Haplotype tag single nucleotide polymorphism selection

We first consulted the HapMap database (release #16c.1, *www.hapmap.org*) and determined the LD block with the criteria $D' > 0.8$ using HAPLOVIEW ver. 3.2 software [9]. All single nucleotide polymorphisms (SNPs) listed in the entire coding region as well as the 500 bp upstream 5′-flanking region and 500 bp downstream 3′-UTR region (minor allele frequency > 0.05) were included in the LD analysis. Haplotype tag SNPs (htSNPs) were defined as those capturing 90% of the haplotype diversity within each LD block using the same program. The Japanese portion of the HapMap data was used for this procedure (Fig. 1).

Single nucleotide polymorphism genotyping

Genotyping of the htSNPs was carried out using TaqMan assays (Applied Biosystems, Foster City, California, USA) and the PCR-restriction fragment length polymorphism (RFLP) method (Table 1). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. A 5-μl total reaction volume was used, and allelic-specific fluorescence was measured using the ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Detailed information on the PCR method is available upon request.

Statistical analysis

Genotype deviation from the Hardy–Weinberg equilibrium (HWE) was evaluated by the χ^2 test. Single marker association and haplotype analyses were performed with SPSS version 11.0J (Tokyo, Japan) and COCAPHASE version 2.403 (*http://portal.litbio.org/Registered/Option/unphased*; Dudbridge, 2003), respectively. The significance level for all statistical tests was 0.05. Power calculations were performed using the genetic statistical package Genetic Power Calculator (*http://pngu.mgh.harvard.edu/~purcell/gpc/*; Purcell 2001–2005).

Results

The *TACR3* gene was composed of six LD blocks. One nonblock SNP and eight htSNPs were finally selected according to the criteria (Fig. 1). The genotype and allele frequency of each htSNP in schizophrenic patients and controls are summarized in Table 1. The observed genotype frequencies of all SNPs were within the distribution expected according to HWE. Neither the genotype nor allele frequency of any SNP differed significantly between the schizophrenia group and the control group (Table 1). The distribution of haplotype frequencies did not differ significantly between the schizophrenic patients and controls (Table 2). More than 80% power in detecting any association with schizophrenia was obtained when the genotype relative risk was set at 1.33–1.66 under a multiplicative model of inheritance.

Discussion

Our study indicates that the *TACR3* gene does not play a major role in the development of schizophrenia in the Japanese population, as no significant differences in allele, genotype, or haplotype frequencies of the selected SNPs were found between schizophrenic patients and controls. As it is, however, suspected that genetic risk factors for schizophrenia may differ between races or ethnicities, a replication study including different ethnic populations is needed to validate these results.

As mentioned in the Introduction, the NK3 receptor was reported to regulate the DA and 5-HT release or concentration at the synapse. It would therefore be valuable to investigate the gene–gene interactions between *TACR3* and other DA or 5-HT signaling related genes [10]. Furthermore, the 5-HT1A receptor partial agonist tandospirone is reported to be effective as an adjunctive treatment to improve cognition in patients with schizophrenia [11]. As the NK3 receptor is thought to have the potential for indirect influence on the 5-HT1A receptor through 5-HT release, association analysis using samples with data on the cognitive function might help elucidate the pathogenesis of schizophrenia.

A couple of limitations in this study should be considered. First, the male/female ratios and average ages did not match between schizophrenic patients and controls. When we performed a multiple regression analysis, there were no effects of age or sex on the disease status (data not shown). Additionally, these effects might be small because not likely

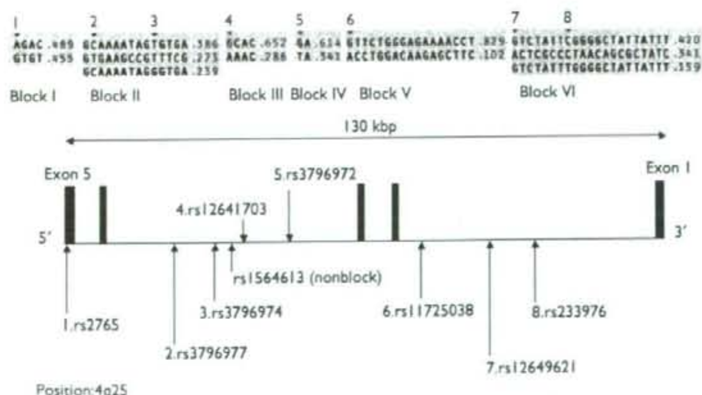

Fig. 1 Genomic structure of *TACR3* with haplotype tag single nucleotide polymorphisms (SNPs) and haplotype frequencies in each linkage disequilibrium block provided by HapMap database V.16. Numbers under or above the arrows represent the SNPs we selected in this study.

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Table 1 Association analyses of haplotype tag SNPs

					Allelic distribution[a]			Genotypic distribution[a]			
SNP	Block	Method of genotyping		GRR	M	m	P value	M/M	M/m	m/m	P value
rs2765	I	TaqMan	SCZ	1.33	411	345	0.685	114	183	81	0.861
			CONT		401	351		112	178	87	
rs3796977	II	TaqMan	SCZ	1.41	611	153	0.471	249	113	20	0.79
			CONT		598	164		240	120	22	
rs3796974	II	TaqMan	SCZ	1.34	502	264	0.992	165	172	46	0.563
			CONT		493	259		169	156	52	
rs12641703	III	TaqMan	SCZ	1.35	506	260	0.669	167	172	44	0.536
			CONT		485	261		164	157	52	
rs3796972	IV	PCR-RFLP	SCZ	1.33	406	350	0.45	106	195	78	0.397
			CONT		414	330		121	177	77	
rs11725038	V	TaqMan	SCZ	1.37	556	210	0.899	204	149	31	0.954
			CONT		548	210		201	146	33	
rs12649621	VI	TaqMan	SCZ	1.33	430	336	0.284	127	178	79	0.53
			CONT		407	355		113	183	87	
rs233976	VI	TaqMan	SCZ	1.44	639	123	0.833	270	100	12	0.614
			CONT		642	120		269	106	8	
rs1564613	Non-block	TaqMan	SCZ	1.66	710	52	0.799	331	48	2	0.793
			CONT		705	49		332	43	3	

CONT, control; GRR, genotype relative risk; M, major allele; m, minor allele; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]In absolute numbers.

Table 2 Haplotype analyses

Block	SNP	Haplo-type	SCZ[a]	CON[a]	P value[b]	Global P value[b]
		AT	0.455	0.44	0.558	
2	rs3796977-rs3796974	AC	0.345	0.344	0.993	0.734
		CT	0.2	0.215	0.467	
		GG	0.439	0.466	0.284	
6	rs12649621-rs233976	AG	0.4	0.377	0.348	0.531
		AA	0.161	0.157	0.837	

CONT, control; SCZ, schizophrenia; SNP, single nucleotide polymorphism.
[a]Estimated frequencies.
[b]P values were calculated by log-likelihood ratio test.

more than four participants given a lifetime morbidity risk of 1% will eventually develop schizophrenia. Second, we selected htSNPs so as to cover 90% of the haplotypes within each LD block. It is, however, possible that the htSNPs used in this study did not capture all haplotypes in the gene, as the LD block structure of *TACR3* was not tight. In other words, there may be SNPs not found in the LD, for which we did not investigate the possible association with schizophrenia. Thus, further analysis based on more comprehensive and detailed SNP coverage of *TACR3* is required to make conclusive results.

Conclusion

The present results suggest that *TACR3* itself is unlikely to be related to the development of schizophrenia in the Japanese population. Further studies including pharmacogenetic investigations are required, however, for conclusive results on the exact roles of *TACR3* in the pathophysiology of schizophrenia.

Acknowledgements

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Genetic analysis of the gene coding for DARPP-32 (*PPP1R1B*) in Japanese patients with schizophrenia or bipolar disorder

Akira Yoshimi [a], Nagahide Takahashi [b], Shinichi Saito [b,*], Yoshihito Ito [b], Branko Aleksic [b], Hinako Usui [b], Yukiko Kawamura [a], Yukari Waki [a], Takeo Yoshikawa [c], Tadafumi Kato [d], Nakao Iwata [e], Toshiya Inada [f], Yukihiro Noda [a], Norio Ozaki [b]

[a] *Division of Clinical Science and Neuropsychopharmacology, Graduate School of Pharmacy, Meijo University, Aichi, 468-8503, Japan*
[b] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Aichi, 466-8550, Japan*
[c] *Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, 351-0198, Japan*
[d] *Laboratory for Molecular Dynamics of Mental Disorders, RIKEN Brain Science Institute, Saitama, 351-0198, Japan*
[e] *Department of Psychiatry, Fujita Health University School of Medicine, Aichi, 470-1192, Japan*
[f] *Department of Psychiatry, Teikyo University School of Medicine, Chiba Medical Center, Chiba, 299-0111, Japan*

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Abstract

Several lines of evidence, including genome-wide linkage scans and postmortem brain studies of patients with schizophrenia or bipolar disorder, have suggested that DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a key regulatory molecule in the dopaminergic signaling pathway, is involved in these disorders. After evaluating the linkage disequilibrium pattern of the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), we conducted association analyses of this gene with schizophrenia and bipolar disorder. Single-marker and *haplotypic* analyses of four single nucleotide polymorphisms (SNPs; rs879606, rs12601930, rs907094, and rs3764352) in a sample set (subjects with schizophrenia = 384, subjects with bipolar disorder = 318, control subjects = 384) showed that *PPP1R1B* polymorphisms were not significantly associated with schizophrenia, whereas, even after Bonferroni corrections, significant associations with bipolar disorder were observed for rs12601930 (corrected genotypic $p=0.00059$) and rs907094 (corrected allelic $p=0.040$). We, however, could not confirm these results in a second independent sample set (subjects with bipolar disorder = 366, control subjects = 370). We now believe that the significant association observed with the first sample set was a result of copy number aberrations in the region surrounding these SNPs. Our findings suggest that *PPP1R1B* SNPs are unlikely to be related to the development of schizophrenia and bipolar disorder in the Japanese population.
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Keywords: Schizophrenia; Bipolar disorder; Dopamine- and cAMP-regulated phosphoprotein; 32 kDa; Japanese population

1. Introduction

A number of studies have proposed that disruption of monoaminergic pathways, and in particular the dopaminergic pathway, contributes to both schizophrenia and bipolar disorder (Catapano and Manji, 2007; Murray

* Corresponding author. Department of Psychiatry, Nagoya University, Graduate School of Medicine, 65 Tsuruma-cho, Showa-Ku, Nagoya 466-8550, Japan. Tel.: +81 52 744 2282; fax: +81 52 744 2293.
E-mail address: s-saito@med.nagoya-u.ac.jp (S. Saito).

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et al., 2004). DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, 32 kDa), a critical molecule in *the* striatal neurons, regulates the dopaminergic signaling pathway through phosphorylation of protein phosphatase-1 and protein kinase A (Fienberg et al., 1998). Recently, it has been revealed that DARPP-32 also plays an important role in the regulation of *glutamatergic* signaling pathway (Nishi et al., 2005), which is also thought to contribute to the development of these disorders (Beneyto et al., 2007; Svenningsson et al., 2003).

DARPP-32 knockout mice have been shown to have abnormal responses to psychoactive drugs, such as the decrease of cage climbing behavior induced by dopamine agonists (Fienberg et al., 1998) and the decrease of attenuating effect of antidepressants on immobility (Svenningsson et al., 2002).

Moreover, reduced expression of DARPP-32 has been observed in the postmortem brain of schizophrenic patients (Albert et al., 2002). This is suggested to be related to neostriatal volume, activation, and functional connectivity in the prefrontal cortex, all of which are thought to be abnormal in patients with schizophrenia (Meyer-Lindenberg et al., 2007).

Additionally, several lines of evidence have demonstrated that genetic factors contribute to the development of schizophrenia and bipolar disorder, and genome-wide linkage scans have shown that several chromosomal regions are simultaneously linked to the development of these disorders. Namely, a chromosomal region within 17q, which includes the gene encoding DARPP-32 (*PPP1R1B*; located on 17q12), has been demonstrated to have high logarithm of the odds scores for schizophrenia (Cardno et al., 2001) and bipolar disorder (Dick et al., 2003), i.e. 2.54 and 3.63, respectively.

Therefore, *PPP1R1B* is considered to be one of the candidate genes that contribute to these disorders. In the present study, we performed linkage disequilibrium analysis of *PPP1R1B*, and investigated the association of polymorphisms in this gene with schizophrenia and bipolar disorder in Japanese patients. We employed a two-stage analysis using two independent sets of samples as a previous report (Ikeda et al., 2005). Additionally, copy number variations (CNVs), which have been observed for many genes (Lee and Lupski, 2006; Redon et al., 2006) can affect the accuracy of genotyping with single nucleotide polymorphisms (SNPs). Therefore, we also explored copy number differences of this gene to test the accuracy of genotyping with the SNPs, which deviated from the Hardy–Weinberg equilibrium (HWE).

2. Materials and methods

2.1. Subjects

The subjects for the case-control analysis consisted of 384 patients with schizophrenia (226 males and 158 females; 52.1±15.3 years old), 318 patients with bipolar disorder (162 males and 156 females; 44.0±20.7 years old), and 384 control subjects (159 males and 225 females; 43.9±15.9 years old). To confirm a significant association with bipolar disorder, a second sample set was used, which consisted of 366 patients with bipolar disorder (181 males and 185 females; 50.1±13.4 years old), and 370 control subjects (185 males and 185 females; 50.6±12.6 years old).

For the analysis of copy number differences, we selected 12 male and 12 female subjects (schizophrenia patients: 54.9±12.5 years old; bipolar disorder patients: 46.3±17.4 years old; control subjects: 43.0±12.6 years old) for screening, and another independent sample set of 36 male and 36 female subjects (schizophrenia patients: 41.1±12.2 years old; bipolar disorder patients: 47.1±15.6 years old; control subjects: 43.5±12.7 years old) was used to confirm the results. The subjects used for the copy number analysis were also included in the first sample set used for the association study.

All subjects were unrelated and ethnically Japanese. The patients were diagnosed by at least two experienced psychiatrists according to the DSM-IV-TR (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision) criteria for schizophrenia and bipolar disorder on the basis of unstructured interviews and reviews of their medical records. All healthy control subjects were also psychiatrically screened on the basis of unstructured interviews.

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine, Fujita Health University, and the RIKEN Brain Science Institute. Written informed consent was obtained from each subject.

2.2. Linkage disequilibrium (LD) analysis and tagging SNP selection

For LD analysis, we consulted the HapMap database (release #21a; population: Japanese in Tokyo; minor allele frequency: more than 0.05) in order to obtain SNPs throughout the entire coding region of *PPP1R1B* (GenBank accession No. NM_032192) as well as in flanking regions 500 base pairs (bp) upstream and 500 bp downstream of the coding regions. For the gene-spanning analysis, we selected four SNPs (rs2271309,

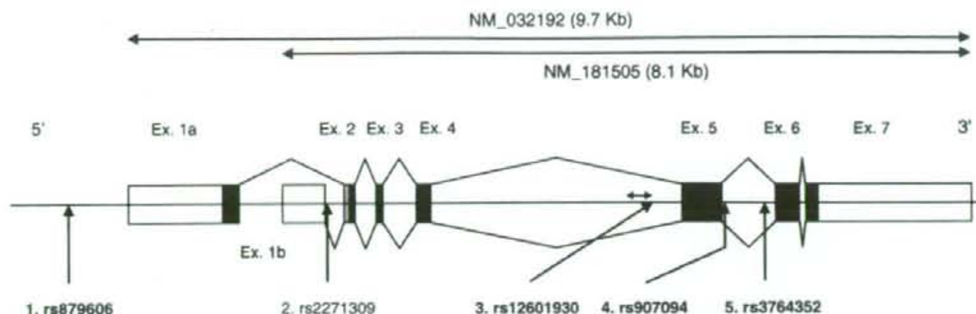

Fig. 1. Genomic structure of *PPP1R1B*. Black boxes indicate protein-coding regions, whereas open boxes denote untranslated regions (UTR). Each box represents the *PPP1R1B* exons. Numbers under the arrows represent the SNP IDs. Bold numbers represent tagging SNPs (pairwise tagger; $r^2>0.8$; Haploview 3.32). All SNPs in the coding region are listed as well as those within the 500-bp upstream 5′-flanking region and the 500-bp downstream 3′ UTR of *PPP1R1B*. Arrows ~200 bp upstream of rs12601930 shows the sites that were PCR amplified for copy number analysis.

rs12601930, rs907094, and rs3764352) in addition to a dbSNP (rs879606) located in the promoter region (Fig. 1). After evaluating the LD pattern with 48 control subjects using Haploview version 3.32, rs2271309 was excluded according to the criterion for pairwise tagging, $r^2>0.8$ (de Bakker et al., 2005) (see Fig. 2).

2.3. SNP genotyping

Genotyping of tagging SNPs was carried out primarily using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. TaqMan assays were used when available (Applied Biosystems, California, USA). TaqMan probes and Universal PCR Master Mix were obtained from Applied Biosystems. Allelic-specific fluorescence was measured using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). Information about each primer pair and enzyme is available upon request.

2.4. Quantitative real-time PCR

The *PPP1R1B* copy number was analyzed using real-time PCRs with a specific primer set and FAM-labeled fluorescent probe or TaqMan expression assays (Applied Biosystems). The test region for *PPP1R1B* was ~200 bp upstream of the SNP that produced results that deviated from the HWE (Fig. 1) and the prostaglandin transporter gene (*SLCO2A1*) was used as a single-copy control gene (Wilson et al., 2006). We did not use glucose-6-phosphate dehydrogenase (G6PD), which is located on the X chromosome and was used as single-copy control gene in the study from Wilson et al., because it showed an unstable copy number value (not an integral value) in their report.

To determine the relative copy number, 10 ng of genomic DNA was assayed in triplicate in 20 μL of reaction solution containing 1×final concentration TaqMan Universal Master Mix (Applied Biosystems) and 1×final concentration TaqMan probe (Applied Biosystems) specific for *PPP1R1B* or 700 nM each primer and 200 nM probe specific for *SLCO2A1*.

Each experiment was performed using an ABI PRISM 7900 Sequence Detector System (Applied Biosystems). To evaluate the relative copy number of *PPP1R1B*, we calculated the relative quantity of the dose of *PPP1R1B* using a comparative C_T method (*PPP1R1B* vs. *SLCO2A1*). The TaqMan specific primers and FAM-labeled fluorescent probe used for the PCR amplifications were as follows: *PPP1R1B*-FAM probe (5′-FAM-CCCCTTGCTCCTTTCC-MGBNFQ-3′), *PPP1R1B*-for (5′-GCCTTGGCCCCTTTCTCTAA-3′), *PPP1R1B*-rev (5′-GCAGCTGGAGACAAGTTTCC-3′), *SLCO2A1*-FAM (5′-FAM-CCATCCATGTCCTCATCTC-

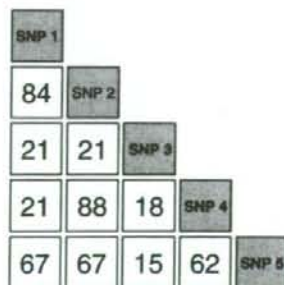

Fig. 2. LD analysis of *PPP1R1B*. Numbers in the top gray boxes correspond to the SNP ID numbers in Fig. 1. Numbers in the white boxes represent the r^2 values after the decimal point.

Table 1
Genotype and allele frequency of *PPP1R1B* in schizophrenia and controls

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a] SCZ[b]			CON[b]			*p* value	Armitage's *p* value	Allele SCZ		CON		*p* value	Global *p* value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 62 (34%)	G/A 81 (45%)	A/A 39 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.631	0.985	G 205 (56%)	A 159 (44%)	G 207 (56%)	A 161 (44%)	0.985	0.697
	Rs12601930 (C/T)	real time PCR	C/C 256 (68%)	C/T 110 (29%)	T/T 11 (3%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.190	0.271	C 622 (83%)	T 132 (18%)	C 614 (80%)	T 150 (20%)	0.287	
	rs907094 (T/C)	real time PCR	T/T 99 (27%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.518	0.673	T 400 (54%)	C 344 (46%)	T 421 (55%)	C 347 (45%)	0.681	
	rs3764352 (A/G)	RFLP	A/A 86 (24%)	A/G 193 (54%)	G/G 77 (22%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.582	0.419	A 365 (51%)	G 347 (49%)	A 403 (53%)	G 353 (47%)	0.434	

[a]M: major allele, m: minor allele [b]SCZ: schizophrenia, CON: control [c]global *p* value: haplotypic analysis.

MGBNFQ-3′), *SLCO2A1*-for (5′-ATCCCCAAAG-CACCTGGTTT-3′), and *SLCO2A1*-rev (5′-AGAGGC-CAAGATAGTCCTGGTAA-3′).

2.5. Statistical analysis

Genotype deviations from the HWE and single-marker association were analyzed using Haploview software. We evaluated the allelic and genotypic associations by the χ^2-test. Genotypic association of SNPs that deviated from the HWE was analyzed using Cochran-Armitage trend tests for multiplicative model of inheritance (Balding, 2006). Haplotypic analyses were performed with Unphased version 2.403 (Dudbridge, 2003). The significance level for all statistical tests was 0.05. Bonferroni corrections were used for multiple comparisons. Power calculations were performed using the genetic statistical package on a genetic power calculator (Purcell et al., 2003).

3. Results

The genotype and allele frequency of each SNP in schizophrenic patients, bipolar disorder patients, and control subjects are summarized in Table 1 and Table 2-1, respectively. The observed genotype frequencies of the tagging SNPs were within the distribution expected from the HWE except for rs12601930.

Neither the genotype nor the allele frequency of any of the examined *PPP1R1B* SNPs in the schizophrenic patients differed significantly from those observed for the control subjects (Table 1). Moreover, the distribution of haplotypic frequencies in the schizophrenia patients did not differ significantly from that in control subjects. Power analysis showed that more than 80% power in detecting an association with schizophrenia was obtained when the genotype relative risk (GRR) was set from 1.35 to 1.51 in a multiplicative model of inheritance.

A significant association was observed with both the genotype and the allele containing rs907094 ($p=0.036$ and $p=0.010$, respectively), whereas a significant association was only found with the genotype for rs12601930 ($p=0.000147$). Haplotypic analysis supported this association (global $p=0.030$; Table 2-1). After Bonferroni corrections, the observed positive associations were no longer significant for the rs907094 genotype (corrected $p=0.144$) and haplotype (corrected global $p=0.120$), whereas, even after the corrections, the associations remained significant for the rs907094 allele (corrected $p=0.040$) and the rs12601930 genotype (corrected $p=0.000588$). We, however, could not confirm these

Table 2-1
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (first sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						p value	Armitage's	Allele				p value	Global
			BP[b]			CON[b]				p value	BP		CON			p value[c]
PPP1R1B	rs879606 (G/A)	RFLP	G/G 87 (30%)	G/A 146 (50%)	A/A 61 (21%)	G/G 58 (32%)	G/A 91 (49%)	A/A 35 (19%)	0.857	0.580	G 320 (54%)	A 268 (46%)	G 207 (56%)	A 161 (44%)	0.306	0.030
	rs12601930 (C/T)	real time PCR	C/C 169 (69%)	C/T 57 (23%)	T/T 18 (7%)	C/C 240 (63%)	C/T 134 (35%)	T/T 8 (2%)	0.000147	0.804	C 395 (81%)	T 3 (19%)	C 614 (80%)	T 150 (20%)	0.802	
	rs907094 (T/C)	real time PCR	T/T 63 (23%)	T/C 202 (54%)	C/C 71 (19%)	T/T 114 (30%)	T/C 193 (50%)	C/C 77 (20%)	0.036	0.010	T 267 (48%)	C 293 (52%)	T 421 (55%)	C 347 (45%)	0.010	
	rs3764352 (A/G)	RFLP	A/A 64 (22%)	A/G 170 (58%)	G/G 58 (20%)	A/A 104 (28%)	A/G 195 (52%)	G/G 79 (21%)	0.176	0.385	A 298 (51%)	G 286 (49%)	A 403 (53%)	G 353 (47%)	0.407	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 2-2
Genotype and allele frequency of *PPP1R1B* in bipolar disorder and controls (second sample set)

Gene symbol	SNP ID (M/m)	Method of genotyping	Genotype[a]						p value	Armitage's	Allele				p value	Global
			BP[b]			CON[b]				p value	BP		CON			p value[c]
PPP1R1B	Rs12601930 (C/T)	real time PCR	C/C 232 (63%)	C/T 119 (33%)	T/T 15 (4%)	C/C 248 (68%)	C/T 105 (29%)	T/T 12 (3%)	0.419	0.192	C 583 (80%)	T 149 (20%)	C 601 (82%)	T 129 (18%)	0.191	0.307
	Rs907094 (T/C)	real time PCR	T/T 102 (28%)	T/C 190 (53%)	C/C 70 (19%)	T/T 107 (29%)	T/C 180 (49%)	C/C 81 (22%)	0.565	0.731	T 394 (54%)	C 330 (46%)	T 394 (54%)	C 342 (46%)	0.734	

[a]M: major allele, m: minor allele [b]BP: bipolar disorder, CON: control [c]global p value: haplotypic analysis.

Table 3-1
PPP1R1B copy number variations detected by qPCR[a] (first sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.81 ± 0.14	0.87 ± 0.10	1.11 ± 0.34
Decrease	6	3	2
Normal	17	21	15
Increase	1	0	7

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

results in a second independent sample set as shown in Table 2-2. When the two sample sets were merged, power analysis showed the level for detecting association was higher than 80% for bipolar disorder at the genotype relative risk of 1.24 to 1.39 under a multiplicative model of inheritance.

DNA copy number analysis revealed aberrations in the copy number in the schizophrenic patients (increase=1 patient, decrease=6), the bipolar patients (decrease=3), and the control subjects (increase=7, decrease=2; Table 3-1). In the second independent sample set, we observed aberrations in the schizophrenic patients (increase=2, decrease=1), the bipolar patients (increase=2, decrease=1), and the control subjects (increase=2, decrease=5; Table 3-2). All analyses were performed in duplicate.

4. Discussion

According to the common disease-common variants hypothesis (Chakravarti, 1999), the present study showed that *PPP1R1B* was unlikely to be related to be the development of schizophrenia and bipolar disorder in Japanese patients. These results were consistent with a recently published study that examined Chinese patients (Li et al., 2006).

The SNPs used in the association analysis, which covered the entire gene, included all of the common SNPs (more than 5% frequency) listed in the dbSNP database; therefore, it is unlikely that there are other common variants related to these disorders. Because we did not perform mutation screening of this gene, however, the possibility that rare variants could be causal to the development of these disorders cannot be excluded. The GRR value calculated using power analysis was appropriate when compared to other promising candidate genes for schizophrenia (Schwab et al., 2005; Schwab et al., 2003; Shifman et al., 2002).

Recently, Meyer-Lindenberg et al. (2007) tested for an association in schizophrenia with SNPs in this gene using a relatively small sample of Caucasian families, and found a strong association with rs879606 and mild association with two other SNPs (rs3764352 and rs3794712). Two of these three SNPs (rs879606 and rs3764352), however, did not show any significant association in our samples, suggesting that ethnic differences might play a role in these associations.

rs3794712 was not further pursued for two reasons. *Firstly*, this SNP is not validated and the frequency of this SNP is not reported in the dbSNP database. *Secondly*, this SNP is unlikely to affect the function of this gene considering its genomic position in the middle of an intron and the results from *in silico* analysis (RegRNA; http://regrna.mbc.nctu.edu.tw/index.html).

In the present study, we found that copy number differences in the region that includes the SNPs deviated from the HWE and were significantly associated with bipolar disorder (Tables 3-1 and 3-2), suggesting that the presence of copy number alterations gives rise to the deviation from the HWE due to a high frequency of heterozygotes. Therefore, caution must be taken when interpreting results from SNP analysis. These results, however, must be regarded as preliminary because we did not observe a definite association between copy number differences and the disorders. Because accumulating evidence has highlighted that CNVs are observed in many chromosome regions, *including 17q12* (Sharp et al., 2006), and copy number alterations can affect gene expression (Redon et al., 2006), further investigations of the CNVs as well as the SNPs may be an effective complementary approach to elucidate the genetic risk factors underlying the complex phenotypes associated with psychiatric disorders, including schizophrenia and bipolar disorder (Lee and Lupski, 2006).

A couple of limitations should be considered in the present study. Firstly, we observed a strong association between rs12601930 and bipolar disorder in the first sample set even after a Bonferroni correction (corrected genotype p=0.000588); the genotype frequency of the minor allele homozygotes (TT) in the control subjects, however, was only 2% (Table 2-1). This low frequency may have resulted in a false-positive association. When we used a Cochran–Armitage trend test (Balding, 2006)

Table 3-2
PPP1R1B copy number variations detected by qPCR[a] (second sample set)

	SCZ[b] (n=24)	BP[b] (n=24)	CON[b] (n=24)
Relative quantity	0.95 ± 0.10	0.98 ± 0.14	1.03 ± 0.27
Decrease	2	2	2
Normal	69	69	65
Increase	1	1	5

[a]qPCR: quantitative real-time PCR.
[b]SCZ: schizophrenia, BP: bipolar disorder, CON: control.

to correct the observed association with rs12601930, which deviated from the HWE, there was no significance in the genotype frequency (Armitage's $p=0.804$). Thus, the possibility of a type I error derived from an insufficient sample size should be considered. Secondly, although the method used for copy number analysis has been widely used and is thought to be highly reliable due to the consistency of results obtained in our duplicate experiments, supplementary methods, such as Southern blotting, fluorescence in situ hybridization, or array comparative genomic hybridization, would have provided further confirmation of our results. Finally, the male/female ratios and average ages were not completely consistent between the schizophrenic patients and the control subjects. Based on results from an exploratory analysis using a logistic regression model, however, these variables do not appear to contribute to the results obtained in the present association study (data not shown).

In conclusion, our findings suggest that SNPs within *PPP1R1B* do not elevate the risk for either schizophrenia or bipolar disorder in the Japanese population. Further functional analysis of the CNVs and association studies using other endophenotypes including cognitive function should be needed to clarify the exact role of this gene in the pathophysiology of these disorders.

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Contributors

Author Akira Yoshimi, Nagahide Takahashi, and Shinichi Saito designed the study and wrote the protocol. Author Norio Ozaki and Yukihiro Noda performed the literature searches and analyses. Author Akira Yoshimi wrote the first draft of the manuscript and Nagahide Takahashi and Shinichi Saito revised it. All of the authors contributed to and have approved the final version of the manuscript.

Conflict of interest

All authors declare that they have no conflicts of interests.

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Failure to replicate the association between *NRG1* and schizophrenia using Japanese large sample

Masashi Ikeda [a,*,1], Nagahide Takahashi [b,c,1], Shinichi Saito [c], Branko Aleksic [a,c], Yuichiro Watanabe [d], Ayako Nunokawa [d], Yoshio Yamanouchi [a], Tsuyoshi Kitajima [a], Yoko Kinoshita [a], Taro Kishi [a], Kunihiro Kawashima [a], Ryota Hashimoto [e,f], Hiroshi Ujike [g], Toshiya Inada [h], Toshiyuki Someya [d], Masatoshi Takeda [e,f], Norio Ozaki [c], Nakao Iwata [a]

[a] *Department of Psychiatry, Fujita Health University School of Medicine, Aichi, Japan*
[b] *Laboratory of Molecular Neuropsychiatry, Department of Psychiatry, Mount Sinai School of Medicine, New York, USA*
[c] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[d] *Department of Psychiatry, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
[e] *The Osaka-Hamamatsu Joint Research Center For Child Mental Development, Osaka University Graduate School of Medicine, Osaka, Japan*
[f] *Department of Psychiatry, Osaka University Graduate School of Medicine, Osaka, Japan*
[g] *Department of Neuropsychiatry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
[h] *Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, Japan*

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Abstract

Systematic linkage disequilibrium (LD) mapping of 8p12–21 in the Icelandic population identified neuregulin 1 (*NRG1*) as a prime candidate gene for schizophrenia. However, results of replication studies have been inconsistent, and no large sample analyses have been reported. Therefore, we designed this study with the aim of assessing this putative association between schizophrenia and *NRG1* (especially HAP_{ICE} region and exon region) using a gene-based association approach in the Japanese population.

This study was a two-stage association analysis with a different panel of samples, in which the significant association found in the first-set screening samples (1126 cases and 1022 controls) was further assessed in the confirmation samples (1262 cases and 1172 controls, and 166 trio samples). In the first-set scan, 60 SNPs (49 tagging SNPs from HapMap database, four SNPs from other papers, and seven SNPs detected in the mutation scan) were examined.

One haplotype showed a significant association in the first-set screening samples (Global P-value = 0.0244, uncorrected). However, we could not replicate this association in the following independent confirmation samples. Moreover, we could not find sufficient evidence for association of the haplotype identified as being significant in the first-set samples by imputing ungenotyped SNPs from HapMap database.

Abbreviations: *NRG1*, neuregulin 1; SNP, single nucleotide polymorphism; GGF2, glial growth factor 2; LD, linkage disequilibrium; dHPLC, denaturing high performance liquid chromatography; MAF, minor allele frequency; TDT, transmission disequilibrium test; UTR, untranslated region.

* Corresponding author. Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan. Tel.: +81 562 93 9250; fax: +81 562 93 1831.

E-mail address: ikeda-ma@fujita-hu.ac.jp (M. Ikeda).

[1] These authors contributed equally to this work.

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These results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover, the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases.
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Keywords: Schizophrenia; Neuregulin 1; Association study; False positive; Linkage disequilibrium

1. Introduction

Schizophrenia is a common psychiatric disorder with a lifetime prevalence of 1% worldwide. Family, twin and adoption studies show conclusive evidence of a substantial genetic component in this disorder. Progress towards detecting these genetic elements is now being made (Harrison and Weinberger, 2005).

The neuregulin 1 gene (*NRG1*) was first reported to be a prime candidate gene for schizophrenia in the Icelandic population (Stefansson et al., 2002). The significant association of a haplotype was detected in the 5′-region of glial growth factor 2 (GGF2) isoforms, and this at-risk haplotype, consisting of five single nucleotide polymorphisms (SNPs) and two microsatellites, was named as HAP_{ICE}. Several subsequent studies provided the following evidence to support this association with schizophrenia.

Firstly, the location of this gene corresponds to the linkage regions for schizophrenia (8p12–21, OMIM: SCZ5), which were identified by recent meta-analyses of genome-wide linkage studies (Badner and Gershon 2002; Lewis et al., 2003). Secondly, recent evidence suggests that mutation within the *NRG1* region might give rise to functional alterations that are in line with the neurodevelopmental hypothesis and glutamate/GABA hypothesis of schizophrenia (Corfas et al., 2004).

Thirdly, several independent association studies have replicated the original significant association found by Stefansson et al. (2002). However, the results of replication studies using the identical number or fewer sets of markers have been inconsistent. Thus, while some research groups did not report any association (Iwata et al., 2004), other studies showed a positive association but showed different 'at-risk' haplotypes to be associated with schizophrenia (Harrison and Law, 2006).

These inconsistent results could stem from the possibility that *NRG1* is not involved in the etiology of schizophrenia in all populations. However, this inconsistency could be a consequence of the unique structure of the human genome. In other words, differences in linkage disequilibrium (LD) among populations may also be responsible for the differences in the results, and the negative findings may only indicate a failure to reflect the actual predisposing variants due to the differences in populations.

Therefore, gene-wide (or region-wide) replication analysis based on LD pattern within the *NRG1* region is essential to detect an association in a certain population setting (Neale and Sham, 2004). In such analyses, particular attention should be paid to selection of genetic variants which adequately reflect the LD background in the targeted population (e.g. tagging SNPs).

Although the above-mentioned LD-based association analysis is based on the common disease–common variant hypothesis, one study reported an association between *NRG1* and schizophrenia from the standpoint of the common disease–rare variant hypothesis (Walss-Bass et al., 2006). The authors scanned the whole exon region, detected a non-synonymous SNP in exon 11, and showed a significant association of this SNP with schizophrenia. Detection of rare but potent functional variants relies on large mutation scan samples; however, such rare variants may also differ among populations (Pritchard, 2001).

Thus, in this study, we first focused on two attractive regions: the 5′ regions of GGF2, where the original study showed the association (henceforth referred to as 'HAP_{ICE} region') and the exon region (henceforth referred to as 'exon region'). In the exon region, prior to association analysis of tagging SNPs, we performed a mutation scan in order to detect the existence of possible potent functional variants in the ethnic samples. In addition, this study was a two-stage association analysis with a different panel of samples, in which the significant association in the first-set screening samples (1126 cases and 1022 controls) was further assessed in confirmation samples (1262 cases, 1172 controls, and 166 trio samples). This approach was adopted in order to avoid the possibility of type I or type II error.

2. Methods and materials

2.1. Subjects

Two independent sample sets were used in this study. For the first-set screening analysis, 1126 patients with schizophrenia (627 male and 499 female; mean age ± standard deviation (SD) 47.0 ± 15.3 years) and 1022

healthy controls (530 male and 492 female; 38.8±14.5 years) were examined. Confirmation analysis was conducted with three samples consisting of: (a) 1262 patients with schizophrenia (662 male and 600 female; 49.1±14.5 years) (b) 1172 controls (576 male and 596 female; 41.7±14.3 years), and (c) 166 family trios samples (of the patients, 91 male and 75 female; 30.0±8.3 years).

The subjects for mutation search were 96 patients with schizophrenia. These subjects were also included in the first-set samples. 385 cases and 336 controls in the first-set samples, and 349 cases (including 84 cases from family samples) and 424 controls in confirmation samples are identical to those in our previous report (Iwata et al., 2004) and Fukui et al.'s (2006) report, respectively.

Characterization details and psychiatric assessment of these subjects were as follows. The patients were diagnosed according to DSM-IV criteria consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. All subjects were ethnically Japanese.

After the study had been described to subjects, written informed consent was requested from each. This study was approved by the ethics committees at Fujita Health University, Teikyo University, Okayama University, Osaka University, Niigata University and Nagoya University Graduate School of Medicine.

2.2. Mutation scan

We performed denaturing high performance liquid chromatography (dHPLC) analysis, details of which can be seen in a previous paper (Ikeda et al., 2005). Primer sequences were designed in accordance with another report (Walss-Bass et al., 2006).

2.3. Tagging SNP selection

We included the three signal SNPs (SNP8NRG221533, SNP8NRG241930 and SNP8NRG243177) from the report of Stefansson et al. (2002) (we excluded SNP8NRG221132 and SNP8NRG433E1006 from the first-set analysis due to low minor allele frequencies (MAFs) in the Japanese population), one positive SNP from the report of Walss-Bass et al. (2006), and SNPs we detected in the mutation scan. Next we consulted the HapMap database (release#19, population: Japanese in Tokyo (JPT), MAF: more than 0.05). In this step, we determined the boundaries of the 'HAP$_{ICE}$ regions' that cover 5′ regions including 19,425 bp and 155,564 bp downstream (3′) from the significant SNPs (SNP8NRG221132 and SNP8NRG433E1006, respectively) in Stefansson's report (Table 1 and Supplementary Fig. 1) (Stefansson et al., 2002), and of the 'exon regions' that cover 5′ regions including 120,576 bp from the first exon and 3510 bp downstream 3′ from the last exon (GenBank accession No. NT_007995: Table 2 and Supplementary Fig. 2). Then fifteen and thirty-four 'tagging SNPs' for the HAP$_{ICE}$ regions and exon regions, respectively were selected with the criterion of an r^2 threshold greater than 0.8 in 'Aggressive tagging: use 2- and 3-markers haplotypes' mode of the 'Tagger' program (de Bakker et al., 2005), a function of HAPLOVIEW software (Barrett et al., 2005).

2.4. SNP genotyping

All SNPs were genotyped by TaqMan assay (Applied Biosystems Japan Ltd, Tokyo).

The genotyping of C#5, C#6, C#7 (which were positive SNPs in the first-set screening analysis) was done with 768 randomly selected samples (384 cases and 384 control subjects) with direct sequencing to check for genotyping error. Detailed information including primer sequences of custom TaqMan SNP genotyping assays can be seen in Supplementary Tables 1 and 2.

Table 1
First-set case control analysis of HAP$_{ICE}$ region

Markers	SNP ID	P-values		
		1-window	2-windows	3-windows
HAP$_{ICE}$#1	rs12674974	.0794	.181	
HAP$_{ICE}$#2	rs4513929	.846	.384	.196
HAP$_{ICE}$#3	SNP8NRG221533	.188	.397	.620
HAP$_{ICE}$#4	rs10096573	.200	.414	.462
HAP$_{ICE}$#5	rs4733263	.310	.616	.267
HAP$_{ICE}$#6	rs4733263	.274	.399	.578
HAP$_{ICE}$#7	SNP8NRG241930	.724	.113	.326
HAP$_{ICE}$#8	SNP8NRG243177	.288	.520	.492
HAP$_{ICE}$#9	rs4733267	.769	.889	.190
HAP$_{ICE}$#10	rs13277456	.862	.736	.847
HAP$_{ICE}$#11	rs13274954	.457	.670	.255
HAP$_{ICE}$#12	rs12677942	.312	.271	.128
HAP$_{ICE}$#13	rs4403369	.0803	.268	.548
HAP$_{ICE}$#14	rs4566990	.625	.628	.525
HAP$_{ICE}$#15	rs13270788	.541	.730	.699
HAP$_{ICE}$#16	rs1503491	.813	.866	.0960
HAP$_{ICE}$#17	rs2202262	.704	.324	.0653
HAP$_{ICE}$#18	rs10087212	.682		
HAP$_{ICE}$#4–#5		.414		
HAP$_{ICE}$#14–#16		.247		
HAP$_{ICE}$#15–#16		.730		

Table 2
First-set case control analysis of exon region

Markers	SNP ID	P-values[a]		
		1-window	2-windows	3-windows
C#1	rs10503915	.116	.0603	
C#2	rs7016691	.231	.371	.349
C#3	rs11782671	.472	.474	.296
C#4	rs10103930	.168	.322	.508
C#5	rs10503917	.699	.628	.0935
C#6	rs10107065	.765	.138	**.0244**
C#7	rs6468118	.138	.154	.174
C#8	rs7000590	.0939	.107	.158
MS1	rs7820838	.110	.142	.181
MS2	rs7834206	.149	.0879	.145
C#9	rs4236709	.0786	.187	.352
C#10	rs13260545	.0994	.248	.403
C#11	rs4316112	.948	.144	.0984
C#12	rs2439305	.196	.130	.132
C#13	rs7826814	.715	.851	.129
C#14	rs2466064	.690	.313	.436
MS3	rs3924999	.162	.113	.0699
C#15	rs10954864	.803	.969	.602
C#16	rs2439281	.965	.0725	.301
C#17	rs9642729	.0680	.0988	.137
C#18	rs12547858	.0801	.457	.523
C#19	rs10098373	.801	.835	.654
C#20	rs10095694	.380	.727	.872
MS4	rs3735774	.762	.727	.718
C#21	rs2466058	.372	.526	.587
C#22	rs2466052	.379	.286	.509
C#23	rs2466046	.187	.372	.431
C#24	rs10503923	.546	.473	.203
C#25	rs2466084	.310	.551	.197
C#26	rs2976515	.253	.654	.563
C#27	rs4445183	.702	.484	.500
C#28	rs2919377	.151	.341	.455
C#29	rs2919375	.819	.222	.182
MS5	rs3735776	.740	.758	.129
C#30	rs7007436	.711	.815	.866
C#31	rs3757934	.758	.421	.562
MS7	rs4733376	.379	.336	.357
C#32	rs4360253	.357	.893	.789
C#33	rs7005288	.864	.812	.738
C#34	rs6992642	.569		
MS6 (C#24–#30)[b]	rs17731664	.772		
C#5–#11–#14		1.00		
C#5–#14		.180		
C#16–#27		.751		
C#23–#26–#28		.245		

[a] Bold number represents significant P-value.
[b] MS6 could be represented by the haplotypes constructed by C#24–30.

2.5. Statistical methods for conventional association analysis

In the case–control samples, the marker–trait association was evaluated with the χ^2 test in allele- and genotype-wise analyses. Haplotype frequencies were estimated in a 2- to 3-marker sliding window fashion by EM algorithm and Log likelihood ratio tests were performed for Global P-values with COCAPHASE program version 3.06 (Dudbridge, 2003). In the family samples, the transmission disequilibrium test (TDT) and 3-marker haplotype analyses were performed with the TDTPHASE program version 3.06 (Dudbridge, 2003). In these haplotype-wise analyses, rare haplotypes (less than 0.05) of cases and controls were excluded from the association analysis in order to provide greater sensitivity and accuracy.

The significance level was set at $P<0.05$.

2.6. Imputation of ungenotyped SNPs

Our conventional haplotype-wise analysis was done in a sliding window fashion, since our selection for tagging SNPs was not based on the haplotype block concept. Although this type of haplotype-wise analysis does not adapt to the degree of LD, so that it is unclear which markers should be considered jointly, it results in a higher level of statistical power since it can reflect unknown SNPs that were not included in the analysis. Considering this, we included a recently developed method, imputation, to test for any SNPs that reflect the significant haplotypes (Marchini et al., 2007). The IMPUTE program imputes the genotypic distribution of un-observed SNPs using observed SNP information (60 SNPs used in the screening scan) and the HapMap database (fine-scale recombination map, haplotype for JPT/CHP).

The targeted region for imputation was limited to within known recombination hot spots, because our data targeted only the HAP_{ICE} and exon regions.

After imputation, we applied a Bayesian test with an additive model to assess the association using SNPTEST software (Marchini et al., 2007). Default values were used in all settings needed in IMPUTE and SNPTEST (e.g. effective population size for JPT/CHP, buffer, call threshold for calling genotyped SNPs and number of samples of genotypes that should be used for Bayesian tests).

Table 3
Individual haplotype analyses from significant Global P-values in first-set samples

	haplotypes	Case Freq (%)	Con Freq (%)	P-value	Global P-value
C#5–6–7	1–1–1	9.36	11.8	.0104	.0244
	1–1–2	15.6	13.6	.0896	
	1–2–2	65.8	65.5	.886	
	2–1–1	7.21	6.27	.300	

2.7. Power calculation

Power calculation was performed with a web-based statistical program, Genetic Power Calculator (Purcell et al., 2003). Power was estimated under a multiplicative model of inheritance, assuming the disease prevalence to be 1% and the population susceptibility allele frequencies to be the values observed in control samples.

3. Results

3.1. Mutation scan and first-set association analysis

We detected seven SNPs through dHPLC analysis of the exon region (MS1–7: Table 2). One of them, MS3 (rs3924999), is a non-synonymous SNP (Gly38Arg) and had shown a significant association in the Chinese population (Yang et al., 2003). The other SNPs were located in an untranslated region (UTR) or branch site, and may therefore have a functional effect (Table 2).

Next, 49 SNPs and 7 haplotypes were selected as Tagging SNPs from the HapMap database. These SNPs are located in the HAP_{ICE}- and coding regions based on the HapMap database (Tables 1 and 2).

Consequently, by involving 11 SNPs (the 7 SNPs we detected and 4 SNPs reported in other papers (Stefansson et al., 2002; Walss-Bass et al., 2006)), a total of 60 SNPs were genotyped in the first-set screening samples (however, since we were unable to design a genotyping method for one SNP that we detected (MS6) by TaqMan Assay by Design (Applied Biosystems), we determined the genotype distribution of some samples (192 cases and 192 controls) using a direct sequencing method. With these samples we confirmed that MS6 could be represented by the haplotypes constructed by C#24–30 in LD evaluation.).

The SNP for which significance was shown in the report of Walss-Bass et al. (2006) was not polymorphic in our samples.

Allele- and genotype-wise analyses did not show association either the HAP_{ICE} region or the exon region. In this haplotype-wise analysis, 3-marker haplotypes of C#5–6–7 were associated with schizophrenia (Global P-value=0.0244, uncorrected: Tables 1, 2 and 3, Supplementary Tables 3 and 4). The genotyping of C#5, C#6, C#7 in a subset of the screening samples was re-confirmed by direct sequencing, and the results were perfectly identical to those shown by TaqMan assay. Hence, we speculate that it was unlikely that genotyping error had occurred.

3.2. Imputation of ungenotyped SNP for first-set samples

Data for ungenotyped SNPs could not provide sufficient evidence for association in either region (Fig. 1). In particular, the weights of evidence for the regions near the significant haplotypes in first-set samples were less than one. Since weights of evidence of at least four are required for evidence for association

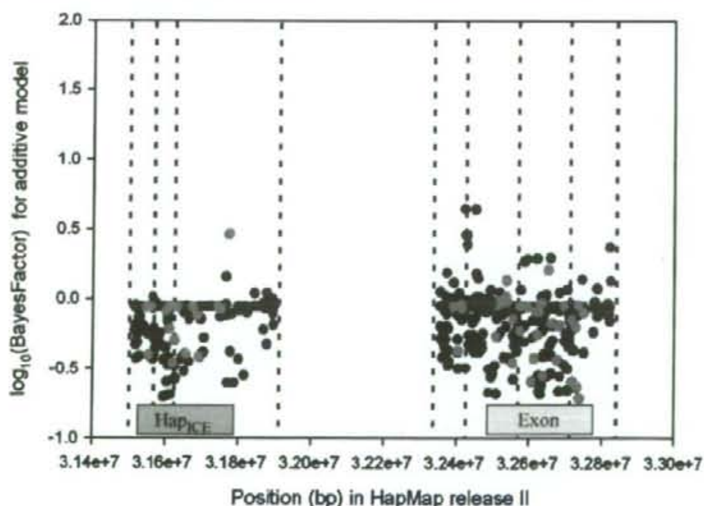

Fig. 1. Results of imputing SNP in the *NRG1* gene. The weights of evidence were calculated using imputed genotypes (red circles) and observed genotypes (black circles). Data from SNPs that constructed the significant haplotype in the first-set samples are shown in blue circles. Dotted lines indicate the estimated hot spots from the HapMap database. The SNP position from the HapMap release II database is plotted on the X axis.

Table 4
Confirmation analysis of significant haplotypes from first-set analysis

Samples	SNPID	1-window	2-windows	3-windows
Case–control	C#5	.408	.101	
	C#6	.362	.601	.120
	C#7	.371		
Family samples	C#5	.107	.323	
	C#6	.964	.846	.505
	C#7	.499		
Combined samples	C#5	.976	.591	
	C#6	.389	.303	.478
	C#7	.801		

(if 1000 SNPs of 10,000,000 common human SNPs might be associated with a disease, we may assign a prior odds of association of 1/10,000. Therefore, a Bayes factor more than 10,000 (or $\log_{10}$ [Bayes factor] more than 4) is required (Balding, 2006)). Thus, these results indicate a low probability for association in our sample.

3.3. Confirmation analysis of the positive haplotypes using different case–control samples and family samples

To confirm the significance of exon region C#5–6–7 in the first-set samples, we conducted a confirmation analysis using independent case–control samples and family samples. In these analyses, we could not replicate this association. To increase the power, we combined samples (first-set and confirmation samples) but again we could not detect an association in this explorative analysis (Table 4).

4. Discussion

In the present study, using three large and independent samples, our data did not provide sufficient evidence for associations between tagging SNPs in the HAP_{ICE} and exon regions of *NRG1* and schizophrenia in the Japanese population.

We could not replicate previous reports for the HAP_{ICE} region (Stefansson et al., 2002; Stefansson et al., 2003); however, the results of this study are in concordance with our previous replication study in the Japanese population (schizophrenia = 607, controls = 515) (Iwata et al., 2004). Another study (Fukui et al., 2006), however, examined independent Japanese samples (belonging to one-third of confirmation case–control samples) and reported a positive association. Specifically, that study reported a significant association of haplotypes constructed by three core SNPs from Stefansson et al. (SNP8NRG221533 (HAP_{ICE}#3), SNP8NRG241930 (HAP_{ICE}#7) and SNP8NRG243177 (HAP_{ICE}#8)), and one more intronic SNP (rs1081062), as well as a trend for association of rs1081062. Since our tagging SNPs could not involve this SNP (rs1081062), we found by consulting the latest HapMap database (release#21a) that rs1081062 is tagged by rs13274954 (HAP_{ICE}#11); moreover, neither HAP_{ICE}#10 nor its haplotypes (HAP_{ICE}#3–7–8–11) were associated with schizophrenia (Global P-value = 0.540). Therefore, the aforementioned positive report could have been the result of type I error due to inadequate sample size (schizophrenia = 349, controls = 424) (Fukui et al., 2006). Or, as the authors speculated (Fukui et al., 2006), the different clinical backgrounds (e.g. genetic loading) in each sample could have led to inconsistent results. In this regard, a recent study reported that *DAOA/G30*, which is also a strong candidate gene for schizophrenia, influences susceptibility to the symptomatology of psychiatric disorders including schizophrenia and bipolar disorder, but not to diagnosis itself (Williams et al., 2006).

In the coding region, our results indicated the importance of controlling inflation of the type I error rate due to multiple testing, when a significant association is obtained in an analysis that involves several markers. In this study we found significant associations only from haplotype-wise analysis, not from allele- or genotype-wise analysis. It is generally accepted that a haplotype-wise analysis gives high power. At the same time, haplotype-wise analysis, especially multi-marker analysis or sliding-window analysis, tends to increase the chance of false positive results, since numerous hypotheses are examined. Bonferroni correction is typically used for solving multiple testing problems; however, since markers are not independent due to the existence of LD, Bonferroni correction is thought to be too conservative.

Therefore, we adopted two methods to validate the observed association; firstly, we imputed ungenotyped SNPs that might reflect a significant haplotype based on observations including our genotypic distribution of tagging SNPs and LD structure from the HapMap database. However, our simulation suggests that results for ungenotyped SNPs do not provide sufficient evidence for association. In other words, there was no SNP which could reflect a significant haplotype in the current data in HapMap release II. Secondly, we examined independent sets of samples for which a significant association was obtained in the initial screening analysis. We considered this to be the best strategy at present; however, the former significance of the exon region haplotype could not be replicated though independent case–control and family trios samples.

It is unlikely that negative results are due to type II error since a large sample size was used in this study; moreover, power analyses showed that the power was more than 80% when genotype relative risk (GRR) was set at 1.2–1.65 and 1.6–3.1 for confirmation case–

control samples and family samples, respectively (MAF=2.4% and 47%), under a multiplicative model of inheritance in first-set screening samples.

Regarding interpretation of the results from this study, several limitations should be mentioned: Firstly, we did not screen the entire region of *NRG1*. On that point, Corvin et al. showed an independent 'at-risk' haplotype close to an EST cluster of unknown function (*Hs*.97362) within intron 1 of *NRG1* (Corvin et al., 2004). Secondly, our samples were not assessed with the use of the standard structured interview, and therefore have the possibility of false negatives due to misdiagnosis or sampling bias. Detailed association analyses with dense markers in the entire region of *NRG1* in well-phenotyped samples, including symptomatology, are essential in future study.

In conclusion, these results indicate that the positionally and functionally attractive regions of *NRG1* are unlikely to contribute to susceptibility to schizophrenia in the Japanese population. Moreover the nature of our results support that two-stage analysis with large sample size is appropriate to examine the susceptibility genes for common diseases; independent samples for examination of significance found in screening results should be an integral part of experimental design in genetic association analysis. Imputation methods should also be used when only haplotype association shows significance, in order to check for possible causal SNPs that can reflect the haplotype.

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Contributors

MI and NT designed the study, wrote the protocol and drafted the manuscript. MI, NT, SS, BA, YW, AN, YY, TK, YK, TK, and KK performed laboratory assays and the data-analysis. RH, HU, TI, TS, and MT advised on data-analysis. NO and NI participated in the design of the study, interpretation of the data, and drafting of the manuscript. All authors contributed to and have approved the final manuscript.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2008.01.010.

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PNAS

Glucocorticoid receptor interaction with TrkB promotes BDNF-triggered PLC-γ signaling for glutamate release via a glutamate transporter

Tadahiro Numakawa[1], Emi Kumamaru, Naoki Adachi, Yuki Yagasaki, Aiko Izumi, and Hiroshi Kunugi

Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, Japan

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An increase in glucocorticoid level, and down-regulation of BDNF (brain-derived neurotrophic factor) are supposed to be involved in the pathophysiology of depressive disorders. However, possible crosstalk between glucocorticoid- and BDNF-mediated neuronal functions in the CNS has not been elucidated. Here, we examined whether chronic glucocorticoid exposure influences BDNF-triggered intracellular signaling for glutamate release via a glutamate transporter. We found that chronic exposure to dexamethasone (DEX, a synthetic glucocorticoid) suppressed BDNF-induced glutamate release via weakening the activation of the PLC-γ (phospholipase C-γ)/Ca^{2+} system in cultured cortical neurons. We demonstrated that the GR (glucocorticoid receptor) interacts with receptor tyrosine kinase for BDNF (TrkB). Following DEX treatment, TrkB-GR interaction was reduced due to the decline in GR expression. Corticosterone, a natural glucocorticoid, also reduced TrkB-GR interaction, BDNF-stimulated PLC-γ, and BDNF-triggered glutamate release. Interestingly, BDNF-dependent binding of PLC-γ to TrkB was diminished by DEX. SiRNA transfection to induce a decrease in endogenous GR mimicked the inhibitory action of DEX. Conversely, DEX-inhibited BDNF-activated PLC-γ signaling for glutamate release was recovered by GR overexpression. We propose that TrkB-GR interaction plays a critical role in the BDNF-stimulated PLC-γ pathway, which is required for glutamate release, and the decrease in TrkB-GR interaction caused by chronic exposure to glucocorticoids results in the suppression of BDNF-mediated neurotransmitter release via a glutamate transporter.

BDNF | GR | PLC | depression | cortical neurons

Most patients with depression exhibit prolonged elevation of a glucocorticoid stress hormone, cortisol (1, 2). The blood level of glucocorticoids (cortisol in humans and corticosterone in rodents) is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (2). When excessive stress is prolonged, abnormally increased amounts of glucocorticoids may damage the CNS and cause depressive symptoms, which can be decreased with antidepressants (3–5).

Glucocorticoids function via the glucocorticoid receptor (GR), which regulates gene transcription. Glucocorticoids contribute to glucose homeostasis, cell differentiation, and inflammation (6). Additionally, glucocorticoids and the GR influence neuronal functions such as hippocampal long-term potentiation/depression (7–9) and cognitive function governed by the prefrontal cortex (10). The GR potentiates the response to NMDA in dopamine-sensitive neurons in the ventral tegmental area (11) and modulates the NMDA receptor function in the spinal cord following peripheral nerve injury (12), suggesting that the GR is involved in synaptic plasticity.

Beyond the promotion of cell differentiation, nerve growth, and neuronal survival, brain-derived neurotrophic factor (BDNF) plays a crucial role in synaptic function (13–15). For instance, BDNF increases neurotransmitter release (16, 17). We reported that BDNF rapidly induces glutamate transporter-mediated glutamate release via phospholipase C-γ (PLC-γ)/Ca^{2+} signaling and that antidepressants enhance PLC-γ/Ca^{2+} signaling (18, 19). Growing evidence has suggested a close relationship between BDNF and the pathophysiology of depression (20, 21). The BDNF level was low in the brains of suicide victims, most of whom had depressive disorders (22). BDNF plays a critical role in cognition, learning, and memory, and patients with depression exhibit deficits in these brain functions (23, 24). Both BDNF and glucocorticoids/GR are involved in synaptic function and the pathophysiology of depression. However, the possible influence of glucocorticoids on the acute action of BDNF is poorly understood.

Here we report that chronic treatment with glucocorticoids suppressed BDNF-triggered PLC-γ signaling for glutamate release via a glutamate transporter. We found that the GR interacted with receptor tyrosine kinase for BDNF (TrkB), playing an important role in BDNF action.

Results

Chronic Dexamethasone (DEX) Treatment Suppressed BDNF-Induced Glutamate Release by Inhibiting PLC-γ/Ca^{2+} Signaling. We examined BDNF-induced glutamate release in cultured cortical neurons after exposure to DEX (a synthetic GR-selective agonist). DEX pretreatment (48 h) suppressed BDNF-induced glutamate release in a dose-dependent manner (Fig. 1*Ai*). After various durations of DEX (1 μM) exposure, we found that DEX exposure for 24 or 48 h inhibited BDNF-induced glutamate release, whereas shorter exposure times (10 min to 12 h) did not (Fig. 1*Aii*). When the dose-dependency of BDNF on glutamate release was examined, DEX inhibited BDNF-induced release at any dose of BDNF (Fig. 1*Aiii*). In this study, the following experiments were performed with 100 ng/ml of BDNF. Cell viability [supporting information (SI) Fig. S1 *A* and *B*] and the number of glutamatergic and GABAergic synapses (Fig. S1 *C* and *D*) were unchanged by DEX exposure for 48 h. The endogenous GR expression in vitro and in vivo during neuronal maturation is shown in Fig. S2 *A* and *B*. In this study, we applied DEX at days in vitro (DIV) 4–5 because the expressions of the GR and synaptic proteins markedly increase (Fig. S2*A*), and BDNF-induced glutamate release begins at approximately DIV 5 (18). We confirmed the inhibitory effects of TTX (a Na^+ channel blocker) and TBOA (a glutamate transporter inhibitor) on BDNF-induced glutamate release (Fig. S3*A*). Tetanus toxin, an exocytosis inhibitor, had no effect on BDNF-induced glutamate release (Fig. S3*B*). These results suggest that BDNF-

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The authors declare no conflict of interest.

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[1]To whom correspondence should be addressed. E-mail: numakawa@ncnp.go.jp.

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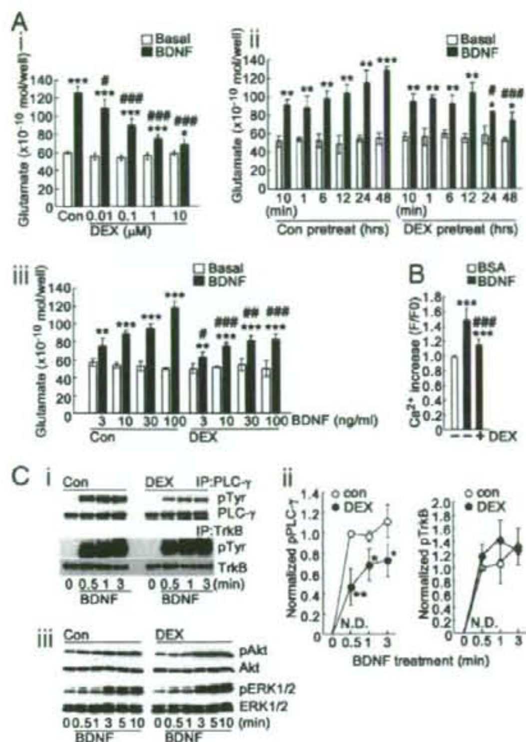

Fig. 1. Chronic DEX treatment suppressed BDNF-activated PLC-γ/Ca^{2+} signaling for glutamate release in cultured cortical neurons. (*Ai*) Dose-dependent inhibitory effect of DEX on BDNF-induced glutamate release. DEX (0.01–10 μM) was applied at DIV 4. After 48 h, BDNF (100 ng/ml, 1 min) was added. Con means no DEX treatment. Data represent mean ± SD. ($n = 4$). (*ii*) Time-course analysis of the DEX effect on BDNF-induced glutamate release. DEX (1 μM) was added at DIV 5 for the indicated durations. Data represent mean ± SD. ($n = 3$). (*iii*) Dose-dependency of BDNF on glutamate release after DEX treatment. DEX (1 μM, at DIV 4) was applied for 48 h. Then, BDNF (3–100 ng/ml) was added. Data represent mean ± SD. ($n = 5$). $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ versus basal, $^{###}P < 0.001$, $^{##}P < 0.01$, $^{\#}P < 0.05$ versus BDNF-induced release in Con. (*t* test). (*B*) DEX prevented BDNF-increased intracellular Ca^{2+}. Data from 61 randomly selected cells for each experimental condition. The fluorescence ratio (F/F_0; BDNF-induced/basal) was calculated. DEX (1 μM) was applied at DIV 4. After 24 h later, Ca^{2+} imaging was performed. $***P < 0.001$ versus vehicle (BSA) in the control. $^{###}P < 0.001$ versus BDNF-induced increase in the control. (*t* test). (*C*) Immunoprecipitation with anti-PLC-γ (*Upper*) or anti-TrkB (*Lower*) antibodies was carried out. Blotting was performed with anti-pTyr, anti-PLC-γ, or anti-TrkB antibodies. DEX (1 μM) was applied at DIV 4. After 48 h, BDNF was applied for the indicated duration. (*ii*) Quantification of pPLC-γ or pTrkB. Data represent mean ± SD. ($n = 4$). Data were normalized to the level of BDNF (0.5 min) in Con. N.D.: not detected. $**P < 0.01$, $*P < 0.05$ versus BDNF-induced in Con. (*t* test). (*iii*) DEX did not affect pAkt or pERK1/2. BDNF was applied for the indicated duration.

induced glutamate release occurs via a glutamate transporter in a Na^+-dependent manner as we previously reported (18).

BDNF-induced glutamate release depends on an intracellular Ca^{2+} increase via IP_3-sensitive Ca^{2+} channels (IP_3 receptor) (18). As expected, chronic DEX treatment weakened BDNF-increased Ca^{2+} (Fig. 1*B* and Fig. S4 *A* and *B*). We confirmed that U73122 (a PLC-γ inhibitor) and xestospongin C (Xest C, an IP_3 receptor inhibitor) blocked BDNF-increased Ca^{2+}, although BDNF still increased Ca^{2+} in the presence of APV (an NMDA receptor inhibitor), CNQX (an AMPA receptor inhibitor), or bicuculline (a $GABA_A$ receptor inhibitor) (Fig. S4 *C* and *D*), indicating the importance of the PLC-γ/IP_3 pathway. We confirmed that both U73122 and Xest C blocked BDNF-induced glutamate release in the control and DEX-treated cultures (Fig. S4*E*). These results suggest that BDNF-induced glutamate release depends on the PLC-γ pathway.

Next, we focused on PLC-γ activation (phosphorylation). A significant decline in BDNF-activated PLC-γ following chronic DEX exposure was observed, although TrkB (upstream of PLC-γ) was equally activated by BDNF with or without DEX (Fig. 1 *Ci* and *ii*). In other pathways activated by TrkB, DEX did not change activation of Akt (pAkt, phosphorylated Akt) or ERK1/2 (pERK1/2) stimulated by BDNF (Fig. 1*Ciii*).

Recently, activation of TrkB signaling within several hours of glucocorticoid exposure was reported (25). Indeed, TrkB, PLC-γ, Akt, and ERK1/2 were activated by short-term application of glucocorticoids (DEX or corticosterone) (Fig. S5 *A* and *B*). These activations reached their maximum at 2–4 h after the application and returned to the basal level at 6 h. As expected, BDNF induced much higher activation of TrkB signaling (including PLC-γ, Akt, and ERK1/2) compared with that induced by sole acute DEX (2 h) or corticosterone (2 h) exposure (Fig. S6 *A*–*C*). In contrast to chronic exposure, such a short-term treatment with DEX (2 h) or corticosterone (2 h) did not affect the exogenous BDNF-stimulated TrkB signaling, including PLC-γ. Subsequently, we focused on the suppression of BDNF-dependent PLC-γ signaling for glutamate release after long-lasting glucocorticoid exposure.

DEX-Dependent GR Down-Regulation Was Involved in the Suppressed Responses to BDNF. To investigate the mechanisms underlying the DEX-suppressed responses to BDNF, the possible involvement of the GR was examined. When DEX was coapplied with RU486 (a GR antagonist), BDNF-stimulated PLC-γ activation and glutamate release were not inhibited (Fig. 2 *Ai* and *ii* and Fig. 2*B*), suggesting that DEX acts via the GR. Thus, endogenous GR expression after DEX addition was examined. Marked down-regulation of the GR was observed following DEX application for 24 to 48 h (Fig. 2*C*). DEX induced GR down-regulation in a dose-dependent manner (Fig. 2 *Di* and *ii*), while the mineral corticoid receptor (MR, the other receptor for glucocorticoid) and TUJ1 (class III β-tubulin, a neuronal marker) expression was intact (Fig. 2*Di*). Immunocytochemistry with anti-microtubule-associated protein 2 (MAP2) and anti-GR antibodies was performed, and the quantification of the staining indicated a DEX-dependent GR reduction in neurons (Fig. 2 *Ei*–*vii*). As expected, RU486 recovered GR down-regulation by DEX (Fig. 2 *Fi* and *ii*). Corticosterone also down-regulated the GR and BDNF-induced glutamate release (Fig. S7 *A*–*C*). Moreover, the suppression of BDNF-activated PLC-γ by corticosterone was also observed (Fig. S7 *D* and *E*). We confirmed that the GR was markedly reduced in the homogenates of the cerebral cortex prepared from rats after DEX administration, although the MR level was intact (Fig. S7 *F* and *G*). These results suggested that the inhibitory action of glucocorticoid results from the down-regulation of the GR.

Then, the effects of GR overexpression were examined. After viral infection, about 85% of MAP2-positive cells in either the control (GFP) or GR-overexpressing (GR and GFP) cultures were GFP-positive, indicating that the majority of neurons were infected (Fig. 3*Ai*). Blotting with anti-GR and anti-GFP antibodies showed GR overexpression (Fig. 3*Aii*), which enhanced BDNF-induced glutamate release (Fig. 3*B*). DEX failed to reduce BDNF-activated PLC-γ in GR-infected cultures (Fig. 3 *Ci* and *ii*). Next, siRNA was used to examine the function of endogenous GR. Approximately 60% of endogenous GR was depleted by GR-siRNA (Fig. 3*D*), and BDNF-induced glutamate