disorder: BDNF is a potential risk locus. Brain-derived neutrophic factor. *Mol Psychiatry* 2002; **7**: 579–593.
61 Neves-Pereira M, Mundo E, Muglia P, King N, Macciardi F, Kennedy JL. The brain-derived neurotrophic factor gene confers susceptibility to bipolar disorder: evidence from a family-based association study. *Am J Hum Genet* 2002; **71**: 651–655.
62 Niculescu III AB, Segal DS, Kuczenski R, Barrett T, Hauger RL, Kelsoe JR. Identifying a series of candidate genes for mania and psychosis: a convergent functional genomics approach. *Physiol Genom* 2000; **4**: 83–91.
63 Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S *et al.* Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 2003; **301**: 805–809.
64 Cordeiro ML, Umbach JA, Gundersen CB. Lithium ions up-regulate mRNAs encoding dense-core vesicle proteins in nerve growth factor-differentiated PC12 cells. *J Neurochem* 2000; **75**: 2622–2625.
65 Bunney WE, Bunney BG. Molecular clock genes in man and lower animals: possible implications for circadian abnormalities in depression. *Neuropsychopharmacology* 2000; **22**: 335–345.
66 Cermakian N, Boivin DB. A molecular perspective of human circadian rhythm disorders. *Brain Res Brain Res Rev* 2003; **42**: 204–220.
67 Shimomura K, Low-Zeddies SS, King DP, Steeves TD, Whiteley A, Kushla J *et al.* Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. *Genome Res* 2001; **11**: 959–980.
68 Hannibal J, Vrang N, Card JP, Fahrenkrug J. Light-dependent induction of cFos during subjective day and night in PACAP-containing ganglion cells of the retinohypothalamic tract. *J Biol Rhythms* 2001; **16**: 457–470.
69 Hannibal J, Jamen F, Nielsen HS, Journot L, Brabet P, Fahrenkrug J. Dissociation between light-induced phase shift of the circadian rhythm and clock gene expression in mice lacking the pituitary adenylate cyclase activating polypeptide type 1 receptor. *J Neurosci* 2001; **21**: 4883–4890.
70 Lewy AJ, Wehr TA, Goodwin FK, Newsome DA, Rosenthal NE. Manic-depressive patients may be supersensitive to light. *Lancet* 1981; **1**: 383–384.
71 Wetterberg L, Aperia B, Beck-Friis J, Kjellman BF, Ljunggren JG, Nilsonne A *et al.* Melatonin and cortisol levels in psychiatric illness. *Lancet* 1982; **2**: 100.
72 Kramer A, Yang FC, Snodgrass P, Li X, Scammell TE, Davis FC *et al.* Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science* 2001; **294**: 2511–2515.
73 Cheng MY, Bullock CM, Li C, Lee AG, Bermak JC, Belluzzi J *et al.* Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 2002; **417**: 405–410.
74 Hubble JP, Cao T, Hassanein RE, Neuberger JS, Koller WC. Risk factors for Parkinson's disease. *Neurology* 1993; **43**: 1693–1697.
75 Leentjens AF, Van den AM, Metsemakers JF, Lousberg R, Verhey FR. Higher incidence of depression preceding the onset of Parkinson's disease: a register study. *Mov Disord* 2003; **18**: 414–418.
76 Giasson BI, Lee VM. Are ubiquitination pathways central to Parkinson's disease? *Cell* 2003; **114**: 1–8.
77 Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 2001; **105**: 891–902.
78 Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc Natl Acad Sci USA* 2000; **97**: 13354–13359.
79 Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI *et al.* CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* 2002; **10**: 55–67.
80 Nurnberger Jr J, Guroff JJ, Hamovit J, Berrettini W, Gershon E. A family study of rapid-cycling bipolar illness. *J Affect Disord* 1988; **15**: 87–91.
81 Gershon ES, Hamovit J, Guroff JJ, Dibble E, Leckman JF, Sceery W *et al.* A family study of schizoaffective, bipolar-I, bipolar-II, unipolar, and normal control probands. *Arch Gen Psychiatry* 1982; **39**: 1157–1167.
82 Maier W, Lichtermann D, Minges J, Hallmayer J, Heun R, Benkert O *et al.* Continuity and discontinuity of affective disorders and schizophrenia. Results of a controlled family study. *Arch Gen Psychiatry* 1993; **50**: 871–883.
83 Kendler KS, Gruenberg AM, Tsuang MT. Psychiatric illness in first-degree relatives of schizophrenic and surgical control patients. A family study using DSM-III criteria. *Arch Gen Psychiatry* 1985; **42**: 770–779.
84 Kendler KS, Gruenberg AM, Tsuang MT. A DSM-III family study of the nonschizophrenic psychotic disorders. *Am J Psychiatry* 1986; **143**: 1098–1105.
85 Kendler KS, McGuire M, Gruenberg AM, O'Hare A, Spellman M, Walsh D. The Roscommon Family Study. I. Methods, diagnosis of probands, and risk of schizophrenia in relatives. *Arch Gen Psychiatry* 1993; **50**: 527–540.
86 Kendler KS, McGuire M, Gruenberg AM, O'Hare A, Spellman M, Walsh D. The Roscommon Family Study. IV. Affective illness, anxiety disorders, and alcoholism in relatives. *Arch Gen Psychiatry* 1993; **50**: 952–960.
87 Erlenmeyer-Kimling L, Adamo UH, Rock D, Roberts SA, Bassett AS, Squires-Wheeler E *et al.* The New York High-Risk Project. Prevalence and comorbidity of axis I disorders in offspring of schizophrenic parents at 25-year follow-up. *Arch Gen Psychiatry* 1997; **54**: 1096–1102.
88 Farmer AE, McGuffin P, Gottesman II. Twin concordance for DSM-III schizophrenia. Scrutinizing the validity of the definition. *Arch Gen Psychiatry* 1987; **44**: 634–641.
89 Cardno AG, Rijsdijk FV, Sham PC, Murray RM, McGuffin P. A twin study of genetic relationships between psychotic symptoms. *Am J Psychiatry* 2002; **159**: 539–545.
90 Badner JA, Gershon ES. Meta-analysis of whole-genome linkage scans of bipolar disorder and schizophrenia. *Mol Psychiatry* 2002; **7**: 405–411.
91 Berrettini WH. Are schizophrenic and bipolar disorders related? A review of family and molecular studies. *Biol Psychiatry* 2000; **48**: 531–538.
92 Segurado R, Detera-Wadleigh SD, Levinson DF, Lewis CM, Gill M, Nurnberger Jr JI *et al.* Genome scan meta-analysis of schizophrenia and bipolar disorder, part III: bipolar disorder. *Am J Hum Genet* 2003; **73**: 49–62.
93 Hodgkinson CA, Goldman D, Jaeger J, Persaud S, Kane JM, Lipsky RH *et al.* Disrupted in schizophrenia 1 (DISC1): association with schizophrenia, schizoaffective disorder, and bipolar disorder. *Am J Hum Genet* 2004; **75**: 862–872.
94 Tkachev D, Mimmack ML, Ryan MM, Wayland M, Freeman T, Jones PB *et al.* Oligodendrocyte dysfunction in schizophrenia and bipolar disorder. *Lancet* 2003; **362**: 798–805.
95 Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS *et al.* Genetic analysis of genome-wide variation in human gene expression. *Nature* 2004; **430**: 743–747.
96 Monks SA, Leonardson A, Zhu H, Cundiff P, Pietrusiak P, Edwards S *et al.* Genetic inheritance of gene expression in human cell lines. *Am J Hum Genet* 2004; **75**: 1094–1105.
97 Chesler EJ, Lu L, Shou S, Qu Y, Gu J, Wang J *et al.* Complex trait analysis of gene expression uncovers polygenic and pleiotropic networks that modulate nervous system function. *Nat Genet* 2005; **37**: 233–242.
98 Bystrykh L, Weersing E, Dontje B, Sutton S, Pletcher MT, Wiltshire T *et al.* Uncovering regulatory pathways that affect hematopoietic stem cell function using 'genetical genomics'. *Nat Genet* 2005; **37**: 225–232.
99 Dick DM, Foroud T, Flury L, Bowman ES, Miller MJ, Rau NL *et al.* Genomewide linkage analyses of bipolar disorder: a new sample of 250 pedigrees from the National Institute of Mental Health Genetics Initiative. *Am J Hum Genet* 2003; **73**: 107–114.
100 Schildkraut JJ. The catecholamine hypothesis of affective disorders: a review of supporting evidence. *Am J Psychiatry* 1965; **122**: 509–522.
101 Cohen RM, Campbell IC, Cohen MR, Torda T, Pickar D, Siever LJ *et al.* Presynaptic noradrenergic regulation during depression

and antidepressant drug treatment. *Psychiatry Res* 1980; **3**: 93–105.
102 Ogren SO, Fuxe K, Agnati L. The importance of brain serotonergic receptor mechanisms for the action of antidepressant drugs. *Pharmacopsychiatry* 1985; **18**: 209–213.
103 Charney DS, Manji HK. Life stress, genes, and depression: multiple pathways lead to increased risk and new opportunities for intervention. *Sci STKE* 2004; **2004**: re5.
104 Tanoue A, Koshimizu TA, Tsujimoto G. Transgenic studies of alpha(1)-adrenergic receptor subtype function. *Life Sci* 2002; **71**: 2207–2215.
105 Feng J, Sobell JL, Heston LL, Goldman D, Cook Jr E, Kranzler HR *et al.* Variants in the alpha2A AR adrenergic receptor gene in psychiatric patients. *Am J Med Genet* 1998; **81**: 405–410.
106 Philipp M, Brede M, Hein L. Physiological significance of alpha(2)-adrenergic receptor subtype diversity: one receptor is not enough. *Am J Physiol Regul Integr Comp Physiol* 2002; **283**: R287–295.
107 Ewald H, Degn B, Mors O, Kruse TA. Support for the possible locus on chromosome 4p16 for bipolar affective disorder. *Mol Psychiatry* 1998; **3**: 442–448.
108 Khaitan L, Calabrese JR, Stockmeier CA. Effects of chronic treatment with valproate on serotonin-1A receptor binding and function. *Psychopharmacology (Berlin)* 1994; **113**: 539–542.
109 Frisch A, Postilnick D, Rockah R, Michaelovsky E, Postilnick S, Birman E *et al.* Association of unipolar major depressive disorder with genes of the serotonergic and dopaminergic pathways. *Mol Psychiatry* 1999; **4**: 389–392.
110 Bellivier F, Leboyer M, Courtet P, Buresi C, Beaufils B, Samolyk D *et al.* Association between the tryptophan hydroxylase gene and manic-depressive illness. *Arch Gen Psychiatry* 1998; **55**: 33–37.
111 Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. *Biochem Pharmacol* 2003; **66**: 1673–1680.
112 Zill P, Baghai TC, Zwanzger P, Schule C, Eser D, Rupprecht R *et al.* SNP and haplotype analysis of a novel tryptophan hydroxylase isoform (TPH2) gene provide evidence for association with major depression. *Mol Psychiatry* 2004; **9**: 1030–1036.
113 Vincent JB, Masellis M, Lawrence J, Choi V, Gurling HM, Parikh SV *et al.* Genetic association analysis of serotonin system genes in bipolar affective disorder. *Am J Psychiatry* 1999; **156**: 136–138.
114 Tatarczynska E, Klodzinska A, Stachowicz K, Chojnacka-Wojcik E. Effect of combined administration of 5-HT1A or 5-HT1B/1D receptor antagonists and antidepressants in the forced swimming test. *Eur J Pharmacol* 2004; **487**: 133–142.
115 Whale R, Clifford EM, Bhagwagar Z, Cowen PJ. Decreased sensitivity of 5-HT(1D) receptors in melancholic depression. *Br J Psychiatry* 2001; **178**: 454–457.
116 Lin Z, Walther D, Yu XY, Drgon T, Uhl GR. The human serotonin receptor 2B: coding region polymorphisms and association with vulnerability to illegal drug abuse. *Pharmacogenetics* 2004; **14**: 805–811.
117 Lerer B, Macciardi F, Segman RH, Adolfsson R, Blackwood D, Blairy S *et al.* Variability of 5-HT2C receptor cys23ser polymorphism among European populations and vulnerability to affective disorder. *Mol Psychiatry* 2001; **6**: 579–585.
118 Niesler B, Flohr T, Nothen MM, Fischer C, Rietschel M, Franzek E *et al.* Association between the 5′ UTR variant C178T of the serotonin receptor gene HTR3A and bipolar affective disorder. *Pharmacogenetics* 2001; **11**: 471–475.
119 Davies PA, Pistis M, Hanna MC, Peters JA, Lambert JJ, Hales TG *et al.* The 5-HT3B subunit is a major determinant of serotonin-receptor function. *Nature* 1999; **397**: 359–363.
120 Ohtsuki T, Ishiguro H, Detera-Wadleigh SD, Toyota T, Shimizu H, Yamada K *et al.* Association between serotonin 4 receptor gene polymorphisms and bipolar disorder in Japanese case–control samples and the NIMH Genetics Initiative Bipolar Pedigrees. *Mol Psychiatry* 2002; **7**: 954–961.
121 Anguelova M, Benkelfat C, Turecki G. A systematic review of association studies investigating genes coding for serotonin receptors and the serotonin transporter: I. Affective disorders. *Mol Psychiatry* 2003; **8**: 574–591.
122 Kirov G, Murphy KC, Arranz MJ, Jones I, McCandles F, Kunugi H *et al.* Low activity allele of catechol-*O*-methyltransferase gene associated with rapid cycling bipolar disorder. *Mol Psychiatry* 1998; **3**: 342–345.
123 Kirov G, Jones I, McCandless F, Craddock N, Owen MJ. Family-based association studies of bipolar disorder with candidate genes involved in dopamine neurotransmission: DBH, DAT1, COMT, DRD2, DRD3 and DRD5. *Mol Psychiatry* 1999; **4**: 558–565.
124 Ni X, Trakalo JM, Mundo E, Macciardi FM, Parikh S, Lee L *et al.* Linkage disequilibrium between dopamine D1 receptor gene (DRD1) and bipolar disorder. *Biol Psychiatry* 2002; **52**: 1144–1150.
125 Massat I, Souery D, Del-Favero J, Van Gestel S, Serretti A, Macciardi F *et al.* Positive association of dopamine D2 receptor polymorphism with bipolar affective disorder in a European Multicenter Association Study of affective disorders. *Am J Med Genet* 2002; **114**: 177–185.
126 Elvidge G, Jones I, McCandless F, Asherson P, Owen MJ, Craddock N. Allelic variation of a BalI polymorphism in the DRD3 gene does not influence susceptibility to bipolar disorder: results of analysis and meta-analysis. *Am J Med Genet* 2001; **105**: 307–311.
127 Muglia P, Petronis A, Mundo E, Lander S, Cate T, Kennedy JL. Dopamine D4 receptor and tyrosine hydroxylase genes in bipolar disorder: evidence for a role of DRD4. *Mol Psychiatry* 2002; **7**: 860–866.
128 Iwayama-Shigeno Y, Yamada K, Toyota T, Shimizu H, Hattori E, Yoshitsugu K *et al.* Distribution of haplotypes derived from three common variants of the NR4A2 gene in Japanese patients with schizophrenia. *Am J Med Genet* 2003; **118B**: 20–24.
129 Jahnes E, Muller DJ, Schulze TG, Windemuth C, Cichon S, Ohlraun S *et al.* Association study between two variants in the DOPA decarboxylase gene in bipolar and unipolar affective disorder. *Am J Med Genet* 2002; **114**: 519–522.
130 Preisig M, Bellivier F, Fenton BT, Baud P, Berney A, Courtet P *et al.* Association between bipolar disorder and monoamine oxidase A gene polymorphisms: results of a multicenter study. *Am J Psychiatry* 2000; **157**: 948–955.
131 Sands SA, Guerra V, Morilak DA. Changes in tyrosine hydroxylase mRNA expression in the rat locus coeruleus following acute or chronic treatment with valproic acid. *Neuropsychopharmacology* 2000; **22**: 27–35.
132 Furlong RA, Rubinsztein JS, Ho L, Walsh C, Coleman TA, Muir WJ *et al.* Analysis and metaanalysis of two polymorphisms within the tyrosine hydroxylase gene in bipolar and unipolar affective disorders. *Am J Med Genet* 1999; **88**: 88–94.
133 Massat I, Souery D, Del-Favero J, Nothen M, Blackwood D, Muir W *et al.* Association between COMT (Val(158)Met) functional polymorphism and early onset in patients with major depressive disorder in a European multicenter genetic association study. *Mol Psychiatry* 2004: (Epub ahead of print).
134 Ralph-Williams RJ, Paulus MP, Zhuang X, Hen R, Geyer MA. Valproate attenuates hyperactive and perseverative behaviors in mutant mice with a dysregulated dopamine system. *Biol Psychiatry* 2003; **53**: 352–359.
135 Greenwood TA, Alexander M, Keck PE, McElroy S, Sadovnick AD, Remick RA *et al.* Evidence for linkage disequilibrium between the dopamine transporter and bipolar disorder. *Am J Med Genet* 2001; **105**: 145–151.
136 Cordeiro ML, Gundersen CB, Umbach JA. Lithium ions modulate the expression of VMAT2 in rat brain. *Brain Res* 2002; **953**: 189–194.
137 Picciotto MR. Common aspects of the action of nicotine and other drugs of abuse. *Drug Alcohol Depend* 1998; **51**: 165–172.
138 Graham AJ, Martin-Ruiz CM, Teaktong T, Ray MA, Court JA. Human brain nicotinic receptors, their distribution and participation in neuropsychiatric disorders. *Curr Drug Targets CNS Neuro Disord* 2002; **1**: 387–397.
139 Wang JC, Hinrichs AL, Stock H, Budde J, Allen R, Bertelsen S *et al.* Evidence of common and specific genetic effects: association of the muscarinic acetylcholine receptor M2 (CHRM2) gene with alcohol dependence and major depressive syndrome. *Hum Mol Genet* 2004; **13**: 1903–1911.
140 Horiuchi Y, Nakayama J, Ishiguro H, Ohtsuki T, Detera-Wadleigh SD, Toyota T *et al.* Possible association between a haplotype of

the GABA-A receptor alpha 1 subunit gene (GABRA1) and mood disorders. *Biol Psychiatry* 2004; **55**: 40–45.
141 Sieghart W. Unraveling the function of GABA(A) receptor subtypes. *Trends Pharmacol Sci* 2000; **21**: 411–413.
142 Sieghart W, Sperk G. Subunit composition, distribution and function of GABA(A) receptor subtypes. *Curr Top Med Chem* 2002; **2**: 795–816.
143 Edenberg HJ, Dick DM, Xuei X, Tian H, Almasy L, Bauer LO *et al.* Variations in GABRA2, encoding the alpha 2 subunit of the GABA(A) receptor, are associated with alcohol dependence and with brain oscillations. *Am J Hum Genet* 2004; **74**: 705–714.
144 Mombereau C, Kaupmann K, Froestl W, Sansig G, van der PH, Cryan JF. Genetic and pharmacological evidence of a role for GABA(B) receptors in the modulation of anxiety- and antidepressant-like behavior. *Neuropsychopharmacology* 2004; **29**: 1050–1062.
145 Borden LA. GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem Int* 1996; **29**: 335–356.
146 Volk D, Austin M, Pierri J, Sampson A, Lewis D. GABA transporter-1 mRNA in the prefrontal cortex in schizophrenia: decreased expression in a subset of neurons. *Am J Psychiatry* 2001; **158**: 256–265.
147 Ohnuma T, Augood SJ, Arai H, McKenna PJ, Emson PC. Measurement of GABAergic parameters in the prefrontal cortex in schizophrenia: focus on GABA content, GABA(A) receptor alpha-1 subunit messenger RNA and human GABA transporter-1 (HGAT-1) messenger RNA expression. *Neuroscience* 1999; **93**: 441–448.
148 Gasnier B. The SLC32 transporter, a key protein for the synaptic release of inhibitory amino acids. *Pflugers Arch* 2004; **447**: 756–759.
149 Heckers S, Stone D, Walsh J, Shick J, Koul P, Benes FM. Differential hippocampal expression of glutamic acid decarboxylase 65 and 67 messenger RNA in bipolar disorder and schizophrenia. *Arch Gen Psychiatry* 2002; **59**: 521–529.
150 Guidotti A, Auta J, Davis JM, Giorgi-Gerevini V, Dwivedi Y, Grayson DR *et al.* Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. *Arch Gen Psychiatry* 2000; **57**: 1061–1069.
151 Berrettini WH, Nurnberger JI, Post RM, Gershon ES. Platelet 3H-imipramine binding in euthymic bipolar patients. *Psychiatry Res* 1982; **7**: 215–219.
152 Zhou L, Chillag KL, Nigro MA. Hyperekplexia: a treatable neurogenetic disease. *Brain Dev* 2002; **24**: 669–674.
153 Du J, Gray NA, Falke CA, Chen W, Yuan P, Szabo ST *et al.* Modulation of synaptic plasticity by antimanic agents: the role of AMPA glutamate receptor subunit 1 synaptic expression. *J Neurosci* 2004; **24**: 6578–6589.
154 Kamboj RK, Schoepp DD, Nutt S, Shekter L, Korczak B, True RA *et al.* Molecular cloning, expression, and pharmacological characterization of humEAA1, a human kainate receptor subunit. *J Neurochem* 1994; **62**: 1–9.
155 Mundo E, Tharmalingham S, Neves-Pereira M, Dalton EJ, Macciardi F, Parikh SV *et al.* Evidence that the *N*-methyl-D-aspartate subunit 1 receptor gene (GRIN1) confers susceptibility to bipolar disorder. *Mol Psychiatry* 2003; **8**: 241–245.
156 Itokawa M, Yamada K, Iwayama-Shigeno Y, Ishitsuka Y, Detera-Wadleigh S, Yoshikawa T. Genetic analysis of a functional GRIN2A promoter (GT)*n* repeat in bipolar disorder pedigrees in humans. *Neurosci Lett* 2003; **345**: 53–56.
157 Miyamoto Y, Yamada K, Nagai T, Mori H, Mishina M, Furukawa H *et al.* Behavioural adaptations to addictive drugs in mice lacking the NMDA receptor epsilon1 subunit. *Eur J Neurosci* 2004; **19**: 151–158.
158 Chaki S, Yoshikawa R, Hirota S, Shimazaki T, Maeda M, Kawashima N *et al.* MGS0039: a potent and selective group II metabotropic glutamate receptor antagonist with antidepressant-like activity. *Neuropharmacology* 2004; **46**: 457–467.
159 Wieronska JM, Branski P, Szewczyk B, Palucha A, Papp M, Gruca P *et al.* Changes in the expression of metabotropic glutamate receptor 5 (mGluR5) in the rat hippocampus in an animal model of depression. *Pol J Pharmacol* 2001; **53**: 659–662.
160 Cryan JF, Kelly PH, Neijt HC, Sansig G, Flor PJ, van Der Putten H. Antidepressant and anxiolytic-like effects in mice lacking the group III metabotropic glutamate receptor mGluR7. *Eur J Neurosci* 2003; **17**: 2409–2417.
161 O'Shea RD. Roles and regulation of glutamate transporters in the central nervous system. *Clin Exp Pharmacol Physiol* 2002; **29**: 1018–1023.
162 Smith RE, Haroutunian V, Davis KL, Meador-Woodruff JH. Expression of excitatory amino acid transporter transcripts in the thalamus of subjects with schizophrenia. *Am J Psychiatry* 2001; **158**: 1393–1399.
163 McCullumsmith RE, Meador-Woodruff JH. Striatal excitatory amino acid transporter transcript expression in schizophrenia, bipolar disorder, and major depressive disorder. *Neuropsychopharmacology* 2002; **26**: 368–375.
164 Chen L, Muhlhauser M, Yang CR. Glycine tranporter-1 blockade potentiates NMDA-mediated responses in rat prefrontal cortical neurons *in vitro* and *in vivo*. *J Neurophysiol* 2003; **89**: 691–703.
165 Hashimoto K, Fukushima T, Shimizu E, Komatsu N, Watanabe H, Shinoda N *et al.* Decreased serum levels of D-serine in patients with schizophrenia: evidence in support of the *N*-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia. *Arch Gen Psychiatry* 2003; **60**: 572–576.
166 Kim SJ, Young LJ, Gonen D, Veenstra-VanderWeele J, Courchesne R, Courchesne E *et al.* Transmission disequilibrium testing of arginine vasopressin receptor 1A (AVPR1A) polymorphisms in autism. *Mol Psychiatry* 2002; **7**: 503–507.
167 Douglass AB. Narcolepsy: differential diagnosis or etiology in some cases of bipolar disorder and schizophrenia? *CNS Spectr* 2003; **8**: 120–126.
168 Caberlotto L, Hurd YL. Reduced neuropeptide Y mRNA expression in the prefrontal cortex of subjects with bipolar disorder. *Neuroreport* 1999; **10**: 1747–1750.
169 Kinkead B, Binder EB, Nemeroff CB. Does neurotensin mediate the effects of antipsychotic drugs? *Biol Psychiatry* 1999; **46**: 340–351.
170 Ribeiro SJ, De Lima TC. Naloxone-induced changes in tachykinin NK3 receptor modulation of experimental anxiety in mice. *Neurosci Lett* 1998; **258**: 155–158.
171 Carlezon Jr WA, Thome J, Olson VG, Lane-Ladd SB, Brodkin ES, Hiroi N *et al.* Regulation of cocaine reward by CREB. *Science* 1998; **282**: 2272–2275.
172 Deckert J, Nothen MM, Albus M, Franzek E, Rietschel M, Ren H *et al.* Adenosine A1 receptor and bipolar affective disorder: systematic screening of the gene and association studies. *Am J Med Genet* 1998; **81**: 18–23.
173 DeteraWadleigh SD, Badner JA, Goldin LR, Berrettini WH, Sanders AR, Rollins DY *et al.* Affected-sib-pair analyses reveal support of prior evidence for a susceptibility locus for bipolar disorder, on 21q. *Am J Hum Genet* 1996; **58**: 1279.
174 Muller M, Holsboer F, Keck ME. Genetic modification of corticosteroid receptor signalling: novel insights into pathophysiology and treatment strategies of human affective disorders. *Neuropeptides* 2002; **36**: 117–131.
175 Wang JF, Bown CD, Chen B, Young LT. Identification of mood stabilizer-regulated genes by differential-display PCR. *Int J Neuropsychopharmacol* 2001; **4**: 65–74.
176 Futamura T, Toyooka K, Iritani S, Niizato K, Nakamura R, Tsuchiya K *et al.* Abnormal expression of epidermal growth factor and its receptor in the forebrain and serum of schizophrenic patients. *Mol Psychiatry* 2002; **7**: 673–682.
177 Bezchlibnyk YB, Wang JF, McQueen GM, Young LT. Gene expression differences in bipolar disorder revealed by cDNA array analysis of post-mortem frontal cortex. *J Neurochem* 2001; **79**: 826–834.
178 Faivre L, Gosset P, Cormier-Daire V, Odent S, Amiel J, Giurgea I *et al.* Overgrowth and trisomy 15q26.1-qter including the IGF1 receptor gene: report of two families and review of the literature. *Eur J Hum Genet* 2002; **10**: 699–706.
179 Zubenko GS, Maher B, Hughes III HB, Zubenko WN, Stiffler JS, Kaplan BB *et al.* Genome-wide linkage survey for genetic loci that influence the development of depressive disorders in families with recurrent, early-onset, major depression. *Am J Med Genet* 2003; **123B**: 1–18.

180 Toyota T, Yamada K, Saito K, Detera-Wadleigh SD, Yoshikawa T. Association analysis of adenylate cyclase type 9 gene using pedigree disequilibrium test in bipolar disorder. *Mol Psychiatry* 2002; **7**: 450–452.
181 Vuoristo JT, Berrettini WH, Overhauser J, Prockop DJ, Ferraro TN, Ala-Kokko L. Sequence and genomic organization of the human G-protein Golfalpha gene (GNAL) on chromosome 18p11, a susceptibility region for bipolar disorder and schizophrenia. *Mol Psychiatry* 2000; **5**: 495–501.
182 Ye Y, Conti M, Houslay MD, Farooqui SM, Chen M, O'Donnell JM. Noradrenergic activity differentially regulates the expression of rolipram-sensitive, high-affinity cyclic AMP phosphodiesterase (PDE4) in rat brain. *J Neurochem* 1997; **69**: 2397–2404.
183 Suda S, Nibuya M, Ishiguro T, Suda H. Transcriptional and translational regulation of phosphodiesterase type IV isozymes in rat brain by electroconvulsive seizure and antidepressant drug treatment. *J Neurochem* 1998; **71**: 1554–1563.
184 Zhang HT, Huang Y, Jin SL, Frith SA, Suvarna N, Conti M *et al*. Antidepressant-like profile and reduced sensitivity to rolipram in mice deficient in the PDE4D phosphodiesterase enzyme. *Neuropsychopharmacology* 2002; **27**: 587–595.
185 Chang A, Li PP, Warsh JJ. cAMP-Dependent protein kinase (PKA) subunit mRNA levels in postmortem brain from patients with bipolar affective disorder (BD). *Brain Res Mol Brain Res* 2003; **116**: 27–37.
186 Chang A, Li PP, Warsh JJ. Altered cAMP-dependent protein kinase subunit immunolabeling in post-mortem brain from patients with bipolar affective disorder. *J Neurochem* 2003; **84**: 781–791.
187 Garzon J, Rodriguez-Munoz M, Lopez-Fando A, Garcia-Espana A, Sanchez-Blazquez P. RGSZ1 and GAIP regulate mu- but not delta-opioid receptors in mouse CNS: role in tachyphylaxis and acute tolerance. *Neuropsychopharmacology* 2004; **29**: 1091–1104.
188 Mirnics K, Middleton FA, Stanwood GD, Lewis DA, Levitt P. Disease-specific changes in regulator of G-protein signaling 4 (RGS4) expression in schizophrenia. *Mol Psychiatry* 2001; **6**: 293–301.
189 Smith FD, Oxford GS, Milgram SL. Association of the D2 dopamine receptor third cytoplasmic loop with spinophilin, a protein phosphatase-1-interacting protein. *J Biol Chem* 1999; **274**: 19894–19900.
190 Frankland PW, O'Brien C, Ohno M, Kirkwood A, Silva AJ. Alpha-CaMKII-dependent plasticity in the cortex is required for permanent memory. *Nature* 2001; **411**: 309–313.
191 Chandy KG, Fantino E, Wittekindt O, Kalman K, Tong LL, Ho TH *et al*. Isolation of a novel potassium channel gene hSKCa3 containing a polymorphic CAG repeat: a candidate for schizophrenia and bipolar disorder? *Mol Psychiatry* 1998; **3**: 32–37.
192 Coyle JT, Duman RS. Finding the intracellular signaling pathways affected by mood disorder treatments. *Neuron* 2003; **38**: 157–160.
193 Manji HK, Lenox RH. Ziskind–Somerfeld Research Award. Protein kinase C signaling in the brain: molecular transduction of mood stabilization in the treatment of manic-depressive illness. *Biol Psychiatry* 1999; **46**: 1328–1351.
194 Jacobsen NJ, Franks EK, Owen MJ, Craddock NJ. Mutational analysis of phospholipase A2A: a positional candidate susceptibility gene for bipolar disorder. *Mol Psychiatry* 1999; **4**: 274–279.
195 Papadimitriou GN, Dikeos DG, Souery D, Del Favero J, Massat I, Avramopoulos D *et al*. Genetic association between the phospholipase A2 gene and unipolar affective disorder: a multicentre case–control study. *Psychiatr Genet* 2003; **13**: 211–220.
196 Turecki G, Grof P, Cavazzoni P, Duffy A, Grof E, Ahrens B *et al*. Evidence for a role of phospholipase C-gamma1 in the pathogenesis of bipolar disorder. *Mol Psychiatry* 1998; **3**: 534–538.
197 Zill P, Baghai TC, Zwanzger P, Schule C, Minov C, Riedel M *et al*. Evidence for an association between a G-protein beta3-gene variant with depression and response to antidepressant treatment. *Neuroreport* 2000; **11**: 1893–1897.
198 Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR *et al*. Molecular basis for interactions of G protein betagamma subunits with effectors. *Science* 1998; **280**: 1271–1274.
199 Manji HK, Chen G. PKC, MAP kinases and the bcl-2 family of proteins as long-term targets for mood stabilizers. *Mol Psychiatry* 2002; **7**(Suppl 1): S46–S56.
200 Toyota T, Watanabe A, Shibuya H, Nankai M, Hattori E, Yamada K *et al*. Association study on the DUSP6 gene, an affective disorder candidate gene on 12q23, performed by using fluorescence resonance energy transfer-based melting curve analysis on the LightCycler. *Mol Psychiatry* 2000; **5**: 489–494.
201 Mathews R, Li PP, Young LT, Kish SJ, Warsh JJ. Increased G alpha q/11 immunoreactivity in postmortem occipital cortex from patients with bipolar affective disorder. *Biol Psychiatry* 1997; **41**: 649–656.
202 Yoshikawa T, Kikuchi M, Saito K, Watanabe A, Yamada K, Shibuya H *et al*. Evidence for association of the myo-inositol monophosphatase 2 (IMPA2) gene with schizophrenia in Japanese samples. *Mol Psychiatry* 2001; **6**: 202–210.
203 Stopkova P, Saito T, Fann CS, Papolos DF, Vevera J, Paclt I *et al*. Polymorphism screening of PIP5K2A: a candidate gene for chromosome 10p-linked psychiatric disorders. *Am J Med Genet* 2003; **123B**: 50–58.
204 Saito T, Stopkova P, Diaz L, Papolos DF, Boussemart L, Lachman HM. Polymorphism screening of PIK4CA: possible candidate gene for chromosome 22q11-linked psychiatric disorders. *Am J Med Genet* 2003; **116B**: 77–83.
205 Benedetti F, Serretti A, Colombo C, Barbini B, Lorenzi C, Campori E *et al*. Influence of CLOCK gene polymorphism on circadian mood fluctuation and illness recurrence in bipolar depression. *Am J Med Genet* 2003; **123B**: 23–26.
206 Taheri S, Mignot E. The genetics of sleep disorders. *Lancet Neurol* 2002; **1**: 242.
207 Nurnberger Jr JI, Adkins S, Lahiri DK, Mayeda A, Hu K, Lewy A *et al*. Melatonin suppression by light in euthymic bipolar and unipolar patients. *Arch Gen Psychiatry* 2000; **57**: 572–579.
208 Partonen T, Lonnqvist J. Seasonal affective disorder. *Lancet* 1998; **352**: 1369–1374.
209 Liu C, Weaver DR, Jin X, Shearman LP, Pieschl RL, Gribkoff VK *et al*. Molecular dissection of two distinct actions of melatonin on the suprachiasmatic circadian clock. *Neuron* 1997; **19**: 91–102.
210 Nyegaard M, Borglum AD, Bruun TG, Collier DA, Russ C, Mors O *et al*. Novel polymorphisms in the somatostatin receptor 5 (SSTR5) gene associated with bipolar affective disorder. *Mol Psychiatry* 2002; **7**: 745–754.
211 Jin X, von Gall C, Pieschl RL, Gribkoff VK, Stehle JH, Reppert SM *et al*. Targeted disruption of the mouse Mel(1b) melatonin receptor. *Mol Cell Biol* 2003; **23**: 1054–1060.
212 Hannibal J. Neurotransmitters of the retino-hypothalamic tract. *Cell Tissue Res* 2002; **309**: 73–88.
213 Ishiguro H, Ohtsuki T, Okubo Y, Kurumaji A, Arinami T. Association analysis of the pituitary adenyl cyclase activating peptide gene (PACAP) on chromosome 18p11 with schizophrenia and bipolar disorders. *J Neural Transm* 2001; **108**: 849–854.
214 Johansson C, Willeit M, Smedh C, Ekholm J, Paunio T, Kieseppa T *et al*. Circadian clock-related polymorphisms in seasonal affective disorder and their relevance to diurnal preference. *Neuropsychopharmacology* 2003; **28**: 734–739.
215 Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E *et al*. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 2003; **299**: 256–259.
216 Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA *et al*. Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum Mol Genet* 2000; **9**: 1415–1423.
217 Ozeki Y, Tomoda T, Kleiderlein J, Kamiya A, Bord L, Fujii K *et al*. Disrupted-in-schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. *Proc Natl Acad Sci USA* 2003; **100**: 289–294.
218 Costa E, Chen Y, Davis J, Dong E, Noh JS, Tremolizzo L *et al*. REELIN and schizophrenia: a disease at the interface of the genome and the epigenome. *Mol Intervent* 2002; **2**: 47–57.
219 Tokuoka SM, Ishii S, Kawamura N, Satoh M, Shimada A, Sasaki S *et al*. Involvement of platelet-activating factor and LIS1 in neuronal migration. *Eur J Neurosci* 2003; **18**: 563–570.

220 Sakurai K, Migita O, Toru M, Arinami T. An association between a missense polymorphism in the close homologue of L1 (CHL1, CALL) gene and schizophrenia. *Mol Psychiatry* 2002; **7**: 412–415.
221 Poltorak M, Wright R, Hemperly JJ, Torrey EF, Issa F, Wyatt RJ *et al.* Monozygotic twins discordant for schizophrenia are discordant for N-CAM and L1 in CSF. *Brain Res* 1997; **751**: 152–154.
222 Kurumaji A, Nomoto H, Okano T, Toru M. An association study between polymorphism of L1CAM gene and schizophrenia in a Japanese sample. *Am J Med Genet* 2001; **105**: 99–104.
223 Arai M, Itokawa M, Yamada K, Toyota T, Arai M, Haga S *et al.* Association of neural cell adhesion molecule 1 gene polymorphisms with bipolar affective disorder in Japanese individuals. *Biol Psychiatry* 2004; **55**: 804–810.
224 Liu H, Heath SC, Sobin C, Roos JL, Galke BL, Blundell ML *et al.* Genetic variation at the 22q11 PRODH2/DGCR6 locus presents an unusual pattern and increases susceptibility to schizophrenia. *Proc Natl Acad Sci USA* 2002; **99**: 3717–3722.

Hum Genet (2005) 117: 520–527
DOI 10.1007/s00439-005-1342-8

ORIGINAL INVESTIGATION

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Genetic association analyses of *PHOX2B* and *ASCL1* in neuropsychiatric disorders: evidence for association of *ASCL1* with Parkinson's disease

Received: 15 March 2005 / Accepted: 2 May 2005 / Published online: 14 July 2005

Abstract We previously identified frequent deletion/insertion polymorphisms in the 20-alanine homopolymer stretch of *PHOX2B* (*PMX2B*), the gene for a transcription factor that plays important roles in the development of oculomotor nerves and catecholaminergic neurons and regulates the expression of both tyrosine hydroxylase and dopamine β-hydroxylase genes. An association was detected between gene polymorphisms and overall schizophrenia, and more specifically, schizophrenia with ocular misalignment. These prior results implied the existence of other schizophrenia susceptibility genes that interact with *PHOX2B* to increase risk of the combined phenotype. *ASCL1* was considered as a candidate interacting partner of *PHOX2B*, as ASCL1 is a transcription factor that co-regulates catecholamine-synthesizing enzymes with PHOX2B. The genetic contributions of *PHOX2B* and *ASCL1* were examined separately, along with epistatic interactions with broader candidate phenotypes. These phenotypes included not only schizophrenia, but also bipolar affective disorder and Parkinson's disease (PD), each of which involve catecholaminergic function. The current case-control analyses detected nominal associations between polyglutamine length variations in *ASCL1* and PD ($P = 0.018$), but supported neither the previously observed weak association between *PHOX2B* and general schizophrenia, nor other gene-disease correlations. Logistic regression analysis revealed the effect of *ASCL1* dominant × *PHOX2B* additive ($P = 0.008$) as an epistatic gene–gene interaction increasing risk of PD. ASCL1 controls development of the locus coeruleus (LC), and accumulating evidence suggests that the LC confers protective effects against the dopaminergic neurodegeneration inherent in PD. The present genetic data may thus suggest that polyglutamine length polymorphisms in *ASCL1* could influence predispositions to PD through the fine-tuning of LC integrity.

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Introduction

Paired-like homeobox 2b (PHOX2B, also known as PMX2B or NBPhox) is a homeodomain transcription factor, and is known to determine noradrenergic phenotype (Pattyn et al. 2000) and play a role in the development of cranial motor nerves, including the oculomotor (nIII) and trochlear (nIV) nerves (Pattyn et al. 1997) controlling ocular alignment and movement. As a transcription factor, PHOX2B regulates the expression of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) genes. TH catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA), a precursor of dopamine, and DBH catalyzes the conversion of dopamine to noradrenaline. The protein structure of PHOX2B is characterized by two

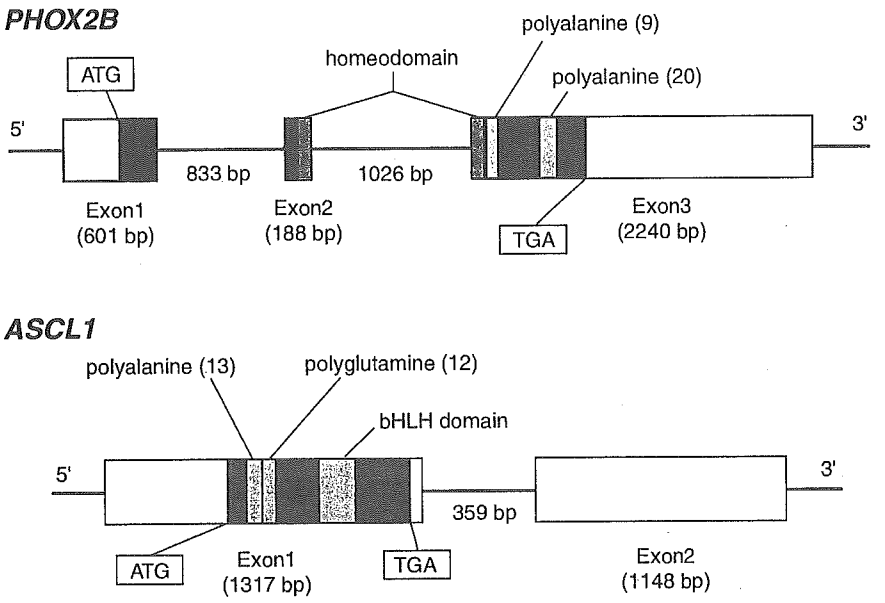

Fig. 1 Schematic representation of the *PHOX2B* (NM_003924) (*above*) and *ASCL1* (NM_004316) genes (*below*). Exons are *boxed*, and initiation and stop codons and protein domains are indicated

homopolymeric stretches of alanine residues: one consisting of nine alanines located downstream of the homeodomain; the other comprising 20 alanines (Ala20) on the C-terminal side (Fig. 1). Our prior genomic screening of *PHOX2B* identified frequent length variations in the Ala20 stretch in the general population, representing an unusual phenomenon compared with other polyalanine-containing transcription factors (Toyota et al. 2004). Variations included −3Ala, −5Ala, −7Ala, −13Ala and +2Ala. These alterations in alanine length resulted in decreased transcriptional ability of the protein and represented the only functional polymorphisms found in the gene. In accordance with the known function of PHOX2B and the functional consequences of these variations, associations between the polymorphisms and general schizophrenia were detected, particularly for schizophrenia manifesting with strabismus (ocular misalignment) (Toyota et al. 2004). That study also raised a possibility of interactions between *PHOX2B* and other schizophrenia-precipitating factors (genes) for increased risk of the combined phenotype (Toyota et al. 2004).

Human achaete-scute homologue 1 (HASH1; ASCL1 in HUGO nomenclature), a human orthologue of mouse Mash1, is a basic helix–loop–helix (bHLH) transcription factor that is known to co-regulate differentiation of the autonomic system along with PHOX2B (Pattyn et al. 2000). Cross-regulation by the *Phox2* and *Mash1* genes, and the importance of the HASH1-PHOX pathway in the development of neurons in the noradrenergic lineage have been demonstrated in both mice (Pattyn et al. 1999, 2000), and a human disease mechanism (De Pontual et al. 2003). We therefore speculated that *PHOX2B* and *ASCL1* may affect predispositions to broad catecholamine-related diseases both separately and in combination. The present study examined genetic associations between *PHOX2B* and *ASCL1* and schizophrenia, bipolar disorder and Parkinson's disease (PD).

Materials and methods

Study subjects

Subjects included 715 schizophrenic patients (394 men, mean age 48.3 ± 12.3 years; 321 women, mean age 50.7 ± 13.3 years), 249 bipolar disorder patients (118 men, mean age 52.6 ± 13.2 years; 131 women, mean age 55.8 ± 12.9 years), 100 PD patients (32 men, mean age 67.3 ± 7.8 years; 68 women, mean age 67.8 ± 7.0 years) and 801 healthy controls (369 men, mean age 40.9 ± 11.4 years; 432 women, mean age 41.3 ± 13.7 years). Compared with the prior study (Toyota et al. 2004), the number of schizophrenia patients was increased by 369 and the number of controls was increased by 260, but these newly added subjects were not screened for strabismus. All subjects were recruited from a geographic area located in central Japan. Diagnosis of schizophrenia and bipolar disorder was based on the *Diagnostic and statistical manual of mental disorders* (American Psychiatric Association 1994). PD was diagnosed according to the standardized criteria. All PD patients underwent brain computed tomography examination to exclude organic abnormalities. Control subjects were recruited from hospital staff and company employees who were documented as free of psychoses or any kind of neurodegenerative disorder. None of the current subjects displayed mental retardation or congenital central hypoventilation syndrome (De Pontual et al. 2003). This study was approved by the Ethics Commit-

tees of RIKEN, Hamamatsu University and Juntendo University, and all subjects provided written informed consent to participate.

Mutation screening of *ASCL1*

ASCL1 is located on human chromosome 12q22-q23 (Renault et al. 1995) and comprises two exons, with the first exon including both the initiation and stop codons (Fig. 1). The protein-coding region contains a poly-alanine stretch comprising 13 alanines, and a polyglutamine tract of 12 glutamine residues (Gln12), in addition to the bHLH. The two exons and their flanking genomic stretches were screened using polymerase chain reaction (PCR) amplification and subsequent direct sequencing of genomic DNA from 24 randomly chosen patients. Sequencing was performed using a DYEnamic ET terminator cycle sequencing kit (Amersham, Piscataway, N.J., USA). Information on primer sequences and PCR conditions employed in this study is available on request. Screening detected the insertion of three CAG repeats (coding glutamine) into the polyglutamine stretch. This was the only non-synonymous polymorphism identified, and we therefore focused on this Gln12 length polymorphism in subsequent analyses.

Genotyping

Genotyping of Ala20 length variations in the *PHOX2B* was performed according to the methods described elsewhere (Toyota et al. 2004). To genotype Gln12 polyglutamine length variations in *ASCL1*, template DNA was amplified using fluorescently labeled forward (5′-AGCTCTGCCAAGATGGAGAG; 3′ end at nt c.26) and reverse (5′- gttttcttTTGCTTGGGCGC-TGACTTGT; 3′ end at nt c.236) primers. The *underlined* tail sequence was added because Taq DNA polymerase catalyzes the non-templated addition of adenosine to the 3′ end of PCR products to varying degrees. This phenomenon is primer-specific and represents a potential source of genotyping error. Placing the gtttctt sequence at the 5′ end of reverse primers produces nearly 100% adenylation of the 3′ end of the forward strand, facilitating accurate genotyping (Brownstein et al. 1996; Itokawa et al. 2003). PCR products were run on an ABI 3700 genetic analyzer (Applied Biosystems, Foster City, Calif., USA), and the resulting data were analyzed using GeneScan software (Applied Biosystems). Genotypes were confirmed by subcloning the amplicons into a TA vector (Invitrogen, Carlsbad, Calif., USA) and sequencing. Primers were designed to produce a 249-bp DNA fragment for the wild-type allele (Gln12), but GeneScan analysis yielded a band approximately 14 bp shorter than expected (Fig. 2a), with occasional inconsistent genotype results compared with those obtained by subcloning, which could not be resolved by applying

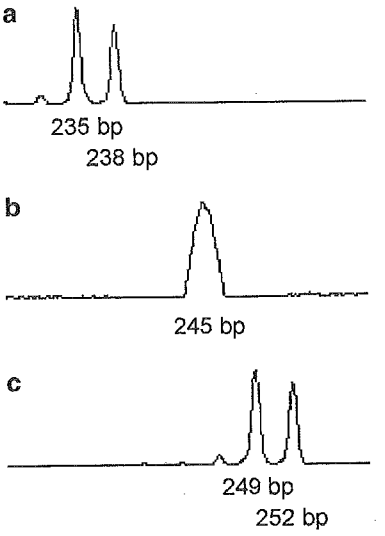

Fig. 2a–c GeneScan migration patterns of *ASCL1* Gln12 length polymorphisms. DNA fragments with Gln12 or Gln13 genotypes were run after PCR under varying concentrations of c^7 dGTP. Exact sizes of the Gln12 and Gln13 alleles were 249 and 252 bp, respectively. **a** The c^7 dGTP was not added to the PCR mixture. Note that displayed allele sizes were 14 bp shorter than actual sizes. **b** Addition of c^7 dGTP to 25% resulted in fusion of the two peaks. **c** When all dGTP in the PCR reaction mixture was replaced with c^7dGTP, peaks appeared at expected sizes with good separation of the two adjacent alleles

the constant 14-bp difference to GeneScan results. This phenomenon was attributed to the secondary DNA structure generated by abundant GCs in the PCR products (Toyota et al. 2004). When 7-deaza-2′-deoxyguanosine triphosphate (c^7 dGTP) was added to the PCR reaction mixture (c^7 dGTP:dGTP = 1:3) to breakdown hydrogen bonds in the GC-rich templates, GeneScan peaks were broadened and two adjacent peaks merged (Fig. 2b). We replaced all dGTP in the PCR reaction mixture with c^7 dGTP, and obtained sharp and correctly sized bands, enabling accurate genotyping (Fig. 2c).

Statistical analysis

Associations of either *PHOX2B* or *ASCL1* polymorphisms with each neuropsychiatric disorder were evaluated using the Monte–Carlo method implemented in the CLUMP program (T1–T4 modes; number of simulations set to 10,000; random number seed, 100) (Sham and Curtis 1995) or Fisher's exact test when appropriate. Rare alleles or genotypes showing frequencies of $< 1\%$ in both comparison groups were removed from the analysis. Hardy–Weinberg equilibrium was evaluated using Arlequin software (http://lgb.unige.ch/arlequin/) (Schneider and Excofier 2000). Logistic regression analysis in the SPSS Regression Models software (SPSS Japan, Tokyo, Japan) was performed to test the joint

effects of the two genes. Letting P represent the probability of an individual being a case rather than a control, we modeled P as

$$\log it(P) = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{2} \sum_{j=3}^{4} \beta_{ij} x_i x_j$$

where x_1, x_2, x_3 and x_4 represent covariants depending on the genotypes of the individual, β_0 is the intercept, and β_i and β_{ij} are coefficients to be estimated. When applied to the formula, genotypes were dichotomized into two groups: wild-type (*w*); and mutant (*m*). Following the approach of Cordell and Clayton (2002) for the possible genotypes of *w*/*w*, *m*/*w* and *m*/*m*, we coded −1, 0 and 1, respectively, to represent the additive effects of allele m and −0.5, 0.5, −0.5, respectively, to represent the dominant effect of allele *m* over allele *w*.

Results

Table 1 shows the results of association analyses between *PHOX2B* Ala20 length polymorphisms and the three disease categories. We detected six different genotypes, and distributions of genotypes in each group were all in Hardy–Weinberg equilibrium. None of the modes T1–T4 on CLUMP analysis displayed significant associations for any disease groups. The number of different alleles observed in this study was the same as in our previous study (Toyota et al. 2004), although much larger cohorts were examined here. Again, no allelic associations were detected for any of the three neuropsychiatric disorders.

Tables 2 and 3 show the results of genotypic and allelic analyses of *ASCL1* Gln12 stretch polymorphisms, respectively. Analysis of the 1,866 subjects yielded 13 different length variations in the Gln12 homopolyer repeat region of *ASCL1*. These polymorphisms were not genotypically associated with schizophrenia or bipolar disorder, but displayed associations with PD ($P < 0.05$ in T2, T3 and T4) (Table 2). Allelic analysis demonstrated that the allele containing 12 glutamine repeats, the most common of these alleles, was more frequent in PD than in the control group (2×2 Fisher's exact test, two-sided, $P = 0.015$; odds ratio = 1.68, 95% CI = 1.10–2.54), while the allele containing 15 glutamine repeats, as the second most common allele, exhibited an opposite distribution pattern ($P = 0.011$; odds ratio = 0.57, 95% CI = 0.36–0.89) (Table 3). These results suggest that the *ASCL1* allele harboring 15 glutamine repeats may play a protective role against PD manifestation.

Logistic regression analysis was then performed to test the joint effect of the two genes on PD. The Ala20 allele of *PHOX2B* and the Gln12 allele of *ASCL1* were classified as *w*, with the remaining alleles as *m*. As a result, only the effect of *ASCL1* dominant × *PHOX2B* additive was found to be significant ($P = 0.008$), among the effects of all possible interaction modes (Table 4).

Discussion

PHOX2B/*ASCL1* and psychiatric disorders

We have previously reported genotypic associations between Ala20 polymorphisms in *PHOX2B* and overall

Table 1 Genotypic and allelic distributions of the *PHOX2B* Ala20 repeat polymorphism

	Schizophrenia ($n = 715$)	Bipolar disorder ($n = 249$)	Parkinson's disease ($n = 100$)	Controls ($n = 802$)
Genotype[a]	Genotype counts (% frequency)			
15/15	0 (0)	0 (0)	1 (1.0)	3 (0.4)
20/7	1 (0.2)	0 (0)	0 (0)	0 (0)
20/13	6 (0.9)	2 (1.2)	0 (0)	7 (0.9)
20/15	57 (8.8)	14 (8.4)	9 (9.3)	59 (7.4)
20/20	579 (89.8)	151 (90.4)	87 (89.7)	727 (91.2)
20/22	2 (0.3)	0 (0)	0 (0)	1 (0.1)
$P^{b,c}$				
T1	0.35	0.81	0.50	
T2	0.50	0.76	0.71	
T3	0.54	0.83	0.60	
T4	0.46	0.89	0.79	
Allele[a]	Allele counts (% frequency)			
7	1 (0.1)	0 (0)	0 (0)	0 (0)
13	6 (0.5)	2 (0.6)	0 (0)	7 (0.4)
15	57 (4.4)	14 (4.2)	11 (5.7)	65 (4.1)
20	1224 (94.9)	318 (95.2)	183 (94.3)	1521 (95.4)
22	2 (0.2)	0 (0)	0 (0)	1 (0.1)
$P^{b,d}$	0.64	0.88	0.34	

[a]Number of alanine repeats
[b]Minor genotypes and alleles with frequencies (< 1% in both comparison groups were omitted from analyses
[c]Calculated using the Monte Carlo method
[d]Calculated using Fisher's exact test

Table 2 Genotypic distribution of the *ASCL1* Gln12 repeat polymorphism

	Schizophrenia ($n=715$)	Bipolar disorder ($n=249$)	Parkinson's disease ($n=100$)	Controls ($n=802$)
Genotype[a]	Genotype counts (% frequency)			
6/12	1 (0.1)	0 (0)	0 (0)	0 (0)
6/15	1 (0.1)	0 (0)	0 (0)	0 (0)
7/12	0 (0)	0 (0)	0 (0)	1 (0.1)
8/12	0 (0)	0 (0)	0 (0)	1 (0.1)
9/12	1 (0.1)	0 (0)	0 (0)	2 (0.3)
9/15	1 (0.1)	0 (0)	0 (0)	0 (0)
11/12	1 (0.1)	0 (0)	0 (0)	1 (0.1)
12/12	429 (61.5)	144 (60.0)	74 (75.5)	481 (61.0)
12/13	21 (3.0)	8 (3.3)	3 (3.1)	21 (2.7)
12/14	2 (0.3)	0 (0)	1 (1.0)	1 (0.1)
12/15	186 (26.6)	66 (27.5)	16 (16.3)	232 (29.4)
12/16	6 (0.9)	4 (1.7)	0 (0)	8 (1.0)
12/17	2 (0.3)	0 (0)	0 (0)	1 (0.1)
12/18	0 (0)	0 (0)	0 (0)	1 (0.1)
12/19	1 (0.1)	0 (0)	0 (0)	1 (0.1)
13/13	1 (0.1)	0 (0)	0 (0)	0 (0)
13/15	9 (1.3)	3 (1.3)	1 (1.0)	3 (0.4)
14/15	1 (0.1)	0 (0)	0 (0)	0 (0)
15/15	34 (4.9)	14 (5.8)	3 (3.1)	31 (3.9)
15/16	1 (0.1)	0 (0)	0 (0)	3 (0.4)
15/17	0 (0)	1 (0.4)	0 (0)	0 (0)
P[b,c]				
T1	0.41	0.50	0.052	
T2	0.28	0.33	*0.016*	
T3	0.25	0.61	*0.010*	
T4	0.33	0.39	*0.046*	

[a] Number of glutamine repeats
[b] Minor genotypes and alleles with frequencies < 1% in both comparison groups were omitted from analyses
[c] Calculated using the Monte Carlo method

schizophrenia ($P=0.012$), with a more prominent association for schizophrenia with strabismus ($P=0.004$) (Toyota et al. 2004). However, the present study did not detect this association in a larger case-control panel with a 2.2-fold increase in the schizophrenia population and a 1.6-fold increase in control samples. This discrepancy may be partly due to the fact that prior control samples had undergone ocular examinations, and only those subjects who did not suffer from strabismus were chosen, while the present study used control samples with-

Table 3 Allelic distribution of the *ASCL1* Gln12 repeat polymorphism

	Schizophrenia ($n=715$)	Bipolar disorder ($n=249$)	Parkinson's disease ($n=100$)	Controls ($n=802$)
Allele[a]	Allele counts (% frequency)			
6	2 (0.1)	0 (0)	0 (0)	0 (0)
7	0 (0)	0 (0)	0 (0)	1 (0.1)
8	0 (0)	0 (0)	0 (0)	1 (0.1)
9	2 (0.1)	0 (0)	0 (0)	2 (0.1)
11	1 (0.1)	0 (0)	0 (0)	1 (0.1)
12	1079 (77.3)	366 (76.3)	168 (85.7)	1232 (78.2)
13	32 (2.3)	11 (2.3)	4 (2.0)	24 (1.5)
14	3 (0.2)	0 (0)	1 (0.5)	1 (0.1)
15	267 (19.1)	98 (20.4)	23 (11.7)	300 (19.0)
16	7 (0.5)	4 (0.8)	0 (0)	11 (0.7)
17	2 (0.1)	1 (0.2)	0 (0)	1 (0.1)
18	0 (0)	0 (0)	0 (0)	1 (0.1)
19	1 (0.1)	0 (0)	0 (0)	1 (0.1)
P[b,c]				
T1	0.30	0.40	*0.036*	
T2	0.29	0.40	*0.022*	
T3	0.27	0.51	*0.018*	
T4	0.27	0.51	*0.026*	

[a] Number of glutamine repeats
[b] Minor genotypes and alleles with frequencies < 1% in both comparison groups were omitted from analyses
[c] Calculated using the Monte Carlo method

Table 4 Logistic regression analysis of effects of *PHOX2B* and *ASCL1* genes on Parkinson's disease

Variable	β[a]	SE[b]	Wald[c]	df[d]	*P*	Exp (β)[e]	95% CI[f]
ASCL1 dominant by *PHOX2B* additive	0.71	±0.27	7.0	1	0.008	2.0	1.2–3.4

[a]Logistic regression coefficient in the model
[b]Standard error of the coefficient
[c]Wald statistic to test significance of the coefficient
[d]Degrees of freedom for the Wald chi-square test
[e]Exponentiation of the β coefficient (odds ratio)
[f]95% confidence interval of exponentiation (β)

out determining the presence of ocular misalignment. The newly added schizophrenic samples in this study were also not screened for ocular misalignment. While the genetic contributions of *PHOX2B* Ala20 variations to general schizophrenia are more likely to be very weak or even negligible, even by considering genetic interactions with *ASCL1* (data not shown), these contributions may be evident only in a subset of schizophrenia (i.e., schizophrenia with strabismus). As might be expected according to this hypothesis, no association was apparent between *PHOX2B* and schizophrenia without strabismus ($P=0.076$) in our previous study (Toyota et al. 2004). We also tested here *ASCL1* as a singleton or *PHOX2B-ASCL1* epigenetic interaction (data not shown) for altered risk of another major psychosis, bipolar disorder, but no significant signals were detected. As a whole, the current results do not support these genetic mechanisms in the manifestation of functional psychoses.

PHOX2B/ASCL1 and Parkinson's disease

PD is a common neurodegenerative disorder, characterized clinically by resting tremor, rigidity and bradykinesia. Neuropathological studies have revealed degeneration of the dopamine-producing substantia nigra and various other regions, including the basal ganglia, brainstem, autonomic nervous system and cerebral cortex (Dekker et al. 2003). Clinically defined PD represents an etiologically heterogeneous group of conditions encompassing a small population of individuals with Mendelian-type inheritance and a larger population of apparently sporadic cases (Hattori et al. 2003). Accumulating evidence has suggested that genetic predispositions exist even for sporadic PD (Marder et al. 1996). Dopamine deficiency is a primary pathomechanism in PD, and genes involved in dopamine neurotransmission, such as those for dopamine transporter, dopamine receptors, tyrosine hydroxylase, catechol-it O-methyltransferase and monoamine oxidase, have been examined in population-based association studies over the past decade. However, few of these genes have been definitively established as conferring susceptibility to sporadic PD (reviewed in Warner and Schapira 2003).

Perturbation of *PHOX2B* and *ASCL1* function has the potential to disturb catecholaminergic neurons, as these genes control the expression of the *TH* and *DBH* genes, which encode enzymes for the biosynthesis of dopamine (TH) and noradrenalin (TH and DBH) biosynthesis. Ludecke et al. (1996) reported a female infant who manifested L-dopa responsive Parkinsonism and carried a $Leu^{205}Pro$ mutation in exon 5 of the *TH* gene, reducing the catalytic ability of TH. The current study identified a positive association between PD and *ASCL1* polymorphisms. However, whether these *ASCL1* variants result in a predisposition to PD through direct effects on dopamine neurons remains unclear, as *ASCL1* expression in the human substantia nigra has not yet been confirmed. In contrast, expression of *ASCL1* in developing noradrenergic neurons in the human brainstem (locus coeruleus: LC) has been reported (De Pontual et al. 2003). The LC is known to play an important role in the pathophysiology of PD (reviewed in Gesi et al. 2000). Zarow et al. (2003) found more severe neuronal loss in the LC than in the substantia nigra in a postmortem examination of brains from PD patients. Mavridis et al. (1991) demonstrated that monkeys with LC lesions displayed impaired recovery from Parkinsonism induced using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Other studies have also shown that animals with LC lesions exhibit marked dopamine loss on administration of MPTP or methamphetamine (Bing et al. 1994; Fornai et al. 1997). These data suggest a protective role of the LC against the development of PD. Indeed, Srinivasan and Schmidt (2004) reported that the enhancement of noradrenergic transmission in the LC by β_2-adrenoceptor antagonists exerts a prophylactic effect against 6-hydroxydopamine-induced Parkinsonism. The present finding that the *ASCL1* allele containing 15 glutamines is less represented in PD than in controls might suggest that the 15-repeat allele could confer protective benefits compared to the most common 12-repeat allele, perhaps allowing the development of a well-functionalized LC that in turn helps to protect the substantia nigra from various insults.

Because of the presumed multigenic nature of complex traits, it would be desirable to analyze several polymorphisms jointly and investigate their effects and possible interactions on disease outcome (Ott 2001). One of the statistical methods that can be used to resolve this problem is logistic regression analysis. When applied to the current data, this analysis indicated that the dominant effect of *ASCL1* with the additive effect of *PHOX2B* was positive. The biological consequences resulting from the interaction between *ASCL1* and *PHOX2B* might thus offer useful insights into the pathogenesis of PD. Further studies elucidating the detailed mechanisms of this interaction are thus warranted.

Polyglutamine length variations in *ASCL1*

Polyglutamine expansion has been found in various neurodegenerative disorders, including Huntington's disease, spinocerebellar ataxia types 1, 2, 3 and 7, dentatorubral-pallido-luysian atrophy and spinobulbar muscular atrophy (Lipinski and Yuan 2004). The aggregation or accumulation of proteins with expanded polyglutamine sequences is considered to represent a critical contribution to neurodegeneration in these diseases. Generally these aggregate-forming proteins display more than 30 glutamine repeats, while *ASCL1* displays repeats of less than 20 glutamines. None of the Gln12 length variations for *ASCL1* detected in this study are thus likely to exert deteriorative effects on neurons. However, the functional consequences evoked by variations of the polyglutamine stretch in *ASCL1* are yet to be examined.

In summary, we performed an association study for *PHOX2B* and *ASCL1*, genes that are functionally closely related and display imperative roles in the development of neurons in the noradrenergic (dopaminergic) lineage, in three major neuropsychiatric diseases. Significant contributions of *ASCL1* and *ASCL1-PHOX2B* interactions to PD were detected. These results require genetic replication studies in different populations and further biological investigations to clarify the precise mechanisms and effects.

Acknowledgements This work was supported by RIKEN BSI Funds, Research on Brain Science Funds from the Ministry of Health Labor and Welfare, CREST funds from the Japan Science and Technology Agency and grants from the MEXT of Japan. We wish to thank Ms Ohba for technical assistance and the members of the Research Resource Center at the RIKEN Brain Science Institute for genotyping services.

References

American Psychiatric Association (1994) Diagnostic and statistical manual of mental disorders, 4th edn. American Psychiatric Association, Washington, D.C.

Bing G, Zhang Y, Watanabe Y, McEwen BS, Stone EA (1994) Locus coeruleus lesions potentiate neurotoxic effects of MPTP in dopaminergic neurons of the substantia nigra. Brain Res 668:261–265

Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping Biotechniques 20:1004–1006, 1008–1010

Cordell HJ, Clayton DG (2002) A unified stepwise regression procedure for evaluating the relative effects of polymorphisms within a gene using case/control or family data: application to HLA in type 1 diabetes. Am J Hum Genet 70:124–141

De Pontual L, Nepote V, Attie-Bitach T, Al Halabiah H, Trang H, Elghouzzi V, Levacher B, Benihoud K, Auge J, Faure C, Laudier B, Vekemans M, Munnich A, Perricaudet M, Guillemot F, Gaultier C, Lyonnet S, Simonneau M, Amiel J (2003) Noradrenergic neuronal development is impaired by mutation of the proneural *HASH-1* gene in congenital central hypoventilation syndrome (Ondine's curse). Hum Mol Genet 12:3173–3180

Dekker MC, Bonifati V, Van Duijn CM (2003) Parkinson's disease: piecing together a genetic jigsaw. Brain 126:1722–1733

Fornai F, Alessandri MG, Torracca MT, Bassi L, Corsini GU (1997) Effects of noradrenergic lesions on MPTP/MPP+ kinetics and MPTP-induced nigrostriatal dopamine depletions. J Pharmacol Exp Ther 283:100–107

Gesi M, Soldani P, Giorgi FS, Santinami A, Bonaccorsi I, Fornai F (2000) The role of the locus coeruleus in the development of Parkinson's disease. Neurosci Biobehav Rev 24:655–668

Hattori N, Kobayashi H, Sasaki-Hatano Y, Sato K, Mizuno Y (2003) Familial Parkinson's disease: a hint to elucidate the mechanisms of nigral degeneration. J Neurol 250(Suppl 3):III2–10

Itokawa M, Yamada K, Yoshitsugu K, Toyota T, Suga T, Ohba H, Watanabe A, Hattori E, Shimizu H, Kumakura T, Ebihara M, Meerabux JMA, Toru M, Yoshikawa T (2003) A microsatellite repeat in the promoter of the NMDA receptor 2A subunit (*GRIN2A*) gene suppresses transcriptional activity and correlates with chronic outcome in schizophrenia. Pharmacogenetics 13:271–278

Lipinski MM, Yuan J (2004) Mechanisms of cell death in polyglutamine expansion diseases. Curr Opin Pharmacol 4:85–90

Ludecke B, Knappskog PM, Clayton PT, Surtees RA, Clelland JD, Heales SJ, Brand MP, Bartholome K, Flatmark T (1996) Recessively inherited L-DOPA-responsive Parkinsonism in infancy caused by a point mutation (L205P) in the tyrosine hydroxylase gene. Hum Mol Genet 5:1023–1028

Marder K, Tang MX, Mejia H, Alfaro B, Cote L, Louis E, Groves J, Mayeux R (1996) Risk of Parkinson's disease among first-degree relatives: a community-based study. Neurology 47:155–160

Mavridis M, Degryse AD, Lategan AJ, Marien MR, Colpaert FC (1991) Effects of locus coeruleus lesions on parkinsonian signs, striatal dopamine and substantia nigra cell loss after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine in monkeys: a possible role for the locus coeruleus in the progression of Parkinson's disease. Neuroscience 41:507–523

Ott J (2001) Neural networks and disease association studies. Am J Hum Genet 105:60–61

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1997) Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis. Development 124:4065–4075

Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene *Phox2b* is essential for the development of autonomic neural crest derivatives. Nature 399:366–370

Pattyn A, Goridis C, Brunet JF (2000) Specification of the central noradrenergic phenotype by the homeobox gene *Phox2b*. Mol Cell Neurosci 15:235–243

Renault B, Lieman J, Ward D, Krauter K, Kucherlapati R (1995) Localization of the human achaete-scute homolog gene (*ASCL1*) distal to phenylalanine hydroxylase (*PAH*) and proximal to tumor rejection antigen (*TRA1*) on chromosome 12q22-q23. Genomics 30:81–83

Schneider RD, Excofier L (2000) Arlequin: a software for population genetics data analysis, 2 edn, version 2.000. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva

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

Srinivasan J, Schmidt WJ (2004) Treatment with alpha2-adrenoceptor antagonist, 2-methoxy idazoxan, protects 6-hydroxydopamine-induced Parkinsonian symptoms in rats: neurochemical and behavioral evidence. Behav Brain Res 154:353–363

Sriuranpong V, Borges MW, Strock CL, Nakakura EK, Watkins DN, Blaumueller CM, Nelkin BD, Ball DW (2002) Notch signaling induces rapid degradation of achaete-scute homolog 1. Mol Cell Biol 22:3129–3139

Toyota T, Yoshitsugu K, Ebihara M, Yamada K, Ohba H, Fukasawa M, Minabe Y, Nakamura K, Sekine Y, Takei N, Suzuki K, Itokawa M, Meerabux JMA, Iwayama-Shigeno Y, Tomaru Y, Shimizu H, Hattori E, Mori N, Yoshikawa T (2004) Association between schizophrenia with ocular misalignment and polyalanine length variation in PMX2B. Hum Mol Genet 13:551–561

Warner TT, Schapira AH (2003) Genetic and environmental factors in the cause of Parkinson's disease. Ann Neurol 53 (Suppl 3):S16–S23

Zarow C, Lyness SA, Mortimer JA, Chui HC (2003) Neuronal loss is greater in the locus coeruleus than nucleus basalis and substantia nigra in Alzheimer and Parkinson diseases. Arch Neurol 60:337–341

Molecular Psychiatry (2005) 10, 1045–1055

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ORIGINAL RESEARCH ARTICLE

Gene expression and association analyses of *LIM (PDLIM5)* in bipolar disorder and schizophrenia

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We previously reported that expression level of *LIM* (*ENH*, *PDLIM5*) was significantly and commonly increased in the brains of patients with bipolar disorder, schizophrenia, and major depression. Expression of *LIM* was decreased in the lymphoblastoid cells derived from patients with bipolar disorders and schizophrenia. LIM protein reportedly plays an important role in linking protein kinase C with calcium channel. These findings suggested the role of *LIM* in the pathophysiology of bipolar disorder and schizophrenia. To further investigate the role of *LIM* in these mental disorders, we performed a replication study of gene expression analysis and performed genetic association studies. Upregulation of *LIM* was confirmed in the independent sample set obtained from Stanley Array Collection. No effect of sample pH or medication was observed. Genetic association study revealed the association of single nucleotide polymorphism (SNP)1 (rs10008257) with bipolar disorder. In an independent sample set, SNP2 (rs2433320) close to SNP1 was associated with bipolar disorder. In total samples, haplotype of these two SNPs was associated with bipolar disorder. No association was observed in case–control analysis and family-based association analysis in schizophrenia. These results suggest that SNPs in the upstream region of *LIM* may confer the genetic risk for bipolar disorder.

Molecular Psychiatry (2005) **10**, 1045–1055. doi:10.1038/sj.mp.4001719; published online 26 July 2005

Keywords: bipolar disorder; schizophrenia; *PDLIM5*; association study; gene expression; enigma homolog (ENH)

The role of genetic factors in bipolar disorder has been well established from twin, adoption, and family studies.[1] Extensive linkage analyses suggested many candidate loci.[2] In such loci, genes having functions related to bipolar disorder were examined as candidate genes, and several promising results have been reported. Among them, association with *G72* at 13q34 has been replicated in several studies.[3–5]

The other strategy to identify candidate genes is gene expression analysis. Mirnics *et al*[6]performed gene expression analysis using cDNA micoarray and reported that *RGS4* was downregulated in the postmortem brains of patients with schizophrenia. They further examined the association of *RGS4* with schizophrenia and found a positive association.[7] Several studies confirmed this finding.[8–10] A similar approach to identify candidate genes may also be effective for bipolar disorder.

We have performed comprehensive gene expression analysis of the frontal lobes obtained from Stanley Foundation Brain Bank using oligonucleotide microarray.[11] By analyzing 50 brains, we found that two genes, *LIM* and *PRPF4B*, were commonly altered in three mental disorders, bipolar disorder, schizophrenia, and major depression. Of the two genes, upregulation of *LIM* in the postmortem brain was confirmed by RT-PCR. Subsequently, we also found that *LIM* was significantly downregulated in the lymphoblastoid cell lines from patients with bipolar disorder. Since we cultured lymphoblastoid cells for more than 1 month after blood collection, effects of drugs and secondary effects of other confounding factors, such as endocrinological abnormalities, can be ruled out in this analysis.

Next, we performed a replication study of *LIM* expression in lymphoblastoid cells.[12] Reduced expression was confirmed in the extended samples with bipolar I disorder ($N=26$). We also found that *LIM* was significantly downregulated in bipolar II disorder ($N=10$) and schizophrenia ($N=13$). Thus, we speculated that regulation of *LIM* might be genetically impaired in bipolar disorder and other mental disorders.

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Received 25 March 2005; revised 11 May 2005; accepted 23 May 2005; published online 26 July 2005

LIM encodes an adopter protein connecting protein kinase C (PKC) ε and N-type calcium channel.[13] Altered PKC activity in peripheral blood cells of bipolar patients is reported.[14] Furthermore, altered calcium signaling has been postulated as an important pathophysiological mechanism of this disorder.[15] Thus, it is reasonable to hypothesize that genetic variation of *LIM* causes genetically determined dysregulation of *LIM*, which causes calcium-signaling abnormalities in bipolar disorder.

LIM is located at 4q22,[16] for which some linkage signal has been detected in bipolar disorder[17] and schizophrenia.[18] Only a few studies revealed the loci in 4q for bipolar disorder[19–21] or schizophrenia.[22–24] Although the support by linkage studies is marginal, above-mentioned findings by gene expression analyses seemed strong enough to start genetic association analysis of this gene in bipolar disorder.

Here, we performed a replication study of altered expression levels of *LIM* in a larger number of samples of postmortem prefrontal cortex of bipolar disorder and schizophrenia obtained from the Stanley Array Collection, and analyzed possible confounding factors. We further performed association study of *LIM* in bipolar disorder and schizophrenia. While *LIM* was not associated with schizophrenia, it was associated with bipolar disorder, which was replicated in a different sample set. These results suggest that polymorphisms of *LIM* may confer a genetic risk for bipolar disorder.

Subjects and methods

RNA samples

RNA samples extracted from the prefrontal cortices (Broadmann's Area 46) were donated by the Stanley Array Collection. They contain total RNA samples from 35 individuals in each of three diagnostic groups (BD, SZ, and controls). Diagnoses was made according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (American Psychiatric Association). Detailed information about the diagnosis, and summary of demographic variables of each diagnostic group can be found at the website (http://www.stanleyresearch.org/programs/brain_collection.asp).

Real-time quantitative RT-PCR

In all, 3–5 μg of total RNA was used for cDNA synthesis by olgo(dT) and SuperScript II reverse transcriptase (Invitrogen). RT-PCR using SYBER/GREEN I (Applied Biosystems, Foster city, CA, USA) was performed with an ABI PRISM 7900HT (Applied Biosystems). The comparative C_t method was used for quantification according to the manufacture's protocol (Applied Biosystems). Measurement of delta C_t was carried out at least in triplicate. Amplification of the single product was confirmed by monitoring the dissociation curve and by gel electrophoresis. We used two control genes (*GAPDH* and *CFL1*) for normalization to control for possible fluctuations in quantitative values of the target transcripts. The validity of the use of *CFL1* as an internal control gene in postmortem brain samples was shown previously. Primer pairs used in this study were according to the previous report.[11] Among the 105 samples, four samples showing poor RNA qualities were not analyzed.

Subjects for genetic analyses: bipolar disorder

The first sample set was collected in the Shiga University of Medical Science Hospital, University of Tokyo Hospital, and Laboratory for Molecular Dynamics of Mental Disorders (called 'MDMD' samples). These include 128 patients with bipolar disorder (47.8±13.6 years old, 50 males and 78 females) and 130 controls (48.8±15.3 years old, 65 males and 65 females). They were diagnosed with the consensus of two senior psychiatrists without using any structured interviews, or were diagnosed by a senior psychiatrist after an interview using SCID-IV (Structured Clinical Interview for DSM-IV). Controls were selected from students, nurses, office workers, and doctors in participating institutes, and their friends. A senior psychiatrist interviewed them and they did not have major mental disorders. Only a part of them were interviewed using a structured interview, Mini-International Neuropsychiatric Interview (M.I.N.I.).[25]

The replication sample set was collected in the Tokyo Medical and Dental University, Hamamatsu University School of Medicine, and Lab. for Molecular Psychiatry ('MPS' samples). These include 240 patients with bipolar disorder (51.2±13.1 years old, 132 males and 108 females) and 240 controls (51.4±10.7 years old, 120 males and 120 females).

For the quantification of copy number of *LIM* gene, 28 patients with bipolar disorder were selected from 'MDMD' samples.

Subjects for genetic analyses: schizophrenia

Subjects for the case–control analysis consist of 570 patients with schizophrenia (48.6±12.0 years old, 285 males and 285 females) and an equal number of control subjects (48.4±11.8 years old, 285 males and 285 females) collected by the Laboratory for Molecular Psychiatry. Control subjects were recruited from hospital staff and their acquaintances. They were interviewed by an experienced psychiatrist without using structured interviews and found not to have psychoses. Most of the controls in the MPS samples are included in this control group. All were Japanese. Diagnosis of the patients by DSM-IV criteria was made by consensus of two psychiatrists based on unstructured interviews of the patients, chart reviews, and information from family members and hospital staff.

We presumed that all these subjects were unrelated to each other, but it cannot be totally ruled out that some of the patients were related, because the ethics policy of the Japanese Government requires stringent anonymity.

The subjects for TDT analysis consisted of 124 families: 80 trios (schizophrenic offspring and their parents), 15 probands with one parent, and 13 probands with affected siblings, and 30 probands with discordant siblings.[26] They were diagnosed according to DSM-IV criteria by at least two experienced psychiatrists, on the basis of direct interviews, available medical records, and information from hospital staff and relatives.

The ethics committee of RIKEN and participating institutes approved the present study, and written informed consent was obtained from all participants.

Genotyping

Genotyping was performed using commercially available TaqMan probes and ABI7900HT according to the protocol recommended by the manufacturer.

Quantitative genomic PCR (gQ-PCR)

The copy number of *LIM* gene was analyzed by the real-time PCR method using SYBR/GREEN dye (Applied Biosystems). *MLC1* was used as a single copy control gene. For the gQ-PCR, DNA solution was once quantified by the ultraviolet spectrophotometer, and again quantified by TaqMan assay using *RnaseP* (Applied Biosystems). For the quality control, a gene on the X chromosome (*PF2K*) was also examined using SYBR/GREEN dye, and separation between males and females was confirmed. The DNA samples with intermediate copy number of X chromosome gene were regarded as having poor quality and were not used for further analysis. Sequences of primers and probes for these analyses except for *RNaseP* will be provided upon request.

Data analysis

The Mann–Whitney U test was used for comparison of expression level of *LIM* between control and bipolar disorder or schizophrenia.

Family-based association analysis was performed by pedigree disequilibrium test (PDT) program, v3.12.[27] Extended transmission disequilibrium test (ETDT) algorithm, v2.2,[28] was also performed in 80 complete trios. Detailed methods for data analysis were described elsewhere.[26] For the haplotype-based TDT analysis, the TRANSMIT program, v2.5.4,[29,30] was used.

Linkage disequilibrium (LD) patterns were assessed in Japanese controls by the standardized disequilibrium coefficient (D') and the squared correlation

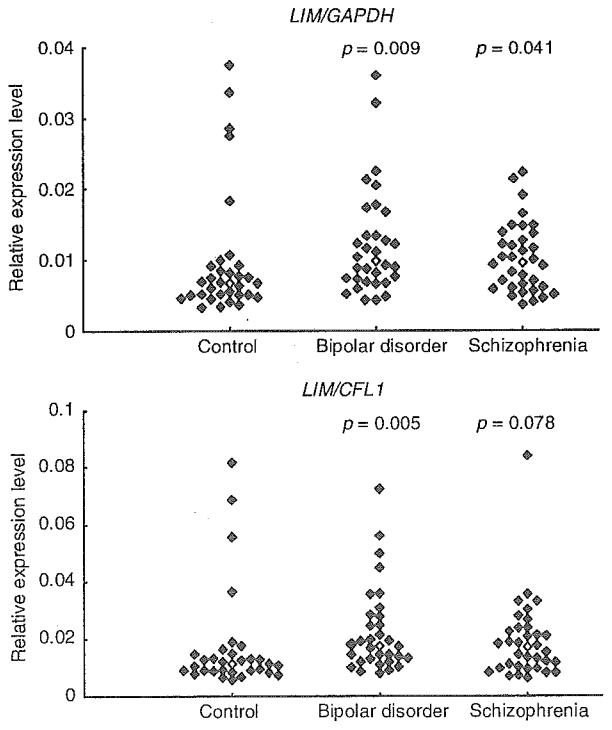

Figure 1 Increased expression levels of *LIM* (*PDLIM5*) in the postmortem brain samples of bipolar disorder and schizophrenia. Each closed diamond represents each subject. Open diamonds indicate the average of each group. In (b), a control subject with extremely high value of *LIM/CFN1* (0.23) is not shown.

Table 1 Effects of medication and suicide status on the expression levels of *LIM*

	Drug	*N*	*Mean*	*SD*	*P-value*
Valproate					
LIM/GAPDH	−	85	0.0103	0.0075	0.206
	+	16	0.0128	0.0057	
LIM/CFN1	−	85	0.0217	0.0287	0.896
	+	16	0.0227	0.0103	
Antidepressants					
LIM/GAPDH	−	74	0.0107	0.0079	0.855
	+	27	0.0104	0.0049	
LIM/CFN1	−	74	0.0227	0.0305	0.578
	+	27	0.0194	0.0103	
Lithium					
LIM/GAPDH	−	90	0.0105	0.0075	0.644
	+	11	0.0116	0.0042	
LIM/CFN1	−	90	0.0220	0.0281	0.861
	+	11	0.0205	0.0083	
Antipsychotics					
LIM/GAPDH	−	51	0.0094	0.0073	0.071
	+	50	0.0120	0.0069	
LIM/CFN1	−	51	0.0210	0.0340	0.737
	+	50	0.0228	0.0162	
Suicide					
LIM/GAPDH	−	80	0.0108	0.0076	0.684
	+	21	0.0101	0.0056	
LIM/CFN1	−	80	0.0226	0.0293	0.592
	+	21	0.0191	0.0121	

coefficient (r^2) calculated by the COCAPHASE program.[31]

Assessment of sample stratification

For population homogeneity assessment, a total of 20 single nucleotide polymorphisms (SNPs) were genotyped for all participants in this study, except for recently recruited 'sample Set C ($N=196$ each for schizophrenia and controls)'. *STRUCTURE* software[32] (http://pritch.bsd.uchicago.edu/software.html) was used to identify genetically similar diploid subpopulations by grouping individuals. In the application of this Markov chain Monte Carlo method, 1 000 000 replications were used for the burn-in period of the chain and for parameter estimation. The number of populations present in the sample (K) was unknown, so analysis was run at $K=1$, 2, 3, 4, and 5. From these results, best estimate of K was found by calculating posterior probabilities, Pr ($K=1$, 2, 3, 4, or 5), as described by Pritchard *et al.*[32] No evidence for stratification was identified in our samples, with a Pr ($K=1$) >0.99.

Results

Gene expression analysis

Patients with bipolar disorder ($P<0.01$) and schizophrenia ($P<0.05$) showed significantly higher expression levels of *LIM* normalized by *GAPDH* in the postmortem cortex (Figure 1). This difference was also confirmed using the normalization by *CFN1* (bipolar disorder, $P<0.01$, schizophrenia, $P=0.07$, respectively). There is a critical pH threshold in these

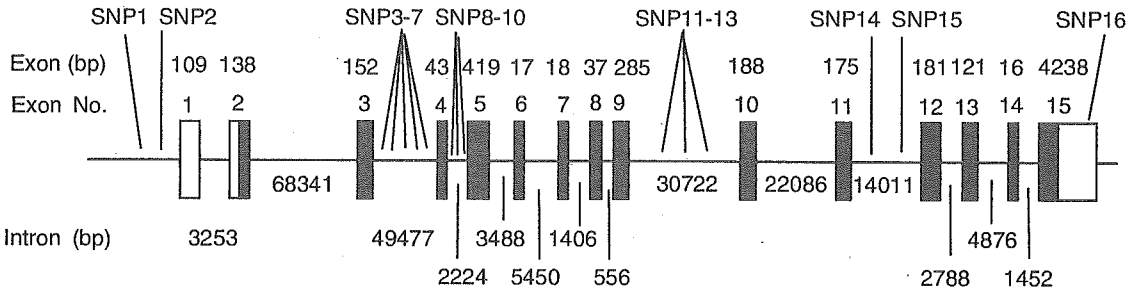

Figure 2 Genomic structure and the location of single nucleotide polymorphisms of *LIM* (*PDLIM5*) gene.

Table 2 Intermarker linkage disequilibrium (LD) patterns in Japanese controls

D'

	SNP1 rs10008257	SNP2 rs2433320	SNP3 rs2433327	SNP4 rs2438146	SNP5 rs2438140	SNP6 rs2452583	SNP7 rs2433324	SNP8 rs2452574	SNP9 rs2452578	SNP10 rs902981	SNP11 rs4634230	SNP12 rs12510147	SNP13 rs6854173	SNP14 rs12641023	SNP15 rs951613	SNP16 rs14082
r2 SNP1 rs10008257		0.776	0.687	0.376	0.205	0.153	0.055	0.321	0.299	0.283	0.31	0.316	0.298	0.207	0.211	0.208
SNP2 rs2433320	0.021		1	1	1	1	0.805	0.659	0.348					0.41	0.402	0.455
SNP3 rs2433327	0.039	0.687		1	1	0.869	0.674	0.621	0.4	0.4	0.4	0.4	0.475	0.308	0.293	0.322
SNP4 rs2438146	0.016	1	0.649		1	1	0.685	0.926		0.824				0.377	0.373	0.421
SNP5 rs2438140	0.011	0.444	0.664	0.451		0.946	0.441				0.419	0.419	0.452	0.181	0.174	0.175
SNP6 rs2452583	0.007	0.395	0.494	0.403	0.806		0.681	0.811	0.641			0.75		0.103	0.094	0.092
SNP7 rs2433324	0.002	0.442	0.341	0.462	0.461	0.532		1	0.876	0.864	0.884	0.81	0.844	0.151	0.132	0.156
SNP8 rs2452574	0.086	0.103	0.09	0.118	0.163	0.22	0.222		0.953	0.831	0.89	0.935		0.197	0.201	0.203
SNP9 rs2452578	0.048	0.129	0.099	0.134	0.098	0.154	0.245	0.58		1	0.935	1	0.921	0.242	0.245	0.232
SNP10 rs902981	0.067	0.101	0.057	0.133	0.121	0.172	0.173	0.697	0.626		1	1	0.941	0.279	0.281	0.279
SNP11 rs4634230	0.077	0.086	0.051	0.096	0.117	0.17	0.179	0.669	0.588	0.953		1	0.955	0.26	0.267	0.257
SNP12 rs12510147	0.051	0.098	0.081	0.102	0.088	0.137	0.252	0.53	0.905	0.603	0.632		1	0.176	0.189	0.17
SNP13 rs6854173	0.068	0.067	0.051	0.077	0.093	0.134	0.173	0.59	0.587	0.829	0.882	0.635		0.318	0.32	0.297
SNP14 rs12641023	0.018	0.05	0.041	0.041	0.021	0.007	0.011	0.037	0.036	0.073	0.061	0.017	0.087		1	1
SNP15 rs951613	0.018	0.048	0.037	0.04	0.019	0.006	0.008	0.038	0.037	0.073	0.063	0.02	0.088	1		1
SNP16 rs14082	0.019	0.059	0.043	0.05	0.018	0.006	0.011	0.041	0.035	0.077	0.062	0.017	0.081	0.939	0.939	

1>=D'>0.8
0.8>=D'>0.6
0.6>=D'>0.4

Table 3 Association of SNPs in *LIM* with bipolar disorder (MDMD sample)

MDMD sample (BP)		*HWE*	*n*	*Allele*		*P-value**	*Genotype*			*P-value**	*Frequency*
				A	G		A/A	A/G	G/G		
SNP1	BP	0.3736	127	116	138		24	68	35		0.4567
rs10008257	CT	0.5661	130	84	176	**0.0021**	15	54	61	**0.0048**	0.3231
				A	G		A/A	A/G	G/G		
SNP2	BP	0.6976	128	36	220		2	32	94		0.1406
rs2433320	CT	0.0958	129	33	225	0.6994	0	33	96	0.5953	0.1279
				T	C		T/T	I/C	C/C		
SNP3	BP	0.3424	126	205	47		85	35	6		0.1865
rs2433327	CT	0.0660	130	207	53	0.6564	79	49	2	0.1214	0.2038
				T	C		T/T	T/C	C/C		
SNP4	BP	0.7688	128	35	221		2	31	95		0.1367
rs2438146	CT	0.0974	130	33	227	0.7952	0	33	97	0.5942	0.1269
				T	C		T/T	I/C	C/C		
SNP5	BP	0.8914	125	58	192		7	44	74		0.2320
rs2438140	CT	0.6031	130	69	191	0.4133	8	53	69	0.6170	0.2654
				A	G		A/A	A/G	G/G		
SNP6	BP	0.9953	128	75	181		11	53	64		0.2930
rs2452563	CT	0.7341	129	71	187	0.6960	9	53	67	0.8758	0.2752
				A	C		A/A	A/C	C/C		
SNP7	BP	0.6115	128	196	60		74	48	6		0.2344
rs2433324	CT	0.4598	130	213	47	0.1579	86	41	3	0.3226	0.1808
				T	G		T/T	I/G	G/G		
SNP8	BP	0.2880	127	139	115		41	57	29		0.4528
rs2452574	CT	0.4650	130	153	107	0.3735	43	67	20	0.2948	0.4115
				A	G		A/A	A/G	G/G		
SNP9	BP	0.9446	128	138	118		37	64	27		0.5391
rs24 52578	CT	0.9562	130	139	121	0.9299	37	65	28	1.0000	0.5346
				A	G		A/A	A/G	G/G		
SNP10	BP	0.4634	127	117	137		29	59	39		0.4606
rs902981	CT	0.7237	130	114	146	0.6576	24	66	40	0.6480	0.4385
				A	G		A/A	A/G	G/G		
SNP11	BP	0.4491	127	135	119		38	59	30		0.4685
rs4634230	CT	0.6387	130	143	117	0.7234	38	67	25	0.6405	0.4500
				A	G		A/A	A/G	G/G		
SNP12	BP	0.8575	128	112	144		24	64	40		0.4375
rs12510147	CT	0.4476	130	112	148	0.9293	22	68	40	0.9033	0.4308
				A	G		A/A	A/G	G/G		
SNP13	BP	0.2922	125	138	112		41	56	28		0.4480
rs6854173	CT	0.8438	129	139	119	0.7896	38	63	28	0.7897	0.4612
				A	G		A/A	A/G	G/G		
SNP14	BP	0.1297	127	103	151		25	53	49		0.4055
rs12641023	CT	0.1252	129	102	156	0.8570	16	70	43	0.0989	0.3953
				T	C		T/T	T/C	C/C		
SNP15	BP	0.1027	128	105	151		26	53	49		0.4102
rs951613	CT	0.1636	129	101	157	0.7189	16	69	44	0.0944	0.3915
				A	G		A/A	A/G	G/G		
SNP16	BP	0.4590	127	108	146		25	58	44		0.4252
rs14082	CT	0.0581	130	105	155	0.6547	16	73	41	0.1574	0.4038

**P*-values are calculated by Fisher's exact test.
Bold values indicate statistically significant results.

Table 4 Association of SNPs in *LIM* with bipolar disorder (MPS samples)

MPS sample (BP)		*HWE*	*n*	*Allele*		*P-value**	*Genotype*			*P-value**	*Frequency*
				A	*G*		*A/A*	*A/G*	*G/G*		
SNP1	BP	0.8970	238	189	287		38	113	87		0.3971
rs10008257	CT	0.7163	237	183	291	0.7400	34	115	88	0.8995	0.3861
				A	G		A/A	A/G	G/G		
SNP2	BP	0.1322	239	58	420		6	46	187		0.1213
rs2433320	CT	0.5375	239	84	394	**0.0227**	6	72	161	**0.0198**	0.1757
				T	C		T7T	T/C	C/G		
SNP3	BP	0.0931	239	390	88		163	64	12		0.1841
rs2433327	CT	0.4510	238	369	107	0.1274	141	87	10	0.0692	0.2248
				T	C		T/T	T/C	C/C		
SNP4	BP	0.3856	236	58	414		5	48	183		0.1229
rs2438146	CT	0.6472	240	82	398	**0.0438**	6	70	164	0.0676	0.1708
				T	C		T/T	I/C	C/C		
SNP5	BP	0.2800	238	119	357		18	83	137		0.2500
rs2438140	CT	0.5346	240	142	338	0.1273	19	104	117	0.1351	0.2958
				A	G		A/A	A/G	G/G		
SNP6	BP	0.3733	235	131	339		21	89	125		0.2787
rs2452563	CT	0.4785	240	153	327	0.1791	22	109	109	0.2147	0.3188
				A	C		A/A	A/C	C/C		
SNP7	BP	0.4042	240	378	102		151	76	13		0.2125
rs2433324	CT	0.2328	240	358	122	0.1470	130	98	12	0.1139	0.2542
				T	G		T/T	T/G	G/G		
SNP8	BP	0.7851	237	263	211		74	115	48		0.4451
rs2452574	CT	0.1186	239	275	203	0.5563	85	105	49	0.5400	0.4247
				A	G		A/A	A/G	G/G		
SNP9	BP	0.0261	239	252	226		75	102	62		0.5272
rs2452578	CT	0.3695	240	245	235	0.6057	66	113	61	0.5706	0.5104
				A	G		A/A	A/G	G/G		
SNP10	BP	0.6862	239	213	265		49	115	75		0.4456
rs902981	CT	0.0939	240	207	273	0.6961	51	105	84	0.5990	0.4313
				A	G		A/A	A/G	G/G		
SNP11	BP	0.6294	240	265	215		75	115	50		0.4479
rs4634230	CT	0.0826	240	269	211	0.8455	82	105	53	0.6524	0.4396
				A	G		A/A	A/G	G/G		
SNP12	BP	**0.0147**	239	220	258		60	100	79		0.4603
rs12510147	CT	0.8353	240	223	257	0.8971	51	121	68	0.1728	0.4646
				A	G		A/A	A/G	G/G		
SNP13	BP	0.8049	236	259	213		72	115	49		0.4513
rs6854173	CT	0.1356	238	266	210	0.7939	80	106	52	0.6525	0.4412
				A	G		A/A	A/G	G/G		
SNP14	BP	0.3156	237	207	267		49	109	79		0.4367
rs12641023	CT	0.6105	240	203	277	0.6949	41	121	78	0.5245	0.4229
				T	C		T/T	T/C	C/C		
SNP15	BP	0.0880	238	199	277		48	103	87		0.4181
rs951613	CT	0.6557	239	202	276	0.8958	41	120	78	0.3171	0.4226
				A	G		A/A	A/G	G/G		
SNP16	BP	**0.0388**	239	210	268		54	102	83		0.4393
rs14082	CT	0.7510	240	206	274	0.7943	43	120	77	0.2378	0.4292

**P*-values are calculated by Fisher's exact test.
Bold values indicate statistically significant results.

samples.[33] When only the samples with pH of 6.4 or more were used for the analysis, patients with bipolar disorder and schizophrenia still showed higher levels of *LIM/GAPDH* (bipolar disorder, $P<0.006$, schizophrenia, $P=0.050$) and *LIM/CFN1* (bipolar disorder, $P<0003$, schizophrenia, $P=0.081$).

To examine the effects of medication on the expression levels of *LIM*, we performed exploratory *t*-test between subjects with or without a particular class of drugs, antipsychotics, antidepressants, lithium, and valproate. However, none of them were significantly associated with mRNA expression of *LIM* (Table 1). There was no significant difference of *LIM* expression levels between suicide cases and nonsuicide cases (Table 1).

Genetic association study

We genotyped 16 SNP markers surrounding *LIM* gene (Figure 2). The markers covering the genomic region and having higher heterozygocity were selected to enhance the information content. Minor allele frequency was between 0.23 and 0.50. Linkage disequilibrium was assessed by the D' and r^2 in the control subjects. Although the entire gene is within weak linkage disequilibrium, there seems to be several haplotype blocks in this region (Table 2).

All SNP markers were within the Hardy–Weinberg Equilibrium (HWE) in the MDMD samples. Allele and genotype frequencies of the SNP1 (rs10008257) were significantly different between bipolar patients and controls (allele, $P=0.0021$; genotype, $P=0.0048$, by Fisher's exact probability test) (Table 3). The difference of allele and genotype frequencies was statistically significant or close to significant even after the Bonferroni correction ($P=0.03$ and 0.07 after Bonferroni correction of 16 SNP markers).

To examine whether or not this is a false-positive finding, we further genotyped an independent sample set (MPS samples). Most of SNP markers were within HWE, except for three SNP markers (SNPs 9, 12, and 16) showing some deviation from HWE only in bipolar disorder subjects. Allele and genotype frequencies of SNP2 (rs2433320), close to SNP1, were significantly different between bipolar disorder patients and control subjects (allele, $P=0.02$; genotype, $P=0.01$). The other marker, SNP4 (rs2438146), was also associated with bipolar disorder (allele $P=0.04$, genotype, $P=0.06$) (Table 4).

Since the analyses in two independent sample sets showed the association in the upstream region of *LIM*, we further performed haplotype analysis (Table 5). Haplotype of two SNPs (SNPs 1 and 2) showed significant association with bipolar disorder ($P=0.03$, global *P*-value by permutation test). Haplotype of three SNPs (SNPs 1, 2, and 3) also showed a tendency of association with bipolar disorder ($P=0.06$).

Quantitative genomic PCR

Recent studies suggested that copy number polymorphisms could be observed in many genes.[34] In the analysis of bipolar disorder, genotype frequency was deviated from HWE only in bipolar disorder in MPS samples. This was mainly derived from a higher number of homozygotes than expected. This could implicate that copy number polymorphism or deletion of this gene might be associated with bipolar disorder. To further explore this possibility, we performed quantitative genomic PCR analysis to measure the copy number of this genomic region. In all, 22 patients homozygous for the SNP15 (rs951613) (C/C, $n=5$; T/T, $n=17$) and six patients heterozygous for this SNP (C/T, $n=6$) were examined.

While copy number difference of *PF2K* on X chromosome between females and males was clearly

Table 5 Association of *LIM* haplotype with bipolar disorder (total)

		2SNPs			3SNPs			
SNP1	rs10008257	0.0359			0.0645			
SNP2	rs2433320		0.2157			0.2523		
SNP3	rs2433327			0.2760			0.3643	
SNP4	rs2438146	0.1955						0.3401
SNP5	rs2438140		0.2239		0.3222			
SNP6	rs2452563			0.5116		0.1803		
SNP7	rs2433324	0.0916					0.1488	
SNP8	rs2452574		0.3219					0.5976
SNP9	rs2452578			0.5255	0.6055			
SNP10	rs902981	0.4650				0.5641		
SNP11	rs4634230		0.6942				0.8982	
SNP12	rs12510147			0.8782				0.9802
SNP13	rs6854173	0.9799			0.9897			
SNP14	rs12641023		0.7014			0.2313		
SNP15	rs951613			0.2083				
SNP16	rs14082							

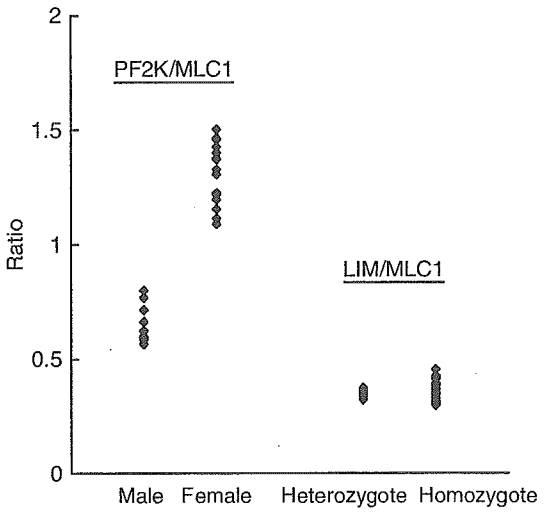

Figure 3 Quantitative genomic PCR of *LIM*. *PF2K* on X chromosome were also measured to verify the experimental procedures. *PF2K/MLC1* clearly differentiate males and females. There is no difference of *LIM/MLC1* between homozygotes and heterozygotes, suggesting that there is no copy number polymorphism or deletion of this gene.