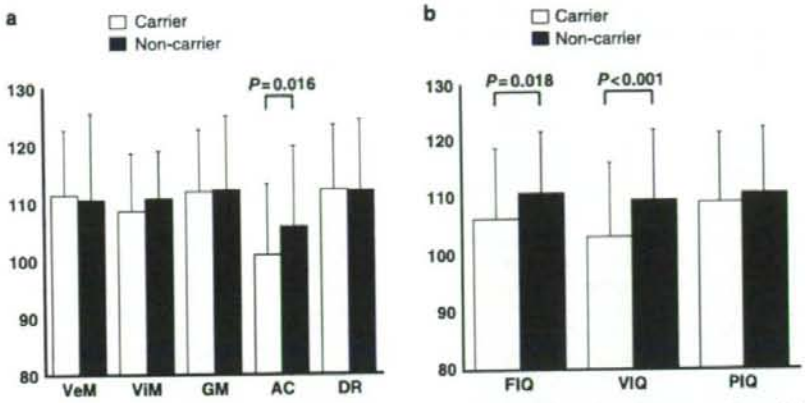

Figure 3 Relationship of memory and IQ with genotype of SNP23. Memory and IQ were compared between those who carried the G allele (carrier: G/G or A/G) of SNP23 and those who did not (non-carrier: A/A). (a) Memory and genotype. VeM, verbal memory; ViM, visual memory; GM, general memory; AC, attention and concentration; DR, delayed recall. (b) IQ and genotype. FIQ, full-scale IQ; VIQ, verbal IQ; PIQ, performance IQ.

we did not conduct structured interview for diagnosis of the patients. However, consensus diagnosis was made by at least two psychiatrists one of whom was in charge of the patients; thus, the possibility of misdiagnosis might be minimal. In addition, our sample size (207 bipolar disorder subjects and 727 controls) was not very large, and thus further investigations in other samples are required to draw any conclusion. With respect to schizophrenia or major depression, we did not obtain any evidence for association with hNP.

Interestingly, we found significant association of memory and IQ with the hNP gene in healthy subjects. Carrying the G-allele of SNP23, the risk allele for bipolar disorder, was associated with lower score in attention/concentration assessed with the WMS-R ($P=0.016$) and lower verbal IQ assessed with WAIS-R ($P<0.001$). The evidence for the former association (with attention/concentration) was weak and it would not be significant any more after correcting for multiple testing; however, the latter association (with verbal IQ) was highly significant and remained significant even when multiple testing was taken into consideration. Since bipolar disorder shows a wide range of cognitive deficits, including memory and IQ (Schretlen *et al*, 2007; Daban *et al*, 2006), the observed impact on intelligence may have some relevance to susceptibility to bipolar disorder. However, given that deficits in intelligence and memory are generally worse in schizophrenia than in bipolar disorder, alterations in hNP may have some effects specific to molecular mechanisms of bipolar disorder.

NP is a secretory serine protease that degrades cell adhesion molecule L1 (CAM-L1) (Matsumoto-Miyai *et al*, 2003) and is possibly involved in the synaptogenesis and maturation of orphan and small synapses (Nakamura *et al*, 2006). Furthermore, NP has been shown to be involved in activity-dependent synaptic plasticity, that is, LTP and kindling epileptogenesis (Komai *et al*, 2000; Okabe *et al*, 1996). As mentioned above, the type 2 splice variant has been shown to be expressed as abundant as the type 1 in human brain (Mitsui *et al*, 1999) and the hominoid-specific form (Li *et al*, 2004), which occurred through a human-specific T-to-A mutation (c.71–127T>A) during primate evolution (Lu *et al*, 2007). Taken together, NP is involved in synaptic plasticity via modulation of synaptic structure, and may play an important role in brain function of higher order such as learning, memory, and mental disorders. With respect to psychiatric diseases, indeed, altered expression levels of CAM-L1 mRNA and protein have been reported in postmortem brains of depressed patients (Laifenfeld *et al*, 2005). In line with this, chronic antidepressants increase expression levels of CAM-LI in rats (Sairanen *et al*, 2007; Laifenfeld *et al*, 2002). It would be intriguing to examine the expression levels of NP in postmortem brains of psychiatric patients.

We found that SNP23 is most associated with bipolar disorder among the examined SNPs. Haplotype-based analysis did not yield any stronger results, suggesting that SNP23 may be responsible for giving susceptibility to bipolar disorder. In addition, SNP23 showed strong impact on verbal IQ in healthy subjects. SNP23 is located 69 bp downstream to the 3′ end of exon 6 (the final exon). Thus SNP23 is on the 3′ regulatory region of the hNP gene. Growing evidence has shown that 3′ regulatory regions of human genes play an important role in regulating mRNA 3′ end formation, stability/degradation, nuclear export, subcellular localization, and translation, and are consequently rich in regulatory elements. Indeed, several diseases have been reported to be associated with variants in the 3′ regulatory region (Chen *et al*, 2006). Notably, the major allele (A) of SNP23 differs from the corresponding base (G) in monkeys or apes (ie, rhesus macaques or chimpanzees), according to the UCSC database, and thus it is not evolutionally conserved. It is interesting that carriers of the G allele were found to be poorer in memory and IQ subscales than individuals with A/A genotype in the present study. Although SNP23 is not located on obvious motifs or conserved sequence elements, it is also possible that this human-specific mutation may contribute to the higher memory and intelligence functions in humans. If our results

are replicated in other samples, it is important to elucidate the possible functional effects of SNP23 on regulation of hNP mRNA, which may contribute to understanding of the pathogenesis of bipolar disorder and brain function of higher order specific to human beings.

ACKNOWLEDGEMENTS

This study was supported by Health and Labor Sciences Research Grants (Research on Psychiatric and Neurological Diseases and Mental Health; Research on Human Genome and Tailor-made medicine), the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug innovation), Grant-in-Aid for Scientific Research on Priority Areas of Applied Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Ms Tomoko Shizuno for technical assistance.

DISCLOSURE/CONFLICT OF INTEREST

All authors declare that they have no conflict of interests to disclose.

REFERENCES

American Psychiatric Association (1994). *Diagnostic and Statistical Manual of Mental Disorders*, 4th edn. American Psychiatric Press: Washington, DC.

Badenhop RF, Moses MJ, Scimone A, Mitchell PB, Ewen-White KR, Rosso A *et al* (2002). A genome screen of 13 bipolar affective disorder pedigrees provides evidence for susceptibility loci on chromosome 3 as well as chromosomes 9, 13 and 19. *Mol Psychiatry* 7: 851–859.

Barrett JC, Fry B, Maller J, Daly MJ (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263–265.

Chen JM, Férec C, Cooper DN (2006). A systematic analysis of disease-associated variants in the 3′ regulatory regions of human protein-coding genes I: general principles and overview. *Hum Genet* 120: 1–21.

Chen ZL, Yoshida S, Kato K, Momota Y, Suzuki J, Tanaka T *et al* (1995). Expression and activity-dependent changes of a novel limbic-serine protease gene in the hippocampus. *J Neurosci* 15: 5088–5097.

Daban C, Martinez-Aran A, Torrent C, Tabarés-Seisdedos R, Balanzá-Martínez V, Salazar-Fraile J *et al* (2006). Specificity of cognitive deficits in bipolar disorder *versus* schizophrenia. A systematic review. *Psychother Psychosom* 75: 72–84.

Dudbridge F, Koeleman BP, Todd JA, Clayton DG (2000). Unbiased application of the transmission/disequilibrium test to multilocus haplotypes. *Am J Hum Genet* 66: 2009–2012.

Gan L, Lee I, Smith R, Argonza-Barrett R, Lei H, McCuaig J *et al* (2000). Sequencing and expression analysis of the serine protease gene cluster located in chromosome 19q13 region. *Gene* 257: 119–130.

Green MF (2006). Cognitive impairment and functional outcome in schizophrenia and bipolar disorder. *J Clin Psychiatry* 67: 3–8.

Harvey TJ, Hooper JD, Myers SA, Stephenson SA, Ashworth LK, Clements JA (2000). Tissue-specific expression patterns and fine mapping of the human kallikrein (KLK) locus on proximal 19q13.4. *J Biol Chem* 275: 37397–37406.

Komai S, Matsuyama T, Matsumoto K, Kato K, Kobayashi M, Imamura K *et al* (2000). Neuropsin regulates an early phase of Schaffer-collateral long-term potentiation in the murine hippocampus. *Eur J Neurosci* 12: 1479–1486.

Laifenfeld D, Karry R, Klein E, Ben-Shachar D (2005). Alterations in cell adhesion molecule L1 and functionally related genes in major depression: a postmortem study. *Biol Psychiatry* 57: 716–725.

Laifenfeld D, Klein E, Ben-Shachar D (2002). Norepinephrine alters the expression of genes involved in neuronal sprouting and differentiation: relevance for major depression and antidepressant mechanisms. *J Neurochem* 83: 1054–1064.

Li Y, Qian YP, Yu XJ, Wang YQ, Dong DG, Sun W *et al* (2004). Recent origin of a hominoid-specific splice form of neuropsin, a gene involved in learning and memory. *Mol Biol Evol* 21: 2111–2115.

Lu ZX, Peng J, Su B (2007). A human-specific mutation leads to the origin of a novel splice form of neuropsin (KLK8), a gene involved in learning and memory. *Hum Mutat* 28: 978–984.

Macgregor S, Visscher PM, Knott SA, Thomson P, Porteous DJ, Millar JK *et al* (2004). A genome scan and follow-up study identify a bipolar disorder susceptibility locus on chromosome 1q42. *Mol Psychiatry* 9: 1083–1090.

Magklara A, Scorilas A, Katsaros D, Massobrio M, Yousef GM, Fracchioli S *et al* (2001). The human KLK8 (neuropsin/ovasin) gene: identification of two novel splice variants and its prognostic value in ovarian cancer. *Clin Cancer Res* 7: 806–811.

Matsumoto-Miyai K, Ninomiya A, Yamasaki H, Tamura H, Nakamura Y, Shiosaka S (2003). NMDA-dependent proteolysis of presynaptic adhesion molecule L1 in the hippocampus by neuropsin. *J Neurosci* 23: 7727–7736.

Mitsui S, Tsuruoka N, Yamashiro K, Nakazato H, Yamaguchi N (1999). A novel form of human neuropsin, a brain-related serine protease, is generated by alternative splicing and is expressed preferentially in human adult brain. *Eur J Biochem* 260: 627–634.

Nakamura Y, Tamura H, Horinouchi K, Shiosaka S (2006). Role of neuropsin in formation and maturation of Schaffer-collateral L1cam-immunoreactive synaptic boutons. *J Cell Sci* 119: 1341–1349.

Numakawa T, Yamagishi S, Adachi N, Matsumoto T, Yokomaku D, Yamada M *et al* (2002). Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons. *J Biol Chem* 277: 6520–6529.

Okabe A, Momota Y, Yoshida S, Hirata A, Ito J, Nishino H *et al* (1996). Kindling induces neuropsin mRNA in the mouse brain. *Brain Res* 728: 116–120.

Okada T, Hashimoto R, Numakawa T, Iijima Y, Kosuga A, Tatsumi M *et al* (2006). A complex polymorphic region in the brain-derived neurotrophic factor (BDNF) gene confers susceptibility to bipolar disorder and affects transcriptional activity. *Mol Psychiatry* 11: 695–703.

Otsubo T, Tanaka K, Koda R, Shinoda J, Sano N, Tanaka S *et al* (2005). Reliability and validity of Japanese version of the Mini-International Neuropsychiatric Interview. *Psychiatry Clin Neurosci* 59: 517–526.

Robinson LJ, Thompson JM, Gallagher P, Goswami U, Young AH, Ferrier IN *et al* (2006). A meta-analysis of cognitive deficits in euthymic patients with bipolar disorder. *J Affect Disord* 93: 105–115.

Sairanen M, O'Leary OF, Knuuttila JE, Castrén E (2007). Chronic antidepressant treatment selectively increases expression of plasticity-related proteins in the hippocampus and medial prefrontal cortex of the rat. *Neuroscience* 144: 368–374.

Schretlen DJ, Cascella NG, Meyer SM, Kingery LR, Testa SM, Munro CA *et al* (2007). Neuropsychological functioning in bipolar disorder and schizophrenia. *Biol Psychiatry* 62: 179–186.

Sharma T, Antonova L (2003). Cognitive function in schizophrenia. Deficits, functional consequences, and future treatment. *Psychiatr Clin North Am* **26**: 25–40.

Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E *et al* (1998). The mini-international neuropsychiatric interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry* **59**(Suppl 20): 22–57.

Shih RA, Belmonte PL, Zandi PP (2004). A review of the evidence from family, twin and adoption studies for a genetic contribution to adult psychiatric disorders. *Int Rev Psychiatry* **16**: 260–283.

Shinagawa F, Kobayashi S, Fujita K, Maekawa H (1990). *Japanese Wechsler Adult Intelligence Scale-Revised*. Nihonbunkakagakusha: Tokyo. (in Japanese).

Sugishita M (2001). *Japanese Wechsler Memory Scale-Revised*. Nihonbunkakagakusha: Tokyo. (in Japanese).

Tadokoro K, Hashimoto R, Tatsumi M, Kamijima K, Kunugi H (2004). Analysis of enhancer activity of a dinucleotide repeat polymorphism in the neurotrophin-3 gene and its association with bipolar disorder. *Neuropsychobiology* **50**: 206–210.

Tamura H, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S *et al* (2006). Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus *in vivo*. *J Physiol* **570**: 541–551.

Wechsler D (1981). *Wechsler Adult Intelligence Scale, Revised*. Psychological Corporation: New York.

Wechsler D (1987). *Wechsler Memory Scale Manual, Revised*. Psychological Corporation: San Antonio.

Yoshida S, Taniguchi M, Hirata A, Shiosaka S (1998). Sequence analysis and expression of human neuropsin cDNA and gene. *Gene* **213**: 9–16.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Available online at www.sciencedirect.com

ScienceDirect

Schizophrenia Research 101 (2008) 9–16

SCHIZOPHRENIA RESEARCH

www.elsevier.com/locate/schres

A polymorphism of the metabotropic glutamate receptor mGluR7 (*GRM7*) gene is associated with schizophrenia

Tsuyuka Ohtsuki [a,j], Minori Koga [a,j], Hiroki Ishiguro [a,j,*], Yasue Horiuchi [a,j], Makoto Arai [b], Kazuhiro Niizato [c], Masanari Itokawa [b], Toshiya Inada [d], Nakao Iwata [e], Shyuji Iritani [f], Norio Ozaki [f], Hiroshi Kunugi [g], Hiroshi Ujike [h], Yuichiro Watanabe [i], Toshiuki Someya [i], Tadao Arinami [a,j]

[a] *Department of Medical Genetics, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, Japan*
[b] *Department of Schizophrenia Research, Tokyo Institute of Psychiatry, Tokyo 156-8585, Japan*
[c] *Tokyo Metropolitan Matsuzawa Hospital, Department of Psychiatry, Tokyo 156-0057, Japan*
[d] *Department of Psychiatry, Teikyo University School of Medicine, Chiba Medical Center, Anesaki 3426-3, Ichihara-shi, Chiba 299-0111 Japan*
[e] *Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan*
[f] *Department of Psychiatry, School of Medicine, Nagoya University, Nagoya 466-8550, Aichi, Japan*
[g] *Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan*
[h] *Department of Neuropsychiatry, Okayama University, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan*
[i] *Department of Psychiatry, Niigata University Graduate School of Medical and Denatal Sciences, Niigata 951-8510, Japan*
[j] *CREST, Japan Science and Technology Agency, Kawaguchi-shi, Saitama 332-0012, Japan*

Received 26 October 2007; received in revised form 21 January 2008; accepted 27 January 2008
Available online 10 March 2008

Abstract

Introduction: Glutamate dysfunction has been implicated in the pathophysiology of schizophrenia. The metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors. *GRM7*, the gene that encodes mGluR7, is expressed in many regions of the human central nervous system. The *GRM7* gene is located on human chromosome 3p26, which has been suggested by linkage analysis to contain a susceptibility locus for schizophrenia.
Methods: We screened for mutations in all exons, exon/intron junctions, and promoter regions of the *GRM7* gene in Japanese patients with schizophrenia and evaluated associations between the detected polymorphisms and schizophrenia. We examined the influence of one polymorphism associated with schizophrenia on the expression of *GRM7* by dual-luciferase assay in transfected cells.
Results: Twenty-five polymorphisms/mutations were detected in *GRM7*. Case-control analysis revealed a potential association of a synonymous polymorphism (371T/C, rs3749380) in exon 1 with schizophrenia in our case-control study of 2293 Japanese patients with schizophrenia and 2382 Japanese control subjects (allelic $p=0.009$). Dual-luciferase assay revealed suppression of

* Corresponding author. Department of Medical Genetics, Graduate School of Comprehensive Human Sciences University of Tsukuba, Tsukuba, Ibaraki-ken, 305-8575, Japan. Tel.: +81 298 53 3352; fax: +81 298 53 3333.
E-mail address: hishigur@md.tsukuba.ac.jp (H. Ishiguro).

0920-9964/$ - see front matter
doi:10.1016/j.schres.2008.01.027

transcription activity by exon 1 containing this polymorphism and a statistically significant difference in the promoter activity between the T and C alleles.
Conclusions: Our results support the possible association of a *GRM7* gene polymorphism with genetic susceptibility to schizophrenia.

Keywords: Glutamate; Expression; Luciferase; Prefrontal

1. Introduction

Schizophrenia is a severe psychiatric disorder, which is equally prevalent in men and women and affects approximately 1% of the population worldwide. Several neurotransmitter systems and functional networks within the brain have been found to be affected in patients with schizophrenia. The glutamatergic neuronal dysfunction hypothesis is one of the main explanatory hypotheses (Carlsson et al., 1997). Glutamate is the primary excitatory neurotransmitter in brain. It is contained as a neurotransmitter in approximately 60% of brain neurons, including almost all cortical pyramidal neurons. Further, virtually 100% of brain neurons contain some type of glutamate receptor. Glutamate mediates its effects on the central nervous system via both ionotropic and metabotropic receptors. The metabotropic glutamate receptors (mGluRs), which are G-protein-coupled receptors, are divided into 3 groups on the basis of sequence homology, putative signal transduction mechanisms, and pharmacologic properties (Nakanishi, 1994; Pin and Duvoisin, 1995). The mGluRs in group I are mGluR1 and mGluR5, those in group II are mGluR2 and mGluR3, and those in group III are mGluRs 4, 6, 7, and 8. Group II and group III mGluRs are linked to inhibition of the cyclic AMP cascade but differ in their agonist selectivities.

mGluR7 is the most highly conserved mGluR subtype across mammalian species (Flor et al., 1997). Makoff et al. (1996) observed by in situ hybridization that *GRM7* is expressed in many areas of the human brain, especially the cerebral cortex, hippocampus, and cerebellum. mGluR7 is localized directly in the presynaptic zone of the synaptic cleft of glutamatergic synapses (Kinoshita et al., 1998; Kosinski et al., 1999), where it is thought to act as an autoreceptor that is activated by glutamate released from the presynaptic terminal during action potentials. Furthermore, mGluR7 is thought to be a key player in shaping synaptic responses at glutamatergic synapses as well as in regulating key aspects of inhibitory GABAergic transmission (Kinoshita et al., 1998; Kosinski et al., 1999).

mGluR7 has putative roles in anxiety, emotional responses, and spatial working memory (Callaerts-Vegh et al., 2006; Cryan et al., 2003; Mitsukawa et al., 2006). Cognitive dysfunction is estimated to occur in 75%–85% of patients with schizophrenia, often precedes the onset of other symptoms (Reichenberg et al., 2006). Working memory is one of primary cognitive domains that are crucial for developing targets for the treatment of cognition in schizophrenia (Nuechterlein et al., 2004).

mGluR7 ablation causes dysregulation of the hypothalamic–pituitary–adrenal axis and increases hippocampal BDNF protein levels (Mitsukawa et al., 2005). Dysregulation of BDNF production or release is associated with neuropsychiatric disorders, such as schizophrenia (Harrison and Weinberger, 2005). Association between the val66met polymorphism of the *BDNF* gene and hippocampal volume in human, particularly in patients with schizophrenia (Szeszko et al., 2005).

In the present study, we examined the *GRM7* gene as a candidate for schizophrenia.

2. Materials and methods

2.1. Subjects

All subjects were of Japanese descent and were recruited from the main island of Japan. Patients with schizophrenia were diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV). Control subjects were mentally healthy, unrelated subjects with no self-reported family history of mental illness within second-degree relatives. We sequenced the 5′ region, exons and exon–intron boundaries of the *GRM7* gene in 32 patients (mutation screening patients) with schizophrenia (mean age ± SD, 46.5 ± 10.9 years; 17 men and 15 women) to identify polymorphisms. We then genotyped 576 patients including the mutation screening patients (mean age ± SD, 46.6 ± 14.8 years; 322 men and 254 women) and 576 control subjects (mean age ± SD, 46.8 ± 12.9 years; 268 men and 308 women) (1st association population) with Predesigned TaqMan single nucleotide polymorphism (SNP) genotyping assays. When Predesigned TaqMan SNP genotyping assays were not available, we performed direct sequencing of DNAs from 96 patients including the mutation screening

patients (mean age ± SD, 50.3 ± 13.1 years; 55 men and 40 women) and 96 control subjects (mean age ± SD, 53.6 ± 9.1 years; 42 men and 54 women). For SNPs for which an association with schizophrenia was suggested in the first association population, we performed genotyping in an independent sample of 1817 patients (mean age ± SD, 45.5 ± 14.1 years; 962 men and 855 women) and 1728 control subjects (mean age ± SD, 46.2 ± 13.6 years; 958 men and 770 women) (confirmation population). The present study was approved by the ethics committees of the University of Tsukuba and participating institutes. All participants provided written informed consent.

2.2. DNA isolation and genotyping

DNAs were extracted from peripheral lymphocytes by standard phenol–chloroform extraction. The genomic structure of *GRM7* was determined from the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/cgi-bin/hgGateway) and the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). To screen for polymorphisms, we performed direct sequencing of genomic DNAs with a Big Dye Terminator Cycle Sequencing Kit and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). All exons, exon–intron junctions, and 1.6 kb of the 5′ flanking region of *GRM7* were amplified from the genomic DNAs of 24 randomly selected patients. The sequences of primers and conditions used for amplification for the mutation screening are available upon request. We genotyped polymorphisms with the TaqMan SNP Genotyping Assay (Applied Biosystems) and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

2.3. Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells with the SV Total RNA Isolation System (Promega, Madison, WI, USA). cDNA was synthesized from RNA with Revertra Ace (Toyobo, Tokyo, Japan) and oligo dT primer. Expression of *GRM7* was quantified by real-time quantitative RT-PCR with the TaqMan Gene Expression Assay and ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) per the manufacturer's instructions. Primers and probes were purchased from Applied Biosystems (Assays-on-Demand Assay ID: Hs00179051_m1). *GAPDH* was used as an internal control. Data were collected and analyzed with Sequence Detector Software (SDS) version 2.1 (Applied Biosystems) and the standard curve method.

2.4. Luciferase reporter assay

To assay promoter activity of the 5′-flanking region and exon 1 of the *GRM7* gene, 9 fragments of the 5′ region were cloned into the pGL3-Basic plasmid with and without a Simian virus 40 enhancer sequence (Promega, Madison, WI, USA). The day before transfection, NH-12 cells (Japanese Collection of Research Bioresources Gene Bank, http://genebank.nibio.go.jp/gbank/index_e.html) were plated at 1×10^5 cells/well in a 24-well plate and grown in DMEM (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio, Kerrville, TX, USA). One microgram of each test plasmid was transiently cotransfected into the cells with 0.1 μg of pRL-TK plasmid (an internal standard reporter) (Promega) with Lipofectamine 2000 (Invitrogen) per manufacturer's protocol. After 48 h, the dual-luciferase assay was performed with a PicaGene Dual SeaPansy Kit (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions.

2.5. Statistics

Deviation from predicted Hardy–Weinberg frequencies was examined by chi-square test. Individual allelic associations were examined by Fisher's exact test. Genotypic associations were examined by Armitage's Trend Test for the reasons discussed by Devlin and Roeder (1999). A significant association was defined when the given p value for allelic or genotypic tests was less than 5% (uncorrected $p < 0.05$) and the same association was confirmed in an independent population with $p < 0.05$. Linkage disequilibrium (LD) between polymorphisms and haplotype block structures was evaluated with Haploview software version 3.11 (Barrett et al., 2005). Haplotype blocks were generated with the default algorithm taken from Gabriel et al. (2002). Haplotypic associations with disorders were examined with Haploview software, which performs association tests on the set of blocks selected by obtaining counts for case-control association tests by summing the fractional likelihoods of each individual for each haplotype by the EM algorithm.

3. Results

Twenty-five polymorphisms/mutations were detected in the exons, exon–intron junctions, and 5′-flanking region of the *GRM7* gene (Fig. 1). Genotyping was carried out for all detected polymorphisms except rare variants with allele frequencies < 0.05 and polymorphisms in LD with each other ($r^2 = 1$). Among these SNPs, 1724A/G (rs34373930), 1938C/T (rs7614915), IVS8+

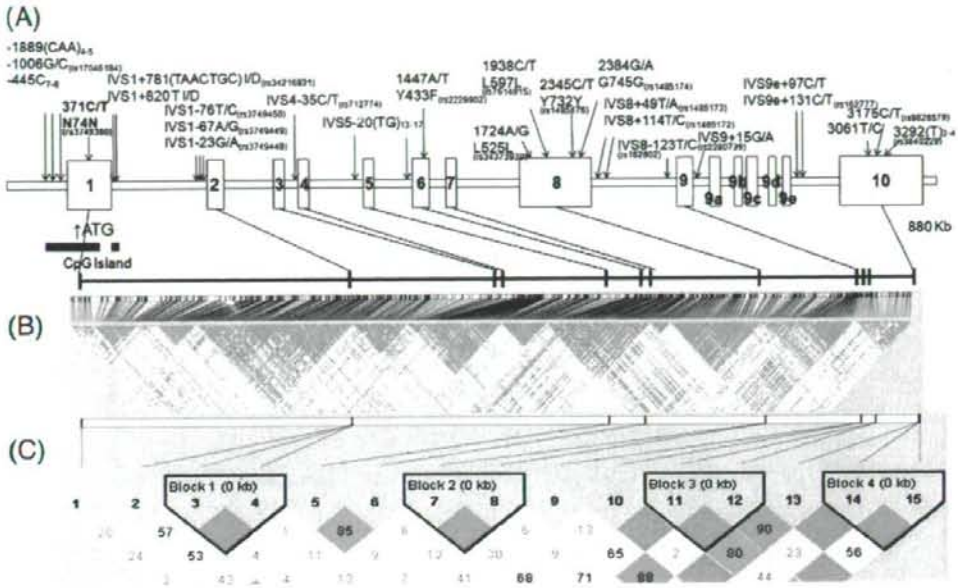

Fig. 1. Schematic representation of the *GRM7* gene and relevant mutations/polymorphisms (A), linkage disequilibrium plot of the Japanese population from HapMap database (B), and between SNPs genotyped in this study shown in Table 1 (C). Figures in rhombus are D' between SNPs and filled rhombus without figures indicates $D'=1$ in (C).

49T/A (T/A at the position of 49 base-pair starting from the G of the donor site of intron 8), and 2345C/T (rs1485175) were in complete LD ($r^2=1$), and IVS8+114T/C and 2384G/A (rs1485174) were also in complete LD ($r^2=1$) in 24 screening samples. Therefore, we genotyped the 2345C/T and 2384G/A polymorphisms as representative SNPs. The IVS9+97C/T, 2912T/C, and 3292(A)3-4 (rs3840229) SNPs were genotyped in 96 patients and 96 controls by direct sequencing, and the other 12 SNPs were genotyped in 576 patients and 575 control subjects by TaqMan SNP Genotyping Assay (Applied Biosystems). The genotypic distributions of these 15 SNPs did not deviate significantly from Hardy–Weinberg equilibrium ($p>0.05$). A synonymous polymorphism (371T/C) in exon 1 of *GRM7* showed a potential association for schizophrenia (allelic uncorrected $p=0.04$) (Table 1). We did not detect a significant association with schizophrenia of any of the other 14 SNPs, including the Tyr433Phe polymorphism (allelic uncorrected $p=0.33$; genotypic uncorrected $p=0.63$), which was previously reported to be associated (Bolonna et al., 2001). The haplotype blocks consisted of IVS1-76T/C, IVS1-67A/G, and IVS1-23G/A and of 2345C/T and 2384A/G. No significant haplotypic association was suggested for these 2 haplotype blocks ($p>0.05$). We confirmed the association of the 371T/C polymorphism with schizophrenia in an independent population of 1717 patients and 1807 control subjects and confirmed the association (allelic $p=0.03$, one-sided) (Table 2). The allelic p value of the association in the total population of 2293 patients with schizophrenia and 2382 control subjects was 0.009 (Table 2). According to the HapMap database (http://www.hapmap.org/index.html), the 371T/C polymorphism (rs3749380) was not in the LD block and was not in LD with other SNPs within 80 kb ($r^2<0.7$). Weak LD was observed with rs458351 ($D'=0.89$ and $r^2=0.69$), which is 30 kb upstream of rs3749380.

Expression of *GRM7* mRNA was assessed by RT-PCR in 7 different human cell lines (IMR-32, NH-12, TN-2, NB-1, SCCH-26, A172, and T98G). *GRM7* was expressed in NH-12 and SCCH-26 cells (data not shown). Because expression in NH-12 cells was higher than in SCCH-26 cells, we used NH-12 cells, a human cell line derived from neuroblastoma, for luciferase assays. Dual-luciferase assay revealed that the strongest promoter activity for *GRM7* was contained in the 1-kb fragment upstream of the ATG site of exon 1. However, a promoter construct containing the sequence from the ATG to the end of exon 1 showed significantly lower activity, indicating that the 371T/C polymorphism is in a regulatory region. When an SV40

Table 1
Genotypic and allelic distributions of polymorphisms in the *GRM7* gene

Polymorphism	Population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
371T/C (rs3749380)			CC		TC		TT			C		T		
	Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
	Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
IVS1-76T/C (rs3749450)			CC		TC		TT			C		T		
	Patients	568	10	(0.02)	148	(0.26)	410	(0.72)		168	(0.15)	968	(0.85)	
	Controls	561	18	(0.03)	129	(0.23)	414	(0.74)	0.95	165	(0.15)	957	(0.85)	0.96
IVS1-67A G (rs3749449)			GG		AG		AA			G		A		
	Patients	570	8	(0.01)	95	(0.17)	467	(0.82)		111	(0.10)	1029	(0.90)	
	Controls	567	7	(0.01)	89	(0.16)	471	(0.83)	0.60	103	(0.09)	1031	(0.91)	0.59
IVS1-23G A (rs3749448)			AA		GA		GG			A		G		
	Patients	569	26	(0.05)	181	(0.32)	362	(0.64)		233	(0.20)	905	(0.80)	
	Controls	566	30	(0.05)	178	(0.31)	358	(0.63)	0.75	238	(0.21)	894	(0.79)	0.75
IVS4-35C/T (rs712774)			CC		CT		TT			C		T		
	Patients	571	121	(0.21)	260	(0.46)	190	(0.33)		502	(0.44)	640	(0.56)	
	Controls	559	117	(0.21)	268	(0.48)	174	(0.31)	0.66	502	(0.45)	616	(0.55)	0.65
1447T/A (rs2229902, Phe433Tyr)			AA		AT		TT			A		T		
	Patients	575	488	(0.85)	81	(0.14)	6	(0.01)		1057	(0.92)	93	(0.08)	
	Controls	569	484	(0.85)	82	(0.14)	3	(0.01)	0.76	1050	(0.92)	88	(0.08)	0.75
2345C/T (rs1485175)			CC		CT		TT			C		T		
	Patients	569	120	(0.21)	275	(0.48)	174	(0.31)		515	(0.45)	623	(0.55)	
	Controls	562	118	(0.21)	282	(0.50)	162	(0.29)	0.69	518	(0.46)	606	(0.54)	0.69
2384A/G (rs1485174)			AA		GA		GG			A		G		
	Patients	569	18	(0.03)	150	(0.26)	401	(0.70)		186	(0.16)	952	(0.84)	
	Controls	561	24	(0.04)	162	(0.29)	375	(0.67)	0.15	210	(0.19)	912	(0.81)	0.14
IVS8-123T/C (rs162802)			CC		TC		TT			C		T		
	Patients	569	3	(0.01)	91	(0.16)	475	(0.83)		97	(0.09)	1041	(0.91)	
	Controls	566	9	(0.02)	88	(0.16)	469	(0.83)	0.49	106	(0.09)	1026	(0.91)	0.48
IVS9+15G/A (rs2280739)			AA		GA		GG			A		G		
	Patients	570	4	(0.01)	84	(0.15)	482	(0.85)		92	(0.08)	1048	(0.92)	
	Controls	564	3	(0.01)	86	(0.15)	475	(0.84)	0.94	92	(0.08)	1036	(0.92)	0.94
IVS9e+97C/T			CC		CT		TT			C		T		
	Patients	96	75	(0.78)	20	(0.21)	1	(0.01)		170	(0.89)	22	(0.11)	
	Controls	95	78	(0.82)	16	(0.17)	1	(0.01)	0.53	172	(0.91)	18	(0.09)	0.62
IVS9e+131C/T (rs162777)			CC		CT		TT			C		T		
	Patients	570	22	(0.04)	186	(0.33)	362	(0.64)		230	(0.20)	910	(0.80)	
	Controls	566	23	(0.04)	178	(0.31)	365	(0.64)	0.82	224	(0.20)	908	(0.80)	0.82
2912T/C			TT		TC		CC			T		C		
	Patients	96	78	(0.81)	18	(0.19)	0	(0.00)		174	(0.91)	18	(0.09)	
	Controls	95	83	(0.87)	12	(0.13)	0	(0.00)	0.32	178	(0.94)	12	(0.06)	0.34
3175C/T (rs9826579)			CC		CT		TT			C		T		
	Patients	574	25	(0.04)	203	(0.35)	346	(0.60)		253	(0.22)	895	(0.78)	
	Controls	567	23	(0.04)	189	(0.33)	355	(0.63)	0.44	235	(0.21)	899	(0.79)	0.44
3292(A)3–4 (rs3840229)			33		34		44			3		4		
	Patients	96	80	(0.83)	16	(0.17)	0	(0.00)		176	(0.92)	16	(0.08)	
	Controls	95	78	(0.82)	17	(0.18)	0	(0.00)	0.85	173	(0.91)	17	(0.09)	0.86

enhancer was added downstream of the luciferase gene, the 371C allele showed significantly higher promoter activity than the 371T allele (Fig. 2).

4. Discussion

In the present study, we examined associations between polymorphisms in the *GRM7* gene and schizophrenia. Weak association was found for a synonymous SNP (371T/C) in exon 1 in the first association population, and this association was replicated in the confirmation population. The T allele, which is associated with schizophrenia, has lower promoter activity than the C allele. On the basis of this finding, we hypothesized that lower expression of mGluR7 may increase risk of developing schizophrenia, though studies of the expression of GRM7 in brains of patients with schizophrenia have not been reported.

Table 2
Association of the 371T/C polymorphism (rs3749380) in the *GRM7* gene with schizophrenia

Polymorphism population	*n*	Genotype count (frequency)						*p*	Allele count (frequency)				*p*
		CC		CT		TT			C		T		
Screening population													
Patients	576	241	(0.42)	256	(0.44)	79	(0.14)		738	(0.64)	414	(0.36)	
Controls	575	274	(0.48)	236	(0.41)	65	(0.11)	0.04	784	(0.68)	366	(0.32)	0.04
Confirmatory population													
Patients	1717	715	(0.42)	771	(0.45)	231	(0.13)		2201	(0.64)	1233	(0.36)	
Controls	1807	799	(0.44)	794	(0.44)	214	(0.12)	0.07	2392	(0.66)	1222	(0.34)	0.03
Total													
Patients	2293	956	(0.42)	1027	(0.45)	310	(0.14)		2939	(0.64)	1647	(0.36)	
Controls	2382	1073	(0.45)	1030	(0.43)	279	(0.12)	0.01	3176	(0.67)	1588	(0.33)	0.009[a]

Genotypic *p* was calculated by Armitage's Trend Test and allelic *p* was calculated by Fisher's exact test.
[a] Odd ratio = 1.12, 95% confidence interval = 1.03–1.22.

mGluR7 was the first group III mGluR found to be enriched presynaptically at active zones of hippocampal pyramidal cells (Shigemoto et al., 1996). The low affinity of mGluR7 for glutamate suggests that mGluR7 might act as a "low-pass filter" that suppresses release of glutamate only when action potentials arriving at a high frequency produce massive glutamate release. The interaction with PICK1 (protein interacting with PRKCA 1) is crucial for

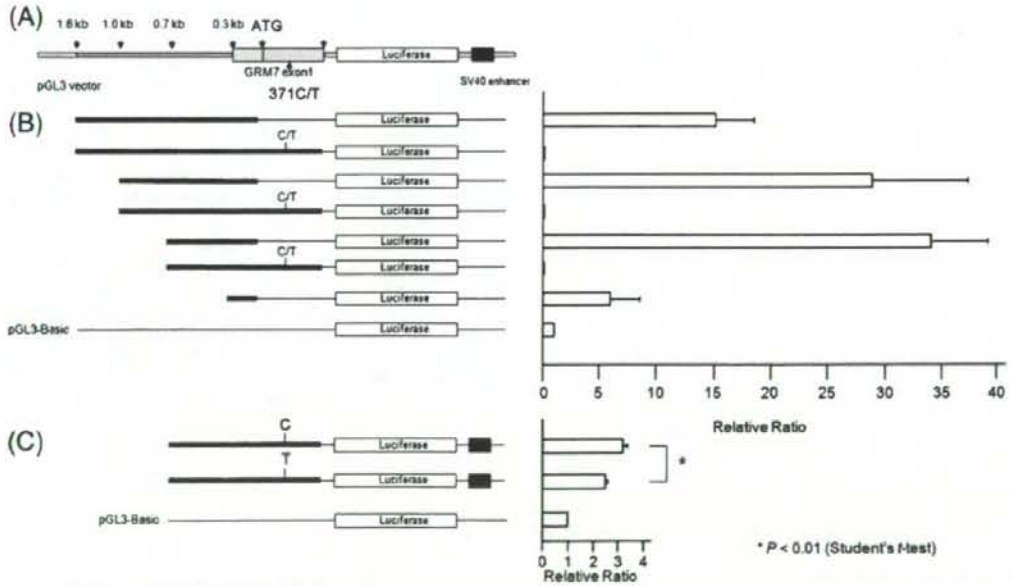

Fig. 2. Luciferase assays. (A) Schematic representation of the *GRM7* gene and reporter gene constructs. (B) Transcriptional activity of various constructs of the 5' region and exon 1 of the human *GRM7* gene in NH-12 cells. The region indicated by fine lines were not included in the constructs. Cotransfections were performed with pRL-TK (*Renilla* luciferase) to normalize transfection efficiency. Luciferase activity was assayed 48 h after transfection. Relative ratio of expression is shown as relative to that of pGL3-Basic, which was set at 1. The mean ± SEM was calculated from triplicate assays. (C) Effect of the 371C/T polymorphism on *GRM7* promoter activity. This assay was performed with pGL3-enhancer vector, because the constructs that contain *GRM7* exon 1 show extremely low luciferase activity.

the clustering of mGluR7 at presynaptic release sites (Boudin et al., 2000). *PICK1* is reported to be associated with schizophrenia (Dev and Henley, 2006; Fujii et al., 2006).

In the present study, we found an association between a functional SNP, 371T/C, in the gene encoding mGluR7 and schizophrenia. Recently, a genome-wide association study of 2000 individuals with bipolar disorder and 3000 control subjects found a strong association of SNP marker rs1485171 ($p=9.7\times10^{-5}$) in *GRM7* with bipolar disorder (Consortium, 2007). Therefore, genetic variations in the *GRM7* gene may be involved in both schizophrenia and bipolar disorder.

An association between Tyr433Phe polymorphism of *GRM7* and schizophrenia was reported (Bolonna et al., 2001). However, we failed to detect the association (allele, $p=0.33$; genotype, $p=0.63$). Recently, a copy number variation of the *GRM7* locus has been reported in patients with schizophrenia (Wilson et al., 2006). In the present study, we did not observe significant deviation from Hardy–Weinberg expectancy of the genotypic distributions of SNPs, indicating that copy number variations at the SNP examined in the present study are not common and are unlikely.

In the present study, the association of 371T/C with schizophrenia was observed in two independent case-control populations. However, its weak association (OR = 1.12) requires replication studies in large sample populations of more than 2000 cases and 2000 control subjects with à power greater than 0.8. We believe that *GRM7* is an interesting target worth such studies for schizophrenia and other psychiatric disorders.

Role of funding source

The present study was supported by Grant-in-Aid for Scientific Research on Priority Areas – Research on Pathomechanisms of Brain Disorders (18023009) – from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Contributors

Author Ohtsuki ran the experiment and wrote the manuscript. Author Horiuchi and Koga prepared the sample analyzed. Author Arai, Niizato, Itokawa, Inada, Iwata, Iritani, Ozaki, Kunugi, Ujike, Watanabe, Someya, managed the sample collection. Author Ishiguro and Arinami designed this study and undertook the statistical analysis and supervised this study.

Conflict of interest

No author has conflict of interest.

Acknowledgements

We thank Ms. Chisato Ishigami for providing technical assistance.

References

Barrett, J.C., Fry, B., Maller, J., Daly, M.J., 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 21 (2), 263–265.

Bolonna, A.A., Kerwin, R.W., Munro, J., Arranz, M.J., Makoff, A.J., 2001. Polymorphisms in the genes for mGluR types 7 and 8: association studies with schizophrenia. Schizophr. Res. 47 (1), 99–103.

Boudin, H., Doan, A., Xia, J., Shigemoto, R., Huganir, R.L., Worley, P., Craig, A.M., 2000. Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron 28 (2), 485–497.

Callaerts-Vegh, Z., Beckers, T., Ball, S.M., Baeyens, F., Callaerts, P.F., Cryan, J.F., Molnar, E., D'Hooge, R., 2006. Concomitant deficits in working memory and fear extinction are functionally dissociated from reduced anxiety in metabotropic glutamate receptor 7-deficient mice. J. Neurosci. 26 (24), 6573–6582.

Carlsson, A., Hansson, L.O., Waters, N., Carlsson, M.L., 1997. Neurotransmitter aberrations in schizophrenia: new perspectives and therapeutic implications. Life Sci. 61 (2), 75–94.

Consortium WTC C, 2007. Genome-wide association study of 14,000 cases of seven common diseases and 3000 shared controls. Nature 447 (7145), 661–678.

Cryan, J.F., Kelly, P.H., Neijt, H.C., Sansig, G., Flor, P.J., van Der Putten, H., 2003. Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. Eur. J. Neurosci. 17 (11), 2409–2417.

Dev, K.K., Henley, J.M., 2006. The schizophrenic faces of PICK1. Trends Pharmacol. Sci. 27 (11), 574–579.

Devlin, B., Roeder, K., 1999. Genomic control for association studies. Biometrics 55 (4), 997–1004.

Flor, P.J., Van Der Putten, H., Ruegg, D., Lukic, S., Leonhardt, T., Bence, M., Sansig, G., Knopfel, T., Kuhn, R., 1997. A novel splice variant of a metabotropic glutamate receptor, human mGluR 7b. Neuropharmacology 36 (2), 153–159.

Fujii, K., Maeda, K., Hikida, T., Mustafa, A.K., Balkissoon, R., Xia, J., Yamada, T., Ozeki, Y., Kawahara, R., Okawa, M., Huganir, R.L., Ujike, H., Snyder, S.H., Sawa, A., 2006. Serine racemase binds to PICK1: potential relevance to schizophrenia. Mol. Psychiatry 11 (2), 150–157.

Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M., Liu-Cordero, S.N., Rotimi, C., Adeyemo, A., Cooper, R., Ward, R., Lander, E.S., Daly, M.J., Altshuler, D., 2002. The structure of haplotype blocks in the human genome. Science 296 (5576), 2225–2229.

Harrison, P.J., Weinberger, D.R., 2005. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol. Psychiatry 10 (1), 40–68.

Kinoshita, A., Shigemoto, R., Ohishi, H., van der Putten, H., Mizuno, N., 1998. Immunohistochemical localization of metabotropic glutamate receptors, mGluR7a and mGluR7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. J. Comp. Neurol. 393 (3), 332–352.

Kosinski, C.M., Risso Bradley, S., Conn, P.J., Levey, A.I., Landwehrmeyer, G.B., Penney Jr., J.B., Young, A.B., Standaert, D.G., 1999. Localization of metabotropic glutamate receptor 7 mRNA and mGluR7a protein in the rat basal ganglia. J. Comp. Neurol. 415 (2), 266–284.

Makoff, A., Pilling, C., Harrington, K., Emson, P., 1996. Human metabotropic glutamate receptor type 7: molecular cloning and mRNA distribution in the CNS. Brain Res. 40 (1), 165–170.

Mitsukawa, K., Yamamoto, R., Ofner, S., Nozulak, J., Pescott, O., Lukic, S., Stoehr, N., Mombereau, C., Kuhn, R., McAllister, K.H.,

van der Putten, H., Cryan, J.F., Flor, P.J., 2005. A selective metabotropic glutamate receptor 7 agonist: activation of receptor signaling via an allosteric site modulates stress parameters in vivo. Proc. Natl. Acad. Sci. U. S. A. 102 (51), 18712–18717.

Mitsukawa, K., Mombereau, C., Lotscher, E., Uzunov, D.P., van der Putten, H., Flor, P.J., Cryan, J.F., 2006. Metabotropic glutamate receptor subtype 7 ablation causes dysregulation of the HPA axis and increases hippocampal BDNF protein levels: implications for stress-related psychiatric disorders. Neuropsychopharmacology 31 (6), 1112–1122.

Nakanishi, S., 1994. Metabotropic glutamate receptors: synaptic transmission, modulation, and plasticity. Neuron 13 (5), 1031–1037.

Nuechterlein, K.H., Barch, D.M., Gold, J.M., Goldberg, T.E., Green, M.F., Heaton, R.K., 2004. Identification of separable cognitive factors in schizophrenia. Schizophr. Res. 72 (1), 29–39.

Pin, J.P., Duvoisin, R., 1995. The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34 (1), 1–26.

Reichenberg, A., Weiser, M., Caspi, A., Knobler, H.Y., Lubin, G., Harvey, P.D., Rabinowitz, J., Davidson, M., 2006. Premorbid intellectual functioning and risk of schizophrenia and spectrum disorders. J. Clin. Exp. Neuropsychol. 28 (2), 193–207.

Shigemoto, R., Kulik, A., Roberts, J.D., Ohishi, H., Nusser, Z., Kaneko, T., Somogyi, P., 1996. Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381 (6582), 523–525.

Szeszko, P.R., Lipsky, R., Mentschel, C., Robinson, D., Gunduz-Bruce, H., Sevy, S., Ashtari, M., Napolitano, B., Bilder, R.M., Kane, J.M., Goldman, D., Malhotra, A.K., 2005. Brain-derived neurotrophic factor val66met polymorphism and volume of the hippocampal formation. Mol. Psychiatry 10 (7), 631–636.

Wilson, G.M., Flibotte, S., Chopra, V., Melnyk, B.L., Honer, W.G., Holt, R.A., 2006. DNA copy-number analysis in bipolar disorder and schizophrenia reveals aberrations in genes involved in glutamate signaling. Hum. Mol. Genet. 15 (5), 743–749.

J Neural Transm (2008) 115:1347–1354
DOI 10.1007/s00702-008-0100-1

BIOLOGICAL PSYCHIATRY - ORIGINAL ARTICLE

Failure to confirm an association between *Epsin 4* and schizophrenia in a Japanese population

Misty Richards · Yoshimi Iijima · Tomoko Shizuno · Yoko Kamegaya · Hiroaki Hori · Mayu Omori · Kunimasa Arima · Osamu Saitoh · Hiroshi Kunugi

Received: 28 March 2008 / Accepted: 20 July 2008 / Published online: 12 August 2008

Abstract Previous studies suggested that genetic variations in the 5′ region of *Epsin 4*, a gene encoding enthoprotin on chromosome 5q33, are associated with schizophrenia. However, conflicting results have also been reported. We examined the possible association in a Japanese sample of 354 patients and 365 controls. Seventeen polymorphisms of *Epsin 4* [3 microsatellites and 14 single nucleotide polymorphisms (SNPs)] were selected. A microsatellite marker (D5S1403) demonstrated a significant difference in the allele frequency between patients and controls (uncorrected $P = 0.04$). However, there was no significant difference in the genotype or allele frequency between the two groups for the other microsatellites or SNPs. Haplotype-based analysis provided no evidence for an association. The positive result at D5S1403 no longer reached statistical significance when multiple testing was taken into consideration. Our results suggest that the examined region of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese.

Keywords Association study · *Epsin 4* · Enthoprotin · Schizophrenia · Polymorphism

M. Richards · Y. Iijima · T. Shizuno · Y. Kamegaya · H. Hori · H. Kunugi (✉)
Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1, Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
e-mail: hkunugi@ncnp.go.jp

M. Richards
Albany Medical College, Albany, NY 12208, USA

M. Omori · K. Arima · O. Saitoh
Department of Psychiatry, National Center of Neurology and Psychiatry Hospital, Tokyo 187-8502, Japan

Introduction

Schizophrenia is a debilitating psychiatric disorder that affects approximately 1% of the world's population (Fenton et al. 2003; Hyman 2000). Patients may suffer from delusions, hallucinations, disorganized speech and behavior, as well as impairment in short-term verbal and nonverbal memory. The complete etiology of the disease remains unknown, though twin and adoption studies have demonstrated that schizophrenia is highly heritable (estimated heritability of >80%) (Cardno et al. 1999). While the contributing genes and pathophysiological mechanisms remain elusive, identifying the susceptibility genes is essential in discovering the true pathogenesis of schizophrenia. The mode of schizophrenia transmission is complex and is thought to be polygenic (Owen et al. 2004). Thus far, linkage and association studies have been successful approaches in searching for complex disease genes, discovering such candidate genes as neuregulin 1, dysbindin, G72, and D-amino acid oxidase (DAAO). Through such analysis, several chromosomal regions have been identified and investigated as potential sources for schizophrenia susceptibility genes (Chumakov et al. 2002; Stefansson et al. 2002; Straub et al. 2002a). As a result, the long arm of chromosome 5q has been identified as a putative chromosomal region of interest and subsequently investigated for susceptibility loci (Lewis et al. 2003; Straub et al. 2002b).

An association study of English, Irish, Welsh, and Scottish populations found *Epsin 4* on chromosome 5q33 as a strong candidate for schizophrenia susceptibility (Pimm et al. 2005). Four associated polymorphisms were discovered at the 5′ end of *Epsin 4*, a gene encoding the clathrin-associated protein enthoprotin. These included two microsatellite markers, D5S1403 and AAAT11, and

two single-nucleotide-polymorphism (SNP) markers, rs10046055 and rs254664. Enthoprotin plays a critical role in the formation, transport and stability of clathrin-coated vesicles (CCVs) and is therefore thought to regulate the transport and storage of neurotransmitters in the brain (McPherson and Ritter 2005; Wasiak et al. 2002). Neuronal CCVs are critical in the insertion and recycling of neurotransmitter receptors at the postsynaptic membrane and have been implicated as a regulatory mechanism for synaptic plasticity (Blanpied et al. 2002; Wang and Linden 2000). Specifically, CCVs facilitate AMPA receptor trafficking on the postsynaptic membrane, therefore affecting overall glutamatergic neurotransmission (Malinow and Malenka 2002; Man et al. 2002). Dysfunction of enthoprotin could, therefore, stimulate disturbances in glutamatergic brain signaling as well as synaptic plasticity, both postulated to be integral components of schizophrenia pathophysiology (Christison et al. 1989; Carter et al. 2006). Moreover, it has been demonstrated that enthoprotin interacts with the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex found in hippocampal pyramidal cells (Antonin et al. 2000; Chidambaram et al. 2004). This suggests a possible role for enthoprotin in calcium-mediated vesicle fusion of excitatory neurotransmitters released from the hippocampus. If the SNARE complex does not function properly in this way, abnormal neural connectivity may result, which is another characteristic of schizophrenia (Honer et al. 2002).

Thus far, two replication studies have been conducted in the Han Chinese population (Liou et al. 2006; Tang et al. 2006). Both studies found no association between the four markers previously reported (D5S1403, AAAT11, rs10046055, or rs254664) and schizophrenia. Tang et al. (2006), however, did detect haplotypes near the 5′ end of *Epsin 4* (252/T consisted of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) showing an association with schizophrenia in Han Chinese family trios. Liou et al. (2006) analyzed nine SNPs on *Epsin 4* in a case–control design and found a significant difference in the allele frequency of a SNP on the 5′ upstream region of *Epsin 4* (rs1186922); however, this difference was not significant after multiple testing. These conflicting results prompted us to examine *Epsin 4* for an association with schizophrenia in a Japanese sample.

Materials and methods

Subjects

Subjects were 354 patients with schizophrenia [212 males, mean age of 44.0 years (SD 13.7)] and 365 healthy controls [113 males, mean age of 39.7 years (SD 14.1)]. All subjects were biologically unrelated Japanese and recruited from the same geographical area (Western part of Tokyo Metropolitan). Consensus diagnosis by at least two psychiatrists was made for each patient according to the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and information from medical records. The controls were healthy volunteers recruited from hospital staffs and their associates. Control individuals were interviewed and those with a current or past history of psychiatric treatment or regular use of psychotropic agents were not enrolled in the study. Participants were excluded from both the patient and control groups if they had prior medical histories of central nervous system disease or severe head injury, or if they met criteria for alcohol/drug dependence or mental retardation. The study protocol was approved by the ethics committee at the National Center of Neurology and Psychiatry, Japan. After description of the study, written informed consent was obtained from every subject.

Genotyping

Venous blood was drawn from the subjects and Genomic DNA was extracted from peripheral leukocytes using the Wizard Genomic DNA Purification System kit (Promega, Madison, WI, USA) based on the solution-based method according to the manufacturer's instructions. Fourteen SNPs (rs1186922, rs10046055, rs1894962, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664) and three microsatellites (D5S1400, D5S1403, AAAT11) were genotyped. Five SNPs (rs1186922, rs10046055, rs1186930, rs1145603, rs254664) were selected based on previous studies conducted by Liou et al. (2006), Tang et al. (2006), and Pimm et al. (2005). The three microsatellites demonstrated to be significantly associated with schizophrenia by Pimm et al. (2005) and Tang et al. (2006) were selected in order to replicate their positive findings. The other nine SNPs (rs1894962, rs6556290, rs7735412, rs1145585, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032) were selected from the international HapMap project (http://hapmap.org/index.html.en) using Paul de Bakker's Tagger algorithm in the Haploview V 3.32 program. The 17 studied polymorphisms cover approximately 193 kb of *Epsin 4* from the 5′ upstream region to intron 1 (Fig. 1).

The SNPs were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay. TaqMan probes of the assay on demand (rs1186922, rs10046055, rs6556290, rs7735412, rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, rs1186998, rs17055032, rs254664)

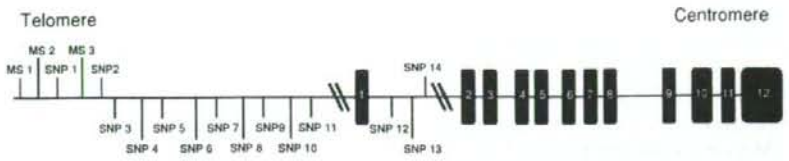

Fig. 1 The genetic structure of *Epsin 4* and location of studied markers. Upper six markers were studied in the previous reports. Lower 11 markers were additionally selected for Tag SNPs in this area

and assay by design (rs1894962) with Universal PCR Master Mix were obtained from Applied Biosystems (Foster City, CA, USA). Thermal cycling conditions for polymerase chain reaction (PCR) were one cycle at 95°C for 10 min followed by 50 cycles of 92°C for 15 s and 60°C for 1 min. After amplification, the allelic specific fluorescence was measured on ABI PRISM 7900 Sequence Detector Systems (Applied Biosystems, Foster City, CA, USA). Genotype data were read blind to the case–control status. Ambiguous genotype data were not included in the analysis.

Primer sequences for PCR amplification of the microsatellite markers D5S1400 and D5S1403 were obtained from the uniSTS database in NCBI (http://www.ncbi.nlm.nih.gov/), while the primer sequence of microsatellite marker AAAT11 was obtained from Pimm et al. (2005). PCR amplification of microsatellite markers was performed using primers fluorescently labeled with Beckman dyes. PCR products were subject to electrophoresis on Beckman CEQ 8000 (Beckman Coulter, Fullerton, CA, USA). As the PCR fragments of microsatellites were not the same length as those in the original work, we adjusted the sizes of our PCR fragments to Pimm et al. (2005).

Statistical analysis

Deviations of genotype distributions from Hardy–Weinberg equilibrium were assessed with the χ^2 test for goodness of fit. Genotype and allele distributions of each SNP were compared between patients and controls by using the χ^2 test for independence. These tests were performed with the SPSS software ver. 11 (SPSS Japan, Tokyo, Japan). The allelic association of microsatellite markers with schizophrenia was examined by use of the CLUMP program (Sham and Curtis 1995), which assesses the significance of departure between the observed and expected values in a $2 \times N$ contingency table using a Monte Carlo approach. The standardized measure of linkage disequilibrium (LD), (D') and r^2, were estimated using the online software SHEsis (http://202.120.7.14/analysis/myAnalysis.php). Haplotype-based association analyses were examined with the COCAPHASE software ver. 2.4 (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/) (Dudbridge et al. 2000). The expectation–maximization (EM) and "droprare" options were used. Haplotypes with frequencies less than 3% were considered to be rare.

Results

All investigated SNPs and microsatellite markers for case and control groups resulted in distributions that did not significantly deviate from Hardy–Weinberg Equilibrium. LD estimates of pairwise markers, expressed in D' and r^2, are presented in Table 1. Pairs in LD are represented as gray-shaded values, with estimates of $D' > 0.8$ and $r^2 > 0.8$. Pairwise LD analysis and haplotype block determination demonstrated that there were two blocks across the studied genomic region (Fig. 2). Measurement of pairwise LD showed that the middle seven SNPs (rs1145585, rs1186930, rs1145603, rs1186934, rs11744778, rs1145602, and rs1186998) were in strong LD with each other and were located in the sample block (haplotype block 2). The remaining block, haplotype block 1, consisted of three SNPs (rs1186922, rs10046055, and rs1894962) in the 5′ upstream region. In the haplotype-based analysis, the haplotypes in the two blocks were analyzed separately.

In the single-marker analysis, the microsatellite 2 marker (D5S1403) demonstrated a significant difference in allele frequency between case and control groups (Table 2: Allele 203, $\chi^2 = 4.26$, $df = 1$, $P = 0.04$ from the "T3" analysis of CLUMP). The remaining microsatellite markers and SNPs did not show any significant allelic association with schizophrenia (Table 2, all $P > 0.05$). The obtained evidence for an association at microsatellite 2 with schizophrenia was weak and non-significant when multiple testing was taken into consideration.

Results of the haplotype-based analysis are also presented in Table 2. None of the haplotypes showed a statistically significant difference between case and control groups. Even when haplotypes were examined according to the groupings established by Pimm et al. (2005), no evidence for an association was obtained. Tang et al. (2006) reported a highly significant transmission of haplotypes consisting of AAAT11 and rs10046055 ($P = 0.0048$) in addition to rs254664 and rs1145603 ($P = 0.0047$) to affected offspring in Han Chinese trios. Our results, again, failed to provide evidence to support this finding.

Table 1 Pair-wise linkage disequilibrium in *Epsin 4*

		MS1 D5S1400	MS2 D5S1403	SNP1 rs1186922	MS3 AAAT11	SNP2 rs10046055	SNP3 rs1894962	SNP4 rs6556290	SNP5 rs7735412	SNP6 rs1145585	SNP7 rs1186930	SNP8 rs1145603	SNP9 rs1186934	SNP10 rs11744778	SNP11 rs1145602	SNP12 rs1186998	SNP13 rs17055032	SNP14 rs254664
MS1	D5S1400		0.071	0.123	0.399	0.347	0.216	*0.979*	0.207	0.026	0.030	0.031	0.038	0.337	0.046	0.087	0.336	0.344
MS2	D5S1403	0.001		0.309	0.250	0.235	0.310	*0.981*	0.246	0.102	0.240	0.243	0.237	0.293	0.114	0.304	0.090	0.294
SNP1	rs1186922	0.005	0.051		*0.879*	*0.999*	*1.000*	*0.999*	0.465	*0.861*	0.579	0.581	0.566	0.032	*0.813*	0.717	0.347	0.062
MS3	AAAT11	0.011	0.014	0.045		*0.897*	0.757	*1.000*	0.741	0.673	0.426	0.430	0.434	0.442	0.668	0.633	0.307	0.441
SNP2	rs10046055	0.008	0.013	0.063	0.775		*0.999*	*1.000*	0.589	*0.993*	0.487	0.490	0.508	0.502	0.581	0.707	0.400	0.516
SNP3	rs1894962	0.007	0.004	0.425	0.015	0.027		0.053	*0.989*	0.772	0.720	0.721	0.699	*0.859*	0.716	*0.855*	0.136	*0.927*
SNP4	rs6556290	0.002	0.004	0.008	0.001	0.001	0.000		*1.000*	0.257	*0.997*	*0.996*	*0.998*	*1.000*	0.259	*0.998*	*1.000*	1.000
SNP5	rs7735412	0.001	0.002	0.013	0.002	0.001	0.003	0.000		*0.923*	*1.000*	*0.999*	*1.000*	*0.955*	*0.835*	*0.999*	*0.987*	*1.000*
SNP6	rs1145585	0.000	0.002	0.415	0.015	0.035	0.455	0.001	0.004		*1.000*	*0.985*	*0.970*	*0.912*	*0.942*	*0.982*	*0.989*	*0.999*
SNP7	rs1186930	0.000	0.041	0.251	0.065	0.092	0.165	0.006	0.046	0.416		*1.000*	*0.997*	*0.984*	*0.931*	*0.992*	*0.996*	*1.000*
SNP8	rs1145603	0.000	0.043	0.254	0.067	0.094	0.166	0.006	0.046	0.406	*0.994*		*0.997*	*0.984*	*0.932*	*1.000*	*0.996*	*1.000*
SNP9	rs1186934	0.000	0.042	0.243	0.068	0.101	0.159	0.006	0.044	0.399	*0.980*	*0.987*		*0.984*	*0.939*	*0.996*	*0.995*	*0.992*
SNP10	rs11744778	0.009	0.035	0.000	0.185	0.249	0.020	0.000	0.110	0.029	0.372	0.375	0.375		*0.999*	*0.991*	0.509	*0.978*
SNP11	rs1145602	0.000	0.003	0.368	0.014	0.012	0.395	0.001	0.003	*0.883*	0.363	0.365	0.374	0.034		*0.937*	0.692	*0.919*
SNP12	rs1186998	0.003	0.050	0.295	0.111	0.150	0.179	0.005	0.036	0.309	0.759	0.766	0.755	0.292	0.284		*1.000*	*1.000*
SNP13	rs17055032	0.003	0.000	0.022	0.034	0.057	0.008	0.000	0.001	0.012	0.030	0.029	0.029	0.003	0.006	0.105		0.514
SNP14	rs254664	0.010	0.034	0.000	0.184	0.262	0.023	0.000	0.116	0.035	0.384	0.386	0.381	0.957	0.029	0.297	0.003	

Upper diagonal figures are D' and lower diagonal figures are r^2

Pairwise LD mesurement using the online software SHEsis

Pairs in LD ($D' > 0.8$ or $r^2 > 0.8$) are shown in italics

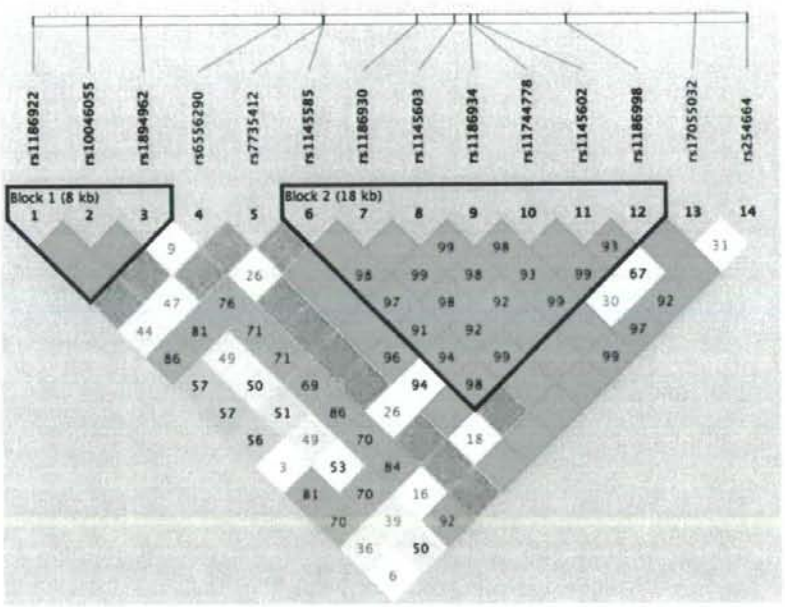

Fig. 2 Linkage disequilibrium (*LD*) block structure estimated from 14 SNPs by using Haploview

Discussion

The purpose of this investigation was to replicate Pimm et al.'s (2005) study of *Epsin 4* and its association with schizophrenia in a Japanese population. Pimm et al. (2005) originally reported that two SNPs, rs254664 (intron 1) and rs10046055 (5′ upstream region), in addition to two microsatellite markers D5S1403 (5′ flanking region) and AAAT11 (5′ upstream region), may be involved in the susceptibility to schizophrenia in the English, Irish, Welsh, and Scottish populations. In the present study, we examined a total of 17 polymorphisms and obtained weak evidence for an association at microsatellite 2 (D5S1403). However, when multiple testing was taken into consideration, the finding no longer reached significance. In addition, the risk-conferring allele was inconsistent between the two studies; we found an enrichment of the 203rd allele of microsatellite 2 in schizophrenics, whereas Pimm et al. (2005) found an enrichment of the same allele in controls. Furthermore, in the haplotype-based analysis, we did not find any evidence for an association.

Thus, we failed to replicate the widespread and highly significant associations with schizophrenia across 200,000 bp of *Epsin 4* reported by Pimm et al. (2005). Though we examined this region thoroughly by using 17 markers, we obtained no evidence for an association. The discrepancy found between Pimm et al.'s (2005) study and ours could result from a combination of factors. The first possibility may be due to the putative difference in allele frequencies between the English, Irish, Welsh, and Scottish population versus the Japanese population. It has been suggested that variations between ethnicities and populations in allele frequencies can dramatically affect the power to detect marginal differences (Marchini et al. 2005). According to the data bank of the International HapMap project (http://www.hapmap.org/index.html.en/), the allele frequency variation for rs10046055 and rs254664 is noticeable. Therefore, ethnic diversity of allele frequency of the SNPs may be a reason for negative findings in our present study. It is possible that there is a potential risk locus that is in strong LD with the investigated genetic variants in Pimm et al.'s (2005) population that is in weak or no LD with the variants in our population. However, to address this issue, we analyzed ten additional SNPs to thoroughly cover the entire genomic region of interest according to the HapMap database for the Japanese population; therefore, such a possibility is unlikely. Another possibility is that our negative results may be a type II error due to the potentially inadequate sample size. Our sample size (354 patients and 365 controls) had a power of 90% to detect an odds ratio of 1.9 or more if the T/A haplotype of HapB was assumed to be a risk (see Table 3). Incidentally, this haplotype gave rise to the most significant result ($P = 0.0005$) in the study of Pimm et al. (2005). This relatively weak power is due to the low frequency (0.06) of the T/A haplotype in our Japanese controls. If the A allele

Table 2 Allelic and haplotypic association analyses between *Epsin 4* and schizophrenia

dbSNP ID	Position[a]	Inter-SNP distance (bp)	Allele frequency			χ^2	P^c	P^d	Haplotype P (global)[e]			
			Allele[b]	Control	Schizophrenia				2 Locus	3 Locus	4 Locus	5 Locus
MS1 D5S1400	157439237	–	368	104 (0.16)	115 (0.18)	1.03	0.31					
			371	304 (0.47)	292 (0.46)	0.07	0.80					
			377	124 (0.19)	101 (0.16)	2.20	0.14					
			380	55 (0.08)	70 (0.11)	2.43	0.12	(T3)				
			383	45 (0.07)	45 (0.07)	0.01	0.90	0.12				
MS2 D5S1403	157346617	92,620	203	417 (0.66)	444 (0.71)	4.26	0.04					
			207	114 (0.18)	99 (0.16)	1.00	0.32					
			211	18 (0.03)	15 (0.02)	0.23	0.63	(T3)				
			215	67 (0.11)	56 (0.09)	0.91	0.34	0.04	0.64			
SNP1	157302083	44,534	A	179 (0.25)	189 (0.27)							
rs1186922			T	547 (0.75)	511 (0.73)	1.02	0.31		0.23	0.43		
MS3 AAAT11	157301697	386	248	567 (0.84)	562 (0.87)	1.48	0.22	(T1)				
			252	100 (0.15)	86 (0.13)	0.71	0.40	0.13	0.47	0.58	0.34	
SNP2	157297912	3,785	A	120 (0.17)	99 (0.14)							
rs10046055			T	606 (0.83)	607 (0.86)	1.74	0.19		0.23	0.43	0.77	0.31
SNP3	UTR	4,191	C	86 (0.12)	99 (0.14)							
rs1894962	157293721		T	642 (0.88)	605 (0.86)	1.61	0.21		0.23	0.26	0.47	0.77
SNP4	UTR	10,737	C	2 (0.00)	2 (0.00)							
rs6556290	157282984		T	726 (1.00)	706 (1.00)	0.00	0.98		0.22	0.23	0.27	0.47
SNP5	UTR	3,395	C	14 (0.02)	16 (0.02)							
rs7735412	157279589		T	712 (0.98)	690 (0.98)	0.20	0.66		1.00	0.21	0.23	0.26
SNP6	UTR	42	C	608 (0.84)	589 (0.83)							
rs1145585	157279547		T	116 (0.16)	117 (0.17)	0.08	0.78		0.76	0.84	0.43	0.38
SNP7	UTR	7,239	T	494 (0.68)	482 (0.68)							
rs1186930	157272308		C	232 (0.32)	222 (0.32)	0.03	0.86		0.78	0.67	0.67	0.52
SNP8	UTR	2,984	C	496 (0.68)	484 (0.69)							
rs1145603	157269324		T	230 (0.32)	222 (0.31)	0.01	0.92		0.92	0.74	0.64	0.63
SNP9	UTR	1,215	A	229 (0.32)	217 (0.31)							
rs1186934	157268109		G	491 (0.68)	479 (0.69)	0.06	0.80		0.89	0.89	0.68	0.57
SNP10	UTR	42	A	114 (0.16)	102 (0.14)							
rs11744778	157268067		G	608 (0.84)	604 (0.86)	0.50	0.48		0.78	0.75	0.75	0.72
SNP11	UTR	517	A	117 (0.16)	115 (0.16)							
rs1145602	157267550		G	609 (0.84)	587 (0.84)	0.02	0.89		0.78	0.58	0.56	0.56
SNP12	Intron 1	6,895	C	279 (0.38)	258 (0.37)							
rs1186998	157260655		G	447 (0.62)	448 (0.63)	0.54	0.46		0.23	0.30	0.42	0.54
SNP13	Intron 1	10,039	A	49 (0.07)	37 (0.05)							
rs17055032	157250616		C	679 (0.93)	671 (0.95)	1.44	0.23		0.39	0.36	0.54	0.51
SNP14	Intron 1	4,036	C	611 (0.84)	603 (0.85)							
rs254664	157246580		T	113 (0.16)	103 (0.15)	0.29	0.59		0.47	0.51	0.48	0.51

[a] Chromosome position was obtained from the HapMap database

[b] Microsatellites alleles presented by PCR fragment size (bp) with frequencies >0.03 in controls in either study

[c] P from 2 × 2 χ^2, with one *df*

[d] P from CLUMP Monte Carlo

[e] P from COCAPHASE-EM, -droprare 0.03 options

Table 3 Replication study of haplotype-based analysis between *Epsin 4* and schizophrenia

Groupe	Markers and haplotype			Case freq.	Cont freq.	*P* value[a]	
						Individual	Global
HapA	rs254664	rs10046055					
	C	A		0.05	0.08	0.098	
	T	A		0.09	0.09	0.743	
	C	T		0.80	0.77	0.151	
	T	T		0.06	0.07	0.664	0.341
HapB	rs1186930	rs10046055					
	C	A		0.10	0.10	0.563	
	T	A		0.04	0.06	0.133	
	C	T		0.22	0.22	0.835	
	T	T		0.64	0.62	0.418	0.471
HapC	rs10046055	AAAT11					
	T	248		0.85	0.82	0.189	
	A	252		0.12	0.14	0.249	0.234
HapD	rs254664	rs10046055	AAAT11				
	G	T	248	0.80	0.76	0.10	
	G	T	248	0.06	0.07	0.42	
	T	A	252	0.05	0.06	0.25	
	T	A	252	0.07	0.08	0.58	0.429
HapE	rs1145603	rs10046055	AAAT11				
	C	T	248	0.64	0.61	0.292	
	T	T	248	0.21	0.22	0.969	
	C	A	252	0.04	0.06	0.131	
	T	A	252	0.08	0.08	0.698	0.440
HapF	rs1145603	rs254664					
	C	C		0.69	0.68	0.940	
	T	C		0.17	0.16	0.671	
	T	T		0.15	0.16	0.591	0.819

[a] *P* from COCAPHASE-EM, -droprare 0.03 options

of rs10046055, which gave rise to the second significant result ($P = 0.002$) by Pimm et al. (2005), was assumed to be a risk, our sample size had a power of 90% to detect an odds ratio of 1.6 or more. Thus our sample size may have been limited by the inadequacy to detect a potentially weak effect of *Epsin 4* in Japanese. It is also possible that there may be a differential effect of *Epsin 4* on the development of schizophrenia between ethnic groups due to unknown factors. Alternatively, the positive association between schizophrenia and *Epsin 4* reported by Pimm et al. (2005) may have arisen by chance.

Subsequent to Pimm et al.'s (2005) investigation, two replication studies have been reported. Tang et al. (2006) reported a significant association of schizophrenia with some haplotypes of *Epsin 4* (252/T composed of AAAT11 and rs10046055, global $P = 0.0021$; T/T of rs1145603 and rs254664, $P = 0.0033$) in Han Chinese family trios, providing evidence for an association between schizophrenia and *Epsin 4*. The other replication study of Liou et al. (2006) found some evidence for an association, discovering a significant difference in allele frequency at rs1186922 ($P = 0.038$) and a significant difference in the frequency of a haplotype composed of two markers (rs1186922 and rs10046055). Liou et al. (2006) conservatively concluded that these findings occurred by multiple testing and chance and that their data did not support an association between schizophrenia and *Epsin 4*. However, Gurling et al. (2007) argued that the data of Liou et al. (2006) should be interpreted as supportive evidence for association. Since these two studies and ours were performed in Asian populations, if the association really exists, the obtained results should be similar. However, we obtained no evidence supporting the positive findings of Tang et al. (2006) or Liou et al. (2006).

In conclusion, we failed to identify a significant association between genetic variations of *Epsin 4* and schizophrenia in a Japanese population, suggesting that the examined region (5′ side) of *Epsin 4* does not have a major influence on susceptibility to schizophrenia in Japanese. Given the inconsistent findings across studies, more studies

should be conducted within different ethnic populations to draw more concrete conclusions. A meta-analysis of such studies and a multicenter study with a very large sample size will eventually be required. Since we examined only the 5′ side of the gene, the possibility remains that other unknown polymorphisms linked to the 3′ region of the *Epsin 4* gene may be associated with schizophrenia.

Acknowledgments We thank all patients for their participation in this study. This study was supported by the Japan Society for Promotion of Science (M. R.), Health and Labor Sciences Research Grants (Research on psychiatric and neurological diseases and mental health) (H. K.), Japan Health Sciences Foundation (Research on health sciences focusing on drug innovation) (H. K.), Grant-in-Aid for Scientific Research on Priority Areas of Applied Genomics from the Ministry of Education, Culture, Sports, Science and Technology of Japan (H. K.).

References

American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders. American Psychiatric Association, Washington

Antonin W, Riedel D, von Mollard GF (2000) The SNARE Vti1a-beta is localized to small synaptic vesicles and participates in a novel SNARE complex. J Neurosci 20:5724–5732

Blanpied TA, Scott DB, Ehlers MD (2002) Dynamics and regulation of clathrin coats at specialized endocytic zones of dendrites and spines. Neuron 36:435–449

Cardno AG, Marshall EJ, Coid B et al (1999) Heritability estimates for psychotic disorders: the Maudsley twin psychosis series. Arch Gen Psychiatry 56:162–168

Carter CJ (2006) Schizophrenia susceptibility genes converge on interlinked pathways related to glutamatergic transmission and long-term potentiation, oxidative stress and oligodendrocyte viability. Schizophr Res 86:1–14

Chidambaram S, Müllers N, Wiederhold K et al (2004) Specific interaction between SNAREs and epsin N-terminal homology (ENTH) domains of epsin-related proteins in trans-Golgi network to endosome transport. J Biol Chem 279:4175–4179

Christison GW, Casanova MF, Weinberger DR et al (1989) A quantitative investigation of hippocampal pyramidal cell size, shape, and variability of orientation in schizophrenia. Arch Gen Psychiatry 46:1027–1032

Chumakov I, Blumenfeld M, Guerassimenko O et al (2002) Genetic and physiological data implicating the new human gene G72 and the gene for D-amino acid oxidase in schizophrenia. Proc Natl Acad Sci USA 99:13675–13680

Dudbridge F, Koeleman BP, Todd JA et al (2000) Unbiased application of the transmission/disequilibrium test to multilocus haplotypes. Am J Hum Genet 66:2009–2012

Fenton WS, Stover EL, Insel TR (2003) Breaking the log-jam in treatment development for cognition in schizophrenia: NIMH perspective. Psychopharmacology (Berl) 169:365–366

Gurling H, Pimm J, McQuillin A (2007) Replication of genetic association studies between markers at the *Epsin 4* gene locus and schizophrenia in two Han Chinese samples. Schizophr Res 89:357–359

Honer WG, Falkai P, Bayer TA (2002) Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. Cereb Cortex 12:349–356

Hyman SE (2000) The NIMH perspective: next steps in schizophrenia research. Biol Psychiatry 47:1–7

Lewis CM, Levinson DF, Wise LH et al (2003) Genome scan meta-analysis of schizophrenia and bipolar disorder, part II: schizophrenia. Am J Hum Genet 73:34–48

Liou YJ, Lai IC, Wang YC et al (2006) Genetic analysis of the human ENTH (Epsin 4) gene and schizophrenia. Schizophr Res 84:236–243

Malinow R, Malenka RC (2002) AMPA receptor trafficking and synaptic plasticity. Annu Rev Neurosci 25:103–126

Man HY, Lin JW, Ju WH et al (2000) Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron 25:649–662

Marchini J, Donnelly P, Cardon LR (2005) Genome-wide strategies for detecting multiple loci that influence complex diseases. Nat Genet 37:413–417

McPherson PS, Ritter B (2005) Peptide motifs: building the clathrin machinery. Mol Neurobiol 32:73–87

Owen MJ, Williams NM, O'Donovan MC (2004) The molecular genetics of schizophrenia: new findings promise new insights. Mol Psychiatry 9:14–27

Pimm J, McQuillin A, Thirumalai S et al (2005) The Epsin 4 gene on chromosome 5q, which encodes the clathrin-associated protein enthoprotin, is involved in the genetic susceptibility to schizophrenia. Am J Hum Genet 76:902–907

Sham PC, Curtis D (1995) Monte Carlo tests for associations between disease and alleles at highly polymorphic loci. Ann Hum Genet 59(Pt 1):97–105

Stefansson H, Sigurdsson E, Steinthorsdottir V et al (2002) Neuregulin 1 and susceptibility to schizophrenia. Am J Hum Genet 71:877–892

Straub RE, Jiang Y, MacLean CJ et al (2002a) Genetic variation in the 6p22.3 gene DTNBP1, the human ortholog of the mouse dysbindin gene, is associated with schizophrenia. Am J Hum Genet 71:337–348

Straub RE, MacLean CJ, Ma Y et al (2002b) Genome-wide scans of three independent sets of 90 Irish multiplex schizophrenia families and follow-up of selected regions in all families provides evidence for multiple susceptibility genes. Mol Psychiatry 7:542–559

Tang RQ, Zhao XZ, Shi YY et al (2006) Family-based association study of *Epsin 4* and Schizophrenia. Mol Psychiatry 11:395–399

Wang YT, Linden DJ (2000) Expression of cerebellar long-term depression requires postsynaptic clathrin-mediated endocytosis. Neuron 25:635–647

Wasiak S, Legendre-Guillemin V, Puertollano R et al (2002) Enthoprotin: a novel clathrin-associated protein identified through subcellular proteomics. J Cell Biol 158:855–862

Open Access

Impaired Secretion of Brain-Derived Neurotrophic Factor and Neuropsychiatric Diseases

Naoki Adachi and Hiroshi Kunugi*

Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, 4-1-1, Ogawahigashi, Tokyo, 187-8502, Japan

Abstract: Recent studies have elucidated mechanisms of brain-derived neurotrophic factor (BDNF) secretion, and impaired secretion of BDNF may be involved in the pathogenesis of several neuropsychiatric diseases. The huntingtin gene, for example, has been shown to regulate vesicular transport of BDNF, which may play a role in the neurodegeneration present in Huntington's disease. In animal studies, mice lacking calcium-dependent activator protein for secretion 2 (CADPS2), which is involved in the activity-dependent release of BDNF, showed several phenotypes including autistic behavior. A single nucleotide polymorphism that results in an amino-acid change (Val66Met) in the BDNF gene has been shown to cause a decline in the function of BDNF vesicular sorting and has been reported to be associated with behavioral and intermediate phenotypes (e.g., episodic memory) in humans. In this review, we introduce recent progress in the molecular mechanisms of BDNF secretion and discuss its possible role in the pathophysiology and treatment of neuropsychiatric diseases.

INTRODUCTION

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, has been implicated in a broad range of processes that are important for neuronal survival and synaptic plasticity in the central nervous system (CNS) [1-3]. Early in the 1950s, nerve growth factor (NGF) was discovered by Levi-Montalcini and Hamburger and Cohen [4,5] as a soluble factor that induced fiber outgrowth of chicken sympathetic neurons. Subsequently, Barde *et al.* [6] isolated BDNF, which was later found to be homologous to NGF [7], from pig brain as a neuronal survival factor. These discoveries motivated homology-based searches for additional family members of which there are currently a total of four in mammals – NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Additional members are conserved in fish – neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) [8,9]. All neurotrophins are secreted from neuronal (partially glial) cells and bind to their receptors in an autocrine/paracrine manner. In the last two decades, a bulk of studies have suggested that neurotrophins, especially BDNF, are involved in the pathophysiology of neuropsychiatric diseases through their role in the regulation of synaptic efficacy (synaptic plasticity) and synaptogenesis in the CNS. In this review, we focus on recent findings of secretion mechanisms of BDNF and their relationship with neuropsychiatric diseases.

I. BIOLOGICAL FUNCTIONS OF BDNF

i. Survival and Synaptic Plasticity

Neurotrophins exert their biological effects through the binding of secreted homodimeric neurotrophins to two types of transmembrane receptor proteins: the tyrosine kinase Trk (tropomyosin-related kinase) receptors and the low affinity common neurotrophin receptor (p75NTR). Neurotrophins are expressed in a precursor form (pro-neurotrophins) and are proteolytically processed to a mature form. Mature neurotrophins preferentially bind to their specific Trk receptor: NGF to TrkA, BDNF and NT-4/5 to TrkB and NT-3 to TrkC. Pro-neurotrophins, however, bind to p75NTR with higher affinity than mature neurotrophins, although they have a lower affinity for Trk receptors [10]. Binding of neurotrophins to Trk receptors immediately generates receptor dimerization and autophosphorylation of tyrosine residues in the intracellular kinase domain. Trk receptor phosphorylation activates intracellular signaling regulated by mitogen-activated protein kinase (MAPK), phosphoinositide-3 (PI3)-kinase/Akt and phospholipase C-γ (PLC-γ) pathways as well as several small G proteins, including Ras, Rap-1, and the CDC-42-Rac-Rho family [11-13]. These intracellular signaling cascades modulate expression of genes and are responsible for most of the long-term effects of neurotrophins related to neuronal growth, survival, and differentiation [14]. On the other hand, binding of pro-neurotrophins to p75NTR leads to antagonistic effects to Trk receptor signaling. Several of these p75NTR-dependent signaling are pro-apoptotic and can be suppressed by Trk receptor-initiated signaling [15,16]. The first evidence of a significant relationship between neurotrophins and synaptic plasticity was obtained by Lohof *et al.* [17]; exogenous BDNF and NT-3 increased synaptic efficacy at the *Xenopus* neuromuscular junction. Subsequently, these neurotrophins were shown to facilitate glutamatergic synaptic transmission, even in the hippocampus of the mammalian CNS [18-20]. There is now substantial evidence implicating BDNF in activity-dependent long-term synaptic plasticity [21,22]. The neurotrophin-binding Trk receptor activates many kinds of signaling pathways that promote neuronal survival and synaptic efficiency, although it is still unclear how the complex signaling pathways are

*Address correspondence to this author at the Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo 187-8502, 4-1-1, Ogawahigashi, Tokyo, 187-8502, Japan; Tel/Fax: +81-42-346-1714; E-mail: hkunugi@ncnp.go.jp

1874-0820/08