Genetic analysis of the calcineurin pathway identifies members of the *EGR* gene family, specifically *EGR3*, as potential susceptibility candidates in schizophrenia

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The calcineurin cascade is central to neuronal signal transduction, and genes in this network are intriguing candidate schizophrenia susceptibility genes. To replicate and extend our previously reported association between the *PPP3CC* gene, encoding the calcineurin catalytic γ-subunit, and schizophrenia, we examined 84 SNPs from 14 calcineurin-related candidate genes for genetic association by using 124 Japanese schizophrenic pedigrees. Four of these genes (*PPP3CC*, *EGR2*, *EGR3*, and *EGR4*) showed nominally significant association with schizophrenia. In a postmortem brain study, *EGR1*, *EGR2*, and *EGR3* transcripts were shown to be down-regulated in the prefrontal cortex of schizophrenic, but not bipolar, patients. These findings raise a potentially important role for *EGR* genes in schizophrenia pathogenesis. Because *EGR3* is an attractive candidate gene based on its chromosomal location close to *PPP3CC* within 8p21.3 and its functional link to dopamine, glutamate, and neuregulin signaling, we extended our analysis by resequencing the entire *EGR3* genomic interval and detected 15 SNPs. One of these, IVS1 + 607A→G SNP, displayed the strongest evidence for disease association, which was confirmed in 1,140 independent case-control samples. An *in vitro* promoter assay detected a possible expression-regulatory effect of this SNP. These findings support the previous genetic association of altered calcineurin signaling with schizophrenia pathogenesis and identify *EGR3* as a compelling susceptibility gene.

genetic association | immediate early gene | postmortem brain | enhancer

Schizophrenia is a devastating psychiatric disease with a complex genetic etiology. Despite a high heritability of ≈80%, the major genetic components underlying disease susceptibility and pathology have remained elusive, although significant progress in this area has been made in recent years (1). Along with genetic studies, a large body of pharmacological evidence suggests that alterations in the regulation of or interactions between the dopamine and glutamate neurotransmitter systems may comprise important contributing factors in the pathophysiology of schizophrenia (2). Recently, we have provided evidence suggesting that variation in the *PPP3CC* gene, encoding the calcineurin A γ-subunit, contributes to schizophrenia susceptibility based on behavioral characterization of CNB1 knockout mice (3) and collaborative human genetic association studies (4). These studies provided support for the idea that alterations in calcineurin function contribute to schizophrenia pathogenesis.

Calcineurin (protein phosphatase 2B) is a member of the serine/threonine protein phosphatase family and the only known phosphatase that can be activated by Ca^{2+} and calmodulin. Calcineurin functions as a heterodimer consisting of a catalytic subunit, calcineurin A (CNA), and regulatory subunit, calcineurin B (CNB). Three mammalian isoforms of CNA [CNAα (*PPP3CA*), CNAβ (*PPP3CB*) and CNAγ] and two of CNB [CNB1 (*PPP3R1*) and CNB2 (*PPP3R2*)] have been identified. The expression of calcineurin is particularly enriched in the CNS, where it plays a critical role in the regulation of a diverse array of neuronal functions (5, 6). Interestingly, calcineurin is positioned downstream of dopaminergic signaling (7) and is involved in NMDA receptor-mediated synaptic plasticity (8) and could therefore provide an important functional link between these two neurotransmitter systems. To further explore the involvement of calcineurin dysfunction in schizophrenia, we have tested for genetic association of a subset of calcineurin-related candidate genes with schizophrenia in Japanese population samples as an extension of the prior study (4). Our results support the association of *PPP3CC* with schizophrenia and provide genetic and biological evidence for the involvement of altered early growth response (EGR) transcription factor signaling in schizophrenia pathogenesis.

Results

Family-Based Association Screening. In this study, we examined the following 14 calcineurin-related genes: *PPP3CA*, *PPP3CB*, *PPP3CC*, *PPP3R1*, *PPP3R2*, *RYR3* (ryanodine receptor 3), *EGR1*, *EGR2*, *EGR3*, *EGR4*, *FKBP5* (FK506 binding protein 5), *FKBP1A* (FK506 binding protein 1A), *CDK5* (cyclin-dependent kinase 5), and *PPP1R1B* (DARPP32). We analyzed a total of 84 SNPs in 124 Japanese schizophrenia pedigrees consisting of 374 individuals. The potential functional interactions of these genes are illustrated in supporting information (SI) Fig. 4. The results of the genetic analyses are shown in SI Table 3 along with the SNP information. We detected nominally significant transmission disequilibrium for *PPP3CC* (8p21.3), *EGR2* (10q21.3), *EGR3* (8p21.3), and *EGR4* (2p13.2), providing confirmatory evidence for the previously reported genetic association of *PPP3CC* and schizophrenia in patient samples from the United States and South Africa (4) (Table 1). The adjacent SNPs in the gene might be in linkage disequilibrium (LD), so applying a Bonferroni correction for the multiple markers would be overly conservative (Bonferroni-corrected $P = 0.0756$). Applying a permutation procedure for multiple test correction yielded a globally significant P value for the multiple markers tested in the

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Abbreviations: LD, linkage disequilibrium; PDT, pedigree disequilibrium test; DLPFC, dorsolateral prefrontal cortex; EGR, early growth response; NFAT, nuclear factor of activated T cells.

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Table 1. Family-based association analysis of SNPs in calcineurin-related genes

Gene (chromosomal location)	rs ID	Our SNP ID	Distance, kb	PDT SUM	PDT AVE	Over-/undertransmitted allele
PPP3CC (8p21.3)	rs1049437	M038 (CC1a)		0.0023	0.0038	C/A*
			60.9			
	rs2449348	M062 (CC33)		0.0348	0.0180	G/A*
			1.7			
	rs2461491	M063 (CCS3)		0.0208	0.0185	T/C*
EGR2 (10q21.3)	rs2297488	EGR2-J2		0.0295	0.0141	A/G
			4.0			
	rs2295814	EGR2-J4		0.0469	0.0214	A/G
EGR3 (8p21.3)	rs35201266	M228		0.0045	0.0009	T/C
			0.9			
	rs3750192	M227		0.0171	0.0115	T/G
EGR4 (2p13.2)	rs6747506	EGR4-J3		0.0195	0.0247	A/T
			3.3			
	rs2229294	EGR4-J6		0.0111	0.0193	T/C
			1.8			
	rs3813226	EGR4-J7		0.0123	0.0234	A/G
			10.1			
	rs6737049	EGR4-J8		0.0208	0.0386	A/G
			NA			
RYR3 (15q14)	rs2303310	RYR3-J18		0.2836	0.0397	T/A

Under PDT, SUM gives more weight to larger families, whereas AVE places equal weight on all families. NA, not applicable.
*These results are consistent with those of our prior study (4).

first screening (adjusted $P = 0.0353$). In addition to the support for *PPP3CC* association, this study provides evidence for the role of *EGR* family genes as potential predisposing factors to schizophrenia. Of the three nominally associated *EGR* genes, *EGR3* lies close to *PPP3CC* on the short arm of chromosome 8 within a 252-kb genomic interval, which raises the question of whether these two genes contribute to schizophrenia onset independently. We addressed this issue by dividing the families into two groups according to the transmission status of the *PPP3CC* risk allele in probands and reanalyzing the data. The results supported independent roles for the two genes, because the significance of association between *EGR3* and schizophrenia increased in families in which the *PPP3CC* risk allele was not transmitted [the most significant $P = 0.0004$ for SNP M228 with pedigree disequilibrium test (PDT) software (PDT-AVE)]. Next, we scrutinized the 564-kb genomic region of 8p21.3 that encompasses both genes by genotyping 49 SNP markers (SI Table 3), determining the LD structure and the resolution of the association signals. These analyses revealed that *PPP3CC* and *EGR3* are located in separate LD islands on the genome (Fig. 1). In addition, the association signal appears to be divided into two major peaks roughly corresponding to the two gene regions (Fig. 1). Therefore, the two genes at 8p21.3, *PPP3CC* and *EGR3*, are likely to confer independent risk for schizophrenia.

Transcript Expression in Postmortem Brains. To examine whether altered expression of *PPP3CC* or *EGR* genes could be associated with schizophrenia pathophysiology, we performed quantitative RT-PCR assays for mRNA levels of the five genes in the dorsolateral prefrontal cortex (DLPFC; Brodmann's area 46) of schizophrenia, bipolar disorder, and control brains. The DLPFC has been implicated in the pathology of schizophrenia by a number of studies. Interestingly, the expression of *EGR1*, *EGR2*, and *EGR3* was down-regulated in a schizophrenia-specific manner, whereas *PPP3CC* mRNA levels did not differ among the three groups (Fig. 2). *EGR4* mRNA could not be reliably detected in the DLPFC.

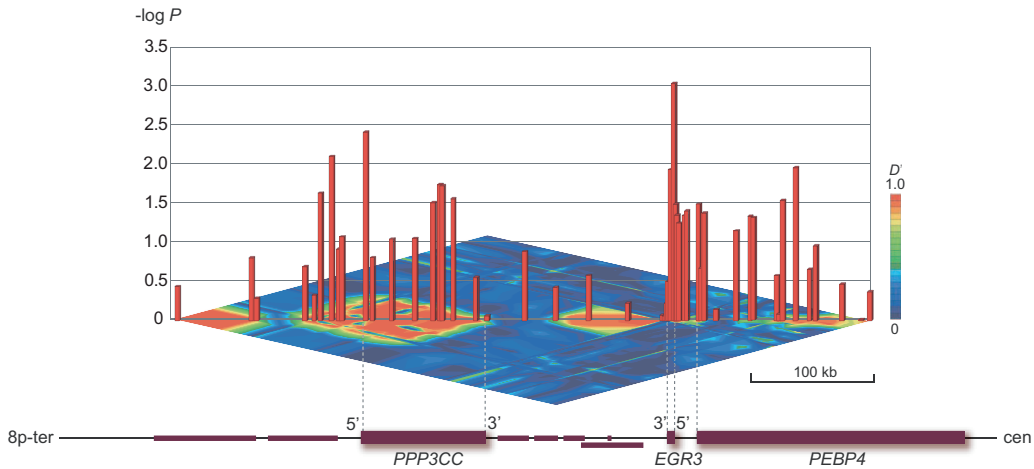

Fig. 1. LD structure of the 8p21.3 locus measured by D' (horizontal surface) and association P values (vertical red bars). D' was calculated by using the genotype data of unrelated individuals in the pedigree sample set. The negative log (P value) computed with PDT-AVE was plotted against the physical location of each SNP.

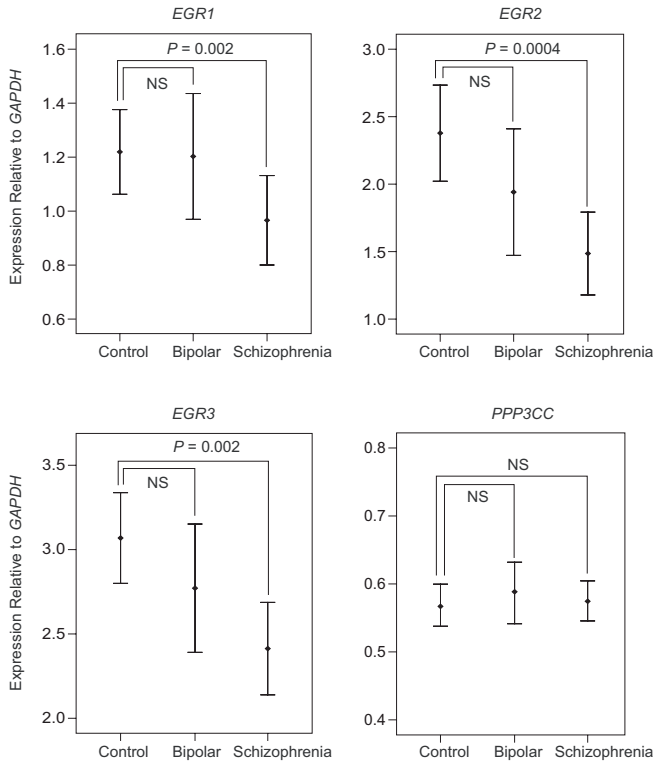

Fig. 2. mRNA levels in the DLPFC region of postmortem brains. The mean and 95% C.I. values are presented. *P* values were calculated by using the Mann–Whitney *U* test (two-tailed). NS, not significant.

Detailed Genetic Analysis of *EGR3* in Schizophrenia Pedigrees. The genetic and expression studies described above provide support for the *EGR* family genes, especially *EGR2* and *EGR3*, as potential schizophrenia susceptibility genes. To further analyze *EGR3*, we resequenced the genomic interval (≈10 kb) spanning the gene and identified 15 SNPs occurring in the gene locus (SI Table 4). From these, we selected six common and informative (minor allele frequency > 10% in our sample) SNPs for further genetic association analyses (Fig. 3*A*). We observed the strongest association with SNP M228 (IVS1 + 607A→G) ($P = 0.001$ for PDT-AVE) in intron 1, with three nearby SNPs (M227, M231, and M232) also exhibiting nominal evidence or a trend for association ($P = 0.012$, 0.044, and 0.056, respectively for PDT-AVE). Because these four SNPs constitute a single haplotype block, we examined the preferential transmission of haplotypes comprising the four SNPs. Four common haplotypes were analyzed, and two of them (haplotypes 2 and 4) were overtransmitted and one (haplotype 1) was undertransmitted to schizophrenic probands (Table 2).

Replication Analysis of *EGR3* in Case-Control Samples. We attempted to replicate the *EGR3* association finding by examining the SNPs in independent case-control samples comprising 1,140 individuals (570 unrelated schizophrenics of Japanese origin and 570 age-/sex-matched controls of the same ethnicity). To avoid confounding results due to population stratification, we first estimated the amount of stratification in our case-control samples by genotyping 30 unlinked genome-wide SNPs. STRUCTURE 2.1 (9) was used to identify genetically similar diploid subpopulations by grouping individuals. The study design has been described in detail elsewhere (10). This analysis confirmed that population stratification should not account for association signals in the present sample panel [$Pr(K = 1) > 0.99$].

We detected a consistent pattern of association, with SNPs M227 and M228 displaying significant genotypic *P* values ($P = 0.037$ and 0.045, respectively) and "haplotype 1" being underrepresented in schizophrenia (Table 2). Importantly, the percentage of transmitted frequency–percentage of nontransmitted frequency relationship of each haplotype in the pedigree samples perfectly matched the overrepresentation/underrepresentation relationship of the haplotypes in the case-control samples (Table 2). Because the four common haplotypes are not independent, we performed 10,000 permutations using the data to compute empirical *P* values for haplotypic association. We obtained a significant global $P = 0.0127$ for the triad sample panel and a significant global $P = 0.0379$ for the case-control panel (Table 2). The odds ratio (approximate relative risk associated with the causative variant) of the putative protective haplotype 1 was 0.821, and that of the putative risk haplotype 4 was 1.371 (Table 2). The replication of the association between the *EGR3* gene and schizophrenia in an independent case-control set, with consistent SNP and haplotype distribution patterns, adds further support to the genetic impact of this gene in schizophrenia.

In the case-control panel, *PPP3CC* showed a trend for allelic association with SNPs M044 ($P = 0.0978$) and M063 ($P = 0.0878$).

Functional Role of *EGR3* SNPs. The IVS1 + 607A→G SNP of *EGR3* demonstrated the strongest and most consistent evidence of association in the two sample sets. It is notable that the intron 1 sequences are well conserved among mouse, rat, and human orthologues, suggesting a functional significance of the *EGR3* intron 1 sequence. Because it is possible that this SNP is located in a transcriptional regulatory sequence, we examined whether the IVS1 + 607A→G genotype affected *EGR3* mRNA levels by quantitative RT-PCR with postmortem brain samples from control subjects to avoid potential confounding factors characteristic of patient samples. A trend for genotype-dependent expression levels of *EGR3* was detected ($P = 0.091$ by Kruskal–Wallis test; expression levels were reduced in the A/A genotype group), but the sample size was too small to draw a definitive conclusion. To further examine this issue, we used an *in vitro* reporter assay system to study the effects of the SNP on transcript levels. The intron 1 sequence with either IVS1 + 607A or IVS1 + 607G was introduced into a vector in which a simian virus 40-derived promoter drives expression of a luciferase-coding sequence. The IVS1 + 607G-containing construct showed higher transcriptional activity relative to the IVS1 + 607A construct in the NB1 cell line ($P = 0.011$ by Student's *t* test), but little difference in transcriptional activity between the two constructs was observed when the IMR32 cell line was used (Fig. 3*B*). The results suggest that IVS1 + 607A→G confers a functional alteration under certain conditions.

We also tested whether the 5′ SNPs, M232 and M231, affected promoter activity. We prepared constructs with the "protective" haplotype A–C (with A at M232 and C at M231) or the opposite haplotype G–G (with the first G at M232 and the second G at M231). There were no significant differences in the transcriptional activities of the two constructs (Fig. 3*C*), suggesting no functional effects of these SNPs in the putative promoter region.

Discussion

In this study, we performed genetic analysis of calcineurin-related genes in two sets of Japanese schizophrenia samples followed by postmortem brain mRNA expression studies and *in vitro* functional assays of identified variants. We obtained genetic evidence for association of the *PPP3CC* gene in the first set of 124 schizophrenia pedigrees. This finding replicates the previous results of association for this gene in 210 ethnically diverse triads from the U.S. and 200 triads from South Africa (4) (i.e., the third replication). Regarding the reported risk haplotype, C-T-G-C-A, this haplotype was overtransmitted in Japanese triad samples (transmitted/nontransmitted = 1.375) and overrepresented in Japanese case-control samples (case/control = 30.8%/27.6%), although these overrepresentations did not achieve significance in these samples ($P = 0.083$ for triads,

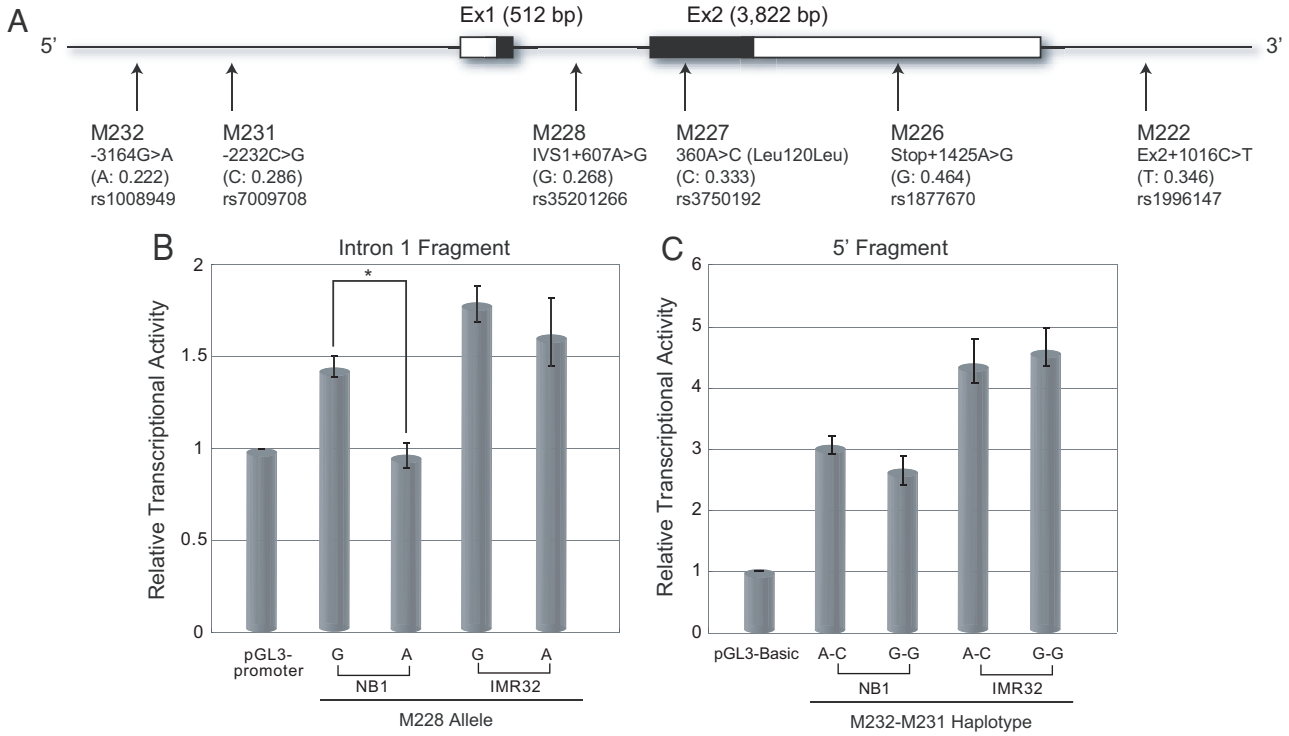

Fig. 3. Location, genetic association, and function of *EGR3* SNPs. (*A*) Genomic structure and location of SNP sites for *EGR3*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons and minor allele frequency of each SNP in the 28 samples used for mutation screening are also shown. (*B*) Luciferase assay for enhancer-like activity of the *EGR3* intron 1. The effects of SNP M228 (IVS1 + 607A→G) were examined in the two cell lines NB1 and IMR32. Values represent the means ± SE of at least two independent transfections, each with triplicate determinations. *, $P = 0.011$ by Student's *t* test. (*C*) Luciferase assay for promoter activity of the 5′ upstream sequence of *EGR3*. The effects of haplotypes constructed by SNPs M232 (−3164A→G) and M231 (−2232C→G) were examined as in *B*.

$P = 0.108$ for case controls). In addition, we obtained evidence for genetic association of *EGR* family genes *EGR2*, *EGR3*, and *EGR4*, with schizophrenia in the Japanese pedigree sample. The association was replicated for *EGR3* in an independent set of Japanese case-control samples. Furthermore, expression of *EGR2* and of *EGR3* was found to be decreased in the DLPFC region of schizophrenic brains relative to healthy controls. In contrast, no change in expression of *PPP3CC* was detected in the DLPFC of schizophrenia patients relative to controls. A recent report, however, showed that *PPP3CC* mRNA levels were decreased in the hippocampus of schizophrenics (11). Together, the genetic and expression studies provide support for the involvement of alterations in EGR signaling in schizophrenia pathogenesis.

Altered EGR function could contribute to schizophrenia pathogenesis through a variety of mechanisms. The *EGR* genes encode transcription factors, sharing a highly conserved DNA binding domain that consists of three zinc finger motifs. EGR family members are known to be important mediators of gene transcription in neuronal development and plasticity and are involved in commitment to mitogenic, other differentiative, and apoptotic pathways (12).

A number of knockout models for members of the EGR family have been described (12–16). Notably, *Egr2* and *Egr3* have been shown to be necessary for normal brain development. *Egr2* (also known as *Krox20*) knockout mice exhibited defects in hindbrain patterning and peripheral nerve myelination (16). *Egr3* is also essential for the development of muscle spindles in mice, and homozygous *Egr3* knockout mice exhibited a number of symptoms, including gait ataxia and scoliosis (14, 15).

EGR2 and *EGR3* are of particular interest as downstream target genes for calcineurin. Calcineurin and interacting proteins are uniquely positioned to regulate dopaminergic, glutamatergic, and other neuronal signaling pathways, alterations of which are observed in schizophrenia (17, 18). *Egr2* and *Egr3* are regulated by calcineurin through activation of nuclear factor of activated T cells (NFAT)-mediated transcription (19). NFAT and Egr2/Egr3 are key

Table 2. Association results for *EGR3* in the pedigree panel and case-control samples

	Marker				Family-based association				Population-based association			
Haplotype	M232	M231	M228	M227	Freq-T	Freq-NT	%T	*P**	Case	Control	OR (95% CI)	*P***
1	A	C	G	C	0.375	0.547	40.7	**0.0057**	0.401	0.450	0.821 (0.695–0.969)	**0.0249**
2	G	G	A	A	0.289	0.148	66.1	**0.0061**	0.237	0.234	1.020 (0.840–1.237)	0.8110
3	G	G	G	C	0.203	0.203	50.0	1.0000	0.212	0.212	1.000 (0.818–1.222)	0.9727
4	G	C	A	C	0.109	0.078	58.3	0.3901	0.102	0.076	1.371 (1.025–1.834)	**0.0435**

The four common haplotypes are shown, with significant *P* values in bold (global *P* values: *, 0.0127; **, 0.0379). The Case and Control columns show corresponding estimated haplotype frequencies in each group. Freq-T (Freq-NT), estimated frequency of the parental haplotype transmitted (nontransmitted) to an affected offspring; %T, estimated frequency with which the haplotype is transmitted from a heterozygous parent to an affected offspring; OR, odds ratio.

molecules in T cell activation (20). A recent study showed that mice deprived of mature T cells manifest cognitive deficits and behavioral abnormalities consistent with those observed in schizophrenia (21). Moreover, the copolymer glatiramer acetate (copolymer-1, Cop-1), a weak agonist of self-antigens, leads to the activation of T suppressor cells that can counteract the psychotomimetic effects of dizolcipine maleate (MK-801) and amphetamine (21). Overexpression of *Egr2* and *Egr3* has been shown to inhibit the transcription of IL-2 (20, 22), and altered IL-2 regulation has been reported in schizophrenia (23, 24).

In addition to their downstream position in calcineurin signaling, EGR genes interact with a number of other factors implicated in schizophrenia. For instance, *neuregulin 1* (*NRG1*) has been identified as a potential susceptibility gene for schizophrenia (25), and alterations in NRG1-ErbB (one of the NRG1 receptors) signaling have been shown to occur in the prefrontal cortex of schizophrenic patients (26, 27). EGR1, EGR2, and EGR3 are downstream targets for NRG1 (28) and have been shown to be regulated by the epidermal growth factor β1 domain of NRG1 in cultured human muscle cells. This regulatory effect of the β1 domain is most pronounced for EGR3 (28). It is intriguing that defects in NRG1-ErbB signaling could affect oligodendrocyte development and myelination (26, 29), given that multiple lines of evidence converge to implicate abnormalities in oligodendroglia and myelin in schizophrenia (30).

Alterations in Akt/GSK3β (glycogen synthase kinase-3β) signaling comprise another proposed contributing factor in schizophrenia pathogenesis (31). Calcineurin is linked to this cascade as a critical regulator of apoptosis through interaction with the Bcl-2 family member, BAD, a substrate of Akt (32). Akt can induce apoptosis by a Fas ligand-dependent mechanism, in which the Fas ligand gene is regulated by a number of transcription factors, including NFAT and Egr3 (33). In addition, GSK3β antagonizes calcineurin signaling by phosphorylation of NFAT proteins, leading to nuclear export of NFAT (34).

It is noteworthy that expression of the entire family of EGRs can be regulated by muscarinic acetylcholine receptors (35). Mounting evidence suggests the alteration of the muscarinic cholinergic system in schizophrenia (36). For example, muscarinic receptors have been shown to be decreased in the cortex of schizophrenia patients (37–39). Moreover, mice lacking the M1 or M4 muscarinic acetylcholine receptor exhibit locomotor behavioral abnormalities (40, 41).

In this study, evidence for the association of *EGR3* with schizophrenia was obtained in the pedigree panel and in an independent case-control sample, revealing consistent risk (excessive transmission) and protective (undertransmission) haplotypes and a significant genetic impact of the intron 1 SNP, IVS1 + 607A→G. The risk A allele of IVS1 + 607A→G elicited decreased transcription, possibly through reduced enhancer-like activity. Given that postmortem brain studies are fraught with confounding factors, this *in vitro* test could be considered a preliminary attempt to assign a function to the potential causative SNP. Reduced function of activity-dependent immediate early genes like *EGR3* may contribute to the well recognized "hypofrontality" of schizophrenia, which is characterized by impairments in neurocognitive domains, such as attention, working memory, language skills, and executive functioning (42). The *Egr3* gene has two alternative translation start sites, and the two derived proteins have distinct transcriptional activation properties (43). A potential mechanistic relationship between the differential function of these two protein forms and schizophrenia is an interesting issue for further investigation.

Transcription of the *EGR* genes has been reported to be regulated by the methylation status of CpG islands in intron 1 of these genes (44, 45). Therefore, epigenetic studies focusing on *EGRs* are of particular interest.

In conclusion, this study provides genetic and biological evidence that *PPP3CC* and *EGR3*, both constituents of the calcineurin signaling pathway, may independently elicit a modestly increased risk for schizophrenia. Hopefully, further exploration of calcineurin-related signal transduction and the downstream signaling cascade of EGR family genes will contribute to our understanding of the molecular basis of schizophrenia pathogenesis, given the genetic and allelic heterogeneities of the disease across different populations.

Materials and Methods

Subjects. Two sets of schizophrenia samples were constructed. The first consisted of 124 nuclear families (376 members, of whom 163 were affected) for a family-based association test. The details of this sample are described elsewhere (46). The second set was an independent case-control sample panel, which comprised 570 unrelated schizophrenics (285 males and 285 females; mean ± SD age, 47.0 ± 11.4 years), and 570 age- and sex-matched controls who showed no history of mental illness in a brief psychiatric interview or structured interview by experts (285 males and 285 females; mean ± SD age, 46.7 ± 11.1 years). All participants were recruited from a geographic area located in central Japan, and all patients were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). The present study was approved by the ethics committee of RIKEN, and all participants provided their written informed consent.

SNP Discovery, Selection, and Genotyping. Analyzed SNPs were selected from University of California (Santa Cruz, CA) Genome Bioinformatics version May 2004 (http://genome.ucsc.edu) and National Center for Biotechnology Information database (http://www.ncbi.nih.gov). DNA was extracted from whole blood according to a standard protocol. Assays-by-Design SNP genotyping products were used to score SNPs based on the TaqMan assay method (Applied Biosystems, Foster City, CA). Each marker was checked for allele-inheritance inconsistency within a pedigree by using PEDCHECK software (47). Samples in which ambiguities could not be resolved after regenotyping by sequencing were omitted from subsequent analyses.

Genetic Statistical Analyses. All members of the 124 families were analyzed with the PDT program, version 5.1 (http://www.chg.duke.edu/research/pdt.html) (48). Allele and genotype distributions between patients and controls were compared by using Fisher's exact test. Haplotype frequencies, normalized LD coefficient D', squared correlation coefficient r^2, and a global test of haplotype transmission were calculated with UNPHASED software (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased) (49). Empirical significance levels were simulated from 10,000 Monte Carlo permutations. Graphical overview of pairwise LD strength between markers was made by using GOLD software (http://well.ox.ac.uk/asthma/GOLD) (50).

Brain Tissues and Quantitative RT-PCR. RNA from the DLPFC (Brodmann's area 46) was obtained from the Stanley Medical Research Institute (http://www.stanleyresearch.org/programs/brain_collection.asp). Samples were taken from 35 schizophrenics [26 males and 9 females; mean ± SD age, 42.6 ± 8.5 years; postmortem interval (PMI), 31.4 ± 15.5 h; brain pH, 6.5 ± 0.2], 35 bipolar disorder patients (17 males and 18 females; mean ± SD age, 45.3 ± 10.5 years; PMI, 37.9 ± 18.3 h; brain pH, 6.4 ± 0.3), and 35 controls (26 males and 9 females; mean ± SD age, 44.2 ± 7.6 years; PMI, 29.4 ± 12.9 h; brain pH, 6.6 ± 0.3). Diagnoses were made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition. There were no significant demographic differences between the schizophrenia, bipolar, and control brains (51). All schizophrenic patients were medicated with antipsychotics. Quantitative RT-PCR analysis was conducted by using an ABI7900HT Fast Real-Time PCR System (Applied Biosystems). TaqMan probes and primers for *PPP3CC*, *EGR*s, and *GAPDH*

(internal control) were from Assay-on-Demand or Assay-by-Design gene expression products (Applied Biosystems). All quantitative RT-PCRs were performed in triplicate, based on a standard curve method. The Mann–Whitney *U* test (two-tailed) was used to detect significant changes in target gene expression levels.

Resequencing Analysis of the *EGR3* Locus. The genomic interval (≈10 kb) encompassing 3.5 kb upstream of exon 1 of *EGR3* to 1.3 kb downstream from exon 2 was screened for SNPs by direct sequencing of PCR products, using 28 unrelated schizophrenia samples. Information on the primers and conditions used for amplification are available upon request. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems) and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). SNPs were detected with the SEQUENCHER program (Gene Codes, Ann Arbor, MI).

Luciferase Reporter Assay. For the assay of intron 1 enhancer-like activity for *EGR3*, the entire intron 1 fragment (1,308 bp) containing either IVS1 + 607A or IVS1 + 607G was cloned into the pGL3-Promoter plasmid containing a simian virus 40 promoter without enhancer sequences (Promega, Madison, WI). For assaying the 5′ upstream sequence promoter activity of *EGR3*, a fragment containing either the (−3164A)-(−2232C) or (−3164G)-(−2232G) haplotype was cloned into the pGL3-Basic plasmid (Promega). The vector pGL3-Control containing the simian virus 40 promoter and enhancer (Promega) was used as a positive control. The day before transfection, NB1 or IMR32 cells (the Japanese Collection of Research Bioresources Gene Bank, http://genebank.nibio.go.jp/gbank/index_e.html) were plated at 1×10^5 cells per well in a 24-well plate and grown in DMEM (Sigma, St Louis, MO) supplemented with 10% FBS (Equitech-Bio, Kerrville, TX). One microgram of a test plasmid was transiently cotransfected into the cells with 0.1 μg of pRL-TK plasmid (an internal standard reporter) (Promega) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 48 h, the dual-luciferase assay was performed by using the PicaGene Dual SeaPansy kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

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