnetic resonance imaging (fMRI) (Gaymard et al., 1999; Connolly et al., 2000; Nobre et al., 2000; Matsuo et al., 2003). These previous studies have indicated that saccadic eye movements are controlled by a cortical network that includes the parietal eye field (PEF) located in the intraparietal sulcus and superior parietal lobule (SPL), the frontal eye field (FEF) located in the precentral gyrus, and the supplementary eye field (SEF) located in the upper medial wall of the frontal lobe. The PEF is thought to be involved in visuospatial integration, the FEF may be involved in the preparation and triggering of intentional saccades, and the SEF is probably involved in the temporal control of sequences of visually guided saccades and eye-hand coordination (Gaymard et al., 1998; Heide and Kompf, 1998). Saccades and voluntary blinks are associated with similar loci of activation patterns (Bodis-Wollner et al., 1999).

Recent research has revealed an association between some psychiatric and neurological disorders and inability to inhibit unwanted reflexive saccades. One task used to investigate the inhibition of saccades is the antisaccade task (Everling and Fischer, 1998), which requires subjects to inhibit a saccade toward a briefly appearing peripheral target, and instead to generate a saccade to an equivalent point in the opposite hemifield. Inhibitory ability can be examined using the same oculomotor task with visually guided saccades by presenting a visual stimulus at one side and asking the subject to look at the opposite side.

In an experiment comparing reflexive saccade and antisaccade generation, a higher BOLD activation was found in the antisaccade than in the reflexive saccade task (Kimmig et al., 2001). The FEF and IPS were reported to be activated during voluntary eye movements, and FEF activity was related to the suppression of reflexive saccades (Mort et al., 2003). Within frontoparietal networks, the human FEF, but not the IPS, was reported to be critically involved in preparatory set, coding both the readiness and intention to perform a particular movement (Connolly et al., 2002; DeSouza et al., 2003). Curtis and D'Esposito (2003) investigated interactions between voluntary top-down and reflexive bottom-up processes using event-related fMRI, and reported that activity in the pre-SMA and SMA was

higher than in the FEF and IPS during the preparatory period, and that FEF and IPS activity was higher than pre-SMA and SMA activity in the stimulus response period. The rostral portions of the SEF and FEF, as well as the rostral and lateral parts of the PEF, were reported to contribute to the suppression of prepotent responses (Merriam et al., 2001; Cornelissen et al., 2002).

In an animal study, saccade-related neurons in the FEF and superior colliculus (SC) were reported to decrease their rate of firing and those in the SEF were reported to increase their rate of firing before antisaccade (Schlag-Rey et al., 1997). Neural activity in the SC is known to be involved not only in the generation of fast saccades but also in antisaccade generation, and this neural activity is concerned with top-down signals from the frontal cortex (Everling et al., 1999).

A deficit in the inhibition of reflexive responses may result in a high number of saccades towards the visual stimulus. Previous studies have shown that patients with frontal brain lesions (Vilis and Hore, 1986), schizophrenia (Fukushima et al., 1988, 1990), and Alzheimer's disease (Currie et al., 1991) display difficulty generating antisaccades. Some authors have argued that the dorsolateral prefrontal cortex (DLPFC) is responsible for the correct performance of antisaccade tasks (Pierrot-Deseilligny et al., 1991).

McDowell et al. (1999) reported that poor anti-saccade performance is related to genetic risk for schizophrenia. Furthermore prefrontal activity was not found during antisaccade tasks in schizophrenic patients (McDowell et al., 2002). However, Raemaekers et al. (2002) reported that schizophrenic patients did not show a selective dysfunction of the prefrontal region, but that they showed abnormalities of a frontostriatal network that is engaged in the suppression of automatic eye movements.

The aim of this study is to use fMRI to investigate the physiological neural bases of antisaccade performance, suppression of reflexive saccades, and generation of voluntary saccades.

## 2. Methods

### 2.1. Subjects

Twenty-one healthy volunteers (mean age  $39.2 \pm 10.2$ ) participated in this study. All subjects were free

from neurological or psychiatric illness, and no abnormalities were observed on brain structural MRI. Written informed consent was obtained from all subjects. The project was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of Nihon University School of Medicine.

## 2.2. Magnetic resonance imaging

MRI data were acquired using a 1.5-T Siemens Symphony system (Siemens, Erlangen, Germany). Gradient-recalled echo planar imaging (EPI) was used for the fMRI sequence to obtain blood oxygen level-dependent (BOLD) contrast. Interleaved multi-slice gradient EPI was used to produce 40 continuous, 3-mm-thick axial slices encompassing the entire brain (echo time = 62 ms, repetition time = 4000 ms, flip angle = 90°, field of view = 192 mm, 64 × 64 matrix). Each subject performed five series contrasting saccade and control tasks and five series contrasting antisaccade and control tasks. For each series, subjects alternated between 40 s of control task and 40 s of oculomotor task. Each series comprised 104 scans with a complete duration of 416 s. The run began with four dummy volumes to allow for T1 equilibration effects. The head of the subject was fixed using cushions to minimize motion artifacts.

## 2.3. Task design

Subjects were instructed to fixate on a central fixation point. A visual stimulus was then presented in the visual periphery, at which point subjects were required to generate a saccade towards the stimulus (saccade task) or towards the horizontal mirror position (antisaccade task). Fixation point offset occurred after 500–1500 ms before a peripheral (randomized left or right on the horizontal axis) target appeared for a duration of 1000 ms. During the control task, subjects were in total darkness and were asked to maintain fixation and not blink. The target size was 1° of visual angle. The number of left and right saccadic eye movements was equated, with position of 10° in either direction. While subjects performed either the saccade or antisaccade task and baseline control tasks, fMRI scans were

obtained. Visual targets were generated using a personal computer (OS: Windows 98) and customized software. The stimulus was projected on a small screen attached to a head coil, using a liquid crystal display projector system customized to our MRI machine (Kiyohara Optics, Tokyo). To measure performance during saccade and antisaccade tasks, electro-oculography (EOG) was undertaken outside the MRI scanner before functional imaging.

## 2.4. Data analysis for fMRI

Activity related to saccades and antisaccades relative to activity during the control task was analyzed independently. Image analysis was performed using an Ultra5 work-station (Sun Microsystems, Palo Alto, CA, USA) using MATLAB (Mathworks Inc., Natick, MA, USA) and statistical mapping (SPM99, Wellcome Department of Cognitive Neurology, London, UK; <http://www.filion.ucl.ac.uk/spm>). Before statistical parametric maps were calculated, EPI images for each time series were realigned to the first functional image to remove residual head movement. Images were then coregistered and transformed into the Montreal Neurological Institute template. Confounding effects of global volume activity and magnetic noise were removed using linear regression and cosine functions (up to a maximum of 1 cycle per 40 scans). Removing the latter confounds corresponds to high-pass filtering of the time series to remove low-frequency artifacts that can arise due to aliased cardiac and other cyclical components. After normalization, three-dimensional spatial smoothing was applied to each volume using a Gaussian kernel of 8 × 8 × 8 mm. Alternating periods of baseline and activation were modeled using a simple delayed box-car reference vector to account for delayed cerebral blood flow after stimulus presentation. Significantly activated pixels were searched for using the General Linear Model approach for time-series data.

Data were analyzed using random-effect analysis. Statistical significance was set at the level of  $P < 0.001$ , uncorrected for multiple comparisons;  $T = 3.35$ . Intra-individual comparisons between saccades and antisaccades were analyzed using paired  $t$ -tests, and statistical significance was set at the level of  $P < 0.029$ , uncorrected for multiple comparisons;  $T = 2.00$ .

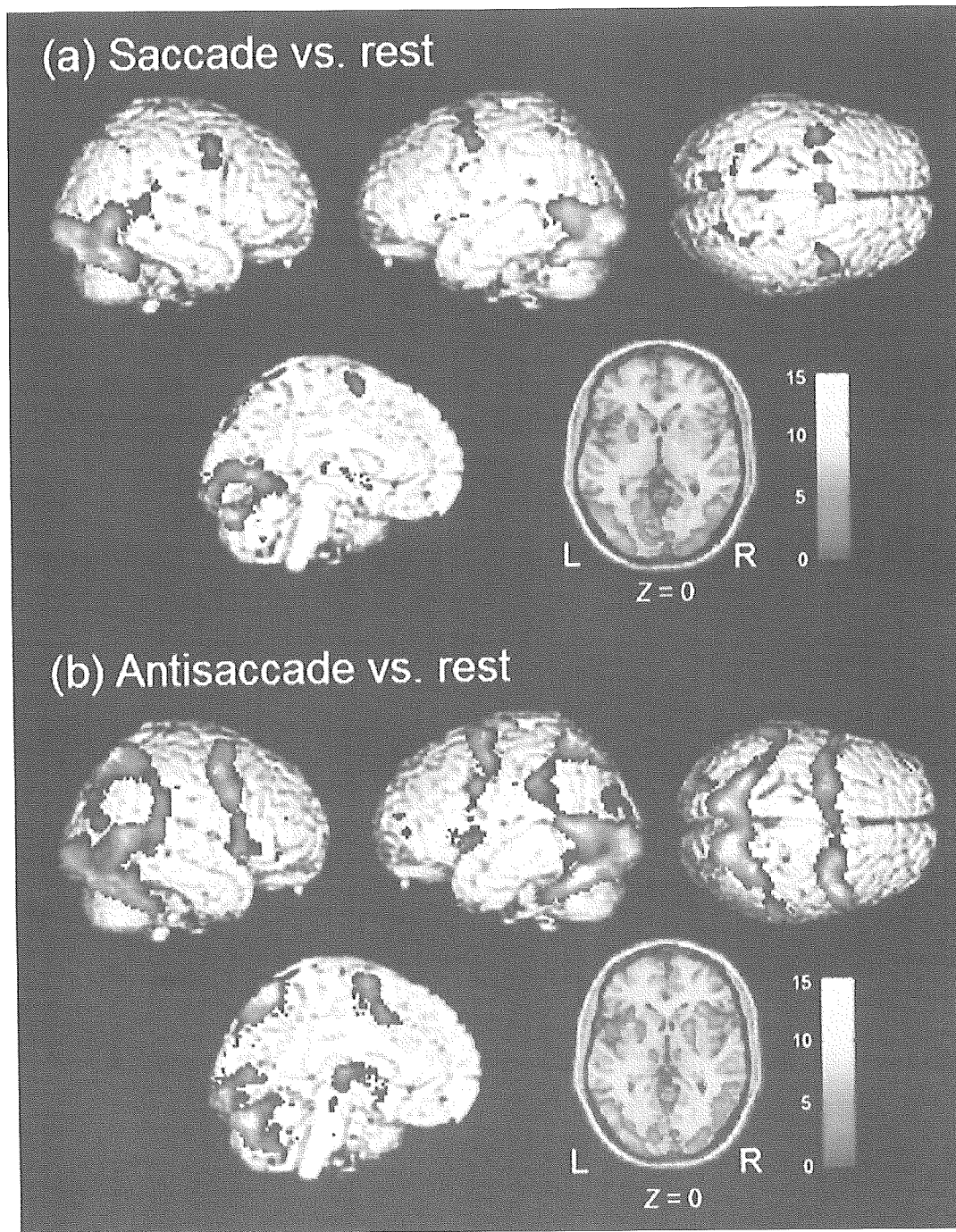


Fig. 1. Brain regions displaying greater activity during saccades (a) or antisaccades (b) than during control conditions. Statistical parametric maps, rendered onto standard brain close to MNI space. Height threshold at  $P < 0.001$ , uncorrected to demonstrate extent of each activated cluster;  $T = 3.35$ .

### 3. Results

Analysis of EOG revealed that no subjects exhibited directional errors during saccade tasks, and the mean percentage of errors during antisaccade tasks was  $1.12 \pm 2.7\%$ .

Activation areas are shown in Fig. 1a for saccade tasks, in Fig. 1b for antisaccade tasks and in Table 1 for both. Montreal Neurological Institute coordinates were determined based on averaged activation maps ( $P < 0.001$ , uncorrected for multiple comparisons:  $T = 3.53$ ). During saccade tasks, regional activation was observed in bilateral FEF, SEF, and PEF, left lenticular nucleus and bilateral occipital cortices (V1). During antisaccade tasks, activation was observed in the same regional areas as in saccade tasks. Additional sites of activation were observed in bilateral inferior

parietal lobules (IPL), ACC and thalamus, right lenticular nucleus and left DLPFC during antisaccade tasks.

Fig. 2 and Table 2 show the regions that were more active during antisaccade than during visually guided saccade tasks ( $P < 0.029$ , uncorrected for multiple comparisons:  $T = 2.00$ ). Activation of bilateral FEF, PEF, IPL, ACC, thalami and DLPFC was observed.

### 4. Discussion

In this study, fMRI was used to reveal thalamic activation during antisaccade tasks, and lenticular nucleus activation during both saccade and antisaccade tasks. A previous PET study (Sweeney et al., 1996) reported task-related activation in the right

Table 1  
Brain regions more active during visually guided saccade and antisaccade than during control tasks

Brain region		Saccade vs. rest coordinates			T-value	Antisaccade vs. rest coordinates			T-value
		X	Y	Z		X	Y	Z	
DLPFC	R				N.S				N.S
	L				N.S	-44	50	4	4.24
FEF	R	46	6	50	5.34	40	-2	50	8.87
	L	-42	-4	58	6.66	-22	-2	68	8.30
SEF	R	6	4	62	3.64	8	8	52	4.20
	L	-4	4	60	5.87	-2	10	46	5.56
PEF	R	22	-68	60	3.80	12	-64	64	12.36
	L	-30	-56	56	4.28	-10	-72	56	11.30
Lenticular nucleus	R				N.S	22	8	-2	6.93
	L	-20	8	2	4.45	-20	6	0	4.71
Visual cortex	R	38	-90	-8	9.81	26	-102	-6	8.12
	L	-22	-102	-8	10.75	-22	-102	-12	8.11
SMG	R				N.S	64	-36	28	6.14
	L				N.S	-64	-40	34	5.75
ACC	R				N.S	8	8	52	4.20
	L				N.S	-2	10	46	5.56
Thalamus	R				N.S	10	-14	8	8.30
	L				N.S	-12	-16	2	5.61

DLPFC: dorsolateral prefrontal cortex, FEF: frontal eye fields, SEF: supplementary eye fields, PEF: parietal eye fields, SMG: supramarginal gyrus, ACC: anterior cingulate cortex.



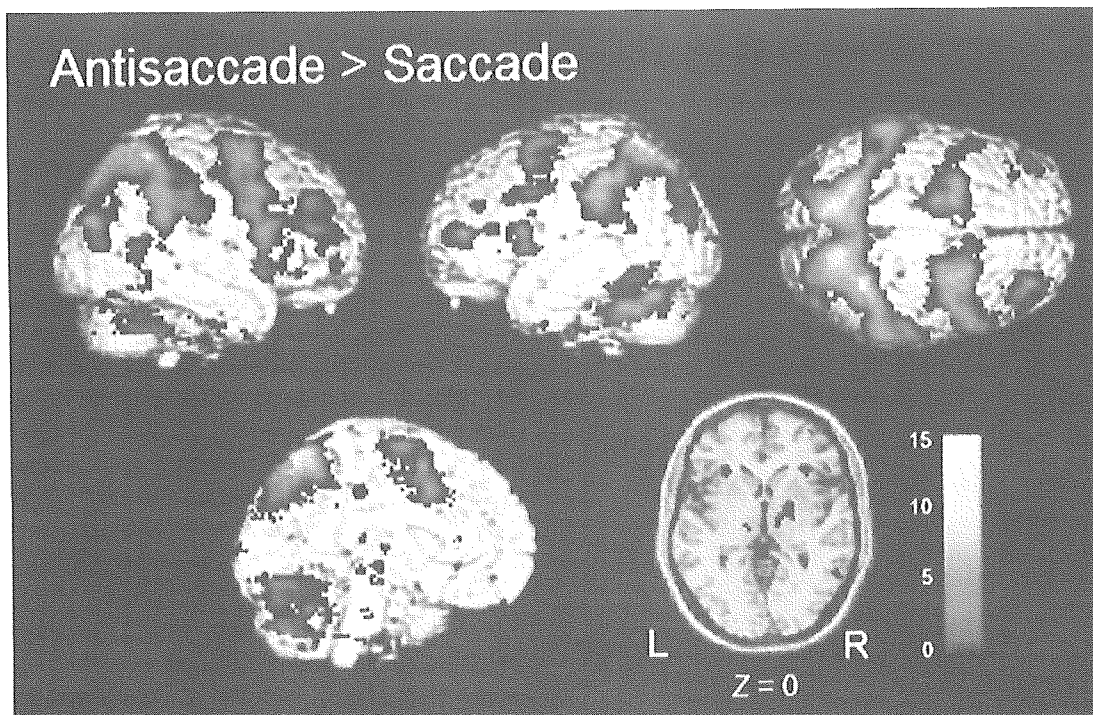


Fig. 2. Brain regions displaying greater activity during antisaccade than during saccade. Statistical parametric maps, rendered onto standard brain close to MNI space. Height threshold at  $P < 0.029$ , uncorrected to demonstrate extent of each activated cluster;  $T = 2.00$ .

posterior thalamus during visually guided saccades and in the left pulvinar during antisaccade tasks. The thalamus is known to subserve visual attention, and monkey studies have revealed thalamic activation during spatial working memory tasks (Petersen et al., 1985). Patients with thalamic infarcts display disrupted saccades (Brigell et al., 1984). The present result also indicated an important role for the thalamus in facilitating and inhibiting voluntary saccadic eye movements.

The basal ganglia tonically inhibit the thalamus through two parallel pathways (Alexander and Crutcher, 1990). A direct pathway runs from the striatum to the thalamus, and activation of the striatum disinhibits the thalamus, thus increasing thalamo-cortical activity. An indirect pathway passes from the lenticular nucleus to the subthalamic nucleus, and finally to the brainstem nuclei. Activation of the indirect pathway further inhibits thalamo-cortical neurons. As a result, activation of the direct pathway facilitates saccades, whereas activation of the indirect pathway inhibits saccades.

Schizophrenia patients display dysfunction of the dopaminergic neural networks (Gerfen et al., 1990) and demonstrate fronto-striato-thalamic circuit dysfunction (Buchsbaum et al., 1992). The direct and indirect pathways from the basal ganglia are affected differently by dopaminergic projections from the substantia nigra pars compacta to the striatum. Striatal neurons that project directly to the two output nuclei possess D1 dopamine receptors that facilitate transmission, while those projecting in the indirect pathway display D2 receptors that reduce transmission (Gerfen et al., 1990). Dysfunction of the striato-thalamo-cortical dopaminergic circuitry may reduce inhibition and thus facilitate saccades in schizophrenia. Our results indicate that this dysfunction has an important role on subtle motor control and therefore affects antisaccade production through both the direct and indirect pathways.

The activation of bilateral DLPFC was observed during antisaccade tasks, but not during visually guided saccade tasks, according to a previous fMRI study (Muri et al., 1998) and a PET study (O'Driscoll

Table 2  
Brain regions more active during antisaccades than visually guided saccades

Brain regions		Coordinates			T-value
		X	Y	Z	
DLPFC	R	42	46	18	2.72
	L	-38	52	12	4.73
FEF	R	28	4	54	6.24
	L	-28	4	48	6.40
PEF	R	16	-66	52	8.74
	L	-26	-50	56	6.84
SMG	R	64	-20	24	4.26
	L	-66	-36	28	6.17
ACC	R	10	18	36	3.04
	L	-12	12	38	5.83
Thalamus	R	12	-14	-4	2.76
	L	-12	-18	-2	2.31

DLPFC: dorsolateral prefrontal cortex, FEF: frontal eye fields, SEF: supplementary eye fields, PEF: parietal eye fields, SMG: supra-marginal gyrus, ACC: anterior cingulate cortex.

et al., 1995). DLPFC activation confirms the results of previous lesion studies, in that patients with DLPFC lesions demonstrate an increased percentage of anti-saccade errors, reflecting difficulties suppressing unwanted reflexive saccades (Guitton et al., 1985; Evdokimidis et al., 1996; Crevits et al., 2000). The fronto-striato-thalamo-cortical network (Alexander et al., 1986; Petit et al., 1993; McFarland and Haber, 2002), including the prefrontal cortex and thalamus, is important in the control of antisaccades. These results suggest schizophrenia patients displaying inhibition errors during antisaccades may have a dysfunction of the fronto-striato-thalamo-cortical network.

Bilateral FEF were activated during both saccade and antisaccade tasks, as in several previous fMRI studies (Muri et al., 1998; Connolly et al., 2000). In a monkey study, the majority of FEF neurons displayed vigorous presaccadic activity (Hanes et al., 1995). Patients with lesions restricted to the FEF demonstrate a normal percentage of directional errors during an antisaccade task, but increased antisaccadic latencies (Rivaud et al., 1994). The FEF is considered responsible for triggering antisaccades and suppressing unwanted reflexive saccades (Merriam et al., 2001;

Cornelissen et al., 2002). The FEF is also concerned with preparatory set, which is involved in readiness and intention to perform a saccade (Connolly et al., 2002; DeSouza et al., 2003).

Bilateral SPL were activated during both saccade and antisaccade tasks, while the IPL, including the SMG, was activated only during antisaccades, in accordance with a previous fMRI study (Connolly et al., 2000). The SPL is active during covert orienting tasks (Nobre et al., 2000), and activation in the SPL might be associated with overt eye movement responses in addition to spatial attention shifts. Saccade tasks require only local attention, while antisaccade tasks require an attentional shift from local to global. Patients with lesions restricted to the right SMG make few saccades to the left, and show abnormal performance on covert attentional shift to the left. The SMG may not carry a topographic representation of visual space, and may instead be involved in switching from local to global features of a stimulus (Perry and Zeki, 2000). Co-activation between SPL and IPL may be needed to perform antisaccade tasks that require attentional shifts from local to global.

The fronto-parietal network, including the FEF, the SPL and the IPL, is considered important for control of attention, and has been implicated in planning saccadic eye movements. These regions also project from the thalamus. These two networks, the fronto-striato-thalamo-cortical and front-parietal networks, are thus considered to be important for accurate control of antisaccades.

In conclusion, saccade and antisaccade tasks commonly activate bilateral FEF, SEF, PEF, lenticular nuclei and V1. Additional activation of bilateral DLPFC, IPL, ACC and thalami were observed during antisaccade tasks. These results indicate the involvement of two important neural networks of fronto-parietal and fronto-striato-thalamo-cortical circuits in the control of inhibition of reflexive saccades and voluntary saccades (Alexander et al., 1986; Petit et al., 1993; McFarland and Haber, 2002). Specific antisaccade errors have been reported in patients with schizophrenia, who are believed to possess abnormalities in the dopaminergic neural network. We speculate that abnormalities in spatial attention and processing of voluntary movement information in schizophrenia stem from dysfunctions in the fronto-

parietal and fronto-striato-thalamo-cortical circuits networks.

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