Table 1
List of primers for genomic DNA fragment amplification and sequencing reactions

Name of primer	Sequence (5′–3′)	Direction	Position[e]		Product size (bp)	Purpose
D4-120F[a]	GTTGTCTGTCTTTTCTCATTGTTTCCATTG	Sense	−1726	−1697	429, 549	Amp[f]
D4-120R[a]	GAAGGAGCAGGCACCGTGAGC	Antisense	−1179	−1199		Amp
D4iF3	CACACCTGTCCCTGGTGCAGG	Sense	−1256	−1236	606	Amp, Seq[g]
D4iR3	CCCACCCGTTGCACAGTTGATC	Antisense	−651	−672		Amp, Seq
D4iiF3	TACCTAGCTCACGGTCTTGGGC	Sense	−765	−744	1160	Amp
D4ivR2	CTGGAAGCTCCGCACCAGAAAG	Antisense	395	374		Amp
D4iiF5	GCTGTCCGCCCAGTTTCGGAG	Sense	−706	−686		Seq
D4pos3[b]	CTCAGGTCTTTCTGCGTCTGGC	Sense	−472	−451		Seq
D4EX1F[c]	CGCCATGGGGAACCGCAG	Sense	−4	14		Seq
D4iiiR1	GTGGCCACGCTCACGCACACG	Antisense	182	162		Seq
D4iiiR2	CGCTGAGCACCGCGGACAACG	Antisense	−17	−37		Seq
D4iiR1	TCGACGCCAGCGCCATCCTAC	Antisense	−346	−366		Seq
D4neg3[a]	CAGGTCACAGGTCACCCCTCTT	Sense	−947	−926	792	Amp, Seq
D4neg4[a]	TTGCTCATCTTGGAATTTTGCG	Antisense	−156	−177		Amp, Seq
D4-48F[d]	AGGTGGCACGTCGCGCCAAGCTGCA	Sense	2612	2636	$174 + (48 \times N^{h})$	Amp
D4-48R[d]	TCTGCGGTGGAGTCTGGGGTGGGAG	Antisense	2929	2905		Amp

[a] Seaman et al. (1999).
[b] Mitsuyasu et al. (1999, 2001).
[c] Catalano et al. (1993).
[d] Nanko et al. (1993).
[e] Relative position to the first nucleotide of initiation codon of the genomic sequence (GenBank Accession No. AC021663).
[f] Amp, these primers were used for PCR amplification.
[g] Seq, these primers were used for direct sequencing.
[h] *N*, number of repeats of the 48-bp sequence in exon 3.

The 48-bp VNTR was genotyped according to published methods (Nanko et al., 1993; Van Tol et al., 1992). PCR products were electrophoresed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., USA). The size of the amplified fragments was 174 bp plus 48 bp multiplied by the repeat number.

DNA sequencing was used to genotype 26 polymorphisms. First DNA sequencing templates were generated by PCR amplification of two DNA fragments (606-bp and 1160-bp) from genomic DNA of each individual (Fig. 1). PCR primers (Table 1) were designed based on GenBank Accession No. AC021663. The 606-bp fragment was amplified in a 10 μl reaction mixture that contained 1 μM of each primer, 0.2 mM of dNTPs (Amersham Biosciences Corporation, USA), 50 ng template DNA, 0.025 U/μl of AmpliTaq polymerase (Applied Biosystems, USA), 5.5 ng/μl of TaqStart Antibody (Clontech, USA), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$ and 10% of dimethylsulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd., Japan). Thermal cycling profile was 1 min at 95 °C for initial denaturation, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C, followed by a final incubation at 72 °C for 5 min. The 1160-bp fragment was amplified in 1 μM of each primer, 0.2 mM of dNTPs, 50 ng template DNA, 0.025 U/μl of KOD Dash polymerase (Toyobo, Japan), KOD Dash PCR buffer supplied by the manufacturer and 10% of DMSO in a total volume of 20 μl. The thermal cycling profile was 1 min at 96 °C for the initial denaturation, followed by 33 cycles of 30 s at 95 °C, 2 s at 63 °C and 30 s at 74 °C followed by a final incubation at 74 °C for 5 min.

These two DNA fragments were then used for 26 minisequencing reactions. First the template fragments were treated with two units of shrimp alkaline phosphatase (Roche Diagnostics Corporation, USA) and exonuclease I (New England Biolabs, USA) at 37 °C for 1 h. Both enzymes were heat inactivated at 80 °C for 15 min. Cycle sequencing was carried out by BigDye Terminator Cycle Sequencing Ready Reaction Kit ver 2.0 (Applied Biosystems, USA) according to the manufacturer's instructions. Depending on the fragments and primers used (Table 1 and Fig. 1), the protocols were slightly modified. Extension products were purified by Multiscreen 96-Well Filter Plates (Millipore, USA). Sample electrophoresis and data analysis were performed on the ABI PRISM 3100 and/or 3700 DNA Analyzer (Applied Biosystems, USA). Duplicate genotypes were generated from 133 individuals using as sequencing template a 792-bp fragment located between position −947 and −156, as previously described (Mitsuyasu et al., 2001). This fragment contains 12 polymorphisms (−713C/T, −616G/C, −615A/G, −603del/T, −600G/C, −598G/T, $-597(G)_{2-5}$, −521T/C, −376C/T, −364A/G, −291C/T and −234C/A) (Table 1 and Fig. 1) and was used to confirm results generated from the 1160-bp fragment.

2.3. Population genetic analyses

Hardy–Weinberg equilibrium of each bi-allelic polymorphism was assessed by χ^2 test. Pairwise LD statistic D' and r^2 were calculated with unphased genotype data by Haploview 3.2 software (Barrett et al., 2005). LD calculations were done for a total of 17 polymorphisms including 14

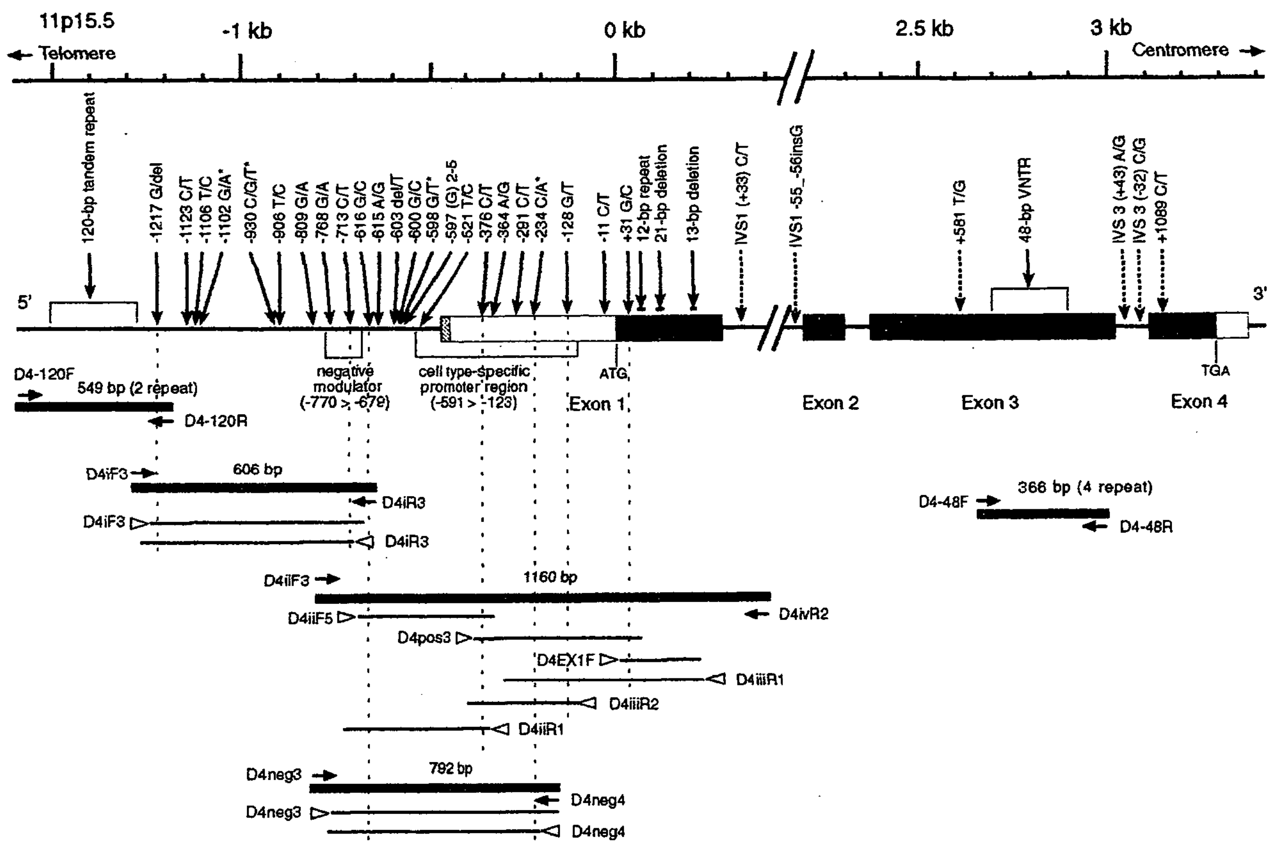

Fig. 1. Schematic representation of polymorphisms of the *DRD4* gene. The *DRD4* gene spans approximately 3.9 kbp consisting of four exons (black boxes: coding regions; white boxes: untranslated regions; hatched box: multiple transcription start sites). The region between position −1217 and +31 nucleotide (the numbering is relative to the first nucleotide of the initiation codon (ATG)) was extensively searched for novel or published SNPs. In total 34 polymorphisms (arrows) were collected from databases (dbSNP; Sherry et al., 1999) and JSNP (Hirakawa et al., 2002), published resources (PubMed) and our experiments. 28 polymorphisms (closed arrows) out of 34 were genotyped, including four novel polymorphisms (asterisks) first reported in this study. For genotyping, five fragments (bold lines) (549-bp, 606-bp, 1160-bp, 792-bp and 366-bp in length) were amplified by five primer sets (closed arrows; details are shown in Table 1) and sequenced by primers as indicated (open arrowheads). Thin lines next to open arrows indicate sequenced regions and orientation of primers. Exact positions of genotyped markers on each sequenced fragment are shown by longitudinal dotted lines. The reference sequence was AC021663 (GenBank).

biallelic polymorphic markers (120-bp TR, −1217G/del, −1106T/C, −906T/C, −809G/A, −768G/A, −713C/T, −616G/C, −603del/T, −600G/C, −521T/C, −376C/T, −291C/T and 12-bp repeat) for which minor allele frequencies exceeded 0.01, and three multi-allelic polymorphisms (−930C/G/T, −597$(G)_{2-5}$ and 48-bp VNTR). Since the Haploview software can analyze only bi-allelic data, we excluded individuals with allele T for −930C/G/T, and individuals with allele $(G)_2$ or $(G)_5$ for −597$(G)_{2-5}$. For the same reason, only individuals with genotype 4/4, 2/4, or 2/2 at the 48-bp VNTR were included. LD blocks were defined according to the confidence intervals described by Gabriel et al. (2002). Haploview LD analysis was carried out by selecting confidence intervals as specified in the software.

LD blocks in the 4.4-kb region of the *DRD4* gene were investigated and tag-SNPs (haplotype tagging markers) selected using Tagger software in Haploview. Markers whose r^2 values were more than 0.8 were selected by Tagger as part of an LD block.

Tag-markers selected using Tagger were used for haplotype estimation by PHASE ver 2.1 software (Stephens and Donnelly, 2003; Stephens et al., 2001). The distribution of the predicted haplotypes was compared between: (i) all schizophrenic patients vs. all controls, (ii) female schizophrenic patients vs. female controls, and (iii) male schizophrenic patients vs. male controls by χ^2 test.

We also carried out a sliding window haplotype analysis using the HTR (Haplotype Trend Regression) program (http://statgen.ncsu.edu/zaykin/htr.html) (Zaykin et al., 2002). This program estimates haplotype frequencies and performs a sliding window mode of haplotype association analysis between cases and controls. In this study, window size was set to be from 2 to 6 markers.

2.4. Statistical methods

Genotype frequencies of 17 polymorphic markers were compared between: (i) all schizophrenic patients vs. all controls, (ii) female schizophrenic patients vs. female controls,

and (iii) male schizophrenic patients vs. male controls by χ^2 test. When the expected number of any cell in a contingency table was less than 5, we employed Fisher's exact test. The significance level (α) for all statistical tests was two sided 0.05. Odds ratios (ORs) were calculated with 95% confidence intervals (CIs).

Following univariate analysis, stepwise logistic regression analyses were carried out using gender, age and the 17 polymorphic markers as independent variables. The binary dependent variable was "schizophrenia affected" = 1 or "control" = 0.

A modified Bonferroni procedure was used to correct for multiple hypothesis testing. According to Bonferroni, since multiple tests were performed, the α level of 0.05 should be divided by the number of tests. However, this correction is almost certainly too strict because of the existence of LD between some of the polymorphisms. Therefore we also used a modified Bonferroni correction (Nyholt, 2004). According to this method, an effective number of independent marker loci is calculated and used in the denominator of the Bonferroni correction. Another adjustment to Bonferroni's method (Li and Ji, 2005) was also used to calculate an effective number of polymorphisms.

Statistical calculations were performed using BMDP statistical software (BMDP Statistical Software, Inc., USA) and SPSS 13.0J software (SPSS Japan Inc., Japan). StatXact (Cytel Software Corporation, USA) was used to compute Fisher's exact test, except for 2×2 contingency tables.

Our sample size had a post-hoc power of 0.848 to detect an effect size of $w = 0.10$ (weak) at the 0.05 significance level (two-tailed), as calculated by software program G*Power (http://www.psycho.uni-duesseldorf.de/aap/projects/gpower/how_to_use_gpower.html) (Erdfelder et al., 1996).

3. Results

3.1. Polymorphism detection and genotyping

Fig. 1 shows the structure of the *DRD4* gene and the locations of all reported polymorphisms (see also Table 2). We collected data on 34 polymorphisms including 28 SNPs and six insertion/deletions within an approximately 4.9 kbp region. The data was obtained from dbSNP (Sherry et al., 1999) (http://www.ncbi.nlm.nih.gov/SNP/snp_summary.cgi), JSNP (Haga et al., 2002; Hirakawa et al., 2002), other published reports and our experiments. As shown in Fig. 1, there are 27 polymorphisms, including 22 SNPs, in the 1.8 kbp region starting 1.5 kbp upstream of the 3′ end of exon 1. This is a much higher SNP density (12.2 SNPs/kbp) than the genome-wide average SNP density [reported to be 0.827 SNPs/kbp in dbSNP or 1.91 kbp/SNP by Sachidanandam et al. (2001)]. Table 2 summarizes data on 27 *DRD4* polymorphisms genotyped in this study, including four novel SNPs (−1102G/A, −930C/G/T, −598G/T and −234C/A) and one novel mononucleotide repeat polymorphism: $-597(G)_{2\text{–}5}$. The $-597(G)_{2\text{–}5}$ polymorphism was previously reported in the database as either −602G/del or $-602(G)_{8\text{–}9}$ (Mitsuyasu et al., 2001; Mitsuyasu et al., 1999; Okuyama et al., 2000). The $-602(G)_7$ and $-602(G)_{10}$ alleles were also identified in our experiments. In addition, a novel SNP (−598G/T) was found within the mononucleotide repeat of $-602(G)_{7\text{–}10}$. Thus, the $-602(G)_{8\text{–}9}$ polymorphism appears to be a combination of a guanine mononucleotide repeat with 2–5 units ($-597(G)_{2\text{–}5}$), together with a SNP at −598G/T and an invariant four guanine nucleotide repeat immediately upstream. Thus we suggest a designation of $-597(G)_{2\text{–}5}$ for this polymorphism instead of −602G/del or $-602(G)_{8\text{–}9}$. The −598G/T SNP was registered as −598G/A/del in the dbSNP database, however, our study showed only the −598G and T genotypes. For this reason, we classified this SNP as novel.

In order to understand the relationship between these polymorphisms, including the four novel SNPs, and the well studied 120-bp TR and 48-bp VNTR polymorphisms we include data on the latter in this study. The 120-bp TR is located approximately 0.8 kb upstream of the 5′ end of exon 1. The 48-bp VNTR is in exon 3. It has been reported that two adjacent intronic SNPs (IVS3(+43)A/G and IVS3(−32)C/G) are in strong LD with the 48-bp VNTR 4 repeat allele (Ding et al., 2002). Based on that data we typed the 48-bp VNTR polymorphism as a representative marker for variation in the 3′ region of the gene.

Twenty-seven polymorphisms were genotyped. (The 13-bp deletion in exon 1 could not be analyzed for technical reasons.) Twenty-one were biallelic SNPs (19 substitution, two insertion/deletion), one triallelic. Five SNPs (−1123C/T, −615A/G, −364A/G, −11C/T and +31G/C) were monomorphic in the study population, as was the 21-bp deletion (Table 2). Four markers (−1102G/A, −598G/T, −234C/A and, −128G/T) were singletons. These polymorphisms were not analyzed for disease association. The seven repeat allele of the 48-bp VNTR was rare; only four heterozygous genotypes (4/7) were found.

The genotype distribution of each biallelic polymorphism was consistent with Hardy–Weinberg equilibrium (data not shown).

3.2. Association with schizophrenia

Uni- and multivariate analyses were carried out with 17 polymorphisms to assess the effect of polymorphism on risk of developing schizophrenia. Specifically, 12 known SNPs (−1217G/del, −1106T/C, −906T/C, −809G/A, −768G/A, −713C/T, −616G/C, −603del/T, −600G/C, −521T/C, −376C/T, and −291C/T), three repeat polymorphisms (120-bp TR, 12-bp repeat, and 48-bp VNTR) and two novel polymorphisms (−930C/G/T and $-597(G)_{2\text{–}5}$) were analyzed. Results from univariate statistical analyses are shown in Table 2.

No polymorphisms differed in frequency between the schizophrenic patients and the controls, even before adjusting for multiple hypothesis testing (Table 2). Comparing the female schizophrenic patients with the female controls, we

Table 2
Comparison of genotype frequencies of polymorphisms of the *DRD4* between schizophrenic patients and controls in Japanese population

Polymorphism[a]		Genotype frequency									db SNP[c]	JSNP[d]	References
		All			Female			Male					
		Control	Schizophrenia	p[b]	Control	Schizophrenia	p	Control	Schizophrenia	p			
120-bp tandem repeat (−1480 to −1240)	n[f]	239	214	0.827	105	95	0.369	134	119	0.798	–	–	Paterson et al. (1996) Seaman et al. (1999)
	2/2	0.582	0.603		0.524	0.621		0.627	0.588				
	2/1	0.364	0.350		0.429	0.347		0.313	0.353				
	1/1	0.054	0.047		0.048	0.032		0.060	0.059				
−1217G/del[e]	n	238	209		105	93		133	116		rs12720364	–	Okuyama et al. (2000), Wang et al. (2004)
	G/G	0.685	0.718	0.713[g]	0.695	0.688	0.566[g]	0.677	0.741	0.497[g]			
	G/del	0.294	0.258		0.295	0.280		0.293	0.241				
	del/del	0.021	0.024		0.010	0.032		0.030	0.017				
−1123C/T	n	241	215		105	94		136	121		–	–	Okuyama et al. (2000)
	C/C	1.000	1.000		1.000	1.000		1.000	1.000				
	C/T	0.000	0.000		0.000	0.000		0.000	0.000				
	T/T	0.000	0.000		0.000	0.000		0.000	0.000				
−1106T/C	n	242	215		105	94		137	121		rs936460	IMS-JST111981	Wang et al. (2004)
	T/T	0.798	0.800	1.000[g]	0.838	0.777	0.226[g]	0.766	0.818	0.488[g]			
	T/C	0.190	0.186		0.162	0.202		0.212	0.174				
	C/C	0.012	0.014		0.000	0.021		0.022	0.008				
−1102G/A	n	239	214		103	94		136	120		–	–	Present study
	G/G	0.996	1.000		0.990	1.000		1.000	1.000				
	G/A	0.004	0.000		0.010	0.000		0.000	0.000				
	A/A	0.000	0.000		0.000	0.000		0.000	0.000				
−930C/G/T	n	240	214		104	94		136	120		–	–	Present study
	C/C	0.979	0.967	0.396[g]	0.981	0.979	0.793[g]	0.978	0.958	0.365[g]			
	C/G	0.017	0.023		0.019	0.011		0.015	0.033				
	C/T	0.000	0.009		0.000	0.011		0.000	0.008				
	G/G	0.004	0.000		0.000	0		0.007	0				
−906T/C	n	239	214		104	94		135	120		rs3758653	IMS-JST111982	Wang et al. (2004)
	T/T	0.669	0.645	0.714	0.654	0.670	0.801[g]	0.681	0.625	0.289			
	T/C	0.280	0.313		0.317	0.287		0.252	0.333				
	C/C	0.050	0.042		0.029	0.043		0.067	0.042				
−809G/A	n	241	215		104	94		137	121		rs936461	IMS-JST111983	Mitsuyasu et al. (1999) Okuyama et al. (2000) Mitsuyasu et al. (2001)
	G/G	0.643	0.605	0.486	0.654	0.606	0.097[g]	0.635	0.603	0.867			
	G/A	0.299	0.349		0.308	0.394		0.292	0.314				
	A/A	0.058	0.047		0.038	0.000		0.073	0.083				
−768G/A	n	240	215		104	94		136	121		rs4987058	–	Mitsuyasu et al. (1999) Mitsuyasu et al. (2001)
	G/G	0.963	0.977	0.544	0.952	0.979	0.449[g]	0.971	0.975	1.000[g]			
	G/A	0.038	0.023		0.048	0.021		0.029	0.025				
	A/A	0.000	0.000		0.000	0.000		0.000	0.000				
−713C/T	n	240	215		104	94		136	121		rs11246224	–	Present study
	C/C	0.996	0.977	0.105[g]	1.000	0.957	0.049[g]	0.993	0.992	1.000[g]			
	C/T	0.004	0.023		0.000	0.043		0.007	0.008				
	T/T	0.000	0.000		0.000	0.000		0.000	0.000				
−616G/C	n	240	210		103	94		137	116		rs747302	–	Mitsuyasu et al. (1999) Okuyama et al. (2000) Mitsuyasu et al. (2001)
	G/G	0.467	0.486	0.503	0.515	0.543	0.486	0.431	0.440	0.876			
	G/C	0.408	0.424		0.359	0.383		0.445	0.457				
	C/C	0.125	0.090		0.126	0.074		0.124	0.103				

−615A/G	n	239	197		103	94		137	116		rs93646	–	
	A/A	1.000	1.000		1.000	1.000		1.000	1.000				
	A/G	0.000	0.000		0.000	0.000		0.000	0.000				
	G/G	0.000	0.000		0.000	0.000		0.000	0.000				
−603del/T	n	240	210		103	94		137	116		rs747303	–	Mitsuyasu et al. (1999)
	del/del	0.500	0.529	0.517	0.553	0.564	0.542	0.460	0.500	0.110			Okuyama et al. (2000),
	del/T	0.388	0.390		0.388	0.340		0.387	0.431				Mitsuyasu et al. (2001)
	T/T	0.113	0.081		0.058	0.096		0.153	0.069				
−600G/C	n	240	210		103	94		137	116		rs109021	–	Mitsuyasu et al. (1999)
	G/G	0.963	0.990	0.107	0.981	1.000	0.499[g]	0.949	0.983	0.186[g]			
	G/C	0.038	0.010		0.019	0.000		0.051	0.017				
	C/C	0.000	0.000		0.000	0.000		0.000	0.000				
−598G/T	n	238	207		100	93		138	114		–	–	Present study
	G/G	1.000	0.995		1.000	0.989		1.000	1.000				
	G/T	0.000	0.005		0.000	0.011		0.000	0.000				
	T/T	0.000	0.000		0.000	0.000		0.000	0.000				
−597(G)$_{2-5}$	n	239	210		103	94		136	116		(rs38422	(IMS-JST186019)	Present study
	G2/G3	0.004	0.000	0.347[g]	0.010	0.000	0.616[g]	0.000	0.000	0.302[g]			
	G3/G3	0.151	0.124		0.097	0.117		0.191	0.129				
	G3/G4	0.481	0.438		0.553	0.479		0.426	0.405				
	G3/G5	0.004	0.010		0.010	0.011		0.000	0.009				
	G4/G4	0.360	0.419		0.330	0.372		0.382	0.457				
	G4/G5	0.000	0.010		0.000	0.021		0.000	0.000				
−521T/C	n	239	206		102	93		137	113		rs1800955	IMS-JST186020	Mitsuyasu et al. (1999),
	T/T	0.389	0.325	0.330	0.402	0.237	0.046	0.380	0.398	0.930			Okuyama et al. (2000)
	T/C	0.481	0.515		0.441	0.548		0.511	0.487				Mitsuyasu et al. (2001)
	C/C	0.130	0.160		0.157	0.215		0.109	0.115				
−376C/T	n	237	212		102	95		135	117		rs916455	IMS-JST186021	Mitsuyasu et al. (1999),
	C/C	0.814	0.835	0.808[g]	0.843	0.832	0.922[g]	0.793	0.838	0.452			Mitsuyasu et al. (2001)
	C/T	0.181	0.160		0.147	0.158		0.207	0.162				
	T/T	0.004	0.005		0.010	0.011		0.000	0.000				
−364A/G	n	237	212		102	95		135	117		rs916456	–	
	A/A	1.000	1.000		1.000	1.000		1.000	1.000				
	A/G	0.000	0.000		0.000	0.000		0.000	0.000				
	G/G	0.000	0.000		0.000	0.000		0.000	0.000				
−291C/T	n	234	214		102	95		132	119		rs916457	IMS-JST186022	Mitsuyasu et al. (1999),
	C/C	0.752	0.743	0.380	0.735	0.789	0.412[g]	0.765	0.706	0.501[g]			Mitsuyasu et al. (2001)
	C/T	0.209	0.238		0.206	0.189		0.212	0.277				
	T/T	0.038	0.019		0.059	0.021		0.023	0.017				
−234C/A	n	237	212		101	95		136	117		–	–	Present study
	C/C	0.996	1.000		1.000	1.000		0.993	1.000				
	C/A	0.004	0.000		0.000	0.000		0.007	0.000				
	A/A	0.000	0.000		0.000	0.000		0.000	0.000				
−128G/T	n	237	214		102	95		135	119		–	–	Mitsuyasu et al. (1999)
	G/G	0.996	1.000		1.000	1.000		0.993	1.000				
	G/T	0.004	0.000		0.000	0.000		0.007	0.000				
	T/T	0.000	0.000		0.000	0.000		0.000	0.000				
−11C/T	n	239	197		93	88		1[illegible]1	109		–	–	Cichon et al. (1995)
	C/C	1.000	1.000		1.000	1.000		1.000	1.000				
	C/T	0.000	0.000		0.000	0.000		0.000	0.000				
	T/T	0.000	0.000		0.000	0.000		0.000	0.000				

Table 2 (*continued*)

Polymorphism[a]		Genotype frequency									db SNP[c]	JSNP[d]	References
		All			Female			Male					
		Control	Schizophrenia	p[b]	Control	Schizophrenia	p	Control	Schizophrenia	p			
+31G/C	*n*	239	197		92	86		130	106		–	–	Cichon et al. (1995)
	G/G	1.000	1.000		1.000	1.000		1.000	1.000				
	G/C	0.000	0.000		0.000	0.000		0.000	0.000				
	C/C	0.000	0.000		0.000	0.000		0.000	0.000				
12-bp repeat	*n*	239	197		104	89		135	108		–	–	
(+64 to +87)	2/2	0.736	0.690	0.240	0.721	0.708	0.797[g]	0.748	0.676	0.205[g]			
	2/1	0.222	0.284		0.231	0.258		0.215	0.306				
	1/1	0.042	0.025		0.048	0.034		0.037	0.019				
21-bp deletion	*n*	239	197		102	88		134	106		–	–	
(+106 to +126)	+/+	1.000	1.000		1.000	1.000		1.000	1.000				
	+/–	0.000	0.000		0.000	0.000		0.000	0.000				
	–/–	0.000	0.000		0.000	0.000		0.000	0.000				
48-bp VNTR	*n*	237	212		102	95		135	117		–	–	Van Tol et al. (1992)
(+2689 to +2880)	4/4	0.696	0.736	0.618	0.716	0.726	0.507	0.681	0.750	0.170			
	4/2	0.186	0.160		0.167	0.158		0.200	0.164				
	4/5	0.055	0.047		0.059	0.063		0.052	0.034				
	4/3	0.013	0.014		0.029	0.000		0.000	0.026				
	4/6	0.017	0.009		0.000	0.011		0.030	0.009				
	4/7	0.013	0.005		0.000	0.000		0.022	0.009				
	2/2	0.004	0.009		0.010	0.021		0.000	0.000				
	5/5	0.008	0.005		0.000	0.011		0.015	0.000				
	3/3	0.000	0.005		0.000	0.011		0.000	0.000				
	5/2	0.000	0.005		0.000	0.000		0.000	0.009				
	5/3	0.004	0.000		0.010	0.000		0.000	0.000				
	5/6	0.004	0.000		0.010	0.000		0.000	0.000				

[a] Polymorphism names of each SNP or the number below names stand for nucleotide varitation and relative position to the first nucleotide of the initiation codon of reference sequence AC021663 (141798 = +1).

[b] p values of χ^2 test (with Yates' correction for 2×2 table) were not corrected for multiple testing. There was no statistical significance after correction. Detailed statistical method was described in the text.

[c] dbSNP, a database of single nucleotide polymorphisms at National Center for Biotechnology Information.

[d] JSNP, a database of common gene variations in the Japanese population (Hirakawa et al., 2002).

[e] del, insertion/deletion polymorphism.

[f] *n*, the number of subject genotyped at each polymorphism.

(*continued on next page*)

found significant differences – before correction for multiple hypothesis testing – in the distribution of both −713C/T ($p = 0.049$) and −521T/C ($p = 0.046$, Table 2). In the case of −713C/T, the minor allele frequency was very low (0.02 in female schizophrenic patients, 0 in controls). There were four heterozygous schizophrenic patients (no rare homozygotes) compared to zero in the female controls. The −521C allele was more frequent in the female schizophrenic patients than the female controls ($p = 0.034$, OR:1.58, 95% CI: 1.06–2.37). When comparing the OR for each genotype using genotype T/T as the referent in the female group, the OR for T/C was 2.11 (95% CI: 1.10–4.07) while the OR for C/C was 2.33 (95% CI: 1.01–5.38). If the −521C allele behaved as a dominant, the OR for the combined C/C and T/C female group would be 2.17 (95% CI: 1.17–4.04, $p = 0.021$) relative to the T/T female group. However, when either the Bonferroni correction, or a less conservative modified Bonferroni that accounts for LD (Li and Ji, 2005; Nyholt, 2004) was applied these results were no longer significant.

There were no significant differences between patients and controls in the male subgroup, even before multiple hypothesis correction (Table 2). Likewise, stepwise logistic regression analyses failed to detect any significant association between polymorphisms and schizophrenia.

Having failed to detect any influence of individual polymorphisms on risk of schizophrenia, we next sought to determine whether *DRD4* haplotypes might influence schizophrenia risk. Before using software to predict haplotypes it is efficient to first remove polymorphisms that are in strong LD with other polymorphisms. Accordingly, we determined the LD coefficient D' and the correlation r^2 between all pairs of 17 polymorphisms (Fig. 2 and Table 3).

The International HapMap Project (http://www.hapmap.org) includes data on only five DRD4 SNPs that are polymorphic in Japanese: rs3758653 (5′ flanking region), rs3889692 (exon 3), rs11246226, rs936465 and rs4331145 (3′ flanking region). These SNPs were analyzed by Haploview. Only one LD block was formed, comprising the three downstream SNPs. Two HapMap SNPs, rs3758653 (−906T/C in this study) and rs3889692 (not genotyped in this study) were not correlated with each other or the other four SNPs (r^2 values 0.024–0.061). These results indicate low LD across the *DRD4* gene.

We used our genotype data to analyze LD in the 4.4-kb region of the *DRD4* gene and select tag-markers using

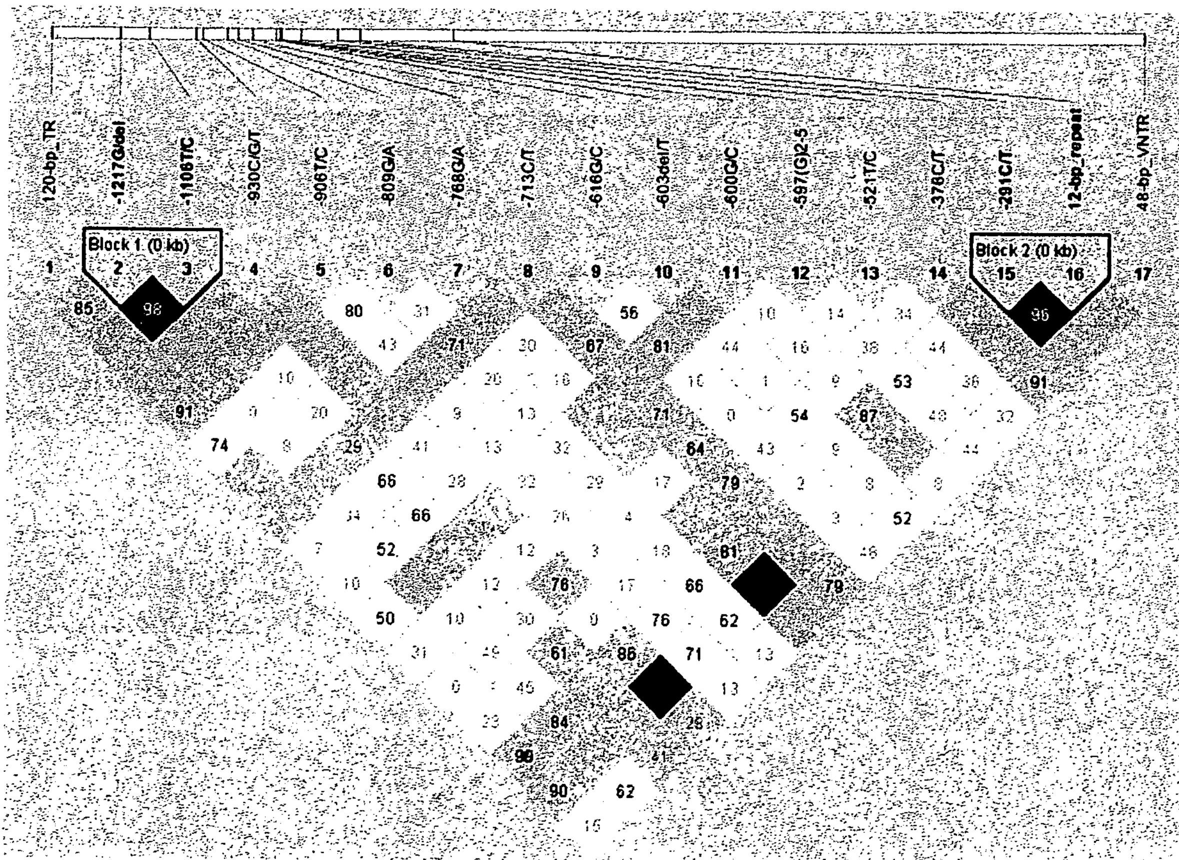

Fig. 2. LD coefficient D' representation of polymorphisms of the *DRD4* gene. The values in the boxes represent D' between pairs of markers. The LD display is from Haploview software. The boxes without values indicate complete LD ($D' = 1.0$). The dark grey boxes indicate strong LD. The light grey boxes indicate uninformative variant pairs. The white boxes indicate low LD. LD blocks were defined according to the algorithm in Haploview (Gabriel et al., 2002).

Table 3
Pairwise LD measure (r^2) of polymorphisms of the *DRD4*

	120-bp TR	−1217G/del	−1106T/C	−930C/G/T	−906T/C	−809G/A	−768G/A	−713C/T	−616G/C	−603del/T	−600G/C	−597(G)$_{2-5}$	−521T/C	−376C/T	−291C/T	12-bp repeat
−1217G/del	0.04															
−1106T/C	0.03	0.59														
−930C/G/T	0.05	0.00	0.00													
−906T/C	0.66	0.04	0.03	0.06												
−809G/A	0.46	0.00	0.01	0.06	0.62											
−768G/A	0.06	0.00	0.01	0.00	0.01	0.01										
−713C/T	0.03	0.00	0.00	0.00	0.03	0.02	0.00									
−616G/C	0.00	0.01	0.02	0.00	0.01	0.03	0.00	0.02								
−603del/T	0.01	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.28							
−600G/C	0.01	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.02	0.04						
−597(G)$_{2-5}$	0.02	0.00	0.00	0.00	0.01	0.01	0.00	0.01	0.02	0.14	0.00					
−521T/C	0.00	0.07	0.02	0.01	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.02				
−376C/T	0.02	0.00	0.00	0.00	0.01	0.01	0.00	0.05	0.05	0.08	0.00	0.03	0.02			
−291C/T	0.43	0.02	0.02	0.06	0.39	0.28	0.07	0.00	0.00	0.00	0.00	0.03	0.02	0.02		
12-bp repeat	0.47	0.03	0.02	0.08	0.37	0.27	0.10	0.00	0.00	0.00	0.00	0.02	0.02	0.02	0.86	
48-bp VNTR	0.01	0.01	0.00	0.00	0.01	0.01	0.00	0.04	0.06	0.08	0.00	0.04	0.02	0.78	0.02	0.02

Pairwise LD measures (r^2) were calculated by Haploview software.

Tagger software in Haploview. Two small LD blocks were detected, one between −1217G/del and −1106T/C ($D' = 0.98$; $r^2 = 0.59$), the other between −291C/T and the 12-bp repeat ($D' = 0.96$; $r^2 = 0.86$). Other polymorphisms were only very weakly correlated, if at all ($r^2 < 0.80$). However, −376C/T and the 48-bp VNTR indicated relatively high correlation value ($r^2 = 0.78$). Based on this analysis 16 markers were selected for haplotype analysis. Thus, as a result of the low LD across the *DRD4* gene, we were only able to decrease the independent polymorphism number from 17 to 16 tag-markers.

The 48-bp VNTR polymorphism is in strong LD with −376C/T ($D' = 0.91$, $r^2 = 0.78$). However, it did not exhibit a high r^2 value with any other polymorphism in the region between the 120-bp TR and the 12-bp repeat of *DRD4* (Table 3). D' values between 120-bp TR and −906T/C ($D' = 0.91$) and −291C/T ($D' = 0.90$) were $\geqslant 0.90$. However, since corresponding r^2 values were less than 0.80, these polymorphisms could not be dropped based on our criteria for removing certain polymorphisms as described in Section 2.

Using 16 markers, a total of 136 haplotypes were estimated by PHASE. We compared the distribution of a total of 20 haplotypes with allele frequencies >0.01 between schizophrenics and controls in: (i) all subjects, (ii) female subgroup, and (iii) male subgroup. When the difference in haplotype frequencies was analyzed by the χ^2 test no significant differences were observed. Using 16 tag-markers, p values of sliding window haplotype analysis with window size 2 and 6 showed no statistically significant difference between schizophrenic patients and controls before adjustment for multiple hypothesis testing. Fig. 3 indicates the results of this analysis only for window sizes 2 and 3 (Fig. 3).

4. Discussion

In order to clarify the structure of genetic variation in the *DRD4* gene and to further explore potential genetic influences on schizophrenia, we genotyped 216 Japanese schizophrenics and 243 healthy controls at 27 polymorphic sites, including four novel SNPs.

Not surprisingly, we found the allele frequencies of some polymorphisms to be different in the Japanese population compared to European or other populations: −615A/G is polymorphic in Caucasians (Ronai et al., 2004), however, it was monomorphic in our study. The same phenomenon was observed with −364A/G, −11C/T and +31G/C (Cichon et al., 1995) and with a 21-bp deletion reported in a single individual suffering from obsessive-compulsive disorder and panic disorder (Cichon et al., 1995). Other polymorphisms (−1102A, −930T, −713T, −598T, −597(G)$_2$, −597(G)$_5$, −234A and −128T) had very low allele frequencies in the Japanese population (Table 2).

In order to assess the relationship between schizophrenia and *DRD4* polymorphisms, we carried out association analyses between Japanese schizophrenic patients and healthy controls. Univariate analyses indicated that none

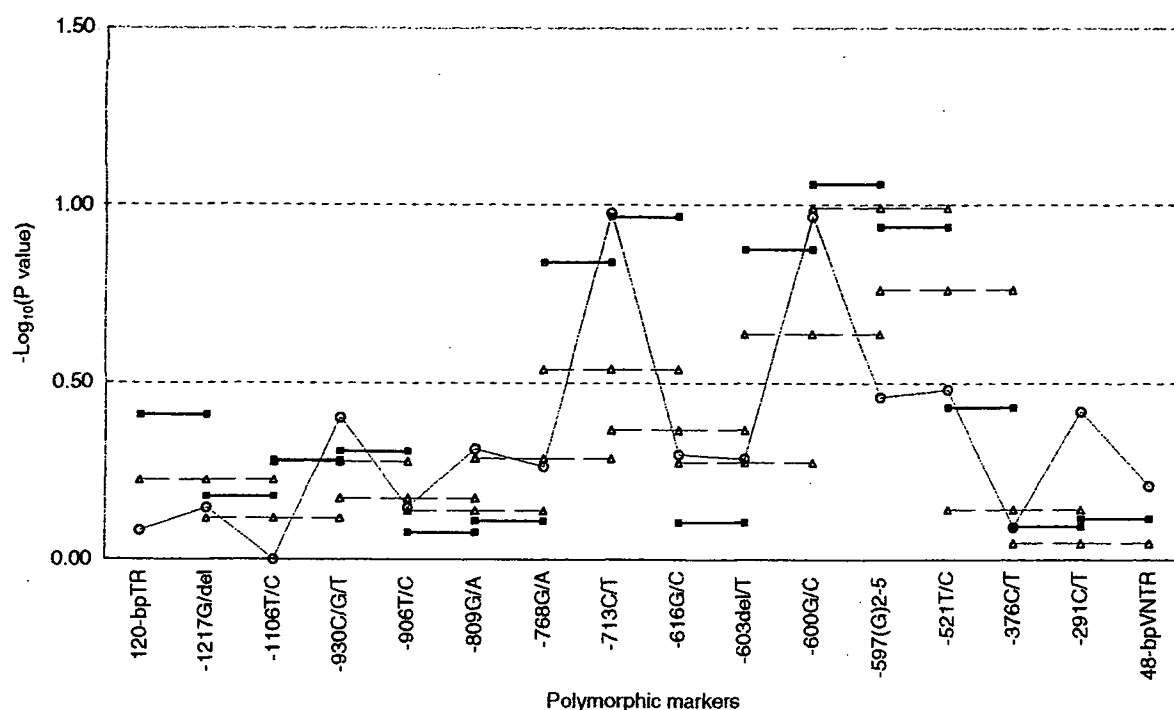

Fig. 3. Sliding window haplotype analysis of the *DRD4* gene. The *X*-axis displays each polymorphism analyzed in this study. The *Y*-axis shows $-\log_{10}(p$ value) of each marker and sliding window (window size 2 and 3) haplotype analysis. Open circles indicated the result of single marker analysis of each polymorphism (univariate analysis). Each line between two closed boxes indicates *p* value of 2-marker sliding window analysis. Each dashed line with three triangles indicates *p* value of 3-marker sliding window analysis.

of the markers was statistically significant after correction for multiple hypothesis testing.

There have been inconsistent reports regarding the −521T/C polymorphism in schizophrenia. Okuyama et al. reported that the T allele of this polymorphism reduces *DRD4* transcriptional efficiency by 40% compared with the C allele, and that, in the Japanese population, this marker is associated with schizophrenia (Okuyama et al., 1999). However, attempts to replicate these results in other populations such as Chinese and Caucasian have failed (Ambrosio et al., 2004; Jonsson et al., 2001; Xing et al., 2003). Based on these results one might speculate that there is heterogeneity in the genetics of schizophrenia. However, our negative findings regarding −521T/C in another Japanese population suggest that the result of Okuyama may reflect type I error.

We also carried out LD and haplotype analyses, however, the *DRD4* region is unusual both in terms of high SNP density and low LD. Consequently the potential power of haplotype based association methods is not much different from SNP based approaches. Only two LD blocks were formed in the *DRD4* region, each consisting of only two Polymorphisms, leaving most polymorphisms as independent variables. These results are consistent with other reports on the population genetic structure of *DRD4* (Wang et al., 2004). No statistically significant haplotype associations with schizophrenia were detected.

There are several limitations of this study that should be borne in mind. One concern is that the control population may not be perfectly matched with the schizophrenic population. Most of the male controls were Japanese Self Defense Forces personnel aged about 50 years old. There might be some characteristics of this population that differ from other healthy control populations. Ideally more detailed socioeconomic information should be collected to guide selection of a balanced control population, and for inclusion in a statistical model along with genetic variables. Also, in view of the effects of environmental factors on the development of schizophrenia, it is important to collect as much information as possible on environmental exposures.

In conclusion, we report in detail the structure of genetic variation across the *DRD4* gene in the Japanese population. LD analysis revealed two small LD blocks, however, the most notable pattern was low LD across most of the gene. Haplotype analysis using 16 tag-markers selected by LD block analysis revealed no associations with risk of schizophrenia. Despite the biological role of DRD4 in dopamine signaling, and reports of functional effects associated with polymorphisms such as the 48-bp repeat, this report contributes to the increasing body of literature suggesting that the gene does not contribute significantly to risk of schizophrenia.

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学　　術

うつ病の現状と薬物治療
― 基本的な診断治療の考え方から、現状まで ―

九州大学病院　精神科神経科　川　嵜　弘　詔

1．はじめに

昨今の社会情勢、文化的価値観の変化を背景として従来のうつ病の概念も近年大きく変化している。加えて、1998年より年間自殺者数が３万人を超え、現在まで減少傾向は認めない。WHOの報告によると自殺者数の98％以上は精神疾患を背景にもつとされ、その約１／３はうつ病とされている。また、従来典型的とされたうつ病の症状も変化し、臨床現場において多彩なうつ病・うつ状態と考えられる関連疾患群の増加が顕著に認められることが報告されている。その中でも、軽症群のうつ病・うつ状態に関しては専門医である精神科、および心療内科を初診するよりも身体症状を不定愁訴として身体科の医療機関に初診することが多いと指摘されている。また、うつ病の薬物療法も従来の薬物療法から大きく変化し、副作用の少ないSSRIの日本への導入（1999年）とともに非精神科医においても処方が可能となっている時代である。このような時代背景の下に、精神疾患のみならず身体疾患を専門とする医師においてもうつ病についての知見を深めることは大変重要なことと思われる。

本講演においては、うつ病・うつ状態の診断、早期発見および治療の現状について述べたいと思う。

本抄録は、2007年４月18日、西鉄グランドホテルにおいて行われた講演を元に作成された。

2．うつ病とは何か
－うつ病の診断について－

典型的なうつ病を理解するための最初の重要なポイントは、うつ病というのは感情障害、つまり気分の障害ということを理解することである。うつ病には気分障害のみならずそれ以外の多彩な症状が認められるが、まず、中核群のうつ病を理解することが現代の複雑なうつ病・うつ状態を理解する重要な基礎となると考えられる。うつ病の気分障害は、抑うつ感（ゆううつである）、悲哀感、絶望感、つらい、苦しい、空虚感などで表現される。次に重要な症状は、欲動障害であり、それらには意欲の障害（意欲低下、億劫）、睡眠欲の障害（不眠）、食欲の低下（体重減少、味覚の変化）、性欲の低下（外界の関心の低下、おしゃれや化粧についての億劫さ）などが認められる。また、精神、および行動の変化として、抑制症状（思考の渋滞（思考抑制））（行動抑制）が認められる。加えて、身体症状が認められるが、これらは非常に多彩な症状として訴えられる。たとえば、そのほと

んどは身体的不定愁訴であり、器質的な原因が特定できない場合が多い。また、疼痛・疲労なども認められる。症状の日内変動も認められ、通常朝が悪く、夕方につれて寛解するというパターンをとることが多い。

鑑別診断としては、精神病症状（幻覚・妄想）を伴う統合失調症、不安障害、一般的身体疾患、脳器質的疾患、アルコール依存、更年期障害（男性、および女性）、躁うつ病が主として考えなければいけない。

米国精神医学会による精神疾患の診断基準（DSM-IV）によると、典型的なうつ病は大うつ病エピソードとして記載されている。その診断基準は1．抑うつ気分、2．興味または喜びの喪失のうちの、少なくとも一つが存在し、さらに、以下のうちの症状（3．食欲の減退あるいは増加、体重の減少あるいは増加、4．不眠あるいは睡眠過多、5．精神運動性の焦燥、または抑止（沈滞）、6．易疲労感または気力の減退、7．無価値感または過剰（不適切）な罪責感、8．思考力や集中力の減退または決断困難、9．死についての反復思考、自殺年慮、自殺企図）をあわせて合計で五つまたはそれ以上が認められるということとなっている。また、上記の症状がほとんど1日中、ほとんど毎日あり、2週間にわたっている、という基準が定められている。

うつ病の原因となりやすい主な身体疾患としては、1．内分泌代謝疾患（甲状腺機能障害など）、2．中枢神経疾患（パーキンソン病、アルツハイマー型認知症など）、3．その他（膠原病、悪性腫瘍など）が考えられる。また、脳血管障害の発症後うつ病になりやすいことが報告されている。また近年、生活習慣病とうつ病との関連が注目されており、糖尿病に罹患する患者群の約30％がうつ病を合併すると報告されている。

大うつ病性障害の成人における障害有病率は日本、および世界において約13％～17％と報告されており、男性では約4％、女性では8％と女性が男性の約2倍となっている。

うつ症状を呈する患者の初診診療科は内科が約65％と圧倒的に多く、専門医に最初に診療を求めない傾向が顕著であることが報告されている。このことに関連して、医師に対して身体症状を訴えるが精神症状に関しては患者自らが訴えることなく医師が聞き出さないと訴えないという傾向がある。という報告がなされている。

WHOによる報告によると2020年における健康な生活を障害する疾患として大うつ病性障害は虚血性心疾患に次いで第二位に位置づけられている。

うつ病の早期発見に関しては二項目質問紙法があり、1．抑うつ気分、2．興味や喜びの喪失について問いその二項目の一つに該当すればうつ病の90％をスクリーニングできるという報告がある。その他に診断に有用な心理テストとして、日本版SDSという自己記入式の心理テストがあり、本テストは保険点数80点が加算されるため、待ち時間の問診と共に使用するとよいだろう。

以上、典型的なうつ病の診断概念について述べたが、現在はうつ病の概念の広範化、混乱が専門医の臨床現場においても報告されており、新たな診断基準、あるいは概念化が必要であろうと論議されている。

3．うつ病の発生機序について。うつ病の原因について

うつ病の発生機序については、生物学的にはセロトニン欠乏説、状況因（対象喪失）、病前性格（執着性格）が関連するといわれている。

4．自殺について

年間自殺者数は1998年より急激に増加し、3万人を超え、厚生労働省もついに昨年度より全国的に対策を講じ始めた。日本の自殺率は人口10万あたり約23.3人と言われており、世界各国の自殺率においては平均値に近い値である。先にも述べたように、厚労省の自殺対策はうつ病に対する啓蒙、早期診断の徹底であり、身体科および精神科専門医の協力が主体となっている。

5．うつ病の治療について

(1)うつ病の精神療法について

うつ病に対する精神療法は基本的には休養と環境調整が主となる。主要な精神療法としては、認知療法、対人関係療法、支持的精神療法がある。

(2)うつ病の薬物療法について

基本的にはうつ病は薬によって治癒する障害であり、患者さんには服薬の重要性を理解して頂き、規則的な服薬の指導を行う。抗うつ剤の種類としては、古典的な三環系抗うつ薬（効果は鋭いが副作用が多い）および最近日本に導入されたSSRIを用いるが、その使用法については、うつ病治療における薬物使用アルゴリズムが制定されており、それに基づいて行うことが推奨されている。主として、どのアルゴリズムにおいても、第一選択剤は、SSRIあるいはSNRIとなっている。今後も、わが国において、多くのSSRI系製剤が治験中であり、近い将来種々の薬剤が認可され、治療の選択肢が拡大することが予想される。

6．抗うつ剤の適応について

SSRIは、抗うつ作用のみならず、抗不安作用も併せ持つことが知られており、パニック障害、更年期障害、強迫性障害、全般性不安障害、社会不安障害、PTSD、月経前不快気分障害への適応が海外でも認可されている。今後わが国でも、適応疾患の拡大がなされると考えられる。

7．抗うつ剤の有効性について

SSRI系薬剤は、三環系抗うつ剤と比較して副作用が少なく同等かそれ以上の効果がうつ病において認められている。しかし、薬剤によっては、消化器症状（嘔気、吐気、下痢）、性機能障害、眠気等が報告されているがそのほとんどは、投与初期に認められるとされている。4～6週間の間に最高用量を使用して効果がなければ他の薬剤に変更することが推奨されている。

8．抗うつ剤の副作用について

上記に述べた所期の副作用以外に最も注意しなければいけない副作用としては、セロト人症候群、断薬症候群、activation syndromeである。

セロトニン症候群は、脳内セロトニン濃度の急激な上昇によって、数分から数時間の間に錯乱や係争状態などの精神症状、発汗、悪寒などの自律神経症状、発熱、下痢、協調運動障害、ミオクローヌスなどの神経症状が出現するもの

である。通常、薬剤の即時中断と補液によって症状は24時間以内に改善することが多く、出現頻度は約1％と低いといわれている。

断薬症候群とは、急激にSSRIを中断することによって出現し、不安焦燥感、イライラの増加が認められる。セロトニン症候群のように重篤ではないが、薬剤減量中に出現することが多いため、急激な中断は避け、徐々に薬剤を減量する必要がある。

activation syndromeは、近年注目されている症候群であるが、SSRI投与後約10日以内に、不安焦燥感、イライラが昂じ自殺にいたる場合があるといわれている。児童青年期に多く見られるとされ、SSRI系薬剤は18歳未満の大うつ病障害への投与は慎重投与（パロキセキンについては警告）となっている。

9．専門医への紹介のポイントは？

専門医に紹介するタイミングとしては、1）診断に迷った場合、2）脳器質性障害が疑われる場合、3）第一選択剤で効果が認められない場合、4）重症のうつ病の場合、5）自殺の危険性がある場合、6）アルコール依存が合併している場合、7）入院の必要性がある場合、8）慢性化している場合、9）そう状態が出現し、躁うつ病が疑われる場合、10）幻覚妄想が認められる場合、11）産後うつ病の場合などが考えられる。

10．終わりに

以上簡単ではあるが、うつ病の概念、診断および治療について概説した。副作用が少なく、治療効果が高いSSRI系薬剤の普及に伴って、プライマリーあるいは身体科において抗うつ剤を処方する機会も増加しているのが現状である。わが国の自殺増加対策もあり、プライマリーあるいは身体科において、うつ病の治療を推進していくという時代の流れがあり、うつ病に対する理解、情報の普及が望まれている。配布資料を参照していただき、本稿がうつ病という障害への理解を深めていただける一助となれば幸いである。

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Research Report

The dopamine D_1 receptor agonist, but not the D_2 receptor agonist, induces gene expression of Homer 1a in rat striatum and nucleus accumbens

Hidetaka Yamada[a,b], *Toshihide Kuroki*[b,c,*], *Tatsuo Nakahara*[b], *Kijiro Hashimoto*[b], *Tetsuyuki Tsutsumi*[b], *Makoto Hirano*[b], *Hisao Maeda*[a]

[a]*Department of Neuropsychiatry, Kurume University School of Medicine, 67 Asahimachi, Kurume, Fukuoka 830-0011, Japan*
[b]*Center for Emotional and Behavioral Disorder, National Hospital Organization Hizen Psychiatric Center, Kanzaki, Saga 842-0192, Japan*
[c]*Department of Neuropsychiatry, Kyushu University Graduate School of Medical Sciences, Maidashi 3-1-1, Higashiku, Fukuoka 812-8582, Japan*

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ABSTRACT

Stimulation of dopamine receptors may induce striatal Homer 1a, an immediate-early gene (IEG) that is involved in the molecular mechanism for the signaling pathway of the group I metabotropic glutamate receptors. This study examined the effects of the agonists for dopamine D_1-like and D_2-like receptors on gene expression of Homer 1a, in comparison with the IEG *c-fos* expression, in the discrete brain regions of rats. The D_1-like agonist SKF38393 (20 mg/kg, s.c.) significantly increased the mRNA levels of Homer 1a in the striatum and nucleus accumbens, but not in the medial prefrontal cortex or hippocampus, 2 h after injection, whereas the D_2-like agonist quinpirole (1 mg/kg, s.c.) had no significant effect on Homer 1a mRNA levels in any brain region examined. Co-administration of SKF38393 and quinpirole significantly increased Homer 1a mRNA levels in the striatum, nucleus accumbens and hippocampus, while this effect was not significantly greater than that of SKF38393 alone. Any treatment did not affect the mRNA levels of other splicing variants, Homer 1b or 1c. In contrast, combination of both dopamine agonists produced a greater increase than SKF38393 did in the mRNA levels of *c-fos* in the nucleus accumbens, striatum and substantia nigra. These results suggest that stimulation of D_1-like receptors, but not D_2-like receptors, may induce gene expression of Homer 1a in the striatum and nucleus accumbens. However, in contrast to *c-fos* expression, it is unlikely that co-activation of both D_1-like and D_2-like receptors exerts a synergic action on Homer 1a expression in these regions.

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1. Introduction

The interactions between dopamine and glutamate within the brain regions including the basal ganglia have significant implications for the pathophysiology of many neuropsychiatric disorders such as schizophrenia, substance abuse and Parkinson's disease (Carlsson and Carlsson, 1990; Pulvirenti

* *Corresponding author.* Department of Neuropsychiatry, Kyushu University Graduate School of Medical Sciences, Maidashi 3-1-1, Higashiku, Fukuoka 812-8582, Japan. Fax: +81 92 642 5644.
E-mail address: toshik@npsych.med.kyushu-u.ac.jp (T. Kuroki).

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and Diana, 2001). Many lines of evidence suggest the glutamatergic control of dopamine transmission and vice versa in the striatum, nucleus accumbens and prefrontal cortex (Morari et al., 1998; Vanderschuren and Kalivas, 2000). The dopamine D_1-like receptor agonist SKF38393 increased striatal Fos induction in 6-hydroxydopamine-lesioned rats following intranigral injection with AMPA/kainite receptor antagonist DNQX (McPherson and Marshall, 2000). In contrast, the D_2-like receptor agonist quinpirole decreased striatal Fos but increased the pallidal Fos induced by intranigral injection with the NMDA receptor antagonist AP5. SKF38393, but not quinpirole, significantly increased amplitude of excitatory postsynaptic current in the prefrontal cortex (Gonzalez-Islas and Hablitz, 2003). Glutamate levels during food consumption are controlled by the dopaminergic system in the nucleus accumbens via not D_1 receptor- but D_2 receptor-mediated mechanism (Mikhailova, 2003). These findings suggest that dopamine receptor subtypes may differentially regulate glutamatergic transmission in a region-specific manner.

The Homer family of proteins has been found to bind selectively to group I metabotropic glutamate receptors (mGluRs) and play an important role in the molecular mechanism for the signaling pathway of these receptors (Brakeman et al., 1997; Kato et al., 1997, 1998). The Homer family consists of three independent genes, Homer 1, 2 and 3. Homer 1 comprises three splicing variants: Homer 1a, 1b and 1c (Xiao et al., 1998). The long form of Homer, Homer 1b/c, is constitutively expressed as a multidimer linking group I mGluRs to calcium-selective endoplasmic inositol triphosphate (IP_3) receptors via the coiled-coil (CC) domain of carboxyl-terminal. In contrast, Homer 1a, a short isoform lacking the CC domain, is an immediate-early gene (IEG) product that is rapidly introduced by neuronal activation (Brakeman et al., 1997; Kato et al., 1997; Berke et al., 1998; Morioka et al., 2001; Bottai et al., 2002; Nielsen et al., 2002). Recently, dopaminergic modulation has been demonstrated to affect gene expression of Homer 1a. Cocaine, a dopamine transporter inhibitor (Brakeman et al., 1997; Swanson et al., 2001), SKF38393, a D_1-like receptor agonist (Berke et al., 1998), and haloperidol, a D_2-like receptor antagonist (de Bartolomeis et al., 2002; Polese et al., 2002), induced the Homer 1a gene expression in the striatum. In contrast, SCH23390, a D_1-like receptor antagonist, attenuated methylphenidate-induced expression of striatal Homer 1a (Yano et al., 2006). Moreover, Homer 1b and 1c-knockout mice displayed enhanced methamphetamine-induced motor behavior (Szumlinski et al., 2005), while behavioral response to amphetamine increased in transgenic mice overexpressing Homer 1a in striatal medium spiny neurons localized predominantly in the striosome (patch) (Tappe and Kuner, 2006). These findings suggest that Homer 1a could play a key role on dopamine–glutamate interactions in the striatum. In line with this assumption, we previously reported that a high dose (40 mg/kg, i.p.) of methamphetamine increased Homer 1a mRNA levels in the striatum and nucleus accumbens, but not in the medial prefrontal cortex or the substantia nigra (Hashimoto et al., 2004). Neither Homer 1b nor 1c mRNAs were affected in any brain regions examined. Striatal Homer 1a was induced to the maximal level 2 h after administration of methamphetamine, which time-dependent profile was similar to that of Homer 1a expression induced by the D_1-like receptor agonist SKF38393 (Berke et al., 1998). Glutamate is reported to induce Homer 1a much slower (the maximal level at 4 h after application) in cerebellar granule cell culture (Sato et al., 2001). Taken into account that dopamine receptor subtypes are thought to play distinct roles in the regulation of glutamatergic transmission, methamphetamine-induced stimulation of D_1-like receptors, but not D_2-like receptors, may increase Homer 1a mRNAs in the striatum. However, it remains to be known whether D_1 and D_2 receptors differently regulate Homer 1a expression in other regions than the striatum.

In this study, we investigated the effects of the D_1-like and D_2-like agonists on gene expression of Homer 1a as well as its splicing variants (Homer 1b and 1c) in the discrete brain regions of rats. Moreover, the effect of combination of both agonists was also examined to elucidate a synergistic action of co-stimulation of both receptor subtypes, which effect has been observed in expression of the IEG *c-fos* (Gerfen et al., 1995).

2. Results

2.1. Analysis of IEG mRNAs

Ethidium bromide staining of a polyacrylamide gel revealed a single band at the expected size of amplification product for each of β-actin, Homer 1a, 1b and 1c cDNAs (Fig. 1). Since no

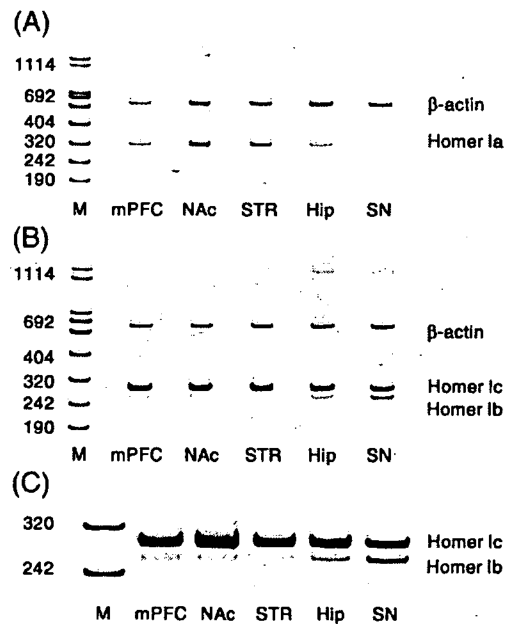

Fig. 1 – Ethidium bromide-stained polyacrylamide gel showing PCR products amplified from rat brain RNA. Total RNA extracted from the medial prefrontal cortex (mPFC), nucleus accumbens (NAc), striatum (STR), hippocampus (Hip) and substantia nigra (SN) was incubated in the absence (left lanes) or in the presence (right lanes) of reverse transcriptase. The reverse transcription products were coamplified with Homer 1a and β-actin primers (A): Homer 1b, Homer 1c and β-actin primers (B), respectively. Homer 1b and Homer 1c mRNAs were also amplified extensively for 32 cycles (C). A DNA standard lane is shown at the left of the gel with bands labeled in bp.

amplified products were observed when the reverse transcriptase step was omitted, the contamination by genomic DNA did not interfere with the signals of PCR products of Homer 1a, 1b or 1c cDNAs. Genomic DNA of Homer 1a was not contaminated to interfere with the signals of PCR products of β-actin, Homer 1b or Homer 1c because these cDNAs were amplified using the pair of primers derived from different exons of the genes.

Homer 1b and 1c mRNAs were amplified with an identical pair of primers and differed by 36 nucleotides. The levels of Homer 1b mRNA in the substantia nigra were significantly higher than those in the other brain regions ($F_{3,12}$=254, P<0.001). The ratios of Homer 1b mRNA levels to Homer 1c mRNA levels were as follows: the medial prefrontal cortex, 0.019±0.003; nucleus accumbens, 0.018±0.003; striatum, 0.020±0.003; and substantia nigra, 0.395±0.023, respectively (Fig. 1C). The Homer 1b mRNA could be quantified only in the substantia nigra.

To determine the optimal amplifications, PCR was performed using different amounts of reverse-transcribed total RNA and different numbers of cycles. These results indicated that amplification was exponential between 18 and 24 cycles for β-actin mRNA. Amplification was also exponential between 26 and 34 cycles for Homer 1a and 1c mRNAs. The PCR products were proportional to RNA input over a range of 5 to 50 ng total RNA for β-actin and Homer mRNAs. Thirty nanograms of reverse-transcribed RNA were amplified for 28 cycles for the quantitation of relative amount of the Homer mRNAs in the rat brain.

2.2. *Effects of SKF38393 and quinpirole on Homer 1a mRNA levels in the discrete brain regions of rats*

The D_1-like receptor agonist SKF38393 (20 mg/kg) significantly increases Homer 1a mRNA levels in the striatum (+97% of saline control levels, $F_{3,33}$=8.294, P<0.001), nucleus accumbens (+61%, $F_{3,31}$=3.03, P<0.05), but not in the medial prefrontal cortex, hippocampus or substantia nigra as compared to saline controls 2 h after injection (Figs. 2 and 4). In contrast, the D_2-like receptor agonist quinpirole (1 mg/kg) had no significant effect on the gene expression of Homer 1a in any brain regions examined. Co-administration of SKF38393 (20 mg/kg) and quinpirole (1 mg/kg) produced a significant increase in the striatum (+83%, $F_{3,33}$=8.294, P<0.001), nucleus accumbens (+59%, $F_{3,31}$=3.03, P<0.05) and hippocampus (+24%, $F_{3,36}$=3.42, P<0.05), but not in the medial prefrontal

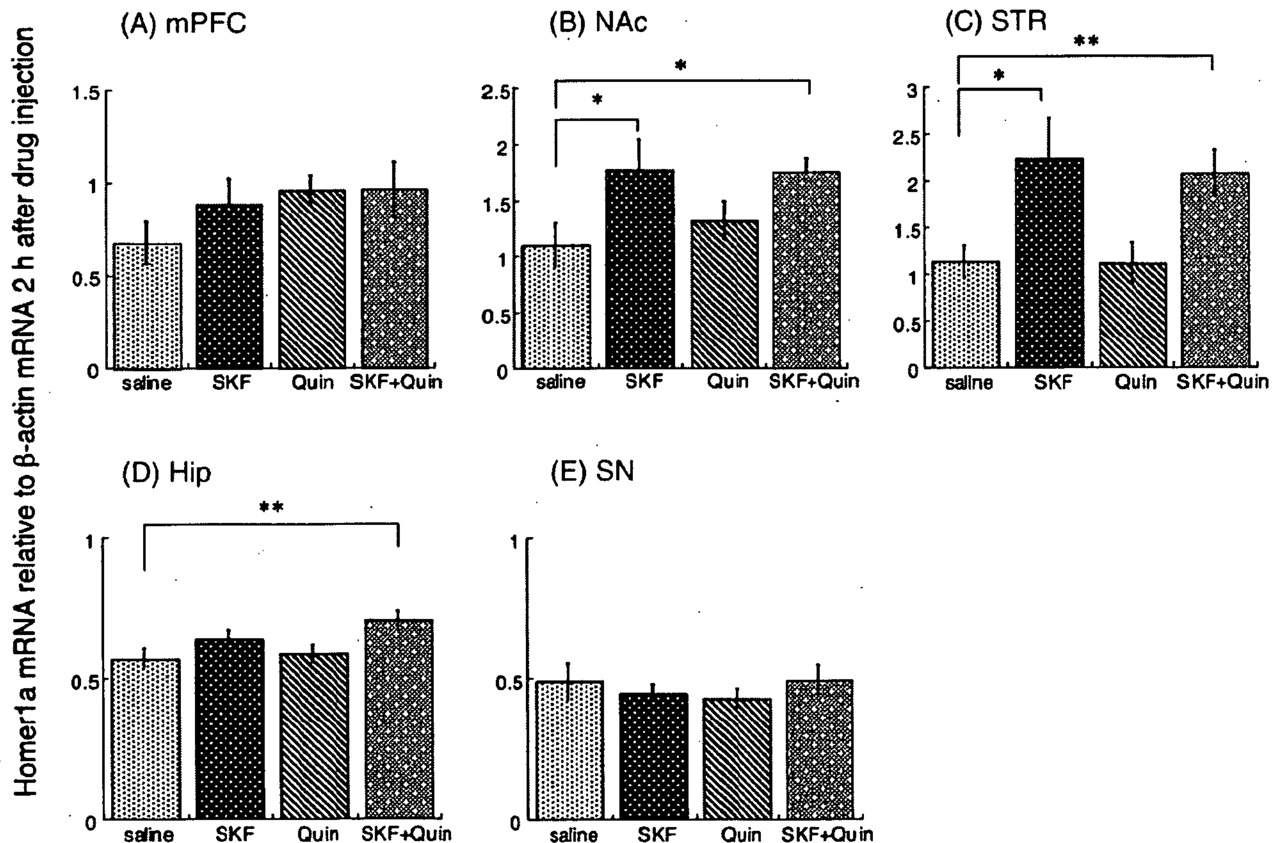

Fig. 2 – Effects of SKF38393 and quinpirole on regional Homer 1a mRNA levels. Homer 1a and β-actin cDNA were coamplified using 30 ng of total RNA for 32 and 20 cycles, respectively, in the five regions; (A) medial prefrontal cortex (mPFC); (B) nucleus accumbens (NAc); (C) striatum (STR); (D) hippocampus (Hip); (E) substantia nigra (SN). Rats were sacrificed 2 h after s.c. injection with SKF38393 (SKF; 20 mg/kg), quinpirole (Quin; 1 mg/kg), combination of SKF38393 and quinpirole (SKF+Quin; 20 mg/kg and 1 mg/kg, respectively) or saline. The values represent Homer 1a mRNA levels relative to β-actin mRNA levels (mean±SEM of 9–10 animals). *P<0.05 and **P<0.01 were considered significant in this study using ANOVA followed by the Fisher's PLSD.

cortex or substantia nigra, compared to saline controls. This effect was not significantly greater than that of SKF38393 alone, while combination of both dopamine agonists, but not single treatment with either drug, significantly increased Homer 1a levels in the hippocampus.

Homer 1b mRNA levels could not be detected in the substantia nigra or other regions examined under the standard experimental condition (Figs. 1B and C). Homer 1c mRNAs were detected with homogeneous distribution in all the brain regions examined, while these were not affected by any drug treatment (Figs. 1B and C).

2.3. *Effects of SKF38393 and quinpirole on c-fos mRNA levels in the discrete brain regions of rats*

SKF38393 (20 mg/kg) significantly increased the level of c-*fos* mRNA in the striatum (+55%, $F_{3,36}$=34.156, P<0.0001), nucleus accumbens (+50%, $F_{3,36}$=16.127, P<0.0001), medial prefrontal cortex (+34%, $F_{3,36}$=11.221, P<0.0001), hippocampus (+55%, $F_{3,36}$=30.076, P<0.0001) and substantia nigra (+44%, $F_{3,36}$=19.256, P<0.0001) 1 h after injection, compared with saline controls (Figs. 3 and 4). Quinpirole (1 mg/kg) also significantly increased the level of c-*fos* mRNA in the nucleus accumbens (+38%, $F_{3,36}$=16.127, P<0.0001), medial prefrontal cortex (+43%, $F_{3,36}$=11.221, P<0.0001), hippocampus (+21%, $F_{3,36}$=30.076, P<0.0001) and substantia nigra (+59%, $F_{3,36}$=19.256, P<0.0001), compared to saline controls. When administered alone, quinpirole (1 mg/kg) had no significant effect on c-*fos* mRNA levels in the striatum. However, combination of SKF38393 and quinpirole produced a greater increase in striatal c-*fos* mRNA levels (+88%, $F_{3,36}$=34.156, P<0.0001) than SKF38393 alone did 1 h after injection. Such a synergic effect of combination of both dopamine agonists on c-*fos* mRNA levels was also found in the nucleus accumbens (+78%, $F_{3,36}$=16.127, P<0.0001 versus SKF38393 treatment group) and substantia nigra (+107%, $F_{3,33}$=19.256, P<0.0001 versus SKF38393 or quinpirole treatment group) (Fig. 3). This synergism still remained in the striatum and substantia nigra 2 h after injection (Fig. 4).

3. Discussion

The major finding of this study is that the dopamine D_1-like receptor agonist SKF38393, but not the D_2-like receptor agonist quinpirole, increased Homer 1a mRNAs in the striatum and

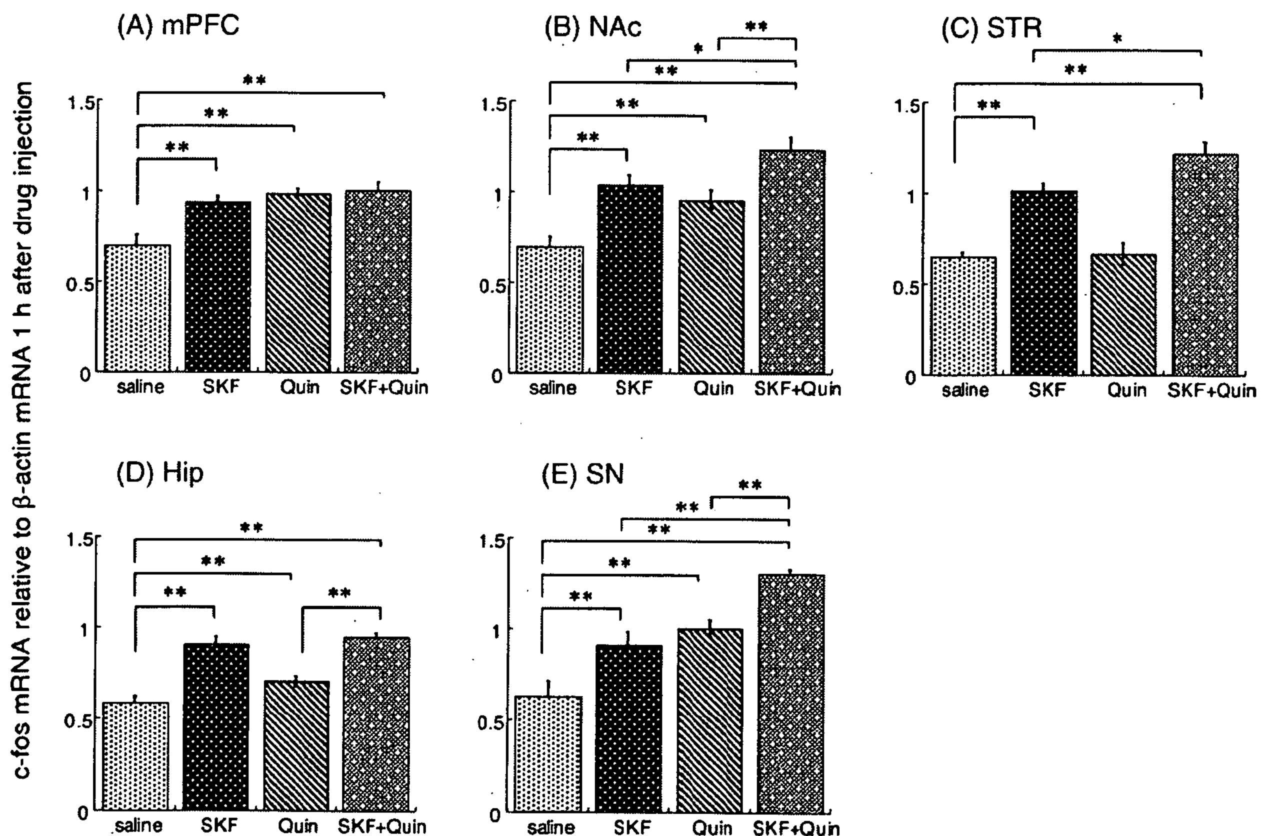

Fig. 3 – Effects of SKF38393 and quinpirole on regional c-*fos* mRNA levels. The IEG c-*fos* and β-actin cDNA were coamplified using 30 ng of total RNA for 30 and 20 cycles, respectively, in five regions; (A) medial prefrontal cortex (mPFC); (B) nucleus accumbens (NAc); (C) striatum (STR); (D) hippocampus (Hip); (E) substantia nigra (SN). Rats were sacrificed 1 h after s.c. injection with SKF38393 (SKF; 20 mg/kg), quinpirole (Quin; 1 mg/kg), combination of SKF38393 and quinpirole (SKF+Quin; 20 mg/kg and 1 mg/kg, respectively) or saline. The values represent c-*fos* mRNA levels relative to β-actin mRNA levels (mean±SEM of 9–10 animals). *P<0.05 and **P<0.01 were considered significant in this study using ANOVA followed by the Fisher's PLSD.

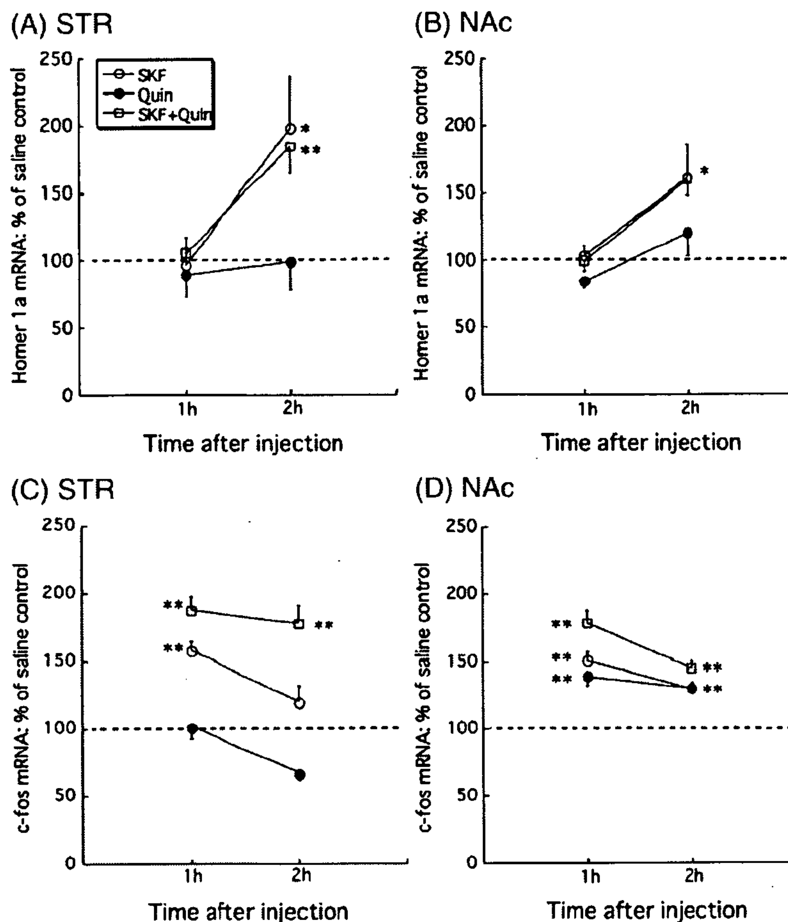

Fig. 4 – Time course of mRNA levels of Homer 1a (A and B) and *c-fos* (C and D) in the striatum (STR, A and C) and nucleus accumbens (NAc, B and D) following the dopamine agonist treatment is plotted, respectively. Rats were sacrificed 1 h or 2 h after s.c. injection with SKF38393 (SKF; 20 mg/kg), quinpirole (Quin; 1 mg/kg), or combination of SKF38393 and quinpirole (SKF + Quin; 20 mg/kg and 1 mg/kg, respectively). The values are expressed as % of saline control (mean ± SEM of 9–10 animals) because the IEG mRNA levels relative to β-actin mRNA levels of saline control rats differed between 1 h and 2 h after injection. Saline control values of IEGs in each region 1 h and 2 h after injection are as follows: Homer 1a, 1.31 ± 0.25 (STR/1 h), 1.13 ± 0.17 (STR/2 h), 1.77 ± 0.16 (NAc/1 h), 1.10 ± 0.20 (Nac/2 h); *c-fos*, 0.65 ± 0.02 (STR/1 h), 0.42 ± 0.06 (STR/2 h), 0.69 ± 0.05 (NAc/1 h) and 1.11 ± 0.06 (NAc/2 h), respectively. When compared with saline control at each time point, *$P < 0.05$ and **$P < 0.01$ were considered significant in this study using ANOVA followed by the Fisher's PLSD.

nucleus accumbens. While SKF38393 is a partial D_1 receptor agonist in terms of in vitro adenylate cyclase stimulation and is often used in dopamine-depleted animals in which D_1 receptors are sensitized (Gerfen et al., 1995; Berke et al., 1998; Pollack and Yates, 1999), the in vivo effect of SKF81297, a full D_1 receptor agonist, on firing rates of the nucleus accumbens neurons has been demonstrated nearly identical to that of SKF38393 (Johansen et al., 1991). In this study, the effects of SKF38393 and its combination with quinpirole on *c-fos* expression, as discussed later, were consistent with the results from the previous studies using 6-hydroxydopamine-lesioned or normal (unlesioned) rats (Paul et al., 1992; LaHoste et al., 1993; Gerfen et al., 1995; Keefe and Gerfen, 1995). Moreover, Berke et al. (1998) reported SKF38393 to induce Homer 1a expression to the maximum 2 h after injection, in the striatum of 6-hydroxydopamine-lesioned rats. Taken together, the present results suggest that D_1-like receptor stimulation by SKF38393 may induce gene expression of Homer 1a in the basal ganglia. Consistently, methylphenidate and methamphetamine are reported to increase Homer 1a expression in the striatum and nucleus accumbens, and these effects are attenuated by SCH23390, a D_1-like receptor antagonist (Hashimoto et al., 2004; Yano et al., 2006).

Further studies employing Western blots would confirm the present finding. In this regard, another experiment in our laboratory demonstrated that methamphetamine enhanced Western blots of Homer 1a protein expression in rat striatum and nucleus accumbens, and this effect correlated to the methamphetamine-induced increase in PCR products of Homer 1a mRNA (unpublished data). This study chose only a single dose of each dopamine receptor agonist on the basis of the previous studies showing it to enhance Fos-like immunoreactivity in rat striatum (LaHoste et al., 1993; Pollack and Yates, 1999). When administered alone, SKF38393 at the dose