the complex genetic diseases like schizophrenia (22). Alternatively, an unknown functional polymorphism, which is in LD with the SNPs and/or haplotypes, may be responsible for providing susceptibility to schizophrenia.

To date, association of dysbindin with schizophrenia has been confirmed across diverse populations. In addition, decreased expression of dysbindin mRNA and protein levels has been observed in prefrontal cortex and hippocampus of postmortem brain in schizophrenic patients (23–25). As dysbindin is distributed at least in part in axonal terminals (17), we focused on the possible role of dysbindin in neuronal transmission. We used two techniques, overexpression and knockdown, to investigate neuronal function of dysbindin. As the overexpression levels of dysbindin using sindbis virus were quite high when compared with the control level (~17-fold), the results could have non-physiological effects. However, the results from the knockdown experiments of the endogenous dysbindin protein were consistent with those from overexpression experiments. Our experiments suggest that dysbindin regulates the expression of SNAP25 and synapsin I proteins in the pre-synaptic machinery and is associated with increased glutamate release. SNAP25 is one of the fundamental molecular components of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) protein complex, which is involved in intracellular vesicle trafficking and neurotransmitter release (18). Synapsin I is localized to the synaptic vesicles that are both docked and located away from the plasma membrane (18). Reduction of SNAP25 protein has been observed in frontal cortex of schizophrenia patients (26) and synapsin I protein was found to be reduced in the hippocampus of patients with schizophrenia (27). Hypofunction of glutamatergic system has been implicated in the neuropathology in schizophrenia (8). The abuse of phencyclidine, an NMDA receptor antagonist, results in positive symptoms, negative symptoms and cognitive impairments, similar to schizophrenic patients. The postmortem brain studies suggested impaired glutamatergic systems, e.g. reduced glutamate level, decreased AMPA receptor binding and expression and reduced NMDA receptor expression in several brain areas, including frontal cortex and hippocampus.

Our experiments also suggest the survival effect of dysbindin protein on cortical neurons against serum deprivation through the PI3-kinase-Akt signaling pathway. Thus, dysbindin might play an important role in neuronal vulnerability. Impaired PI3-kinase-Akt signaling in schizophrenia has been reported recently (28). Dysbindin expression in the brain of schizophrenic patients was reduced (23–25) and our data suggested that the downregulation of dysbindin expression suppressed the phosphorylation levels of Akt. Taken together, impaired PI3-kinase-Akt signaling in the schizophrenic brain might be due, in part, to the decreased expression of dysbindin. As dysbindin may affect neuronal viability through Akt activation, dysbindin-Akt signaling might be involved in early disruptions producing long-term vulnerability that leads to the onset of schizophrenia symptoms. As PI3-kinase-Akt signaling is activated by several growth factors such as brain-derived neurotrophic factor, nerve growth factor and insulin-like growth factors through tyrosine kinase receptors (19), the regulation of this system might be associated with dysbindin.

The Hermansky–Pudlak syndrome defines a group of autosomal recessive disorders characterized by deficiencies in lysosome-related organelles complex-1 (BLOC-1). Hermansky–Pudlak type-7 is caused by a nonsense mutation of dysbindin, which is a component of the BLOC-1 (29). Biological roles of BLOC-1 are still unknown; however, it might be involved in vesicle docking and fusion. Sandy mouse, which has a deleted dysbindin gene, expresses no dysbindin (29). Thus, this mouse could be a powerful tool for investigating brain function of dysbindin *in vivo*. It is of interest to examine the pre-synaptic protein expression, glutamate release, Akt phosphorylation and neuronal vulnerability *in vivo* using this mouse.

We have demonstrated the additional support for the genetic association between dysbindin and schizophrenia in a relatively large sample and the evidence of novel functions of dysbindin in cultured neurons. Our results suggest that an abnormality of dysbindin might influence glutamatergic systems and Akt signaling. Further investigation is necessary to elucidate the mechanisms of Akt activation and upregulation of pre-synaptic molecules by dysbindin.

MATERIALS AND METHODS

Subjects

Subjects for the association study were 670 patients with schizophrenia [males: 50.6%, mean age of 44.2 years (SD 14.6)] and 588 healthy comparison subjects [males: 48.7%, mean age of 36.2 years (SD 12.4)]. All the subjects were biologically unrelated Japanese patients. Consensus diagnosis was made for each patient by at least two psychiatrists according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria. Control subjects were healthy volunteers who had no current or past contact to psychiatric services. After description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethical committees (Fujita Health University School of Medicine, Showa University School of Medicine and National Center of Neurology and Psychiatry).

SNP genotyping

Venous blood was drawn from the subjects and genomic DNA was extracted from whole blood according to the standard procedures. Six SNPs (P1655, P1635, P1325, P1320, P1763 and SNPA) adopted in the work of Straub *et al.* (2) and Williams *et al.* (21) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, described previously (30,31). Briefly, the probes and primers for detection of the SNP were as follows. P1655: forward primer 5′-AGTTTTTAT CACTAATCAAAATGAAACAGCCTTT-3′, reverse primer 5′-CTCATTCTGTTATAACTAGTCTGACATGGT-3′, probe 1 5′-VIC-TATTAGCTATGATAGTGTTTTAT-MGB-3′ and probe 2 5′-FAM-ATTAGCTATGATAGTCTTTTAT-MGB -3′; P1635: forward primer 5′-GGAACTTTTCTTTGAAGA CTTCCTTTCG-3′, reverse primer 5′-ACCACTAACAACC AAAAAGAAAACAAACA-3′, probe 1 5′-VIC-TAAAGCC AATAATTACC-MGB-3′ and probe 2 5′-FAM-AGCCAG

TAATTACC-MGB-3′; P1325: forward primer 5′-GATATG ACTCCTTAATTCACAGGCTACAG-3′, reverse primer 5′-GTTACTGCACACAAGCAACTGTTAA-3′, probe 1 5′-VIC -AATGGATGTTGCATTAGT-MGB-3′ and probe 2 5′-FAM -ATGGATGTTGCGTTAGT-MGB-3′; P1320: forward primer 5′-CCAATCCATTCTTTTATTGACATGGAGTTT-3′, reverse primer 5′-TGATTTTGACCAAGTCCATTGTGTCT -3′, probe 1 5′-VIC-AAAAGCACAAACAACAAG-MGB-3′ and probe 2 5′-FAM-AAAAGCACAAATAACAAG-MGB-3′; P1763: forward primer 5′-GGCAGAAGCAGTGAGTGAGA-3′, reverse primer 5′-TGGGCTCTTATGTCTACCTTTCCTAAA -3′, probe 1 5′-VIC-TCACCTGGATGTCAGC-MGB-3′ and probe 2 5′-FAM-ACCTGGCTGTCAGC-MGB-3′; SNPA: forward primer 5′-TCTGTTATGTGCCATTCACTGTTTT-3′, reverse primer 5′-TAGGGCTGGGATTGGATGA-3′, probe 1 5′-VIC-AGCAGTTTACATCTGGG-MGB-3′ and probe 2 5′-FAM-AGCAGTTTACATCAGGG-MGB-3′. PCR cycling conditions were 95°C for 10 min, 45 cycles of 92°C for 15 s and 60°C for 1 min.

Cell culture

Dissociated cortical cultures were prepared from postnatal 2- or 3-day-old rat (SLC, Shizuoka, Japan) cortex, as described previously (32,33). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 U/ml, Sigma) at 37°C. The dissociated cells were plated at a final density of 5×10^5 per cm^2 on polyethyleneimine-coated 12- or 24-well plates (4 and 2 cm^2 surface area/well, respectively; Corning, NY, USA) or cover glasses (Matsunami, Osaka, Japan) attached to flexiperm (VIVASCIENCE, Gottingen, Germany). The culture medium consisted of 5% precolostrum newborn calf serum, 5% heated-inactivated horse serum and 90% of a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium containing 15 mM HEPES buffer, pH 7.4, 30 nM Na_2SeO_3 and 1.9 mg/ml of $NaHCO_3$.

Sindbis virus

A bicistronic vector plasmid (pSinEGdsp) was provided by Dr Kawamura (Niigata University, Japan). The plasmid was derived from pSinRep5 (Invitrogen, USA) and had two subgenomic promoters followed by a multiple cloning site for arbitrary gene insertion and an EGFP open reading frame, thus the virus can produce arbitrary protein and EGFP independently in the infected cell, as previously described (34). Dysbindin cDNAs amplified by RT–PCR with specific primer pairs (forward 5′-ACGCGTCAATGCTGGAGACCCTTCG-3′ and reverse 5′-GCATGCCAATTTAAGAGTCGCTGTCC-3′) were inserted at the *Mlu*1 and *Sph*1 sites of the plasmid. Each plasmid was cleaved with *Pac*1, and used as a template for mRNA transcription *in vitro* using mMESSAGE mMACHINE kit (Ambion, USA). Pseudovirions were produced according to the experimental procedure of Invitrogen. Baby hamster kidney (BHK) cells were transfected with each mRNA and 26S helper mRNA (Invitrogen) by electroporation (1250 V/cm, 50 μF, single pulse) using Gene Pulser2 (BioRad, USA). The cells were incubated with DMEM supplemented with 10% FCS for 24 h at 37°C, the supernatants were collected as pseudovirion-containing solutions.

Immunocytochemistry

Cultured neurons were fixed with 4% paraformaldehyde for 20 min and then rinsed three times with PBS. Subsequently, cultured cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min at room temperature. The primary antibodies (anti-MAP2; Sigma) with 3% skim milk in PBS were applied overnight at 4°C. After washing, cells were incubated with secondary antibodies (Alexa Fluor, Molecular Probes) for 1 h at room temperature. Fluorescent images were captured by an inverted microscope (Axiovert 200, Zeiss) with a CCD (cool SNAPfx) purchased from Zeiss. Monochrome images were turned into color and analyzed using software (Slide BookTM 3.0, Intelligent Imaging Innovations, Inc., Denver, CO, USA). The images of GFP were analyzed with the same software.

Immunoblotting

Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA (pH 8.0), 10 mM NaF, 2 mM Na_3VO_4, 0.5 mM phenylarsine oxide and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 15 000 rpm for 60 min at 4°C, and the supernatants were collected for analysis. Samples were heat denatured with the standard SDS sample buffer. Immunoblottings were carried out as described previously (35). Briefly, immunoblottings were carried out with anti-SNAP25 antibody (1:3000, mouse monoclonal, Synaptic System, Gottingen, Germany), anti-synapsin I antibody (1:1000, rabbit anti-serum, Chemicon), anti-synaptotagmin antibody (1:1000, mouse monoclonal, BD Transduction Laboratory), anti-syntaxin antibody (1:3000, mouse monoclonal, Sigma), anti-GFP antibody (1:1000, rabbit polyclonal, MBL, Nagoya, Japan), anti-dysbindin antibody (23) (1:100, rabbit polyclonal), anti-TUJ1 antibody (1:5000, mouse monoclonal, Berkeley antibody company, CA, USA), anti-Akt antibody (1:1000, rabbit anti-serum, Cell Signaling) and anti-phospho-Akt antibody (Ser473, 1:1000, rabbit anti-serum, Cell Signaling) in TBS containing 1% non-fat dried milk. The immunoblotting experiments were performed four times and they were quantitatively analyzed by capturing images on films using a scanner (Epson, Tokyo, Japan) in conjunction with the Lane and Spot Analyzer software (version 6.0, ATTO, Tokyo, Japan).

Anti-dysbindin antibody was produced as described previously (36). Briefly, the peptide synthesized (QSDEEEVQVDTALC: 320–333 amino acid residue of human dysbindin, with no homology in any mammalian protein) was conjugated with maleimide-activated keyhole limpet hemocyanin and immunized to two rabbits. The titer was measured by ELISA and sera of high titer against the peptide were obtained from both rabbits. The sera were affinity purified by a column conjugated with the immunized peptide.

Detection of glutamate release

The amount of glutamate released from the cultures was measured as previously reported (33,35). The glutamate released into the modified HEPES-buffered Krebs–Ringer assay buffer (KRH; 130 mM NaCl, 5 mM KCl, 1.2 mM

NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4) were measured by HPLC (Shimadzu, Kyoto, Japan) with a fluorescence detector (excitation wavelength, 340 nm; emission wavelength, 445 nm, Shimadzu). For stimulation of cortical neurons, we used a HK^+ KRH solution consisting of 85 mM NaCl, 50 mM KCl, 1.2 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 10 mM glucose, 1% bovine serum albumin and 25 mM HEPES, pH 7.4. Before exposing the cultures to HK^+ solution (1 min), basal fractions were collected. The glutamate release experiments were performed three times with independent cultures to confirm reproducibility.

MTT assay

To examine the cell viability, the metabolic activity of mitochondria was estimated by measuring the mitochondrial-dependent conversion of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma). We performed the viral infection or transfection of siRNA and then, the serum was deprived from culture medium. MTT (0.5 mg/ml in PBS) was added to each well at 24 h after serum deprivation. MTT was incubated for 1.5 h at 37°C. Then, the medium was carefully aspirated, and 200 µl of acidified isopropyl alcohol was added to solubilize the colored formazan product. Absorbance was determined at 550 nm on a scanning multi-well plate reader (Bio-Rad) after agitating the plates for 5 min on a shaker.

siRNA transfection

We used 23 nt siRNA duplexes with two 3′ overhanging nucleotides targeting position 182–204 (aagugacaagucaaga gaagcaa) of human dysbindin mRNA. Scrambled sequence (aacgaugagaacgaucaagaaga), which had no homology to any mammalian mRNA, was used as a control siRNA. Both sense and antisense strands were synthesized by Dharmacon Research Inc (Lafayette, PA, USA). SiRNA duplexes in the 2′-ACE deprotected and desalted form were dissolved in a 1× universal buffer (Dharmacon Research Inc). Transfection of both siRNAs was performed using NeuroPORTER™ (Gene Therapy Systems, Inc., San Diego, CA, USA), as reported (20).

Statistical analysis

Statistical analysis of association studies was performed using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium was examined by using the χ^2-test for goodness of fit. Allele distributions between patients and controls were analyzed by the χ^2-test for independence. The measure of LD, denoted as D', was calculated from the haplotype frequency using the expectation–maximization algorithm. Case–control haplotype analysis was performed by the permutation method to obtain the empirical significance (37). The global P-values represent the overall significance using the χ^2-test when the observed versus expected frequencies of all the haplotypes are considered together. The individual haplotypes were tested for association by grouping all others together and applying the χ^2-test with 1 df. P-values were calculated on the basis of 10 000 replications. Statistical analysis of neurobiological assays was performed by Students' t-test. All P-values reported are two tailed. Statistical significance was defined at $P < 0.05$. To be conservative, Bonferroni corrections were applied for multiple comparisons, e.g. number of analyzed SNPs and haplotypes, although SNPs were in LD.

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REFERENCES

1. O'Donovan, M.C., Williams, N.M. and Owen, M.J. (2003) Recent advances in the genetics of schizophrenia. *Hum. Mol. Genet.*, **12**, R125–R133.
2. Straub, R.E., Jiang, Y., MacLean, C.J., Ma, Y., Webb, B.T., Myakishev, M.V., Harris-Kerr, C., Wormley, B., Sadek, H., Kadambi, B. *et al.* (2002) 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.
3. Schwab, S.G., Knapp, M., Mondabon, S., Hallmayer, J., Borrmann-Hassenbach, M., Albus, M., Lerer, B., Rietschel, M., Trixler, M., Maier, W. *et al.* (2003) Support for association of schizophrenia with genetic variation in the 6p22.3 gene, dysbindin, in sib-pair families with linkage and in an additional sample of triad families. *Am. J. Hum. Genet.*, **72**, 185–190.
4. Tang, J.X., Zhou, J., Fan, J.B., Li, X.W., Shi, Y.Y., Gu, N.F., Feng, G.Y., Xing, Y.L., Shi, J.G. and He, L. (2003) Family-based association study of DTNBP1 in 6p22.3 and schizophrenia. *Mol. Psychiatr.*, **8**, 717–718.
5. Van Den Bogaert, A., Schumacher, J., Schulze, T.G., Otte, A.C., Ohlraun, S., Kovalenko, S., Becker, T., Freudenberg, J., Jonsson, E.G., Mattila-Evenden, M. *et al.* (2003) The *DTNBP1* (dysbindin) gene contributes to schizophrenia, depending on family history of the disease. *Am. J. Hum. Genet.*, **73**, 1438–1443.
6. van den Oord, E.J., Sullivan, P.F., Jiang, Y., Walsh, D., O'Neill, F.A., Kendler, K.S. and Riley, B.P. (2003) Identification of a high-risk haplotype for the dystrobrevin binding protein 1 (*DTNBP1*) gene in the Irish study of high-density schizophrenia families. *Mol. Psychiatr.*, **8**, 499–510.
7. Morris, D.W., McGhee, K.A., Schwaiger, S., Scully, P., Quinn, J., Meagher, D., Waddington, J.L., Gill, M. and Corvin, A.P. (2003) No evidence for association of the dysbindin gene [*DTNBP1*] with schizophrenia in an Irish population-based study. *Schizophr. Res.*, **60**, 167–172.
8. Tsai, G. and Coyle, J.T. (2002) Glutamatergic mechanisms in schizophrenia. *Annu. Rev. Pharmacol. Toxicol.*, **42**, 165–179.
9. Weinberger, D.R., Egan, M.F., Bertolino, A., Callicott, J.H., Mattay, V.S., Lipska, B.K., Berman, K.F. and Goldberg, T.E. (2001) Prefrontal neurons and the genetics of schizophrenia. *Biol. Psychiatr.*, **50**, 825–844.
10. Moghaddam, B. (2003) Bringing order to the glutamate chaos in schizophrenia. *Neuron*, **40**, 881–884.
11. Harrison, P.J. and Owen, M.J. (2003) Genes for schizophrenia? Recent findings and their pathophysiological implications. *Lancet*, **361**, 417–419.
12. Buonanno, A. and Fischbach, G.D. (2001) Neuregulin and ErbB receptor signaling pathways in the nervous system. *Curr. Opin. Neurobiol.*, **11**, 287–296.
13. Stefansson, H., Sigurdsson, E., Steinthorsdottir, V., Bjornsdottir, S., Sigmundsson, T., Ghosh, S., Brynjolfsson, J., Gunnarsdottir, S.,

Ivarsson, O., Chou, T.T. *et al.* (2002) Neuregulin 1 and susceptibility to schizophrenia. *Am. J. Hum. Genet.*, **71**, 877–892.
14. Mothet, J.P., Parent, A.T., Wolosker, H., Brady, R.O., Jr, Linden, D.J., Ferris, C.D., Rogawski, M.A. and Snyder, S.H. (2000) D-serine is an endogenous ligand for the glycine site of the *N*-methyl-D-aspartate receptor. *Proc. Natl Acad. Sci. USA*, **97**, 4926–4931.
15. Chumakov, I., Blumenfeld, M., Guerassimenko, O., Cavarec, L., Palicio, M., Abderrahim, H., Bougueleret, L., Barry, C., Tanaka, H., La Rosa, P. *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.
16. De Blasi, A., Conn, P.J., Pin, J. and Nicoletti, F. (2001) Molecular determinants of metabotropic glutamate receptor signaling. *Trends Pharmacol. Sci.*, **22**, 114–120.
17. Benson, M.A., Newey, S.E., Martin-Rendon, E., Hawkes, R. and Blake, D.J. (2001) Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. *J. Biol. Chem.*, **276**, 24232–24241.
18. Turner, K.M., Burgoyne, R.D. and Morgan, A. (1999) Protein phosphorylation and the regulation of synaptic membrane traffic. *Trends. Neurosci.*, **22**, 459–464.
19. Brunet, A., Datta, S.R. and Greenberg, M.E. (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.*, **11**, 297–305.
20. Numakawa, T., Nakayama, H., Suzuki, S., Kubo, T., Nara, F., Numakawa, Y., Yokomaku, D., Araki, T., Ishimoto, T., Ogura, A. *et al.* (2003) Nerve growth factor-induced glutamate release is via p75 receptor, ceramide, and Ca(2+) from ryanodine receptor in developing cerebellar neurons. *J. Biol. Chem.*, **278**, 41259–41269.
21. Williams, N.M., Preece, A., Morris, D.W., Spurlock, G., Bray, N.J., Stephens, M., Norton, N., Williams, H., Clement, M., Dwyer, S. *et al.* (2004) Identification in 2 independent samples of a novel schizophrenia risk haplotype of the dystrobrevin binding protein gene (*DTNBP1*). *Arch. Gen. Psychiatr.*, **61**, 336–344.
22. Tokuhiro, S., Yamada, R., Chang, X., Suzuki, A., Kochi, Y., Sawada, T., Suzuki, M., Nagasaki, M., Ohtsuki, M., Ono, M. *et al.* (2003) An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.*, **35**, 341–348.
23. McClintock, W., Shannon Weickert, C., Halim, N.D., Lipska, B.K., Hyde, T.M., Herman, M.M., Weinberger, D.R., Kleinman, J.E. and Straub, R.E. (2003) Reduced expression of dysbindin protein in the dorsolateral prefrontal cortex of patients with schizophrenia. *Program No. 317.9. 2003 Abstract Viewer/Itinerary Planner.* Washington, DC, Society for Neuroscience (Online).
24. Talbot, K., Eidem, W.L., Tinsley, C.L., Benson, M.A., Thompson, E.W., Smith, R.J., Hahn, C.G., Siegel, S.J., Trojanowski, J.Q., Gur, R.E. *et al.* (2004) Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. *J. Clin. Invest.*, **113**, 1353–1363.
25. Weickert, C.S., Straub, R.E., McClintock, B.W., Matsumoto, M., Hashimoto, R., Hyde, T.M., Herman, M.M., Weinberger, D.R. and Kleinman, J.E. (2004) Human dysbindin (*DTNBP1*) gene expression in normal brain and in schizophrenic prefrontal cortex and midbrain. *Arch. Gen. Psychiatr.*, **61**, 544–555.
26. Honer, W.G., Falkai, P., Bayer, T.A., Xie, J., Hu, L., Li, H.Y., Arango, V., Mann, J.J., Dwork, A.J. and Trimble, W.S. (2002) Abnormalities of SNARE mechanism proteins in anterior frontal cortex in severe mental illness. *Cereb. Cortex*, **12**, 349–356.
27. Vawter, M.P., Thatcher, L., Usen, N., Hyde, T.M., Kleinman, J.E. and Freed, W.J. (2002) Reduction of synapsin in the hippocampus of patients with bipolar disorder and schizophrenia. *Mol. Psychiatr.*, **7**, 571–578.
28. Emamian, E.S., Hall, D., Birnbaum, M.J., Karayiorgou, M. and Gogos, J.A. (2004) Convergent evidence for impaired AKT1-GSK3beta signaling in schizophrenia. *Nat. Genet.*, **36**, 131–137.
29. Li, W., Zhang, Q., Oiso, N., Novak, E.K., Gautam, R., O'Brien, E.P., Tinsley, C.L., Blake, D.J., Spritz, R.A., Copeland, N.G. *et al.* (2003) Hermansky–Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat. Genet.*, **35**, 84–89.
30. Hashimoto, R., Yoshida, M., Ozaki, N., Yamanouchi, Y., Iwata, N., Suzuki, T., Kitajima, T., Tatsumi, M., Kamijima, K. and Kunugi, H. (2004) Association analysis of the −308G>A promoter polymorphism of the tumor necrosis factor alpha (TNF-alpha) gene in Japanese patients with schizophrenia. *J. Neural Transm.*, **111**, 217–221.
31. Hashimoto, R., Straub, R.E., Weickert, C.S., Hyde, T.M., Kleinman, J.E. and Weinberger, D.R. (2004) Expression analysis of neuregulin-1 in the dorsolateral prefrontal cortex in schizophrenia. *Mol. Psychiatr.*, **9**, 299–307.
32. Numakawa, T., Matsumoto, T., Adachi, N., Yokomaku, D., Kojima, M., Takei, N. and Hatanaka, H. (2001) Brain-derived neurotrophic factor triggers a rapid glutamate release through increase of intracellular Ca(2+) and Na(+) in cultured cerebellar neurons. *J. Neurosci. Res.*, **66**, 96–108.
33. Numakawa, T., Yamagishi, S., Adachi, N., Matsumoto, T., Yokomaku, D., Yamada, M. and Hatanaka, H. (2002) Brain-derived neurotrophic factor-induced potentiation of Ca(2+) oscillations in developing cortical neurons. *J. Biol. Chem.*, **277**, 6520–6529.
34. Kawamura, M., Namba, H., Otsu, Y., Hayashi, Y., Takei, N. and Nawa, H. (2003) Characterization of novel bicistronic sindbis virus vectors, SinEGdsp and SinIRES-EG, in cultured neurons. *Recent Res. Dev. Neurochem.*, **6**, 105–120.
35. Numakawa, T., Yokomaku, D., Kiyosue, K., Adachi, N., Matsumoto, T., Numakawa, Y., Taguchi, T., Hatanaka, H. and Yamada, M. (2002) Basic fibroblast growth factor evokes a rapid glutamate release through activation of the MAPK pathway in cultured cortical neurons. *J. Biol. Chem.*, **277**, 28861–28869.
36. Hashimoto, R., Nakamura, Y., Komai, S., Kashiwagi, Y., Tamura, K., Goto, T., Aimoto, S., Kaibuchi, K., Shiosaka, S. and Takeda, M. (2000) Site-specific phosphorylation of neurofilament-L is mediated by calcium/calmodulin-dependent protein kinase II in the apical dendrites during long-term potentiation. *J. Neurochem.*, **75**, 373–382.
37. Good, P. (2000) *Permutation Tests. A Practical Guide to Resampling Methods for Testing Hypothesis.* 2nd edn. Springer-Verlag, New York.

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Mitochondrial DNA 3644T→C mutation associated with bipolar disorder

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Abstract

Mitochondrial dysfunction associated with mutant mitochondrial DNA (mtDNA) has been suggested in bipolar disorder, and comorbidity with neurodegenerative diseases was often noted. We examined the entire sequence of mtDNA in six subjects with bipolar disorder having comorbid somatic symptoms suggestive of mitochondrial disorders and found several uncharacterized homoplasmic nonsynonymous nucleotide substitutions of mtDNA. Of these, 3644C was found in 5 of 199 patients with bipolar disorder but in none of 258 controls ($p = 0.015$). The association was significant in the extended samples [bipolar disorder, 9/630 (1.43%); controls, 1/734 (0.14%); $p = 0.007$]. On the other hand, only 5 of 25 family members with this mutation developed bipolar disorder, of which 4 patients with 3644C had comorbid physical symptoms. The 3644T→C mutation converts amino acid 113, valine, to alanine in the NADH-ubiquinone dehydrogenase subunit I, a subunit of complex I, and 113 valine is well conserved from *Drosophila* to 61 mammalian species. Using transmitochondrial cybrids, 3644T→C was shown to decrease mitochondrial membrane potential and complex I activity compared with haplogroup-matched controls. According to human mitochondrial genome polymorphism databases, 3644C was not found in centenarians but was found in 3% of patients with Alzheimer disease and 2% with Parkinson disease. The result of modest functional impairment caused by 3644T→C suggests that this mutation could increase the risk for bipolar disorder.

Keywords: Bipolar disorder; MtDNA 3644T→C; Association study; Mitochondrial membrane potential; Complex I activity

Bipolar disorder is a major mental disorder characterized by recurrent manic and depressive episodes affecting about 1% of the population. The contribution of multiple genetic factors in the etiology of bipolar disorder is known from studies of twins, adoptions, and families. Although recent

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studies suggested several candidate polymorphisms, such as Val 311 of the brain-derived neurotrophic factor [1,2] and the −116G polymorphism of X-box binding protein 1 [3], the pathophysiological mechanisms of bipolar disorder have not yet been totally elucidated. Mitochondrial dysfunction in bipolar disorder was initially suggested by altered brain energy metabolism detected by ^{31}P magnetic resonance spectroscopy [4] and was recently supported by the altered gene expressions of mitochondria-related genes revealed by DNA microarray analysis in the postmortem brain [5]. The comorbidity of bipolar disorder or depression and a mitochondrial disorder, chronic-progressive external ophthalmoplegia (CPEO) [6–8], also suggests that mitochondrial dysfunction can cause bipolar disorder. It was pointed out that some families of bipolar disorder were seen in the maternal lineage [9], suggesting that mitochondrial DNA may have a pathophysiological role in bipolar disorder. The authors previously reported an association between bipolar disorder and two mitochondrial DNA (mtDNA) polymorphisms, 5178C and 10398A, in Japanese subjects [10]. A similar trend of association with 10398A was also reported in Caucasians [11]. These two polymorphisms convert amino acids in the subunits of complex I (NADH:ubiquinone oxidoreductase). NDUFV2, a nuclear-encoded complex I subunit gene, was also associated with bipolar disorder [12]. These results suggest that other genetic variations of complex I subunits in mtDNA are also risk factors for bipolar disorder.

Human mtDNA is inherited only maternally and encodes 13 protein subunits of the respiratory chain, including 7 complex I subunit genes, 22 tRNAs, and rRNAs [13]. It has been reported [14] that heteroplasmic tRNA mutations of mtDNA are related to neuromuscular diseases such as mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS), and myoclonus epilepsy with ragged-red fibers. Large-scale deletions are related to CPEO. On the other hand, there are missense mutations of mtDNA related to diseases, such as neurogenic muscle weakness, ataxia, and retinitis pigmentosa; Leigh encephalopathy; and Leber hereditary optic neuropathy (LHON). Most are heteroplasmic, a mixture of mutant and wild-type mtDNA, but sometimes these mutations can be homoplasmic in patients. The homoplasmic mutation of 1555A→G in the rRNA coding region related to inherited hearing loss caused by aminoglycoside toxicity is well described [15,16]. Alterations in mtDNA have also been studied in patients with Parkinson disease and Alzheimer disease [17,18]. The phenotypes of mitochondrial diseases are diverse and overlapping. The same mtDNA mutation can produce quite different phenotypes, while different mutations can produce similar phenotypes. The mutations or polymorphisms associated with bipolar disorder, if any, may also cause overlapping phenotypes and become a risk factor for other disorders.

In this study, we hypothesized that there are some homoplasmic mutations or polymorphisms increasing the risk for bipolar disorder and other signs and symptoms related to mitochondrial impairment. To identify such nucleotide substitutions of mtDNA, we sequenced the entire 16.6-kb mtDNA of patients with comorbidity of bipolar disorder and somatic symptoms frequently associated with mitochondrial disorders. Among newly identified nonsynonymous nucleotide substitutions in these patients, the 3644T→C at NADH-ubiquinone dehydrogenase subunit 1 (ND1), decreasing mitochondrial membrane potential and complex I activity, was associated with bipolar disorder. The comorbidity with bipolar disorder was present in most of these cases but their phenotypes were various. It was suggested that this mutation could increase risks for bipolar disorder with syndromic comorbidity.

Results and discussion

Unreported homoplasmic mtDNA base substitutions in patients

We examined the entire mtDNA sequence of six patients with bipolar disorder and somatic symptoms suggestive of mitochondrial disorders, such as ptosis, optic neuropathy, cardiomyopathy, and myoclonus (Table 1). None of them could be diagnosed as known mitochondrial diseases, such as MELAS, CPEO, and LHON, because of the reasons as described under Case reports. Five of them had a family history of mood disorder compatible with maternal inheritance. Every patient had several base substitutions compared with the revised Cambridge Reference Sequence [13,19]. The average number of base substitutions in each individual was 32.5 ± 6.9 (mean ± SD), and that of nonsynonymous base substitutions was 5.5 ± 2.1. We consulted the MITOMAP database (http://www.mitomap.org/) [20,21], and two mutations were provisionally reported in relation to mitochondrial diseases, 11084A→G (MELAS) and 12311T→C (CPEO). We also found four nonsynonymous nucleotide substitutions, 3644T→C, 4705T→C, 13651A→G, and 13928G→T, which were not registered in the MITOMAP, all of which were in the complex I subunits. We confirmed that these base substitutions were homoplasmic by the PCR restriction-length polymorphism method (PCR-RFLP).

To identify the mtDNA base substitutions having pathophysiological significance, we examined whether these base substitutions were found in 96 Japanese centenarians using the mtSNP database (Human Mitochondrial Genome Polymorphism Database in Japan, http://www.giib.or.jp/mtsnp/index_e.html) [22]. We regarded the base substitutions found in centenarians as having minimum pathophysiological significance. Base substitutions 4705T→C, 11084A→G, 12311T→C, and 13651A→G were found in centenarians, while two base substitutions, 3364T→C and 13928G→T, were not found in centenarians.

Table 1
Patients and unreported nucleotide substitutions of mitochondria DNA

Case	Diagnosis	Gender	Age at onset	Clinical manifestations		MtDNA substitutions	
				Physical symptoms	Family history	Unreported	Provisionally disease related
1	Bipolar I disorder	F	17	Optic neuritis	Mo, depression	13651A→G	
2	Bipolar I disorder	M	30	Cerebral infarction Dilated cardiomyopathy	Bro, bipolar disorder MoSib, psychotic NOS		
3	Bipolar I disorder	M	50	Ptosis Epilepsy Cardiac arrhythmia	MoSib, depression		12311T→C (CPEO)
4	Bipolar I disorder	F	24	Epileptic EEG	Bro, bipolar disorder Sis, NOS		11084A→G (MELAS)
5	Bipolar I disorder	M	57	Ptosis Muscle weakness NIDDM Multiple cerebral infarction	Sporadic	3644T→C	
6	Bipolar I disorder	M	35	Ptosis	Sib, depression	4705T→C 13928G→T	

Abbreviations: Mo, mother; Bro, brother; Sis, sister; Sib, sibling; MoSib, mother's sibling; psychotic NOS, psychotic disorder not otherwise specified.

Association study of mtDNA base substitutions

To know whether these two base substitutions, 3644T→C and 13928G→T, are associated with bipolar disorder, we used two sets of the study subjects. The initial association study consisted of 199 patients with bipolar disorder and 258 healthy volunteers. An additional independent sample set in COSMO (Collaborative Study of Mood Disorder) consisted of 431 patients with bipolar disorder and 476 healthy volunteers, was also used. To examine whether there is a hidden population structure, we performed stratification analysis on the initial samples using eight polymorphisms [3] using the method of Pritchard et al. [23], and no subpopulation was found for either patients or controls. We performed a similar stratification analysis using 20 SNPs in 169 Japanese samples, including COSMO samples, and found no subpopulation. We further analyzed the stratification in 169 Japanese samples using 374 microsatellite markers and found no hidden subpopulation (Yamada et al., manuscript in preparation). Thus, we concluded that there is no hidden subpopulation in our Japanese samples. Six patients examined for the entire mtDNA sequence were included in the first sample set, because they developed comorbid somatic symptoms after the diagnosis of bipolar disorder.

We genotyped at 3644 and 13928 by PCR-RFLP in the initial sample set (Table 2). Base 3644C was found in 5 of 199 Japanese patients with bipolar disorder in the first sample set, including the proband (case 5 in Table 1, II-1 of family A in Fig. 1), but in none of the controls (p = 0.015) (Table 2). Among other 4 patients, 1 had non-insulin-dependent diabetes mellitus (NIDDM), 1 had headache, and 1 had tremor suggestive of neurological impairment. In their family members, only 5 of 25 members in the same maternal lineages, who were assumed to have the same genotype, 3644C, developed

Table 2
Association study using independent sample sets and haplogroups

Base at 3644:	All samples					Haplogroup D (5178A/10398G)				
	T		C		p value	T		C		p value
Initial sample set										
Patients	97.5%	(194)	2.5%	(5)		94.4%	(68)	5.6%	(4)	
Controls	100.0%	(258)	0.0%	(0)	0.015*	100.0%	(97)	0.0%	(0)	0.003*
Independent sample set										
Patients	99.1%	(427)	0.9%	(4)		98.3%	(171)	1.7%	(3)	
Controls	99.8%	(475)	0.2%	(1)	0.197	100.0%	(192)	0.0%	(0)	0.106
Total sample set										
Patients	98.6%	(621)	1.4%	(9)		97.2%	(239)	2.8%	(7)	
Controls	99.9%	(733)	0.1%	(1)	0.007*	100.0%	(289)	0.0%	(0)	0.004*

Each number in parentheses shows the real number of subjects. The p value was given by Fisher's exact test.
* Statistically significant.

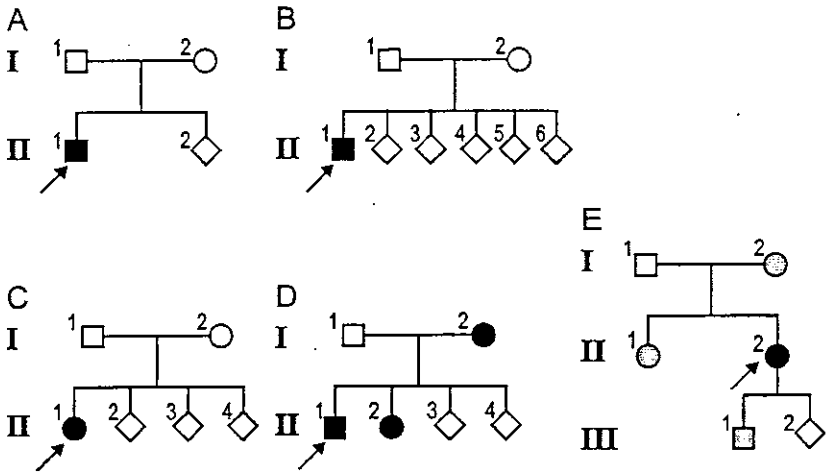

Fig. 1. Pedigrees of the probands with bipolar disorder and mitochondrial 3644C mutation. Arrows indicate the probands with bipolar disorder. Closed squares and circles indicate the patients with bipolar disorder or other mental disorders as follows: D, I-2 had a psychotic disorder not otherwise specified, II-2 had schizotypal personality disorder. Their comorbidities were as follows: A, II-1 had muscle weakness, ptosis, and NIDDM (case 5 in Table 1); C, II-1, had essential tremor; D, II-1, had NIDDM; E, I-2, II-1, II-2, and III-1 had headache. To maintain the anonymity of the pedigrees, the sexes of the unaffected siblings are not shown.

bipolar disorder, of which 4 patients with 3644C had comorbid physical symptoms and one had only bipolar disorder. Mutation 3644T→C converts amino acid 113 valine in the putative third transmembrane region of ND1, the protein subunit of complex I, to alanine. This 113 valine is well conserved from *Drosophila* to 61 mammalian species. There was no difference in the frequency of 13928G→T [13 of 199 patients with bipolar disorder (6.5%) and 19 of 258 controls (7.4%), $p = 0.804$ by Fisher's exact test]. Mutation 13928G→T changes the 531 serine into isoleucine in the ND5 subunit and it was not conserved even among mammalian species.

We further analyzed 3644T→C as a candidate risk factor for bipolar disorder using the independent sample set obtained from COSMO. While 4 additional individuals having 3644C were found among the patients, only 1 of the 476 controls had 3644C. Although this difference in frequency was not statistically significant ($p = 0.197$), this is likely due to the low statistical power to detect the difference (0.29). In the analysis of total samples having higher statistical power (0.79), 3644C was significantly more common in bipolar disorder than in the controls ($p = 0.007$) (Table 2).

Since mtDNA is highly polymorphic, other polymorphisms possibly confounded the association analysis. To minimize the effects of other polymorphisms, we categorized these samples into mitochondrial DNA haplogroups and the association analysis was repeated in each haplogroup. Seven patients with 3644C were assigned to the Asian haplogroup D characterized by 5178A/10398G [22], which we reported as an anti-risk haplotype for bipolar disorder [10]. The 3644C was significantly associated with bipolar disorder in haplogroup D (Table 2). On the other hand, only 1 control subject and 2 patients with 3644C were classified into haplogroup M characterized by 5178C/10398G, and no association was found in haplogroup M [2 of 187 patients (1.1%) and 1 of 233 controls (0.4%), $p = 0.588$].

We concluded that 3644C was associated with bipolar disorder for the following reasons: 3644T→C was associated with bipolar disorder in the initial case-control study; this substitution converts well-conserved amino acid 113 valine to alanine in ND1. A similar trend was observed in the independent samples, although there was no significant difference, possibly due to the small number of subjects replicating the association. In the analysis of the total sample set having enough statistical power to detect a difference, 3644C was significantly associated with bipolar disorder. The significant association between 3644C and bipolar disorder remained in haplogroup-matched case-control analysis.

We called 3644C a "mutation," because its frequency was very low (0.14% in 734 controls and 0.7% in 1364 total samples examined), it converted a well-conserved amino acid, and it appeared in at least two independent haplogroups. However, this mutation is not sufficient to cause bipolar disorder because 3644C was found in 1 healthy volunteer, and only 5 of 25 members in the same maternal lineages, all of whom were assumed to have 3644C, developed bipolar disorder. Among these patients, comorbidity in 4 patients with bipolar disorder was heterogeneous: 2 had NIDDM, 1 headache, and 1 tremor suggestive of neurological impairment. The other patient had only bipolar disorder. It means that 3644C cannot be a risk factor for comorbid symptoms seen in these patients but could be a risk factor for bipolar disorder, if not a causative mutation. Bipolar disorder is a multigenic disease and one type of mutation in mtDNA can cause various phenotypes. We

postulate that synergistic effects of other risk factors and 3644C could cause bipolar disorder.

Functional analyses in cybrids with 3644C

To evaluate the functional consequences of 3644T→C, we generated cell lines of the transmitochondrial hybrids, "cybrids," using the platelets derived from the subjects. Different from heteroplasmic mutations in the regions of tRNAs and protein subunits, functional impairment associated with homoplasmic mutation has not been well established. In the case of heteroplasmic mutation, two cybrid cell lines with different nucleotides at one particular position of mtDNA could be generated and analyzed. On the other hand, in the case of homoplasmic mutation, it was impossible to identify such a pair of cell lines. To minimize the effects of other polymorphisms, we compared cybrids with 3644C with haplogroup-matched controls for functional studies. A total of 24 cybrid cell lines were obtained from the initial sample set, and 9 cybrid cell lines belonged to haplogroup D, 5178A/10398G (Table 3). Among the 9 cell lines, only 2 were from patients with 3644C (II-1 in family D and II-2 in family E, in Fig. 1) and 7 were from subjects with 3644T (3 patients with bipolar disorder and 4 controls). We could not obtain other samples with 3644C because of ethical reasons.

Mitochondrial membrane potential (MMP) was measured using JC-1, a fluorescent cationic dye, which accumulates in mitochondria and changes its emission from wavelength 527 nm (monomer) to 590 nm (aggregates) depending on the mitochondrial membrane potential, and a fluorescence-activated cell sorter (FACS), and it distinguished well the difference between control cybrids and $\rho^0$206 cells lacking mtDNA: while 82.9 ± 9.9% (mean ± SD, N = 12) of the cybrids from control subjects were polarized, only 13.2 ± 7.7 (mean ± SE of three measurements) of the $\rho^0$206 cells were polarized (Fig. 2, left and right, respectively). This indicated that our measurement method is sensitive enough to detect the difference in MMP. The percentage of polarized cells was significantly decreased in cybrids with 3644C [51.7 ± 6.6 and 67.0 ± 4.3% (means ± SE), respectively] compared with haplogroup-matched cybrids (df = 8, p = 0.04 by Mann-Whitney U test) (Table 3). There was no significant difference between cybrids of bipolar disorder and controls nor between cybrids of other haplogroups.

Subsequently, the activities of complexes I (rotenone-insensitive), III, and IV in the electron-transport chain were measured using the citrate synthase activity as the reference (Table 4). The activity of $\rho^0$206 cells was measured to assess nonspecific activity. The 3644C group consisted of two cybrid cell lines. While there was no significant difference between complex III and complex IV activities ($p > 0.1$), complex I activity of the two cybrids with 3644C tended to be lower than four haplogroup-matched control cybrids (df = 5, p = 0.06 by Mann-Whitney U test). Decreased MMP could be explained by reduced complex I activity since MMP is maintained by the efflux of protons from the mitochondrial matrix, in which complex I plays an important role. MMP generated by the proton gradient is the driving force of not only ATP synthesis but also Ca^{2+} uptake across the mitochondrial inner membrane. We hypothesized that impaired mitochondrial Ca^{2+} uptake caused altered calcium signaling in bipolar disorder. Our result of decreased MMP in cybrids with 3644C supports our hypothesis.

Interestingly, the mtSNP database [22] showed that while 3644C was not found in 96 centenarians, it was found in 3.1% (3/96) of patients with Alzheimer disease and 2.0% (2/96) of patients with Parkinson disease. These findings suggested a possibility that 3644C is a risk factor common to bipolar disorder and neurodegenerative disorders, rather than a causative mutation only for bipolar disorder. If 3644C is also a risk factor for neurodegener-

Table 3
Mitochondrial membrane potential (MMP) of 24 cybrid cell lines

	N	Age	(C/B)	Gender	MMP
Diagnosis					
Control	12	48.0 ± 9.2		6/6	82.9 ± 9.9
Bipolar disorder	12	41.8 ±11.4		6/6	77.2 ±11.0
Bipolar disorder with 3644T	10	40.7 ±12.0		5/5	80.8 ± 7.0
Bipolar disorder with 3644C	2	42, 53		1/1	59.4 ±10.9*
Haplogroup					
10398A–5178C–3644T	7	43.6 ± 10.6	4/3	3/4	81.9 ± 8.6
10398G–5178C–3644T	8	41.6 ± 10.7	4/4	4/4	80.8 ± 9.5
10398G–5178A–3644T	7	48.9 ± 10.5	4/3	3/4	83.3 ± 8.5
10398G–5178A–3644C	2	42, 53	0/2	1/1	59.4 ±10.9**
ρ^0 cells	1				13.21

C/B, numbers of control/bipolar disorder; gender, number of men/women. The p value was given by the Mann–Whitney U test.
* p = 0.03 vs 3644T, 0.08 vs controls.
** p = 0.04 vs 3644T, 0.03 vs all other haplogroups.

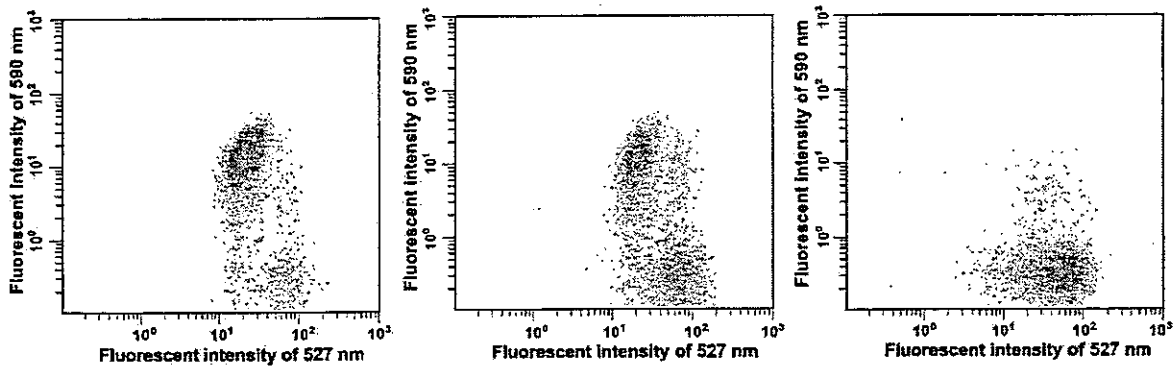

Fig. 2. Measurement of mitochondrial membrane potential using JC-1 and FACS. Vertical line, the fluorescence intensity of 590 nm, reflecting the aggregates and indicating high MMP. Horizontal line, the fluorescence intensity of 527 nm, reflecting the monomer and indicating low MMP. 10,000 cells were examined for one cell line. Representative results of one experiment each from three cell lines are shown. Left, control cybrids whose haplogroups were matched with the cybrids with 3644C; middle, cybrids with 3644C; right, $\rho^0$206 cells. While most control cybrids were polarized, having high 590/527 nm, most $\rho^0$206 cells were depolarized. The cybrids with 3644C were intermediate, having both polarized and depolarized cells.

ative disorders, the mechanism might be explained by a disruption of MMP that causes apoptosis. It is also compatible with the reduction of complex I activity in platelets or altered calcium signaling in cybrids derived from patients with Parkinson disease or Alzheimer disease [24–26]. Neuropathological studies of bipolar disorder also showed a decreased number of neurons in postmortem brains [27,28]. It was pointed out that having bipolar disorder increases the risk of Alzheimer disease [29,30] and Parkinson disease [31]. Two mood stabilizers, lithium and valproate, are known to have antiapoptotic effects by increasing Bcl-2 [32]. These findings are also compatible with the possibility that 3644C is a risk factor common to bipolar disorder and neurodegenerative disorders.

One might have a concern that 3644C is not a risk factor for bipolar disorder but associated with physical symptoms. Although the initial patient had several physical symptoms suggestive of mitochondrial disorder such as ptosis, muscle weakness, NIDDM, and cerebral infarction, other patients carrying 3644C had no or one nonspecific comorbid symptom. Thus, the apparent association between bipolar disorder and 3644C cannot be explained by the secondary phenomenon due to physical symptoms. However, it cannot be ruled out that these patients carrying 3644C have some subtle mitochondria-related symptoms that were not clinically apparent. In fact, there are reports of patients with pathogenic mtDNA mutations such as 3243A→G who showed psychotic symptoms at first and developed mitochondrial diseases later [33,34]. It might be possible that detailed physical examinations, for example, glucose tolerance test or close neurological examinations, would reveal subtle comorbid somatic symptoms. Needless to say, we need to address whether the 3664C substitution is associated with somatic symptoms alone. In the future, it is needed to look carefully at the phenotype and the clinical course of these subjects and investigate whether 3644C is associated with bipolar disorder or a bipolar disorder-somatic symptom subtype.

Functional impairment was reported also in the homoplasmic mutation, 1555A→G, in maternally inherited hearing loss [15,16]. The 11778A mutation of LHON, which is usually heteroplasmic but sometimes homoplasmic, was also shown to cause a modest reduction in complex I activity [35]. It was pointed out that the nuclear background potentially affects the expression of mtDNA polymorphisms [36]. Further study using cybrids with another nuclear background would be interesting. The mechanism of how the V113A amino acid substitution caused by 3644T→C in ND1 decreases complex I activity cannot be explained since the structure and function of each protein subunit are not yet well known. In particular, it remains unclear how complex I translocates protons across the mitochondrial inner membrane coupled to electron transfer. In summary, 3644T→C is a rare base substitution of mtDNA but induces modest impairment of complex I activity and becomes a risk factor for bipolar disorder.

Materials and methods

Subjects

Patients with bipolar disorder were diagnosed according to the DSM-III-R or DSM-IV criteria by at least two

Table 4
Enzyme activities of electron-transport chain of cybrids with 3644C and controls

	Control (mean ± SD), $N = 4$	3644C (mean ± SD), $N = 2$	ρ^0cells	p value*
Complex I/CS	14.47 ± 5.43	7.64 ± 0.08	4.82	0.06
Complex III/CS	32.15 ± 12.78	21.12 ± 2.72	7.36	0.36
Complex IV/CS	39.74 ± 11.24	29.21 ± 6.97	0.76	0.36

* The p value was calculated using the Mann–Whitney U test.

interview sessions by two senior psychiatrists and a consensus diagnosis was made. Their family history of mental disorder was assessed by interviewing the proband and available relatives. Control subjects were recruited from the staff or students of participating institutes and their friends, who reported themselves to be healthy. Written informed consent was obtained from all subjects. This study was approved by the ethics committees of RIKEN and all participating institutes.

The study subjects for the initial association study consisted of 199 patients with bipolar disorder (143 bipolar I and 56 bipolar II, 76 male and 123 female, 49.8 years of age on average) and 258 healthy volunteers (129 male and 129 female, 33.0 years of age on average). An additional independent sample set in COSMO consisted of 431 patients with bipolar disorder (214 male and 217 female, 49.5 years of age on average) and 476 healthy volunteers (226 male and 250 female, 50.4 years of age on average).

Six patients with bipolar disorder with somatic symptoms suggestive of mitochondrial disorders were chosen from the first sample set for examination of the entire mtDNA. They had been recruited in our bipolar disorder study based on our inclusion criteria, having DSM-IV bipolar disorder by consensus diagnoses after two nonstructured interview sessions with senior psychiatrists, and exclusion criteria, having no clinically remarkable neurological diseases, head trauma, or comorbid Axis II diagnoses. Characteristics of these six subjects are listed below and summarized in the Table 1.

The transmitochondrial cybrids for the following functional analyses were generated from 24 subjects in the initial samples, including two patients with 3644C.

Case reports

Case 1, 38-year-old female, is a patient with bipolar I disorder without psychotic features. She had the first episode of mania with psychomotor agitation and confusion at age 17. At age 27, she was admitted to a hospital due to bilateral optic neuritis. She had no other symptoms suggestive of multiple sclerosis. Her optic neuritis was improved by steroid therapy, and final diagnosis was idiopathic optic neuropathy. Because she had no relatives with optic neuropathy and her symptoms were reversible, Leber disease was not considered by the attendant ophthalmologist.

Case 2 is a 61-year-old male diagnosed as having bipolar I disorder. At age 30, he had the onset of mania with mood-incongruent psychotic features. At age 60, after being discharged from a psychiatric hospital, he was admitted to a hospital due to stroke. Brain imaging revealed infarctions in the cerebellum and the brain stem. During this hospitalization, chest X-ray showed enlarged heart and he was diagnosed as idiopathic dilated cardiomyopathy. He also had renal failure. His attendant physician did not suspect mitochondrial disease.

Case 3 is a 56-year-old male diagnosed with bipolar I disorder. At age 49, he had the onset of depression characterized by depressive mood, fatigability, retardation, insomnia, and suicidal thought. At age 52, he suddenly became manic. During this manic episode, he caused a motor vehicle accident. He was admitted to a psychiatric ward for the treatment of mania. After the first admission, he had generalized tonic clonic seizures. Although electroencephalography (EEG) recording showed no signs of epilepsy, he was clinically diagnosed as having epilepsy. At age 54, he complained of swollen eyelid, and medical examination did not show any signs of renal failure. He also complained of muscle weakness and had an episode of falling down due to muscle weakness. A neurologist saw this patient and assessed that ptosis may be present but fluctuating and was not pathological. He also showed some tendency of disturbed movement of the eyes, but it was also fluctuating and he did not have diplopia. Muscle weakness was not objectively present. Based on these clinical examinations, the neurologist ruled out mitochondrial disease from differential diagnosis and judged that further investigation was not necessary. Electrocardiogram indicated supraventricular extrasystole, but it was not clinically remarkable.

Case 4 is a 46-year-old female diagnosed with bipolar I disorder. At age 24, she had the onset of mania. At age 40, she began rapid cycling. During her psychiatric hospitalization, EEG recording showed epileptic abnormality. However, she did not have any signs or symptoms of epilepsy and was not diagnosed as epileptic.

Case 5 is 57-year-old male diagnosed as having bipolar I disorder. He had the onset of a manic episode at age 50. Since his clinical representation resembled confusion caused by organic mental disorder, he received lumbar puncture by a neurologist during psychiatric hospitalization, which showed elevated cerebrospinal fluid protein levels. The neurologist also noted muscle weakness and slight ptosis on the left eyelid. However, these symptoms were improved without any treatment and his subsequent manic episodes were typical manic syndrome without any additional neurological features or psychotic features. He was finally diagnosed as having bipolar I disorder. After the onset of bipolar disorder, he was diagnosed as having non-insulin-dependent diabetes mellitus. His cranial magnetic resonance image showed multiple subcortical silent infarction.

Case 6 is a 38-year-old male having bipolar I disorder. His clinical record was published elsewhere [37]. He complained of ptosis during antipsychotic treatment. Both a neurologist and an ophthalmologist examined and diagnosed him as not having any mitochondrial disease, since his sign was transient and not clinically remarkable.

MtDNA sequencing

Total DNA was extracted from peripheral blood leukocytes by standard protocols. Entire mtDNA sequenc-

ing was performed as previously described [38] with some modifications. In short, each DNA sample was diluted to 10 μg/ml, and nested PCR was performed. PCR was initially performed to obtain two long PCR products, 6 and 11 kb of mtDNA; the second PCR was designed as a set of three overlapping fragments from the first 6-kb PCR product and six fragments from the first 11-kb PCR product. After the second PCR, the products were treated with a SeqDirect PCR Cleaning kit (Qbiogene, Carlsbad, CA, USA) according to the manufacturer's protocol. Both strands of these fragments were then sequenced with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and ABI Prism 3700 DNA sequencer (Applied Biosystems). Each mtDNA site was read at least three times, including at least once for each strand.

Genotyping

The two base substitutions of mtDNA, 3644T→C and 13928G→T, were genotyped using the PCR-RFLP method and sequencing. The enzymes and experimental conditions for the PCR-RFLP were as follows: 3644 was genotyped by primers 5′-GTAGAATGATGGCTAGGGTGACT-3′ and 5′-TCTAGCCACCTCTAGCCTAGACG-3′ and the restriction enzyme *Tai*I (Fermentas), 13928 by 5′-CATACTCGGATTC-TACGCTA-3′, 5′-TTTAGGTAATAGCTTTTCTA-3′, and *Nhe*I (Takara Bio, Inc., Shiga, Japan). MtDNAs 5178C→A and 10398A→G were genotyped to determine the haplogroups. Genotypes of 5178C→A and 10398A→G were examined as previously described [10].

Generation of cybrids

The 143B.TK$^-$ $\rho^0$206 cell line, lacking mtDNA and established by King and Attardi [39], was used for generating cybrids. Platelets of patients and controls were separated from peripheral blood and fused with $\rho^0$206 cells using 40% polyethylene glycol 1500 (Sigma), as previously described [40]. We used DMEM (Gibco BRL) containing 10% FBS (fetal bovine serum; Gibco BRL), penicillin/streptomycin, pyruvate (Gibco BRL), and uridine (Sigma) as the growth medium for ρ^0 cells. For the selection of transmitochondrial cybrid cell lines, we used DMEM containing 10% dialyzed FBS, penicillin/streptomycin, and pyruvate. After the harvest of individual cybrid cell lines, the integration of mtDNA was confirmed by Southern blot analysis using 18S ribosomal RNA repeating units as a reference [41]. The identity of the mtDNA of the cybrids with that of the donor was verified by sequencing the D loop and genotyping several polymorphisms. For Southern blot analysis, we used the ECL Labeling and Detection System according to the manufacturer's protocol (Amersham Biosciences Corp., NJ, USA). Cybrids were stored in liquid nitrogen for further experiments.

Measurement of MMP using JC-1

MMP was estimated using JC-1 (Molecular Probes, Eugene, OR) and flow cytometry. Cybrids stored in liquid nitrogen were thawed and incubated in an atmosphere of 5% CO_2 at 37°C in DMEM containing 10% FBS, penicillin/streptomycin, and pyruvate. Cells (1×10^6) were trypsinized and harvested in 10 ml of DMEM containing 10% FBS, washed with PBS (phosphate-buffered saline) once, and stained with DMEM containing 5 μg/ml JC-1 for 15 min at 37°C. Cells were then washed with PBS and subjected to analysis using a FACS (Epics Elite cell sorter; Beckman Coulter, Fullerton, CA, USA) as previously described [42]. The excitation wavelength was 488 nm by argon ion laser. Emissions at 590 and 527 nm were isolated by each photomultiplier detector and 10,000 cells were measured for each experiment. The experiment was performed in triplicate for each cell line. The cells with polarized mitochondria were defined by an intensity ratio of 590 nm/527 nm above 0.2.

Activities of enzymes in the electron-transport chain

For the sample preparation of the mitochondrial fraction, each line of cybrids was amplified until the cell count was 5×10^7. Cybrids were trypsinized and harvested in DMEM. After being washed once with PBS and once with isolation buffer [210 mM D-mannitol, 71 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin (fatty acid free), 5 mM Hepes, pH 7.2], the cells were suspended in 5 ml of isolation buffer. Using a chilled Dounce glass homogenizer with a loose fitting pestle, 20 passes were applied to the cell suspension on ice, which was centrifuged at 700*g* for 7 min at 4°C. The supernatant was centrifuged at 10,000*g* for 7 min at 4°C, and the mitochondrial pellet was obtained. The pellet was suspended in 250 mM sucrose, divided into aliquots, and kept at −80°C until use. Activities of complexes I, III, and IV were measured as previously described [43]. Rotenone-sensitive complex I activity was measured by the change in absorption of decylubiquinone. All samples were measured within 1 month from preparation. The activity of each complex was corrected by citrate synthase activity. All the chemical products for these assays were obtained from Sigma. We used a UVmini1240 spectrophotometer (Shimadzu, Kyoto, Japan) for this experiment.

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References

[1] P. Sklar, S.B. Gabriel, M.G. McInnis, P. Bennett, Y.M. Lim, G. Tsan, S. Schaffner, G. Kirov, I. Jones, M. Owen, et al., Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. Brain-derived neurotrophic factor, Mol. Psychiatry 7 (2002) 579–593.

[2] M. Neves-Pereira, E. Mundo, P. Muglia, N. King, F. Macciardi, J.L. Kennedy, The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study, Am. J. Hum. Genet. 71 (2002) 651–655.

[3] C. Kakiuchi, K. Iwamoto, M. Ishiwata, M. Bundo, T. Kasahara, I. Kusumi, T. Tsujita, Y. Okazaki, S. Nanko, H. Kunugi, et al., Impaired feedback regulation of XBP1 as a genetic risk factor for bipolar disorder, Nat. Genet. 35 (2003) 171–175.

[4] T. Kato, S. Takahashi, T. Shioiri, T. Inubushi, Alterations in brain phosphorous metabolism in bipolar disorder detected by in vivo ^{31}P and ^{7}Li magnetic resonance spectroscopy, J. Affect. Disord. 27 (1993) 53–59.

[5] C. Konradi, M. Eaton, M.L. MacDonald, J. Walsh, F.M. Benes, S. Heckers, Molecular evidence for mitochondrial dysfunction in bipolar disorder, Arch. Gen. Psychiatry 61 (2004) 300–308.

[6] G. Siciliano, A. Tessa, S. Petrini, M. Mancuso, C. Bruno, G.S. Grieco, A. Malandrini, L. DeFlorio, B. Martini, A. Federico, et al., Autosomal dominant external ophthalmoplegia and bipolar affective disorder associated with a mutation in the ANT1 gene, Neuromuscul. Disord. 13 (2003) 162–165.

[7] J.N. Spelbrink, F.Y. Li, V. Tiranti, K. Nikali, Q.P. Yuan, M. Tariq, S. Wanrooij, N. Garrido, G. Comi, L. Morandi, et al., Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria, Nat. Genet. 28 (2001) 223–231.

[8] G. Van Goethem, B. Dermaut, A. Lofgren, J.J. Martin, C. Van Broeckhoven, Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions, Nat. Genet. 28 (2001) 211–212.

[9] F.J. McMahon, O.C. Stine, D.A. Meyers, S.G. Simpson, J.R. DePaulo, Patterns of maternal transmission in bipolar affective disorder, Am. J. Hum. Genet. 56 (1995) 1277–1286.

[10] T. Kato, H. Kunugi, S. Nanko, N. Kato, Mitochondrial DNA polymorphisms in bipolar disorder, J. Affect. Disord. 62 (2001) 151–164.

[11] F.J. McMahon, Y.S. Chen, S. Patel, J. Kokoszka, M.D. Brown, A. Torroni, J.R. DePaulo, D.C. Wallace, Mitochondrial DNA sequence diversity in bipolar affective disorder, Am. J. Psychiatry 157 (2000) 1058–1064.

[12] S. Washizuka, C. Kakiuchi, K. Mori, H. Kunugi, O. Tajima, T. Akiyama, S. Nanko, T. Kato, Association of mitochondrial complex I subunit gene NDUFV2 at 18p11 with bipolar disorder, Am. J. Med. Genet. 120B (2003) 72–78.

[13] S. Anderson, A.T. Bankier, B.G. Barrell, M.H. de Bruijn, A.R. Coulson, J. Drouin, I.C. Eperon, D.P. Nierlich, B.A. Roe, F. Sanger, et al., Sequence and organization of the human mitochondrial genome, Nature 290 (1981) 457–465.

[14] D.C. Wallace, Mitochondrial diseases in man and mouse, Science 283 (1999) 1482–1488.

[15] T.R. Prezant, J.V. Agapian, M.C. Bohlman, X. Bu, S. Oztas, W.Q. Qiu, K.S. Arnos, G.A. Cortopassi, L. Jaber, J.I. Rotter, et al., Mitochondrial ribosomal RNA mutation associated with both antibiotic-induced and non-syndromic deafness, Nat. Genet. 4 (1993) 289–294.

[16] M.X. Guan, N. Fischel-Ghodsian, G. Attardi, Biochemical evidence for nuclear gene involvement in phenotype of non-syndromic deafness associated with mitochondrial 12S rRNA mutation, Hum. Mol. Genet. 5 (1996) 963–971.

[17] S.W. Chang, D. Zhang, H.D. Chung, H.P. Zassenhaus, The frequency of point mutations in mitochondrial DNA is elevated in the Alzheimer's brain, Biochem. Biophys. Res. Commun. 273 (2000) 203–208.

[18] J.M. van der Walt, K.K. Nicodemus, E.R. Martin, W.K. Scott, M.A. Nance, R.L. Watts, J.P. Hubble, J.L. Haines, W.C. Koller, K. Lyons, et al., Mitochondrial polymorphisms significantly reduce the risk of Parkinson disease, Am. J. Hum. Genet. 72 (2003) 804–811.

[19] R.M. Andrews, I. Kubacka, P.F. Chinnery, R.N. Lightowlers, D.M. Turnbull, N. Howell, Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA, Nat. Genet. 23 (1999) 147.

[20] A.M. Kogelnik, M.T. Lott, M.D. Brown, S.B. Navathe, D.C. Wallace, MITOMAP: a human mitochondrial genome database, Nucleic Acids Res. 24 (1996) 177–179.

[21] A.M. Kogelnik, M.T. Lott, M.D. Brown, S.B. Navathe, D.C. Wallace, MITOMAP: a human mitochondrial genome database—1998 update, Nucleic Acids Res. 26 (1998) 112–115.

[22] M. Tanaka, T. Takeyasu, N. Fuku, G. Li-Jun, M. Kurata, Mitochondrial genome single nucleotide polymorphisms and their phenotypes in the Japanese, Ann. N. Y. Acad. Sci. 1011 (2004) 1–20.

[23] J.K. Pritchard, M. Stephens, P. Donnelly, Inference of population structure using multilocus genotype data, Genetics 155 (2000) 945–959.

[24] Y. Aomi, C.S. Chen, K. Nakada, S. Ito, K. Isobe, H. Murakami, S.Y. Kuno, M. Tawata, R. Matsuoka, H. Mizusawa, J.-I. Hayashi, Cytoplasmic transfer of platelet mtDNA from elderly patients with Parkinson's disease to mtDNA-less HeLa cells restores complete mitochondrial respiratory function, Biochem. Biophys. Res. Commun. 280 (2001) 265–273.

[25] R.H. Swerdlow, J.K. Parks, J.N. Davis II, D.S. Cassarino, P.A. Trimmer, L.J. Currie, J. Dougherty, W.S. Bridges, J.P. Bennett Jr., G.F. Wooten, W.D. Parker, Matrilineal inheritance of complex I dysfunction in a multigenerational Parkinson's disease family, Ann. Neurol. 44 (1998) 873–881.

[26] J.P. Sheehan, R.H. Swerdlow, S.W. Miller, R.E. Davis, J.K. Parks, W.D. Parker, J.B. Tuttle, Calcium homeostasis and reactive oxygen species production in cells transformed by mitochondria from individuals with sporadic Alzheimer's disease, J. Neurosci. 17 (1997) 4612–4622.

[27] M.P. Vawter, W.J. Freed, J.E. Kleinman, Neuropathology of bipolar disorder, Biol. Psychiatry 48 (2000) 486–504.

[28] G. Rajkowska, Cell pathology in bipolar disorder, Bipolar Disord. 4 (2002) 105–116.

[29] L.V. Kessing, E.W. Olsen, P.B. Mortensen, P.K. Andersen, Dementia in affective disorder: a case-register study, Acta Psychiatr. Scand. 100 (1999) 176–185.

[30] R.C. Green, L.A. Cupples, A. Kurz, S. Auerbach, R. Go, D. Sadovnick, R. Duara, W.A. Kukull, H. Chui, T. Edeki, et al., Depression as a risk factor for Alzheimer disease: the MIRAGE Study, Arch. Neurol. 60 (2003) 753–759.

[31] F.M. Nilsson, L.V. Kessing, T.G. Bolwig, Increased risk of developing Parkinson's disease for patients with major affective disorder: a register study, Acta Psychiatr. Scand. 104 (2001) 380–386.

[32] G. Chen, W.Z. Zeng, P.X. Yuan, L.D. Huang, Y.M. Jiang, Z.H. Zhao, H.K. Manji, The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS, J. Neurochem. 72 (1999) 879–882.

[33] E.C. Thomeer, W.M. Verhoeven, C.J. van de Vlasakker, J.L. Klompenhouwer, Psychiatric symptoms in MELAS: a case report, J. Neurol. Neurosurg. Psychiatry 64 (1998) 692–693.

[34] M. Yamazaki, H. Igarashi, M. Hamamoto, T. Miyazaki, I. Nonaka, A case of mitochondrial encephalomyopathy with schizophrenic psychosis, dementia and neuroleptic malignant syndrome, Rinsho Shinkeigaku 31 (1991) 1219–1223.

[35] M.D. Brown, I.A. Trounce, A.S. Jun, J.C. Allen, D.C. Wallace, Functional analysis of lymphoblast and cybrid mitochondria containing the 3460, 11778, or 14484 Leber's hereditary optic neuropathy mitochondrial DNA mutation, J. Biol. Chem. 275 (2000) 39831–39836.

[36] P.L. Roubertoux, F. Sluyter, M. Carlier, B. Marcet, F. Maarouf-Veray, C. Cherif, C. Marican, P. Arrechi, F. Godin, M. Jamon, et al., Mitochondrial DNA modifies cognition in interaction with the nuclear genome and age in mice, Nat. Genet. 35 (2003) 65–69.
[37] T. Kato, Y. Takahashi, Deletion of leukocyte mitochondrial DNA in bipolar disorder, J. Affect. Disord. 37 (1996) 67–73.
[38] J. Akanuma, K. Muraki, H. Komaki, I. Nonaka, Y. Goto, Two pathogenic point mutations exist in the authentic mitochondrial genome, not in the nuclear pseudogene, J. Hum. Genet. 45 (2000) 337–341.
[39] M.P. King, G. Attardi, Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation, Science 246 (1989) 500–503.
[40] A. Chomyn, S.T. Lai, R. Shakeley, N. Bresolin, G. Scarlato, G. Attardi, Platelet-mediated transformation of mtDNA-less human cells: analysis of phenotypic variability among clones from normal individuals—and complementation behavior of the tRNALys mutation causing myoclonic epilepsy and ragged red fibers, Am. J. Hum. Genet. 54 (1994) 966–974.
[41] I. Nishino, O. Kobayashi, Y. Goto, M. Kurihara, K. Kumagai, T. Fujita, K. Hashimoto, S. Horai, I. Nonaka, A new congenital muscular dystrophy with mitochondrial structural abnormalities, Muscle Nerve 21 (1998) 40–47.
[42] A. Sawa, G.W. Wiegand, J. Cooper, R.L. Margolis, A.H. Sharp, J.F. Lawler Jr., J.T. Greenamyre, S.H. Snyder, C.A. Ross, Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization, Nat. Med. 5 (1999) 1194–1198.
[43] I.A. Trounce, Y.L. Kim, A.S. Jun, D.C. Wallace, Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines, Methods Enzymol. 264 (1996) 484–509.

BRIEF REPORT

No Association Between the Val66Met Polymorphism of the Brain-Derived Neurotrophic Factor Gene and Bipolar Disorder in a Japanese Population: A Multicenter Study

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Background: *Two previous studies reported a significant association between a missense polymorphism (Val66Met) in the brain-derived neurotrophic factor (BDNF) gene and bipolar disorder; however, contradictory negative results have also been reported, necessitating further investigation.*

Methods: *We organized a multicenter study of a relatively large sample of 519 patients with bipolar disorder (according to DSM-IV criteria) and 588 control subjects matched for gender, age, and ethnicity (Japanese). Genotyping was done by polymerase chain reaction–based restriction fragment length polymorphism or direct sequencing.*

Results: *The genotype distributions and allele frequencies were similar among the patients and control subjects. Even if the possible relationships of the polymorphism with several clinical variables (i.e., bipolar I or II, presence of psychotic features, family history, and age of onset) were examined, no variable was related to the polymorphism.*

Conclusions: *The Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder, at least in a Japanese population.*

Key Words: Association study, bipolar disorder, brain-derived neurotrophic factor, genetics, single nucleotide polymorphism, susceptibility

Brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family and promotes the development, regeneration, survival, and maintenance of function of neurons (Maisonpierre et al 1991). It modulates synaptic plasticity and neurotransmitter release across multiple neurotransmitter systems, as well as the intracellular signal-transduction pathway (Thoenen 1995). Growing evidence has suggested important roles of BDNF in the pathogenesis of mood disorders and in the mechanism of action of therapeutic agents, such as mood stabilizers and antidepressants (reviewed by Duman 2002). In postmortem brains of patients with bipolar disorder, BDNF protein was reduced compared with control subjects (Knable et al 2004). Chronic electroconvulsive seizure or antidepressant drug treatments increase messenger ribonucleic acid of BDNF and its receptor, tyrosine kinase receptor B (Nibuya et al 1995). Lithium might also exert its neuroprotective effect through enhancing expression of BDNF and trkB (Hashimoto et al 2002).

The BDNF gene is, therefore, an attractive candidate gene that might cause susceptibility to bipolar disorder or influence the clinical phenotype of the illness. Indeed, at least two previous studies reported a significant association between a missense polymorphism (Val66Met; National Center for Biotechnology Information Database of Single Nucleotide Polymorphisms reference number rs6265) of the BDNF gene and bipolar disorder (Neves-Pereira et al 2002; Sklar et al 2002); however, contradictory negative results have also been reported (Hong et al 2003; Nakata et al 2003). One possible reason for this inconsistency is the lack of statistical power due to small sample size. To draw any conclusion with respect to this possible association, we organized a multicenter study in which six laboratories combined their data to ensure adequate statistical power.

Methods and Materials

Subjects

Six laboratories (National Institute of Mental Health, two laboratories of the Brain Science Institute, Showa University, Tokyo Women's Medical College, and Fujita Health University) collected deoxyribonucleic acid (DNA) samples from patients with bipolar disorder and healthy control subjects. Each institute provided DNA samples of patients and control subjects matched for gender, age, and geographic area, which yielded a combined sample of 519 patients with bipolar disorder (244 male) and 588 control subjects (287 male). Mean age (± SD) for the patients was 49.3 ± 14.3 years and for the control subjects was 48.4 ± 12.7 years. All the patients and control subjects were Japanese and biologically unrelated. Consensus diagnosis of bipolar disorder was made for each patient by at least two experienced psychiatrists according to DSM-IV criteria (American Psychiatric Association 1994) on the basis of unstructured interviews and medical records. Among the patients, 347 were diagnosed as bipolar I

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Table 1. Genotype Distributions and Allele Frequencies for the Val66Met Polymorphism of the BDNF Gene Among the Patients with Bipolar Disorder and Control Subjects

	Genotype Distribution				Allele Frequency	
	n	Val/Val	Val/Met	Met/Met	*n*	Val	Met
Patients						
Total	519	188 (36.2)	239 (46.1)	92 (17.7)	1038	615 (59.2)	423 (40.8)
Bipolar I	347	123 (35.4)	166 (47.8)	58 (16.7)	694	412 (59.4)	282 (40.6)
Bipolar II	172	65 (37.8)	73 (42.4)	34 (19.8)	344	203 (59.0)	141 (41.0)
Control subjects	588	216 (36.7)	270 (45.9)	102 (17.3)	1176	702 (59.7)	474 (40.3)

Values in parentheses are percentages. Genotypewise comparisons: total patients vs. control subjects: χ^2 (2) = .0, p = .98; bipolar I vs. control subjects: χ^2 (2) = .3, p = .86; bipolar II vs. control subjects: χ^2 (2) = .8, p = .69. Allelewise comparisons: total patients vs. control subjects: χ^2 (1) = .0, p = .83; bipolar I vs. control subjects: χ^2 (1) = .0, p = .96; bipolar II vs. control subjects: χ^2 (1) = .0, p = .94.

and the remaining 172 as bipolar II. Control subjects were healthy volunteers who had no current or past contact with psychiatric services. The control subjects were recruited from the hospital staffs and their associates at each institution who showed good social functioning and reported themselves to be in good health. They were interviewed, and those individuals who had current or past contact with psychiatric services were excluded. Written informed consent for participation in the study was obtained from all subjects. The study protocol was approved by the institutional ethics committees.

Methods

Venous blood was drawn, and genomic DNA was extracted according to standard procedures. Genotyping was performed according to Neves-Pereira et al (2003). Briefly, the polymorphic site was amplified by polymerase chain reaction (PCR) and then digested with a restriction enzyme, Eco72I. The digested PCR products were visualized with gel electrophoresis and subsequent ethidium bromide staining. Genotyping for a portion of subjects was done by direct sequencing of PCR products encompassing the polymorphic site with an autosequencer (CEQ 8000; Beckman Coulter, Fullerton, California). Genotype data were read blind to the case–control status.

To examine the possible relationships of the Val66Met polymorphism with clinical variables, information on age of onset, family history, and presence of psychotic features (i.e., current or past episode with delusions or hallucinations) was obtained. We defined positive family history as having at least one first-degree relative with a history of contact with psychiatric services with a diagnosis of mood disorder or who was a suicide victim. Individuals with ambiguous clinical data were excluded from statistical analyses.

The presence of Hardy-Weinberg equilibrium for the genotype distributions in the patients and control subjects was examined with the χ^2 test for goodness of fit. The differences in the genotype and allele distributions between patients and control subjects were examined with the χ^2 test for independence. The possible relationships between the polymorphism and clinical variables were examined with the χ^2 test for independence or analysis of covariance (ANCOVA) within the patient group. All p values reported are two-tailed.

Results

Genotype and allele distributions of the Val66Met polymorphism in the patients and control subjects are shown in Table 1. The genotype distributions in the two groups were both in Hardy-Weinberg equilibrium [patients: $\chi^2(1) = 1.1$, $p = .29$; control subjects: $\chi^2(1) = 1.2$, $p = .27$]. The genotype and allele distributions for the patients were quite similar to those for the control group (see Table 1). The genotype and allele distributions of the patients with bipolar I and those with bipolar II were also similar.

When relationships between genotype and clinical variables were examined, genotype and allele distributions were not different according to presence of psychotic features (frequency of the Val66 allele for psychotic patients: .567; for nonpsychotic patients: .579) or family history (positive family history: .602; negative: .603). Age of onset was also similar, irrespective of the genotype (Val/Val: 35.3 ± 13.5 years; Val/Met: 37.7 ± 14.6 years; Met/Met: 36.3 ± 14.0 years). Even when ANCOVA controlling for age and gender was performed, there was no significant difference in age of onset across the three genotypic groups [$F(2) = .99$, $p = .37$].

Discussion

We tried to replicate the studies of Sklar et al (2002) and Neves-Pereira et al (2002), who found a significant association between the Val66Met polymorphism of the BDNF gene with bipolar disorder. They reported excess transmission of the Val66 allele to the patients in their family-based association studies. Contrary to these findings, the genotype and allele frequencies among the patients and control subjects were similar in our sample, which is in turn consistent with more recent studies (Hong et al 2003; Nakata et al 2003), suggesting that the Val66Met polymorphism of the BDNF gene is unrelated to the development of bipolar disorder in our sample. Because our study had adequate statistical power (more than 90% to detect an odds ratio of 1.33 or more in allelic association; power analysis was performed according to Armitage and Berry 1994), the potential type II error due to lack of statistical power is unlikely. One possible explanation for this inconsistency might be a differential effect of the polymorphism depending on ethnicity, given that the majority of the subjects of Sklar et al (2002) and Neves-Pereira et al (2002) were Caucasian, whereas those of Hong et al (2003), Nakata et al (2003), and in our study were Asian. Alternatively, the positive results of Sklar et al (2002) and Neves-Pereira et al (2002) might have arisen by chance.

Concerning the possible effect of the polymorphism on clinical features, Rybakowski et al (2003) reported an earlier age of onset in Val/Val than Val/Met genotype (27 years vs. 38 years) among patients with bipolar disorder. They also found that the performance in all domains of the Wisconsin Card Sorting Test was significantly better for bipolar patients with Val/Val than for those with Val/Met genotype, suggesting a role of the Val66Met polymorphism in prefrontal cognitive function in bipolar disorder. This accords with the findings of Egan et al (2003), who

reported that the Met66 allele was associated with lower activity-dependent secretion of BDNF and poorer human memory and hippocampal function; however, we could not find any significant effect of the genotype on clinical variables of age of onset, subtype (bipolar I or II), psychotic features, or family history. Hong et al (2003) also failed to find significant difference in age of onset or suicidal history across genotypic groups in their Chinese subjects with bipolar disorder.

In conclusion, our results, together with previous two studies (Hong et al 2003; Nakata et al 2003), suggest that the Val66Met polymorphism of the BDNF gene is unrelated to the development or clinical features of bipolar disorder at least in Asian populations; however, the possibility remains that other variants of the BDNF gene might be associated with bipolar disorder in Asian populations, which requires further investigation.

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

Armitage P, Berry G (1994): *Statistical Methods in Medical Research,* 3rd ed. Oxford: Blackwell Science.

Duman RS (2002): Synaptic plasticity and mood disorders. *Mol Psychiatry* 7(suppl 1):S29–S34.

Egan MF, Kojima M, Callicott JH, Goldberg TE, Kolachana BS, Bertolino A, et al (2003): The BDNF val66 met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell* 112:257–269.

Hashimoto R, Takei N, Shimazu K, Christ L, Lu B, Chuang DM (2002): Lithium induces brain-derived neurotrophic factor and activates TrkB in rodent cortical neurons: An essential step for neuroprotection against glutamate excitotoxicity. *Neuropharmacology* 43:1173–1179.

Hong CJ, Huo SJ, Yen FC, Tung CL, Pan GM, Tsai SJ (2003): Association study of a brain-derived neurotrophic-factor genetic polymorphism and mood disorders, age of onset and suicidal behavior. *Neuropsychobiology* 48:186–189.

Knable MB, Barci BM, Webster MJ, Meador-Woodruff J, Torrey EF (2004): Molecular abnormalities of the hippocampus in severe psychiatric illness: Postmortem findings from the Stanley Neuropathology Consortium. *Mol Psychiatry* 9:609–620.

Maisonpierre PC, Le Beau MM, Espinosa RIII, Ip NY, Belluscio L, de la Monte SM, et al (1991): Human and rat brain-derived neurotrophic factor and neurotrophin-3: Gene structures, distributions and chromosomal localizations. *Genomics* 10:558–568.

Nakata K, Ujike H, Sakai A, Uchida N, Nomura A, Imamura T, et al (2003): Association study of the brain-derived neurotrophic factor (BDNF) gene with bipolar disorder. *Neurosci Lett* 337:17–20.

Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, Kennedy JL (2002): The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: Evidence from a family-based association study. *Am J Hum Genet* 71:651–655.

Nibuya M, Morinobu S, Duman RS (1995): Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. *J Neurosci* 15:7539–7547.

Rybakowski JK, Borkowska A, Czerski PM, Skibinska M, Hauser J (2003): Polymorphism of the brain-derived neurotrophic factor gene and performance on a cognitive prefrontal test in bipolar patients. *Bipolar Disord* 5:468–472.

Sklar P, Gabriel SB, McInnis MG, Bennett P, Lim YM, Tsan G, et al (2002): Family-based association study of 76 candidate genes in bipolar disorder: BDNF is a potential risk locus. *Mol Psychiatry* 7:579–593.

Thoenen H (1995): Neurotrophins and neuronal plasticity. *Science* 270:593–598.

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Association Between the Glutathione S-Transferase M1 Gene Deletion and Female Methamphetamine Abusers

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Several lines of evidence suggest that increased generation of auto-oxidized dopamine (DA) *o*-quinone is associated with the neurotoxicity of methamphetamine (MAP) in the brain, and that, as a cellular defenses against DA-derived quinines, glutathione S-transferase (GST) detoxifies auto-oxidized DA *o*-quinone in the brain. Glutathione S-transferase M1 (GSTM1) of the mu-class of GSTs catalyzes reaction between glutathione and catecholamine *o*-quinones under physiological conditions. This study was undertaken to investigate the role of the *GSTM1* gene deletion polymorphism in the neuropathology of MAP abuse. One hundred fifty-seven MAP abusers and 200 healthy comparison subjects were tested for a genetic polymorphism of GSTM1. The difference in the frequency of deletion (D)/non-deletion (N) alleles between the female abusers and female controls was close to statistical significance ($P = 0.071$), although there was no statistical difference ($P = 0.651$) between male abusers and male controls. Furthermore, the number of female abusers with deletion alleles was significantly ($P = 0.007$, odds ratio: 2.77, 95% CI 1.30–5.89) higher than that of male abusers with deletion alleles. These findings suggest that *GSTM1* gene deletion may contribute to a vulnerability to MAP abuse in female subjects, but not in male subjects.

KEY WORDS: methamphetamine; drug abuse; glutathione S-transferase; gender difference

INTRODUCTION

Abuse of methamphetamine (MAP) is a growing problem worldwide. Some lines of evidence have suggested strong genetic contributions to drug abuse vulnerability [Uhl et al., 2002]. The application of brain imaging techniques to the study of drug abuse have demonstrated that the density of dopamine (DA) transporters is significantly reduced in the caudate/putamen of MAP abusers [Sekine et al., 2001; Volkow et al., 2001], suggesting that long-term use of MAP causes damage to dopaminergic neurons in the human brain. Furthermore, it has been shown that MAP-induced neurotoxicity in the brain has been shown to require striatum DA and to involve mechanisms associated with oxidative stress [Cadet and Brannock, 1998]. It is also known that DA is auto-oxidized and the corresponding DA *o*-quinone (aminochrome) is subse-

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quently generated; moreover, aminochrome and its subsequent product, DA *o*-semiquinone, elicit redox cycling which leads to the generation of reactive oxygen species, which in turn degenerate dopaminergic neurons [Graham et al., 1978; Smythies and Galzigna, 1998]. DA oxidation also results in the formation of DA *o*-quinone, which readily participates in nucleophilic addition reactions with sufhydryl groups on free cysteine, glutathione, or cysteine found in protein including DA transporter [Graham et al., 1978; Hastings and Zigmond, 1994; Smythies and Galzigna, 1998; Whitehead et al., 2001]. In addition, it has been reported recently that DA auto-oxidation contributes to MAP-induced neurotoxicity to DA terminals, adding support to the role of DA and oxidative stress in this model [LaVoie and Hastings, 1999]. Taken together, it is likely that increased generation of DA *o*-quinone by DA auto-oxidation is associated with the neurotoxicity of MAP in the brain.

Glutathione S-transferase M1 (GSTM1) is a subtype of GSTs that detoxify xenobiotics by conjugating glutathione. It has been reported that GSTM1 catalyzes a glutathione conjugate of catecholamine *o*-quinones such as aminochrome [Smythies and Galzigna, 1998]. GSTM1 has an entire gene deletion polymorphism and its enzymatic activity is classified into three grades, i.e., a highly active genotype (homozygous non-deletion alleles; NN), a moderately active genotype (heterozygous non-deletion alleles; DN), and a null genotype (homozygous deletion alleles; DD) [McLellan et al., 1997]. Recently, it has been reported that the frequency of D allele of *GSTM1* gene in the patients with schizophrenia was significantly ($P = 0.0075$) higher than that of normal controls, suggesting that *GSTM1* gene may be associated with an increased susceptibility to schizophrenia [Harada et al., 2001a]. Thus, it seems that differences in the GSTM1 genotype may contribute to the development of MAP abuse. In order to verify a potential role of the *GSTM1* gene in the neuropathology by MAP abuse, we analyzed a polymorphism of the *GSTM1* gene in subjects with diagnosed MAP-related disorders and in control groups.

MATERIALS AND METHODS

The research was performed after obtaining approval from the ethics committees of each institute of Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent for the use of their DNA samples for this research. The subjects were 157 patients (125 males, age: 37 ± 11 years (mean ± SD), age range: 19–69 years; and 32 females, age: 28 ± 5 years (mean ± SD), age range: 21–47 years) with MAP dependence and psychotic disorder meeting ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals of JGIDA, and 200 age-, gender- and geographical origin-matched normal controls (157 males, age: 37 ± 11 years (mean ± SD), age range: 19–69 years; and 43 females, age: 36 ± 10 years (mean ± SD), age range: 21–58 years) mostly consisted of medical staffs who had no past history and no family history of drug dependence or psychotic disorders. All subjects were Japanese, born and living in restricted arrears of Japan including northern Kyusyu, Setouchi, Tyukyou, Toukai, and Kantou.

The polymorphism studied in this project was the deletion of the entire *GSTM1* gene. Genotyping for this gene was performed by a combination of two types of polymerase chain reaction (PCR) amplification as reported previously [Harada et al., 2001a,b]. The first type of PCR was used for the detection of a non-deletion allele with the appropriate primers (forward: 5′-CTTCACGTGTTATGGAG GTTC-3′, reverse: 5′-GCGAGTTATTCTGTGTGTAGC-3′). The other type of PCR was used for the detection of a deletion allele with suitable primers (forward: 5′-ACAGAGGAAGGGTG-CATTTGATA-3′, reverse: 5′-GACATTCATTCCCAAA-GCGACCA-3′); both types of PCR were followed by agarose gel electrophoresis with ethidium bromide staining. Allele frequencies were calculated by gene counting and the differences between groups were evaluated by Fisher's exact test. The odds ratio (OD) and 95% confidence intervals were calculated to evaluate the effects of the different genotypes.

RESULTS

The GSTM1 genotypes and the allele frequencies in MAP abusers and controls are shown in Table I. The genotype distribution in both abusers and controls was within the Hardy–Weinberg equilibrium. We found that a difference in the frequency of deletion (D)/non-deletion (N) alleles between the female abusers and female controls was a trend toward a statistical significance ($P = 0.071$). In contrast, there was no significant difference between male abusers and male controls ($P = 0.651$). The frequency of carrying the D allele among female abusers was significantly higher than that in male abusers ($P = 0.007$, odds ratio: 2.77, 95% CI 1.30–5.89), whereas no gender difference was shown among control subjects ($P = 0.297$, odds ratio: 1.36, 95% CI 0.80–2.31). The genotype distribution difference between female abusers and female controls was significant ($P = 0.032$), whereas no significant difference between male abusers and male controls was shown ($P = 0.819$).

DISCUSSION

Our findings suggest that a deletion of the *GSTM1* gene may contribute to MAP abuse vulnerability in female, but not in male, subjects. Based on the role played by GSTM1 in the antioxidant system preventing neurotoxicity, *GSTM1* gene deletion might lead to an excess of catecholamine *o*-quinones (e.g., aminochrome) that are neurotoxic in the brain, including DA neurons. The reason underlying this gender difference is currently unclear. However, recent evidence has been suggestive of gender differences in course of drug dependence and drug abuse [Lynch et al., 2002]. It has been reported that females enter treatment programs after fewer years of amphetamine use, and that females also take less time to become addicted after initial use than do males [Westermeyer and Boedicker,

TABLE I. Allele and Genotype Frequencies of the *GSTM1* Gene Deletion Polymorphism in MAP Abusers and Controls

	Male		Female	
	Abusers (n = 125)	Controls (n = 157)	Abusers (n = 32)	Controls (n = 43)
GSTM1 allele frequency				
D	172 (68.8%)	210 (66.9%)	55 (85.9%)	63 (73.3%)
N	78 (31.2%)	104 (33.1%)	9 (14.1%)	23 (26.7%)
	$P = 0.651$		$P = 0.071$	
GSTM1 genotype frequency				
DD	58 (46.4%)	67 (42.7%)	24 (75.0%)	21 (48.8%)
DN	56 (44.8%)	76 (48.4%)	7 (21.9%)	21 (48.8%)
NN	11 (8.8%)	14 (8.9%)	1 (3.1%)	1 (2.3%)
	$P = 0.819$		$P = 0.032$*	

GSTM1, glutathione S-transferase M1; MAP, methamphetamine; D, deletion allele; N, non-deletion allele.
*$P < 0.05$.

2000]. In addition, positive subjective effects of D-amphetamine are enhanced during the follicular phase, which correlates with changes in estrogen levels [Lynch et al., 2002].

It has been suggested that gonadal hormones such as estrogen play a role in the differences between males and females regarding responses to drugs of abuse [Lynch et al., 2002]. In females, there is an accelerated transition from controlled to uncontrolled use, namely, dependence, and that gonadal hormones, particularly estrogen, may play a role in these processes [Justice and De Wit, 2000]. In studies using rats, estrogen has been revealed to enhance the behavioral and neurochemical responses to MAP by increasing stimulated DA release [Becker, 1999]. Furthermore, recent studies using brain imaging technique revealed that women have higher levels of DA transporters [Mozley et al., 2001] and lower DA D_2 receptor affinity in the striatum than men [Pohjalainen et al., 1998], suggesting a lower baseline of dopaminergic tone and elevated levels of DA released by MAP in females. Therefore, it is likely that gonadal hormones and gender differences in dopaminergic systems may be implicated in gender differences related to susceptibility to addiction to psychomotor stimulants. Thus, it appears that excess DA released by MAP might generate an excess of DA *o*-quinone, rendering it especially difficult for persons with low-activity GST to detoxify a sufficient amount of DA *o*-quinone. Furthermore, the GSTM1 deletion would influence the susceptibility of females to MAP abuse.

In conclusion, our findings suggest that *GSTM1* gene deletion may contribute to a vulnerability to MAP abuse in female subjects, but not in male subjects.

REFERENCES

Becker JB. 1999. Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacol Biochem Behav 64:803–812.

Cadet JL, Brannock C. 1998. Free radicals and the pathobiology of brain dopamine systems. Neurochem Int 32:117–131.

Graham DG, Tiffany SM, Bell WR Jr, Gutknecht WF. 1978. Auto-oxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol Pharmacol 14:644–653.

Harada S, Fujii C, Hayashi A, Ohkoshi N. 2001a. An association between idiopathic Parkinson's disease and polymorphisms of phase II detoxification enzymes: Glutathione S-transferase M1 and quinone oxidoreductase 1 and 2. Biochem Biophys Res Commun 288:887–892.

Harada S, Tachikawa H, Kawanishi Y. 2001b. Glutathione S-transferase M1 gene deletion may be associated with susceptibility to certain forms of schizophrenia. Biochem Biophys Res Commun 281:267–271.

Hastings TG, Zigmond MJ. 1994. Identification of catechol–protein conjugates in neostriatal slices incubated with [^{3}H]dopamine: Impact of ascorbic acid and glutathione. J Neurochem 63:1126–1132.

Justice AJ, De Wit H. 2000. Acute effects of D-amphetamine during the early and late follicular phases of the menstrual cycle in women. Pharmacol Biochem Behav 66:509–515.

LaVoie MJ, Hastings TG. 1999. Dopamine quinone formation and protein modification associated with the strial neurotoxicity of methamphetamine: Evidence against a role for extracellular dopamine. J Neurosci 19:1484–1491.

Lynch WJ, Roth ME, Carroll ME. 2002. Biological basis of sex differences in drug abuse: Preclinical and clinical studies. Psychopharmacol 164:121–137.

McLellan RA, Oscarson M, Alexandrie AK, Seidegård J, Evans DAP, Rannug A, Ingelman-Sundberg M. 1997. Characterization of a human glutathione S-transferase μ cluster containing a duplicated *GSTM1* gene that causes ultrarapid enzyme activity. Mol Pharmacol 52:958–965.

Mozley LH, Gur RC, Mozley PD, Gur RE. 2001. Striatal dopamine transporters and cognitive functioning in healthy men and women. Am J Psychiatry 158:1492–1499.

Pohjalainen T, Rinne JO, Nagren K, Lehikoinen P, Anttila K, Syvalahti EK, Hietala J. 1998. The A1 allele of the human D2 dopamine receptor gene predicts low D2 receptor availability in healthy volunteers. Mol Psychiatry 3:256–260.

Sekine Y, Iyo M, Ouchi Y, Matunaga T, Tsukada H, Okada H, Yoshikawa E, Futatsubashi M, Takei N, Mori N. 2001. Methamphetamine-related psychiatric symptoms and reduced brain dopamine transporters studied with PET. Am J Psychiatry 158:1206–1214.

Smythies J, Galzigna L. 1998. The oxidative metabolism of catecholamines in the brain: A review. Biochim Biophys Acta 1380:159–162.

Uhl GR, Liu QR, Naiman D. 2002. Substance abuse vulnerability loci: Converging genome scanning data. Trends Genet 18:420–425.

Volkow ND, Chang L, Wang GJ, Fowler JS, Leonido-Yee M, Franceschi D, Sedler MJ, Gateley SJ, Hitzemann R, Ding YS. 2001. Association of dopamine transporter reduction with psychomotor impairment in methamphetamine abusers. Am J Psychiatry 158:377–382.

Westermeyer J, Boedicker AE. 2000. Course, severity, and treatment of substance abuse among women versus men. Am J Drug Alcohol Abuse 26:523–535.

Whitehead RE, Ferrer JV, Javitch JA, Justice JB. 2001. Reaction of oxidized dopamine with endogenous cysteine residues in the human dopamine transporter. J Neurochem 76:1242–1251.