Table 2. Rare exonic mutations identified during the resequencing stage.

Genomic Position [a]	Exon	Base Pair Change [b]	AA Change [c]	Frequency	dbSNP Reference	1000 Genomes	ESP Variant Server
20:3016327	Exon 25	171999G>GA	673V>VI	1/382	rs61742029	Registered	Registered
20:2945609	Exon 9	107281T>TC	59L>LP	2/382	Not Registered	Not Registered	Not Registered
20:3018948	3′UTR	174620_174623 het_dupTGAT	—	1/382	Not Registered	Not Registered	Not Registered
20:3019013	3′UTR	174685A>AT	—	2/382	Not Registered	Not Registered	Not Registered
20:2945649	Exon 9	124753A>AG	Synonymous	4/382	rs138210276	Registered	Registered
20:3005207	Exon 21	160879G>GA	Synonymous	1/382	rs150908061	Registered	Registered
20:3017902	Exon 27	173574G>GT	Synonymous	2/382	rs375917163	Not Registered	Not Registered
20:3017903	Exon 27	173575C>CA	Synonymous	2/382	Not Registered	Not Registered	Not Registered

Notes:
[a]: Based on NCBI build 37.1.
[b]: Based on NCBI Reference Sequence NC_000020.10.
[c]: Based on NCBI Reference Sequence NP_001099043. AA: amino acid.
All mutations are heterozygous.
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controls using Fisher's exact test (one-tail), with a threshold of significance set at $p<0.05$.

Results

Mutation Screening Step

Eight rare mutations consisting of 2 missense SNPs, 4 synonymous SNPs and 2 variations located in the 3′UTR area were identified within the target exons (Table 2), 4 of which were not previously reported in dbSNP Build 139 (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1000 Genomes Project (http://www.1000genomes.org), or the NHLBI Exome Sequencing Project (ESP) Variant Server (http://evs.gs.washington.edu/EVS/). All detected mutations were heterozygous.

Association Analysis

Two missense mutations, rs61742029, which had been previously observed only in the Han Chinese population, L59P, a novel variant, as well as the 174620_174623dupTGAT mutation were validated for association with SCZ and/or ASD in stage 2 (Table 3). Although we were unable to detect significance with our sample sets, it is worth noting that L59P was only present in the SCZ patient group.

Evolutionary Conservation Analysis

Conservation status of rs61742029 and L59P in 11 common species was investigated using Mutation Taster (http://www.mutationtaster.org/). Results showed that the amino acids corresponding to the mutations in RPTP-α were highly conserved among different species (Table 4).

In Silico Functional Effects Prediction

Possible functional implications brought by amino acid changes due to the 2 missense mutations were analyzed with PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), PMut (http://www.ngrl.org.uk/Manchester/page/pmut) and SIFT (http://sift.jcvi.org/). (Table 5) According to the results, the mutation L59P, which was only observed in schizophrenia patients, was predicted to be mostly benign, while rs61742029 showed a high probability of pathogenicity in PolyPhen-2.

3′UTR Motif Prediction

174620_174623dupTGAT, a small duplication discovered in the 3′UTR area, was predicted by RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw) to be located within a human Musashi Binding Element (MBE), an evolutionarily conserved region shown to affect neural cell differentiation through its mRNA translation regulator properties [38].

Clinical Information of the Carriers of Mutation L59P and 174620_174623dupTGAT

The patient carrying the *PTPRA* L59P mutation was a male diagnosed with SCZ at the age of 19. The patient was born in 1947 had a normal course of development during childhood. In early 1966, he started to suffer from auditory hallucinations, and soon withdrew into an indoor lifestyle. His family reported him being irritated when visited, as well as behaving improperly in public. He was promptly diagnosed and admitted to a psychiatry ward in the same year, and spent the rest of his life living in a hospital. A remarkable improvement was observed in his positive symptoms after admission and administration of antipsychotic drugs; however, he remained secluded, hardly communicating with people around him. At the time of his enrollment in the study, he was 162 cm tall

Table 3. Association analysis results of two rare missense mutations and one 3′UTR variant.

Mutation	Genotype Counts (Resequencing) [a]	Genotype Counts (Association)			P Value [b]	
		SZ	ASD	Ctrl	SZ	ASD
171999G>GA, 673V>VI	0/3/379	0/2/942	0/2/334	0/4/908	0.3276	0.2829
101281T>TC, 59L>L/P	0/2/380	0/0/944	0/0/336	0/0/912	1.0000	1.0000
174620_174623het_dupTGAT	0/1/381	0/0/944	0/0/336	0/1/911	0.4914	1.0000

Notes:
[a]: Homozygote of minor allele/heterozygote/homozygote of major allele.
[b]: Calculated using Fisher's exact test, one-tailed.
Ctrl: healthy controls.
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and weighed 48 kg. No comorbid physical or mental illnesses were present. He had 3 children, among whom, one daughter had a history of mental disorder. The patient succumbed to pneumonia in the second half of 2013. In a computerized axial tomography (CAT) scan of the head taken a few weeks prior to patient's death, diffuse neocortical atrophy was observed.

The other patient carrying the L59P mutation was a female diagnosed with SCZ at the age of 34. No childhood development abnormalities were reported, but she was noted to have a history of irritability/aggressive tendencies in high school. Since onset, she had experienced auditory hallucinations and persecutory delusions, as well as continued irritability and aggression. Despite the efficacy of antipsychotic drugs on her positive symptoms, the patient suffered numerous relapses throughout her course of illness due to poor insight and lack of adherence to treatment. At the time of recruitment, she was 61 years old, with a chronic condition of diabetes and no comorbid mental conditions. She died in 2012 at the age of 62.

The patient carrying the *PTPRA* 174620_174623dupTGAT mutation was a male diagnosed with SCZ and comorbid intellectual disability at the age of 27, while he was enrolled in our study. He had a normal conception and birth, born to a 28-year-old father and 27-year-old mother. His father died when he was 3. Delayed intellectual development was observed since his childhood, with reports of illiteracy, hyperactivity, poor concentration and low performance at school. He subsequently dropped out of high school in his first year and started attending a technical school. After graduation, not being able maintain a steady position, he changed part-time jobs frequently. He presented at onset with hallucinations, persecutory delusions, and psychomotor excitement, and was subjected to involuntary commitment due to harmful behavior to others as a result of his delusions. At the time of admission, he was 168 cm tall and weighed 74 kg, with a Wechsler Adult Intelligence Scale (WAIS) score of 49 (Verbal IQ = 57, Performance IQ = 48); he also suffered from stuttering (anarthria literalis). After remission under antipsychotic treatment, he was discharged; however, lack of insight or compliance persisted. It was reported that his mother had a history of panic attacks, and one of his maternal relatives was also diagnosed with SCZ.

Discussion

To our knowledge, this is the first study that systematically screened all coding regions and 3′UTR of the *PTPRA* gene for rare variants in SCZ patients and assessed the association of identified mutations in such a study with SCZ/ASD.

Main Findings

In this study, we sequenced the encoding regions, splicing sites, and 3′UTR region of the *PTPRA* gene in 382 SCZ patients using the Sanger sequencing method, and discovered 8 rare variants. We then conducted association analysis in a much larger sample set for the 2 rare, missense mutations and one 3′UTR InDel identified during the mutation-screening phase in order to investigate their relationship with SCZ and/or ASD.

We were unable to detect a statistically significant association for any of the 3 mutations; this may be attributed partially to the low frequency of rare mutations in the population. However, according to our estimation using CaTS, the power calculator for two-stage association studies (http://www.sph.umich.edu/csg/abecasis/CaTS/), it would require a sample size of around 25,000 cases and controls for the study to obtain possible significance [39,40]. Also, L59P was only detected among SCZ patients in our sample, which infers possible connection of this mutation to the disorder. The evolutionary conservation status of the locus also indicates its biological importance.

Recent studies have discussed the limited impact of protein-coding variants detected in exome resequencing projects, attributing it partly to the fact that most associated variants alter gene expression rather than protein structure. These findings may help explain the lack of association for the 2 missense mutations we detected, while hinting that 174620_174623dupTGAT, predicted to be located within an expression-regulating element, may have a more significant effect. [10]

Additionally, an increasing amount of evidence suggests that genetic risks for SCZ and ASD may not be conferred by the effects of individual variants alone, but also the amplifying interactions between multiple susceptibility loci [41–44]. Thus it may be interesting to sequence the mutation carriers for additional related variants in future.

Limitations

Several limitations should be considered when interpreting the results of our study. The single candidate gene paradigm for a gene with less than robust ties to schizophrenia may have been one of the reasons leading to negative results. Besides, the Sanger method it employed predetermined its relatively small sample size and detection power in contrast with next generation resequencing. In addition, we did not have lymphoblastoid cell lines (LCLs) from the mutation carriers for expression analysis or blood samples from their family members for pedigree study. Therefore, we were unable to follow up the results with further biological evidence. Moreover, some potentially interesting regions of the *PTPRA* gene, such as the promoter, 5′UTR, and most of the intronic

Table 4. Evolutionary conservation information for rs61742029 and L59P

Mutation	Species	Match	Gene	AA	Alignment
L59P	Human	—	ENST00000380393	59	K T S N P T S S L T S *L S V A P T F S P N I T L
	Mutant	Not conserved	—	59	K T S N P T S S L T S *P S V A P T F S P N I T
	P. Troglodytes	All identical	ENSPTRG00000033879	59	K T S N P T S S L T S *L S V A P T F S P N I T
	M. Mulatta	All identical	ENSMMUG00000005878	59	K T S N P T S S L T S *L S V A P T F S P N I T
	F. Catus	All identical	ENSFCAG00000019232	59	K T S S P A S S V T S *L S V A P T F S P N L T
	M. Musculus	All identical	ENSMUSG00000027303	59	K T S N S T S S V I S *L S V A P T F S P N L T
	G. Gallus	All identical	ENSGALG00000015995	56	*L N V S - - - S P M T T
	T. Rubripes	All identical	ENSTRUG00000014770	99	P T P S P A S D G T L *L Q A D P N A T G R V L
	D.rerio	Not conserved	ENSDARG00000001769	101	P P V V P P P A V P I *P T V V L P V P P T P T
	D. Melanogaster	No homologue	—	N/A	
	C. Elegans	No alignment	C09D8.1	N/A	
	X. Tropicalis	All conserved	ENSXETG00000017982	71	T T A PF T T T R T A *V I L A P N V T D S I F
rs61742029	Human			664	L K K E E E C E S Y T *V R D L L V T N T R E N
	mutated	all conserved		664	S Y T *L R D L L V T N T R E N
	Ptroglodytes	all identical	ENSPTRG00000033879	673	L K K E E E C E S Y T *V R D L L V T N T R E N
	Mmulatta	all identical	ENSMMUG00000005878	673	L K K E E E C E S Y T *V R D L L V T N T R E N
	Fcatus	all identical	ENSFCAG00000019232	674	L K K E E E C E S Y T *V R D L L V T N T R E N
	Mmusculus	all identical	ENSMUSG00000027303	700	L K K E E E C E S Y T *V R D L L V T N T R E N
	Ggallus	all identical	ENSGALG00000015995	680	L K K E E E C E S Y T *V R D L L V T N T R E N
	Trubripes	all identical	ENSTRUG00000014770	710	Y T *V R D L L V T N N R E N

*Marks the position of the amino acid change due to mutation.

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Table 5. In silico functional effect prediction for rs61742029 and L59P.

Mutation	Prediction Tool		
	PolyPhen-2	Pmut	SIFT
rs61742029	Probably damaging	Neutral	Tolerated
L59P	Benign	Neutral	Tolerated

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areas, were not sequenced (the rare intronic mutations we detected close to the exons can be viewed in Table S1).

Conclusion

In conclusion, our study did not detect any rare missense mutations within the *PTPRA* gene in our samples that showed statistical association with SCZ or ASD. Nonetheless, some potentially interesting variants were identified that might increase the susceptibility of their carriers to the disorders. Also, our results may help provide genetic clues for the involvement of the *PTPRA* gene in the pathogenesis of psychiatric disorders.

Supporting Information

Table S1 Rare intronic mutations identified during the resequencing stage. [a]: Based on NCBI build 37.1. [b]: Based on NCBI Reference Sequence NC_000020.10. All mutations are heterozygous.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JX BA MI NI NO. Performed the experiments: JX CW HK YT. Analyzed the data: JX SK AY YN TK IK BA NO. Contributed reagents/materials/analysis tools: JX YU TO BA MI NI NO. Wrote the paper: JX SK AY YN TK MB IK YU TO BA NO.

References

1. Sullivan PF, Kendler KS, Neale MC (2003) Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. Arch Gen Psychiatry 60: 1187–1192.
2. Stefansson H, Ophoff RA, Steinberg S, Andreassen OA, Cichon S, et al. (2009) Common variants conferring risk of schizophrenia. Nature 460: 744–747.
3. International Schizophrenia C, Purcell SM, Wray NR, Stone JL, Visscher PM, et al. (2009) Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. Nature 460: 748–752.
4. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, et al. (2009) Finding the missing heritability of complex diseases. Nature 461: 747–753.
5. Owen MJ, Craddock N, O'Donovan MC (2010) Suggestion of roles for both common and rare risk variants in genome-wide studies of schizophrenia. Arch Gen Psychiatry 67: 667–673.
6. Association AP (2013) Diagnostic and Statistical Manual of Mental Disorders (Fifth ed.): Arlington, VA: American Psychiatric Publishing.
7. Crespi BJ, Crofts HJ (2012) Association testing of copy number variants in schizophrenia and autism spectrum disorders. J Neurodev Disord 4: 15.
8. Sullivan PF, Daly MJ, O'Donovan M (2012) Genetic architectures of psychiatric disorders: the emerging picture and its implications. Nat Rev Genet 13: 537–551.
9. Ku CS, Polychronakos C, Tan EK, Naidoo N, Pawitan Y, et al. (2013) A new paradigm emerges from the study of de novo mutations in the context of neurodevelopmental disease. Mol Psychiatry 18: 141–153.
10. Schizophrenia Working Group of the Psychiatric Genomics C (2014) Biological insights from 108 schizophrenia-associated genetic loci. Nature 511: 421–427.
11. Bodrikov V, Leshchyns'ka I, Sytnyk V, Overvoorde J, den Hertog J, et al. (2005) RPTPalpha is essential for NCAM-mediated p59fyn activation and neurite elongation. J Cell Biol 168: 127–139.
12. Bodrikov V, Sytnyk V, Leshchyns'ka I, den Hertog J, Schachner M (2008) NCAM induces CaMKIIalpha-mediated RPTPalpha phosphorylation to enhance its catalytic activity and neurite outgrowth. J Cell Biol 182: 1185–1200.
13. Ye H, Tan YL, Ponniah S, Takeda Y, Wang SQ, et al. (2008) Neural recognition molecules CHL1 and NB-3 regulate apical dendrite orientation in the neocortex via PTP alpha. EMBO J 27: 188–200.
14. Wang PS, Wang J, Xiao ZC, Pallen CJ (2009) Protein-tyrosine phosphatase alpha acts as an upstream regulator of Fyn signaling to promote oligodendrocyte differentiation and myelination. J Biol Chem 284: 33692–33702.
15. Fischbach GD (2007) NRG1 and synaptic function in the CNS. Neuron 54: 495–497.
16. Fazzari P, Paternain AV, Valiente M, Pla R, Lujan R, et al. (2010) Control of cortical GABA circuitry development by Nrg1 and ErbB4 signalling. Nature 464: 1376–U1311.
17. Wen L, Lu YS, Zhu XH, Li XM, Woo RS, et al. (2010) Neuregulin 1 regulates pyramidal neuron activity via ErbB4 in parvalbumin-positive interneurons. Proceedings of the National Academy of Sciences of the United States of America 107: 1211–1216.
18. Li B, Woo RS, Mei L, Malinow R (2007) The neuregulin-1 receptor ErbB4 controls Glutamatergic synapse maturation and plasticity. Neuron 54: 583–597.
19. Buxbaum JD, Georgieva L, Young JJ, Plescia C, Kajiwara Y, et al. (2008) Molecular dissection of NRG1-ERBB4 signaling implicates PTPRZ1 as a potential schizophrenia susceptibility gene. Molecular Psychiatry 13: 162–172.
20. Buonanno A (2010) The neuregulin signaling pathway and schizophrenia: from genes to synapses and neural circuits. Brain Res Bull 83: 122–131.
21. Mei L, Xiong WC (2008) Neuregulin 1 in neural development, synaptic plasticity and schizophrenia. Nature Reviews Neuroscience 9: 437–452.
22. Silberberg G, Darvasi A, Pinkas-Kramarski R, Navon R (2006) The involvement of ErbB4 with schizophrenia: Association and expression studies. American Journal of Medical Genetics Part B-Neuropsychiatric Genetics 141B: 142–148.
23. Fanous AH, Neale MC, Webb BT, Straub RE, O'Neill FA, et al. (2008) Novel linkage to chromosome 20p using latent classes of psychotic illness in 270 Irish high-density families. Biological Psychiatry 64: 121–127.
24. Teltsh O, Kanyas K, Karni O, Levi A, Korner M, et al. (2008) Genome-wide linkage scan, fine mapping, and haplotype analysis in a large, inbred, Arab Israeli pedigree suggest a schizophrenia susceptibility locus on chromosome 20p13. American Journal of Medical Genetics Part B-Neuropsychiatric Genetics 147B: 209–215.
25. Ikeda M, Aleksic B, Kinoshita Y, Okochi T, Kawashima K, et al. (2011) Genome-wide association study of schizophrenia in a Japanese population. Biol Psychiatry 69: 472–478.
26. Takahashi N, Nielsen KS, Aleksic B, Petersen S, Ikeda M, et al. (2011) Loss of function studies in mice and genetic association link receptor protein tyrosine phosphatase alpha to schizophrenia. Biol Psychiatry 70: 626–635.
27. Firth HVea (2009) Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources. Am J Hum Genet.
28. Hakak Y, Walker JR, Li C, Wong WH, Davis KL, et al. (2001) Genome-wide expression analysis reveals dysregulation of myelination-related genes in chronic schizophrenia. Proc Natl Acad Sci U S A 98: 4746–4751.
29. Hoistad M, Segal D, Takahashi N, Sakurai T, Buxbaum JD, et al. (2009) Linking white and grey matter in schizophrenia: oligodendrocyte and neuron pathology in the prefrontal cortex. Front Neuroanat 3: 9.
30. Mistry M, Gillis J, Pavlidis P (2013) Meta-analysis of gene coexpression networks in the post-mortem prefrontal cortex of patients with schizophrenia and unaffected controls. BMC Neurosci 14: 105.
31. Martins-de-Souza D (2010) Proteome and transcriptome analysis suggests oligodendrocyte dysfunction in schizophrenia. J Psychiatr Res 44: 149–156.
32. Takahashi N, Sakurai T, Davis KL, Buxbaum JD (2011) Linking oligodendrocyte and myelin dysfunction to neurocircuitry abnormalities in schizophrenia. Prog Neurobiol 93: 13–24.

33. Carmody DP, Lewis M (2010) Regional white matter development in children with autism spectrum disorders. Dev Psychobiol 52: 755–763.
34. Ginsberg MR, Rubin RA, Falcone T, Ting AH, Natowicz MR (2012) Brain transcriptional and epigenetic associations with autism. PLoS One 7: e44736.
35. Kleinhans NM, Pauley G, Richards T, Neuhaus E, Martin N, et al. (2012) Age-related abnormalities in white matter microstructure in autism spectrum disorders. Brain Res 1479: 1–16.
36. Roy SW, Gilbert W (2006) The evolution of spliceosomal introns: patterns, puzzles and progress. Nat Rev Genet 7: 211–221.
37. Ward AJ, Cooper TA (2010) The pathobiology of splicing. J Pathol 220: 152–163.
38. Okano H, Imai T, Okabe M (2002) Musashi: a translational regulator of cell fate. J Cell Sci 115: 1355–1359.
39. Hong EP, Park JW (2012) Sample size and statistical power calculation in genetic association studies. Genomics Inform 10: 117–122.
40. Liu L, Sabo A, Neale BM, Nagaswamy U, Stevens C, et al. (2013) Analysis of rare, exonic variation amongst subjects with autism spectrum disorders and population controls. PLoS Genet 9: e1003443.
41. Vawter MP, Mamdani F, Macciardi F (2011) An integrative functional genomics approach for discovering biomarkers in schizophrenia. Brief Funct Genomics 10: 387–399.
42. Lin Z, Su Y, Zhang C, Xing M, Ding W, et al. (2013) The interaction of BDNF and NTRK2 gene increases the susceptibility of paranoid schizophrenia. PLoS One 8: e74264.
43. Bartlett CW, Flax JF, Fermano Z, Hare A, Hou L, et al. (2012) Gene x gene interaction in shared etiology of autism and specific language impairment. Biol Psychiatry 72: 692–699.
44. Johnson NL, Giarelli E, Lewis C, Rice CE (2013) Genomics and autism spectrum disorder. J Nurs Scholarsh 45: 69–78.

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Identification of Rare, Single-Nucleotide Mutations in NDE1 and Their Contributions to Schizophrenia Susceptibility

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Background: **Nuclear distribution E homolog 1 (NDE1), located within chromosome 16p13.11, plays an essential role in microtubule organization, mitosis, and neuronal migration and has been suggested by several studies of rare copy number variants to be a promising schizophrenia (SCZ) candidate gene. Recently, increasing attention has been paid to rare single-nucleotide variants (SNVs) discovered by deep sequencing of candidate genes, because such SNVs may have large effect sizes and their functional analysis may clarify etiopathology.** *Methods and Results:* **We conducted mutation screening of NDE1 coding exons using 433 SCZ and 145 pervasive developmental disorders samples in order to identify rare single nucleotide variants with a minor allele frequency ≤5%. We then performed genetic association analysis using a large number of unrelated individuals (3554 SCZ, 1041 bipolar disorder [BD], and 4746 controls). Among the discovered novel rare variants, we detected significant associations between SCZ and S214F (P = .039), and between BD and R234C (P = .032). Furthermore, functional assays showed that S214F affected axonal outgrowth and the interaction between NDE1 and YWHAE (14-3-3 epsilon; a neurodevelopmental regulator).** *Conclusions:* **This study strengthens the evidence for association between rare variants within NDE1 and SCZ, and may shed light into the molecular mechanisms underlying this severe psychiatric disorder.**

Key words: YWHAE/SNV/target resequencing/rare variants/DISC1/protein-protein interaction

Introduction

Schizophrenia (SCZ), of which clinical features are characterized by hallucinations, delusions, and cognitive deficits, and cause enormous personal and societal burdens,[1] is a severe psychiatric disorder with a lifetime risk of about 1%. The heritability of SCZ is as high as 80%,[2,3] making this condition a target for human genetics research. Recent studies into the genetic architecture of SCZ have identified both common and rare variants.[4] Several common variants with relatively small odds ratios (ORs) for SCZ have been identified in genome wide association studies (GWASs), and the findings indicate that common variants account for at least a third of the genetic contribution to SCZ risk[3,5] and that genetic risk overlaps with bipolar disorders (BD).[3] Additionally, studies of rare variations of SCZ and autism spectrum disorder (ASD) identified rare copy number variations (CNV) with large effect size,[6] and such variants might be important to understand the etiopathology of SCZ and ASD. Despite their large effect size, the etiopathological roles of CNV remains largely unknown, partially because it remains unclear how the functional change of the genes

within the regions of the CNVs lead to the pathogenesis of neuropsychiatric disorders.

On the other hand, there is growing evidence that rare SNVs, discovered from deep sequencing of candidate genes, may have large effect sizes and account for the missing heritability, as well as contribute to understanding of the pathogenesis of neuropsychiatric disease through functional analysis.[7–10] Intriguingly, recently it has been suggested that some of genetic risk associated with SCZ[11,12] converged on the similar set of genes in terms of their functions. For instance, the SNVs discovered from deep sequencing of ASD[13,14] in the gene encoding the synaptic scaffolding protein SHANK3 (located within the CNVs at 22q13.3 which was associated with ASD),[15] were found to affect dendritic spine morphology via an actin-dependent mechanism.[10] Thus, sequencing the candidate genes from the large scale genome wide analyses may be a promising method for elucidating the pathophysiology of neuropsychiatric disorders such as SCZ and ASD.

There are multiple lines of evidence that deletions and reciprocal duplications at chromosome 16p13.11 are associated with disorders involving abnormal neurodevelopment, such as ASD,[16] epilepsies,[17–19] and SCZ.[20–22] In some cases, microcephaly is a phenotypic manifestation of CNV deletions at 16p13.11.[23,24] Given that the CNVs at 16p13.11 regions contain nuclear distribution E homolog 1 (NDE1), which are closely associated with neurodevelopmental phenotypes, NDE1 is a promising candidate gene. Furthermore, the NDE1 have been associated with SCZ[25] and ASD[26,27] risk by linkage analysis.

NDE1 plays a crucial role in the process of mammalian encephalization and human cerebral cortex growth because of its involvement in mitosis, neuronal migration, and microtubule organization during brain development.[28] In an NDE1-deficient mouse model, in which the mice presented with microcephaly, it was found that NDE1-deficient mice had reduced progenitor cell division and alterations in mitotic spindle formation and in chromosome segregation.[29] NDE1 protein has an N-terminal region with coiled-coil motif known to bind to lissencephaly 1,[30] and a C-terminal region known to harbor several phosphorylation sites involved in subcellular localization, protein-protein interactions and the cell cycle.[31,32] In humans, homozygous loss of function mutations at C-terminal regions have been associated with extreme microlissencephaly,[28,32] suggesting that NDE1 is required for neurogenesis and neuronal migration. NDE1 was also identified as a SCZ susceptibility locus by a genome-wide linkage study on common risk variants of disrupted in schizophrenia 1 (DISC1),[33] which is a scaffold protein indicated in neurodevelopment and synaptic regulation with several interacting proteins and is a suggested risk factor for SCZ, ASD, and BD.[34] NDE1 and NDE-like 1 (NDEL1), which is a paralog of NDE1,[35] are known to directly bind with DISC1.[36,37] Furthermore, in the region of the YWHAE (14-3-3 epsilon), that binds to DISC1 and is involved in neural migration by recognizing the phosphorylation sites of NDEL1,[38] we previously identified a SNP (rs28365859) that showed a significant association with Japanese SCZ.[39]

Considering that NDE1 is essential for neurodevelopment and is included in the SCZ and ASD susceptibility locus at 16p13.11, we hypothesized that mutations in NDE1 might confer susceptibility to SCZ, ASD, and BD pathogenesis. However, there have been no studies focusing only on NDE1 variants by deep sequencing of SCZ and ASD. Therefore, in this study, in order to discover rare variants with large effect size and explore the role in pathogenesis of the discovered rare variants, we performed mutation screening of NDE1 coding exons with SCZ and pervasive developmental disorder (PDD) samples, followed by association analysis and functional analysis of variants with putative large effects. In this study, we found that rare variants of NDE1 may have functional relevance for the pathophysiology of SCZ and BD.

Methods

Samples

Two independent sample groups were used in this study. (1) For mutation screening, we used 433 SCZ (mean age ± SD = 52.3 ± 14.4 years) and 145 PDD (15.6 ± 8.3 years). (2) For genetic association analysis, we used a case control sample set consisting of 3554 SCZ (48.4 ± 14.6 years), 1041 BD (51.0 ± 14.1 years), and 4746 healthy control (HC) subjects (43.2 ± 14.7 years). All subjects were unrelated, living in the mainland of Japan, and self-identified as Japanese. All of the cases were included if they met Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition-Text Revision (TR) criteria for SCZ, BD, and PDD (specifically, Autistic disorder, Asperger's disorder, PDD not otherwise specified). In addition, the patients' capacity to consent was confirmed by a family member when needed. Control subjects were selected from the general population and had no history of mental disorders based on questionnaire responses from the subjects themselves during the sample inclusion step. A general characterization and psychiatric assessment of the subjects is available elsewhere.[40] Written informed consent was obtained from all participants. The Ethics Committees of the Nagoya University Graduate School of Medicine and associated institutes and hospitals approved this study.

Mutation Screening of NDE1 Coding Exons

For the purpose of mutation screening, we designed 8 primer sets (supplementary table S1) to cover the NDE1 coding exons (transcription ID: ENST00000396355 from ensemble database; Chr16: 15,737,124-15,820,210; human reference sequence NCBI (National Center for

Biotechnology Information) build 37.1). Genomic DNA was extracted from peripheral blood using standard methods. Amplicons were generated using standard polymerase chain reaction (PCR) conditions. After PCR amplification, aliquots of PCR products were purified using Illustra Exonuclease I and Alkaline Phosphatase (GE Healthcare and Life Science). These were then sequenced using the Sanger method and a 3130XL Genetic Analyzer (Applied Biosystems). Mutation Surveyor (Softgenetics), which can detect sequencing variations within Sanger sequencing traces, was used to detect mutations in NDE1.[41] We prioritized SNVs for follow-up association analysis as follows. (1) We included rare (minor allele frequency [MAF] ≤ 5%) missense and nonsense SNVs, canonical splicing junction mutations (within 2 bp of intronic sequence at the exon/intron boundaries), and indels. (2) We included only "novel" variants; those that were not registered in dbSNP version 136 (http://www.ncbi.nlm.nih.gov/projects/SNP/). Each rare mutation was reconfirmed using Sanger sequencing. After prioritization, all variants were evaluated in silico for possible structural and functional consequences using the following tools: (1) localization of protein domain and phosphorylation sites was based on the human protein reference database (http://www.hprd.org/index_html) (HPRD); (2) evolutionary conservation was assessed by the HomoloGene database (http://www.ncbi.nlm.nih.gov/homologene/); and (3) analysis of deleterious effects by amino acid substitution was performed by algorithms implemented in Polyphen-2,[42] Sorting Tolerant from Intolerant (SIFT),[43] and PMUT.[44]

Genetic Association Analysis

Genotyping was performed using Taqman (Applied Biosystems) custom probes (details about DNA sequences and PCR conditions are available upon request). Each 384-microtiter plate contained at least 2 non-template controls and the sample(s) in which novel variant was observed. Analysis was performed on an HT7900 instrument (Applied Biosystems) according to the standard protocol. All allele-wise association analyses were carried out by calculating the *P* values for each SNP using Fisher's exact test (one-tail). Differences were considered significant when the *P* value was less than .05. Statistical calculations were done using SPSS v21 (SPSS Inc.) and Plink v1.07.[45] Furthermore, we calculated adjusted *P* value (100 000 permutations) as implemented in Plink v1.07. Calculation of statistical power was performed by the Genetic Power Calculator (http://pngu.mgh.harvard.edu/purcell/gpc/) with the multiplicative model.

Materials and Chemicals

The cDNAs encoding NDE1 and YWHAE/14-3-3ε were isolated from a human fetal brain cDNA library (Clontech). Antibodies against green fluorescent protein (GFP) or glutathione *S*-transferase (GST) were purchased from Nacalai and Sigma, respectively. Other materials and chemicals were obtained from commercial sources.

Plasmid Constructs and Protein Purification

A full-length cDNA encoding human NDE1 (wild type [WT]) (amino acids 1–335) was inserted into pEGFP-C-terminal 1 (C1) (Clontech) and separately into pEFBOS-GST (provided by Dr S. Nagata, Osaka University). Additionally, cDNAs encoding NDE1-S214A (SA), NDE1-S214F (SF), NDE1-T215A (TA), or NDE1-R234C (RC) were generated by site-directed mutagenesis, and were then cloned into pEGFP-C1 and separately into pEFBOS-GST. For protein purification of GST-YWHAE, the cDNA encoding YWHAE was inserted into pGEX-4T1 (GE Healthcare).[46] GST fusion proteins were produced in *Escherichia coli* BL21 (DE3), and purified on glutathione-Sepharose 4B beads according to the manufacturer's instructions (GE Healthcare).

Cell Culture and Transfections

HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) in a 5% CO_2 atmosphere at 37°C. HEK293T cells were transfected with the indicated plasmids using Lipofectamine (Invitrogen Life Technologies). At 36 hours after transfection, the cultured cells were harvested for a GST pull-down assay. For the primary culture of rat hippocampal neurons, all animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of Nagoya University in Japan. Hippocampal neurons were prepared from E16 embryos of WT (C57BL6J) mice as previously described.[47] Briefly, neurons were seeded onto a poly-D-lysine-coated 35-mm glass-based dish and cultured in neurobasal medium (Invitrogen) supplemented with B-27 (Invitrogen) and 1mM L-glutamine.

GST Pull-down Assay

Cells were lysed with the lysis buffer (20mM Tris-HCl, 1mM EDTA, 250mM NaCl, 1mM DTT, 0.1% [wt/vol] Triton X-100, 10μM *p*-amidinophenyl methanesulfonyl fluoride hydrochloride, and 10 μg/ml leupeptin). The lysate was sonicated, and then clarified by centrifugation at 12 000*g* for 10 minutes at 4°C. The soluble supernatant was incubated with the glutathione–Sepharose 4B beads (GE Healthcare) coated with 50 pmol of GST alone or GST-YWHAE. Beads were washed with lysis buffer, and the bound GST-proteins were then eluted with a glutathione-containing buffer (10mM glutathione in Tris-based buffer [pH 7.4]). A portion of each eluate was subjected to SDS-PAGE, followed by immunoblot analyses with the indicated antibodies. We used cell lysates from COS7 that expressed GST-YWHAE or GFP-NDE1 for in vivo GST pull-down assays. The COS7 cell lysates were incubated with glutathione–Sepharose 4B

beads. These beads were washed with lysis buffer, and the bound GST-proteins were then eluted with the glutathione-containing buffer. To quantify the effects of the NDE1 mutations on the interaction with YWHAE, we performed the GST pull-down assay in triplicate. Quantitative densitometric measurement of immunoblots was performed using Image J (http://rsb.info.nih.gov/ij/). The Student's *t* test was used to assess statistical significance.

Neuromorphological Analysis

Hippocampal neurons (6×10^4 cells per well) were seeded onto 24-well plate. At one day in vitro (DIV1), a CalPhos mammalian transfection kit (Clontech) was used to introduce via transfection expression plasmids encoding the indicated cDNA (1.0 μg) into hippocampal neurons. At day three in vitro (DIV3), transfected and control hippocampal neurons were fixed with 3.7% formaldehyde in PBS for 10 minutes and treated with PBS that contained 0.05% Triton X-100 for 10 minutes. Neurons were incubated with anti-GFP and anti-Tau1 antibodies (Sigma) overnight at 4°C, washed, and incubated for 1 hour with secondary antibodies. Immunofluorescent signals were examined with a laser scanning confocal microscope (LSM510; Zeiss). Axon lengths were measured from the cell body to the tip of axonal processes. The Student's *t* test was used to assess statistical significance between groups.

Results

Mutation Screening of NDE1 Coding Exons

To clarify the genetic relationship of NDE1 in the pathology of SCZ and ASD, we performed a mutation screening of NDE1 *coding exons* using the 433 SCZ and 145 ASD samples. We identified 4 rare missense heterozygous mutations within NDE1 coding exons (table 1). No synonymous mutations, nonsense mutations, splicing site mutations, or indels were discovered. In one of the 145 ASD samples, we detected a known rare missense mutation (rs148118152) at exon4 within the N-terminal coiled-coil region. The other 3 rare missense mutations (Q186E, S214F, R234C) were novel and discovered within the 433 SCZ samples (MAF = 0.12%). All of the novel missense mutations were discovered within exon 7, where several phosphorylation sites are located (figure 1A); S214 is also located at a putative phosphorylation site according to the HPRD. S214F was predicted to be deleterious by all of the 3 algorithms (table 1). The result of evolutionary conservation analysis for the novel rare mutations is shown in figure 1D. Case summaries of patients with a novel rare missense mutation are provided in the supplementary results, supplementary tables S2 and S3, and supplementary figures S1 and S2.

Results of Genetic Association Analysis

The results of genetic association analysis of novel missense mutations are shown in table 2. We detected S214F in 6 SCZ cases and 1 HC, and we observed a significant association between SCZ and S214F (OR = 7.1, $P = .039$, adjusted $P = .052$). The clinical feature of 6 SCZ cases with S214F is in supplementary table S4. In addition, we discovered R234C in 1 SCZ case and 2 BD cases, but none in HC, and detected a significant association between BD and R234C ($P = .032$, adjusted $P = .033$). The MAF of Q186E in HC (0.24%) was higher than the MAF in SCZ (0.15%) and BD (0.098%).

Functional Analysis of NDE1 Mutations

Of the 3 novel rare mutations, only S214F and R234C were predicted to be deleterious based on in silico analysis (table 1); they were also the two with a significant association with SCZ or BD (table 2). Notably, even adjusted *P* value (100 000 permutations) showed trend for statistical association. Therefore, we focused on these 2 rare missense mutations for further functional assays.

Table 1. Details of Discovered Rare Missense Mutations and In Silico Analysis

						SCZ (n = 433)		PDD (n = 145)		In Silico Analysis for Amino Acid Substitution		
Chromosome	Physical Position[a]	Exon	Base Change M[b] > m[b]	dbSNP Reference	Variant[c]	MAC[d]	MAF[e]	MAC	MAF	SIFT	Polyphen-2	PMut
16	15761214	4	C > T	Registered	T52M	0	0	1	0.0035	tolerated	benign	pathological
16	15785033	7	C > G	Not registered	Q186E	1	0.0012	0	0	tolerated	benign	neutral
16	15785118	7	C > T	Not registered	S214F	1	0.0012	0	0	damaging	possibly damaging	pathological
16	15785177	7	C > T	Not registered	R234C	1	0.0012	0	0	tolerated	benign	pathological

Note: SCZ, schizophrenia; PDD, pervasive developmental disorder.
[a] Genomic position based on NCBI build 37.1(Transcript ID ENST00000396355).
[b] M, major allele; m, minor allele.
[c] Amino acid change based on NCBI reference sequence NP_060138.1.
[d] Minor allele count.
[e] Minor allele frequency.

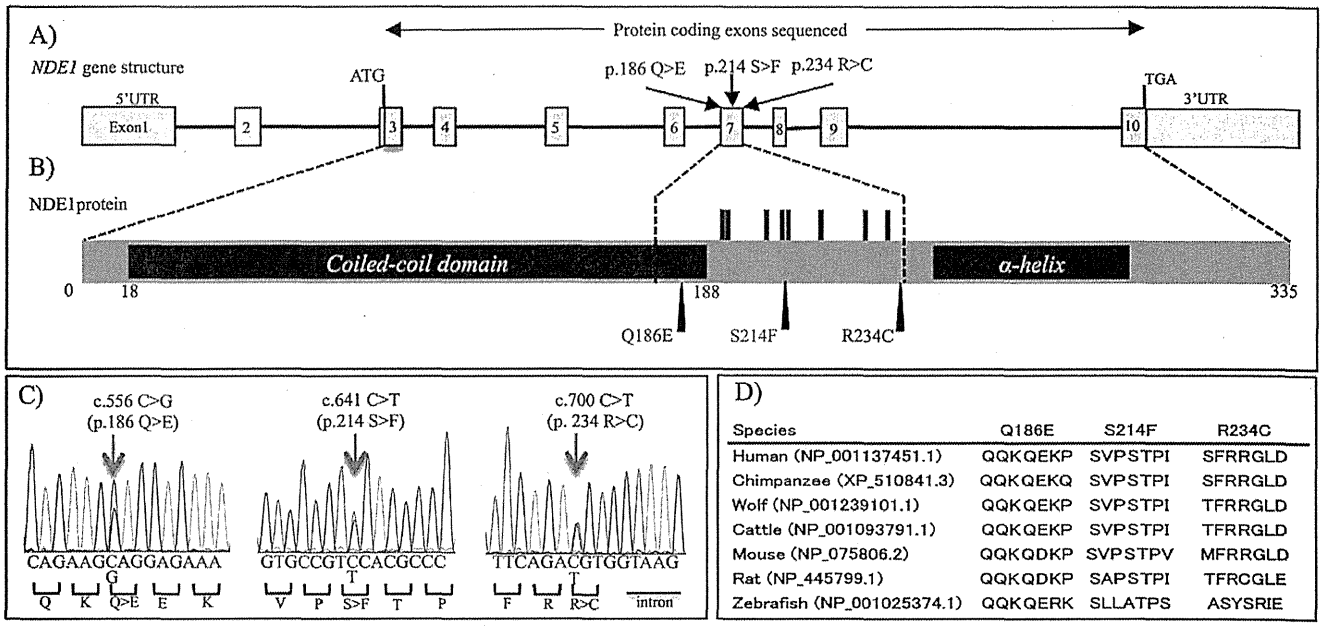

Fig. 1. Locations of novel rare mutations and results of conservation analysis. (A) NDE1 gene structure based on ENST00000396355; yellow boxes indicate the protein-coding exons sequenced in this study. Gray boxes indicate the 5′ and 3′ untranslated regions (UTR). We discovered 3 rare novel mutations in exon7. (B) NDE1 protein structure (335 amino acids); S214F and R234C were located between the N-terminal coiled-coil region and C-terminal α-helix region.[49] Several phosphorylation sites (red sticks) have been identified in the peptide regions encoded by exon7 of NDE1, and S214 is one of those phosphorylation sites. (C) Traces of Sanger sequence illustrating the novel rare variants. Arrows indicate the mutated sites. (D) Multiple alignments of amino acid sequences from NDE1 homologs; these alignments include the novel mutations.

Table 2. Association Analysis of Novel Rare Variants

	Genomic Data		Schizophrenia				Bipolar Disorder				Healthy Control	
Variant	Position	M[a]/m[a]	MAF[b]	Genotype Count[c]	*P* value[d]	Odds Ratio	MAF	Genotype Count	*P* value	Odds Ratio	MAF	Genotype Count
Q186E	16:15785033	C/G	0.0015	0/12/3948	.15	0.65	0.0098	0/2/1025	.17	0.41	0.0024	0/22/4679
S214F	16:15785118	C/T	0.00076	0/6/3954	**.039**	7.1	0	0/0/1034	.82	NA	0.00011	0/1/4693
R234C	16:15785177	C/T	0.00013	0/1/3960	.46	NA[e]	0.0097	0/2/1029	**.032**	NA	0	0/0/4693

Note: [a] M, major; m, minor.
[b] MAF, minor allele frequency.
[c] Genotype count; homozygote of minor allele/heterozygote/homozygote of major allele (Genotype count of SCZ was considering total sample number).
[d] *P* values were calculated by Fisher's exact test (2 × 2 contingency table, one-tail)
[e] NA, not available

We considered that the S214F variant of NDE1 could contribute to the pathophysiology of SCZ by affecting NDE1 protein functions based on the following: (1) NDE1 and the homolog protein NDEL1 are highly similar[35] and are centrosomal phosphoproteins with reported roles in cell proliferation and migration[28,32]; (2) YWHAE, which is one of the DISC1 interacting proteins and is closely related to neuronal migration and brain development, binds to phosphorylated NDEL1 and is required for normal NDEL1 localization and functions[38]; (3) the reported phosphorylation sites of NDEL1 for binding with YWHAE are conserved around S214 of NDE1 (figure 2A); and (4) the aromatic ring of NDE1 F214 might have steric effects on T215, which is suggested to be the phosphorylation site responsible for binding to YWHAE[38] (supplementary figure S3).

To investigate the effect of S214F and that of R234C on the interaction with YWHAE, we used HEK293T cells to perform GST pull-down assays. The results are shown in figures 2B and 2C. As expected from previous studies of NDEL1 (34), NDE1 interacted with YWHAE. We also discovered a reduced interaction between YWHAE

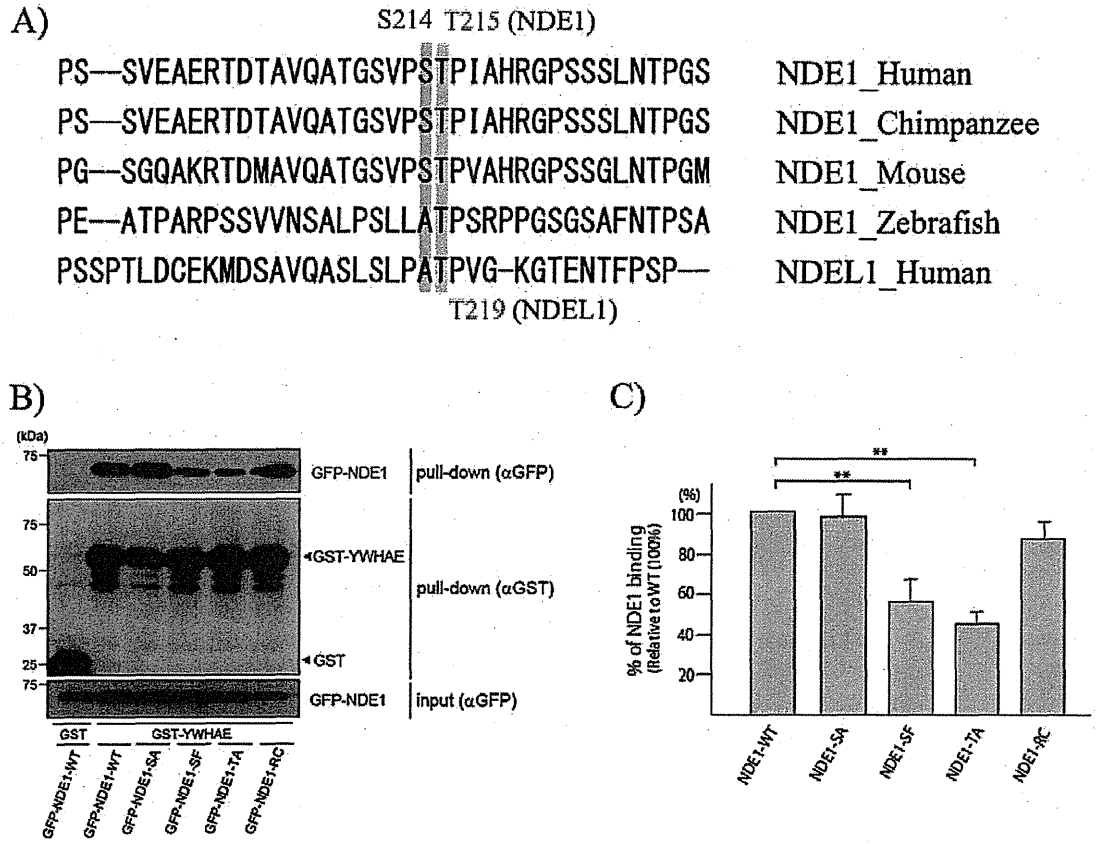

Fig. 2. Evaluation of interaction between YWHAE and NDE1-mutants. Legend: (A) Sequence alignment between the YWHAE-binding site of human NDEL1 and NDE1 orthologs of different species. The T215 residue is highly conserved among some species. T219 of NDEL1 (the counterpart of NDEL1 T219 is NDE1 T215) is also conserved in several species, and had been supposed to be the most important site for binding with YWHAE.[38] (B) Representative immunoblots of total cell lysates and the proteins pulled down by GST-YWHAE. A portion (10%) of each eluate and the inputs (5%) were subjected to SDS-PAGE and subsequent immunoblot analyses with the indicated antibodies. (C) Graph of the relative amount of bound NDE1 normalized to the amount of pulled-down GST-YWHAE. To quantify the effects of each NDE1 mutation on the interaction with YWHAE, we performed the GST-pulldown assay in triplicate. The GST-pulldown assay revealed a reduced interaction of YWHAE with S214F and T215A compared with WT (double asterisks: $P < .01$). No difference was detected between S214A and WT, or between R234C and WT. Each bar represents the mean of the triplicate experiments, and the error bars indicate ±SE.

and S214F or T215A mutants relative to WT ($P < .01$); the T215A mutant was used as a positive control because it cannot be phosphorylated at T215A.[38] However, no significant difference was detected in the interaction of R234C and the S214A (constructed as a mutation with less steric effects than F214), compared with WT. To further examine in vivo interactions between NDE1-WT and the NDE1 mutants, we performed a GST pull-down assay using lysates for COS7 cells that co-expressed GST-YWHAE and GFP-NDE1 (supplementary figure S4). The decreased effects of S214F and T215A on the interaction with YWHAE was also observed in the assay.

To examine the effect of S214F and that of R234C on neurodevelopment, we performed a morphological study of nascent hippocampal neurons, as NDE1 is reportedly known to contribute to neurite extension.[48] We found that endogenous NDE1 localized at nascent axons in rat hippocampal neurons (figure 3A), and that overexpression of NDE1-WT or NDE1-S214A increased the axon length, while that of NDE1-T215A, NDE1-S214F or NDE1-R234C did not (figure 3B).

Discussion

In this study, we conducted exon sequencing of NDE1 as a candidate gene for SCZ, ASD, and BD. After sequencing more than 5.7×10^2 kb, we discovered 3 novel rare missense mutations in exon 7 of the NDE1 gene in SCZ cases. NDE1 exon 7 is located C-terminalα-helix where many phosphorylation sites are located (figures 1A and 1B), and predicted to regulate the NDE1 structure and interactions with other proteins.[31,49] Moreover, it was suggested disease associated mutations cluster within protein interacting domains.[50] In the current study, we could detect a significant association (OR = 7.1, $P = .039$) between SCZ and S214F in the sample comprising 8734

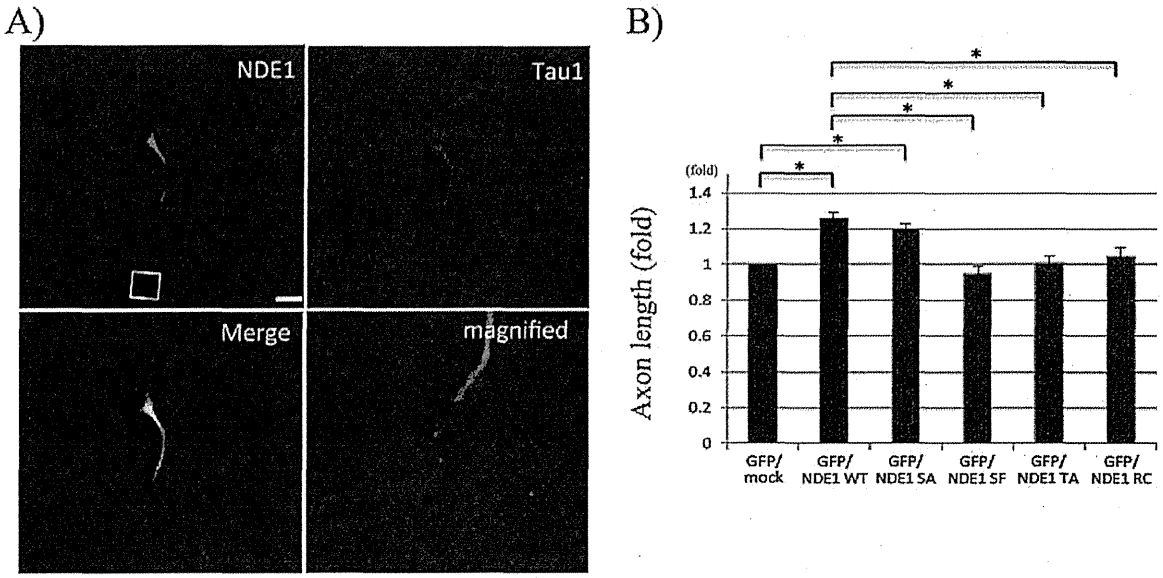

Fig. 3. Effect of NDE1-mutants on axonal outgrowth in hippocampal neurons. (A) Colabeling of NDE1 (green) and Tau1 protein (red) in nascent neurons (DIV2). Tau1 is used as an axon marker. The image in the lower right panel is a magnified view of the tip of an axonal process (rectangle in the upper left panel). Scale bar, 15 μm. (B) Quantitative analysis of axon length in transfected neurons. Hippocampal neurons transfected with the indicated plasmids encoding EGFP and Myc-NDE1 at DIV1 were detected by EGFP fluorescence and staining of Tau-1, which is a maker of axons in DIV3 cells. Each bar shows the mean of axon length in the transfectants. Error bars represent ±SE. $n = 60$, data sets from 3 independent experiments. Asterisks indicate statistical significance ($P < .05$).

unrelated individuals. Post hoc calculations of statistical power showed that our total SCZ sample ($n = 3987$) had sufficient statistical power (1-β > 80%) for SNV with MAF (0.12%) detected within our SCZ resequencing samples (table 1) if the relative risk was >2.7. This result is consistent with the recent exome sequencing study of large SCZ samples, which suggested that rare mutations (MAF < 0.1%) with large effect are significantly enriched in SCZ candidate genes, especially synapse developing related genes.[12] However, the 6 cases with S214F did not seem to share any specific clinical features (supplementary table S4). Considering a high OR, a part of S214F mutations might be a *de novo* (ie the parents of patients with NDE1 S214F did not suffer from SCZ).

Furthermore, we could find the probable biological effect of S214F, which was predicted to be deleterious from several bioinformatics tools, by investigating the interaction with YWHAE. In the pull-down assay using WT and mutant NDE1, the S214A mutation had no effect on the interaction between NDE1 and YWHAE, although the S214 residue is a phosphorylation site of NDE1 according to the PhosphoSitePlus database (http://www.phosphosite.org/homeAction.do). This result suggests that the interaction between NDE1 and YWHAE is independent of the phosphorylation state of NDE1 at S214. In contrast to S214A and R234C, the S214F and T215A mutations impaired the interaction between NDE1 and YWHAE. The mechanism of reduced interaction is probably a steric effect of F214 on T215 (supplementary figure S3), which is suggested to be the phosphorylation site responsible for binding to YWHAE.[38] Furthermore, we found that S214F mutant did not promote axon elongation (figure 3). We believe that changes in axon length, associated with the S214F mutant, could be related to the reduction in the interaction with YWHAE. This hypothesis is based on several findings: (1) NDE1 binds to YWHAE and localizes at axon tips; (2) YWHAE is required for the proper localization of NDEL1, a dimeric partner of NDE1,[38] and is involved in the axon elongation by conveying the protein complex including YWHAE and NDEL1.[47] Importantly, NDE1 and YWHAE are expressed beginning in the early embryonic period (http://hbatlas.org) (supplementary figure S5).[51] Furthermore, we previously identified a SNP (rs28365859) in YWHAE that is significantly associated with SCZ,[39] and subjects carrying the minor allele (C) had increased mRNA transcription and protein expression compared to those with the major allele (G).[39] Thus, in a future study, the epistatic effect of the YWHAE allele (rs28365859) with NDE1 S214F should also be investigated.

R234C missense mutation in NDE1 had an effect on axonal outgrowth (figure 3). Although the contribution of R234C mutation to neurodevelopment remains unclear, the change of the axon length by R234C might relate to NDE1 structure or protein-protein interactions. In fact, several proteins besides YWHAE bind to this region of NDE1, including the S214 and R234 resides; CENPF,[52] Su48,[31] and Katanin p60 are among these.[53] Furthermore, in silico analysis with Splice Aid

2[54] indicates that the nucleotide change causing R234C may be located within an exon-splicing enhancer (ESE) motif[54] and may decrease mRNA transcription because the alternative splicing might result in exon skipping during transcription. Indeed, 16%–20% of missense mutations may disrupt ESEs and change the splicing pattern of the gene.[55] In a preliminary analysis of expression in lymphoblastoid cell lines, transcription levels of the R234C mRNA were between the first quartile and the minimum in 60 SCZ cases (supplementary methods and supplementary figure S6).

Interestingly, we discovered R234C in 2 BD patients and 1 SCZ patient. Therefore, the rare NDE1 variants may be common genetic factor not only for SCZ and BD. Mounting evidence from several types of studies, including GWASs[56,57] and epidemiological family studies,[3] indicates shared genetic components between SCZ and BD. In 1 GWAS, the authors combined SCZ and BD samples so that they could increase the statistical power and detect shared risk SNPs.[56] Therefore, further replication studies for rare variants with putative large effect sizes should be performed in order to find shared pathophysiology between SCZ and BD.

Q186E was located in a highly conserved genomic region, but was discovered more frequently in HC and was judged to be neutral by several software packages for predicting mutation effects. Furthermore, in segregation analysis, we could obtain the DNA from the patient's son who was also suffering from SCZ (supplementary results), but he did not carry the NDE1 Q186E variant.

There are several limitations to be discussed. First, several potentially valuable regions of NDE1, including intronic regions, the promoter, and 5′ and 3′ untranslated regions ends, were not sequenced. Second, we could not fully conduct segregation analyses for mutations due to limited access to the subjects' family members. This made it impossible for us to accurately measure the inheritance of mutation in the pedigree. Furthermore, in the case summary of SCZ with novel rare variants, because we could not obtain detailed clinical information for the developmental period, the effect of discovered rare mutations could not be fully evaluated. Third, we could not perform structural analysis of S214F and R234C because these mutations are located at the C-terminal region, an area known to be difficult to predict the structure of.[49,58] Future studies should focus on the structural effect of the mutations discovered in this study.

In conclusion, screening for mutations in NDE1, a candidate risk gene for SCZ and ASD based on CNV analysis, revealed novel rare missense mutations that might increase susceptibility to SCZ and BD. Importantly, we detected a significant association between SCZ and S214F, which was found to have the biological effects of changing the interaction between NDE1 and YWHAE and the axonal outgrowth. This study therefore strengthens the evidence for the role of rare variants in NDE1 in the etiopathological role of SCZ, and BD. The present results also suggest that deep sequencing of candidate genes will be a promising method, especially using current and developing sequencing technology for elucidating the missing heritability and pathogenesis of neuropsychiatric disorders.

Supplementary Material

Supplementary material is available at http://schizophreniabulletin.oxfordjournals.org.

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References

1. Collins PY, Patel V, Joestl SS, et al. Grand challenges in global mental health. *Nature*. 2011;475:27–30.
2. Sullivan PF, Kendler KS, Neale MC. Schizophrenia as a complex trait: evidence from a meta-analysis of twin studies. *Arch Gen Psychiatry*. 2003;60:1187–1192.
3. Lichtenstein P, Yip BH, Björk C, et al. Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *Lancet*. 2009;373:234–239.
4. Sullivan PF, Daly MJ, O'Donovan M. Genetic architectures of psychiatric disorders: the emerging picture and its implications. *Nat Rev Genet*. 2012;13:537–551.
5. Ripke S, O'Dushlaine C, Chambert K, et al. Genome-wide association analysis identifies 13 new risk loci for schizophrenia. *Nat Genet*. 2013;45:1150–1159.
6. Malhotra D, Sebat J. CNVs: harbingers of a rare variant revolution in psychiatric genetics. *Cell*. 2012;148:1223–1241.
7. Timms AE, Dorschner MO, Wechsler J, et al. Support for the N-methyl-D-aspartate receptor hypofunction hypothesis of schizophrenia from exome sequencing in multiplex families. *JAMA Psychiatry*. 2013;70:582–590.
8. Hamilton PJ, Campbell NG, Sharma S, et al. De novo mutation in the dopamine transporter gene associates dopamine

dysfunction with autism spectrum disorder. *Mol Psychiatry*. 2013;18:1315–1323.
9. Mondal K, Ramachandran D, Patel VC, et al. Excess variants in AFF2 detected by massively parallel sequencing of males with autism spectrum disorder. *Hum Mol Genet*. 2012;21:4356–4364.
10. Durand CM, Perroy J, Loll F, et al. SHANK3 mutations identified in autism lead to modification of dendritic spine morphology via an actin-dependent mechanism. *Mol Psychiatry*. 2012;17:71–84.
11. Szatkiewicz JP, O'Dushlaine C, Chen G, et al. Copy number variation in schizophrenia in Sweden. *Mol Psychiatry*. 2014;19:762–773.
12. Purcell SM, Moran JL, Fromer M, et al. A polygenic burden of rare disruptive mutations in schizophrenia. *Nature*. 2014;506:185–190.
13. Moessner R, Marshall CR, Sutcliffe JS, et al. Contribution of SHANK3 mutations to autism spectrum disorder. *Am J Hum Genet*. 2007;81:1289–1297.
14. Durand CM, Betancur C, Boeckers TM, et al. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet*. 2007;39:25–27.
15. Bonaglia MC, Giorda R, Borgatti R, et al. Disruption of the ProSAP2 gene in a t(12;22)(q24.1;q13.3) is associated with the 22q13.3 deletion syndrome. *Am J Hum Genet*. 2001;69:261–268.
16. Ullmann R, Turner G, Kirchhoff M, et al. Array CGH identifies reciprocal 16p13.1 duplications and deletions that predispose to autism and/or mental retardation. *Hum Mutat*. 2007;28:674–682.
17. Mefford HC, Muhle H, Ostertag P, et al. Genome-wide copy number variation in epilepsy: novel susceptibility loci in idiopathic generalized and focal epilepsies. *PLoS Genet*. 2010;6:e1000962.
18. Heinzen EL, Radtke RA, Urban TJ, et al. Rare deletions at 16p13.11 predispose to a diverse spectrum of sporadic epilepsy syndromes. *Am J Hum Genet*. 2010;86:707–718.
19. de Kovel CG, Trucks H, Helbig I, et al. Recurrent microdeletions at 15q11.2 and 16p13.11 predispose to idiopathic generalized epilepsies. *Brain*. 2010;133:23–32.
20. Ingason A, Rujescu D, Cichon S, et al. Copy number variations of chromosome 16p13.1 region associated with schizophrenia. *Mol Psychiatry*. 2011;16:17–25.
21. Ikeda M, Aleksic B, Kirov G, et al. Copy number variation in schizophrenia in the Japanese population. *Biol Psychiatry*. 2010;67:283–286.
22. Kirov G, Grozeva D, Norton N, et al. Support for the involvement of large copy number variants in the pathogenesis of schizophrenia. *Hum Mol Genet*. 2009;18:1497–1503.
23. Nagamani SC, Erez A, Bader P, et al. Phenotypic manifestations of copy number variation in chromosome 16p13.11. *Eur J Hum Genet*. 2011;19:280–286.
24. Hannes FD, Sharp AJ, Mefford HC, et al. Recurrent reciprocal deletions and duplications of 16p13.11: the deletion is a risk factor for MR/MCA while the duplication may be a rare benign variant. *J Med Genet*. 2009;46:223–232.
25. Williams NM, Norton N, Williams H, et al. A systematic genomewide linkage study in 353 sib pairs with schizophrenia. *Am J Hum Genet*. 2003;73:1355–1367.
26. International Molecular Genetic Study of Autism C. A genomewide screen for autism: strong evidence for linkage to chromosomes 2q, 7q, and 16p. *Am J Hum Genet*. 2001;69:570–581.
27. Lamb JA, Barnby G, Bonora E, et al. Analysis of IMGSAC autism susceptibility loci: evidence for sex limited and parent of origin specific effects. *J Med Genet*. 2005;42:132–137.
28. Bakircioglu M, Carvalho OP, Khurshid M, et al. The essential role of centrosomal NDE1 in human cerebral cortex neurogenesis. *Am J Hum Genet*. 2011;88:523–535.
29. Feng Y, Walsh CA. Mitotic spindle regulation by Nde1 controls cerebral cortical size. *Neuron*. 2004;44:279–293.
30. Tarricone C, Perrina F, Monzani S, et al. Coupling PAF signaling to dynein regulation: structure of LIS1 in complex with PAF-acetylhydrolase. *Neuron*. 2004;44:809–821.
31. Hirohashi Y, Wang Q, Liu Q, et al. Centrosomal proteins Nde1 and Su48 form a complex regulated by phosphorylation. *Oncogene*. 2006;25:6048–6055.
32. Alkuraya FS, Cai X, Emery C, et al. Human mutations in NDE1 cause extreme microcephaly with lissencephaly [corrected]. *Am J Hum Genet*. 2011;88:536–547.
33. Hennah W, Tomppo L, Hiekkalinna T, et al. Families with the risk allele of DISC1 reveal a link between schizophrenia and another component of the same molecular pathway, NDE1. *Hum Mol Genet*. 2007;16:453–462.
34. Brandon NJ, Sawa A. Linking neurodevelopmental and synaptic theories of mental illness through DISC1. *Nat Rev Neurosci*. 2011;12:707–722.
35. Efimov VP, Morris NR. The LIS1-related NUDF protein of Aspergillus nidulans interacts with the coiled-coil domain of the NUDE/RO11 protein. *J Cell Biol*. 2000;150:681–688.
36. Bradshaw NJ, Christie S, Soares DC, et al. NDE1 and NDEL1: multimerisation, alternate splicing and DISC1 interaction. *Neurosci Lett*. 2009;449:228–233.
37. Burdick KE, Kamiya A, Hodgkinson CA, et al. Elucidating the relationship between DISC1, NDEL1 and NDE1 and the risk for schizophrenia: evidence of epistasis and competitive binding. *Hum Mol Genet*. 2008;17:2462–2473.
38. Toyo-oka K, Shionoya A, Gambello MJ, et al. 14-3-3epsilon is important for neuronal migration by binding to NUDEL: a molecular explanation for Miller-Dieker syndrome. *Nat Genet*. 2003;34:274–285.
39. Ikeda M, Hikita T, Taya S, et al. Identification of YWHAE, a gene encoding 14-3-3epsilon, as a possible susceptibility gene for schizophrenia. *Hum Mol Genet*. 2008;17:3212–3222.
40. Ikeda M, Aleksic B, Kinoshita Y, et al. Genome-wide association study of schizophrenia in a Japanese population. *Biol Psychiatry*. 2011;69:472–478.
41. Dong C, Yu B. Mutation surveyor: an in silico tool for sequencing analysis. *Methods Mol Biol*. 2011;760:223–237.
42. Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010;7:248–249.
43. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc*. 2009;4:1073–1081.
44. Ferrer-Costa C, Gelpi JL, Zamakola L, et al. PMUT: a web-based tool for the annotation of pathological mutations on proteins. *Bioinformatics*. 2005;21:3176–3178.
45. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet*. 2007;81:559–575.
46. Funahashi Y, Namba T, Fujisue S, et al. ERK2-mediated phosphorylation of Par3 regulates neuronal polarization. *J Neurosci*. 2013;33:13270–13285.

47. Taya S, Shinoda T, Tsuboi D, et al. DISC1 regulates the transport of the NUDEL/LIS1/14-3-3epsilon complex through kinesin-1. *J Neurosci*. 2007;27:15–26.
48. Bradshaw NJ, Soares DC, Carlyle BC, et al. PKA phosphorylation of NDE1 is DISC1/PDE4 dependent and modulates its interaction with LIS1 and NDEL1. *J Neurosci*. 2011;31:9043–9054.
49. Soares DC, Bradshaw NJ, Zou J, et al. The mitosis and neurodevelopment proteins NDE1 and NDEL1 form dimers, tetramers, and polymers with a folded back structure in solution. *J Biol Chem*. 2012;287:32381–32393.
50. Wang X, Wei X, Thijssen B, et al. Three-dimensional reconstruction of protein networks provides insight into human genetic disease. *Nat Biotechnol*. 2012;30:159–164.
51. Kang HJ, Kawasawa YI, Cheng F, et al. Spatio-temporal transcriptome of the human brain. *Nature*. 2011;478:483–489.
52. Soukoulis V, Reddy S, Pooley RD, et al. Cytoplasmic LEK1 is a regulator of microtubule function through its interaction with the LIS1 pathway. *Proc Natl Acad Sci U S A*. 2005;102:8549–8554.
53. Toyo-Oka K, Sasaki S, Yano Y, et al. Recruitment of katanin p60 by phosphorylated NDEL1, an LIS1 interacting protein, is essential for mitotic cell division and neuronal migration. *Hum Mol Genet*. 2005;14:3113–3128.
54. Piva F, Giulietti M, Burini AB, et al. SpliceAid 2: a database of human splicing factors expression data and RNA target motifs. *Hum Mutat*. 2012;33:81–85.
55. Gorlov IP, Gorlova OY, Frazier ML, Amos CI. Missense mutations in hMLH1 and hMSH2 are associated with exonic splicing enhancers. *Am J Hum Genet*. 2003;73:1157–1161.
56. Smoller JW, Craddock N, Kendler K, et al. Identification of risk loci with shared effects on five major psychiatric disorders: a genome-wide analysis. *Lancet*. 2013;381:1371–1379.
57. Williams HJ, Craddock N, Russo G, et al. Most genome-wide significant susceptibility loci for schizophrenia and bipolar disorder reported to date cross-traditional diagnostic boundaries. *Hum Mol Genet*. 2011;20:387–391.
58. Bradshaw NJ, Ogawa F, Antolin-Fontes B, et al. DISC1, PDE4B, and NDE1 at the centrosome and synapse. *Biochem Biophys Res Commun*. 2008;377:1091–1096.

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Factor Structure of the Japanese Version of the Edinburgh Postnatal Depression Scale in the Postpartum Period

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Abstract

Background: The Edinburgh Postnatal Depression Scale (EPDS) is a widely used screening tool for postpartum depression (PPD). Although the reliability and validity of EPDS in Japanese has been confirmed and the prevalence of PPD is found to be about the same as Western countries, the factor structure of the Japanese version of EPDS has not been elucidated yet.

Methods: 690 Japanese mothers completed all items of the EPDS at 1 month postpartum. We divided them randomly into two sample sets. The first sample set (n = 345) was used for exploratory factor analysis, and the second sample set was used (n = 345) for confirmatory factor analysis.

Results: The result of exploratory factor analysis indicated a three-factor model consisting of anxiety, depression and anhedonia. The results of confirmatory factor analysis suggested that the anxiety and anhedonia factors existed for EPDS in a sample of Japanese women at 1 month postpartum. The depression factor varies by the models of acceptable fit.

Conclusions: We examined EPDS scores. As a result, "anxiety" and "anhedonia" exist for EPDS among postpartum women in Japan as already reported in Western countries. Cross-cultural research is needed for future research.

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Introduction

Postpartum depression (PPD) is a type of major depressive disorder after childbirth and is distinguished from maternity blues in terms of onset, severity and duration of symptoms. The prevalence of PPD is estimated at approximately 13% from meta-analysis [1,2]. Our study shows 10.4% of women in Japan experienced depressive symptomatology assessed by the Edinburgh Postnatal Depression Scale (EPDS) [3]. PPD is a major mental health problem in women with children [4]. First, PPD reduces maternal mental health and quality of life. 5–14% of perinatal and postnatal women have thoughts of self-harm, and suicides account for up to 20% of postpartum deaths [5]. Second, PPD has a negative influence on child health and development [6,7] because it interferes with the mother's ability to care for a baby and handle other daily tasks. Third, the mother-child relationship often worsens because of PPD [8]. Severe depression is also reported to be associated with child abuse [9].

Early detection and intervention are essential for maternal and child health. EPDS, a 10-item self-administered questionnaire for early detection of PPD [10], has been the most widely used screening tool for PPD across countries and cultures. In recent studies, the factor structure of the original English version of EPDS has been reported as shown in Table 1 [11–18]. These results suggest that anxiety symptoms account for a significant part of PPD symptoms, unlike typical major depressive disorders. There are only a few studies about the factor structure of EPDS outside Western countries, but these studies show similar results: that EPDS was found to contain at least two factors, a depressive factor

Table 1. Factor structure of the English version of the EPDS.

First author, Published year	Period	Country	N	Method	Rotation	Factor structure		
						Factor 1	Factor 2	Factor 3
Tuohy & McVey,2008	Postpartum 6.47 months	U.K.	440	EEA	Oblimin	Non-specific depressive symptoms:7, 8, 9, 10	Anhedonia:1, 2	Anxiety symptoms:3, 4, 5
King, 2012	Postpartum 1 week-12 months	U.S.A.	169	CFA	None	Non-specific depressive symptoms:7, 8, 9, 10	Anhedonia:1, 2	Anxiety symptoms:3, 4, 5
Astbury, 1994	Postpartum 8–9 months	Australia	790	PCA	Oblimin	Depression:1, 2, 6, 7, 8, 9, 10	Anxiety:3, 4, 5	-
Matthey, 2008	Postpartum 6 weeks	Australia	238	PCA	Varimax	Depression:1, 2, 6, 7, 8, 9, 10	Anxiety:3, 4, 5	-
Phillips, 2009	Postpartum 0–12 months	Australia	309	EEA/CFA	Oblimin	Depression:1, 2, 6, 7, 8, 9, 10	Anxiety:3, 4, 5	-
Ross, 2003	Pregnant 36 weeks/Postpartum 6 and 16 weeks	Canada	150	PCA	Varimax	Depression:1, 2, 8, 9	Anxiety:3, 4, 5	Suicide 10
Jomeen, 2005	Postpartum 13.6 weeks	U.K.	101	EEA/CFA	Oblimin	Depression:1, 2, 6, 7, 8, 9	Anxiety:3, 4, 5	Suicide 10
Swalm, 2010	Postpartum 26 weeks	Australia	4706	PCA	Varimax	Anhedonia:1, 2	Anxiety:3, 4, 5	-

(EEA: exploratory factor analysis, CFA: confirmatory factor analysis, PCA: principal component analysis).

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and an anxiety factor in Brazil [19], China [19], and the Netherlands [19].

The pathology of PPD has been thought to be caused by biological and psychosocial changes with pregnancy and childbirth. There is no direct evidence that PPD has a common pathology across different populations, ethnicities and cultures; however, the commonality of the prevalence of PPD [20] supports this idea. If a common pathophysiology can be proven and this hypothesis supported, it will become a significant step towards understanding the common pathology of PPD. Because the cross-cultural consistency of the factor structure of EPDS, however, has yet not been examined, particularly outside Western countries, more research is needed to answer the question.

In Japan, the reliability and validity of EPDS in Japanese has been confirmed and the prevalence of PPD is found to be comparable to the Western countries, but the factor structure of the Japanese version of EPDS has not been elucidated. Therefore, we examined the symptomatological structure of PPD measured with the Japanese version of EPDS to compare with the structure of the original English version of EPDS already reported in Western countries.

Methods

Participants

Participants were recruited between August 2004 and October 2012. Every participant was an outpatient in a maternity ward at one of three obstetrics and gynecology hospitals in Nagoya, Japan. The three obstetrics hospitals were a general hospital (Nagoya Teishin Hospital), an obstetrics and gynecology hospital (Kaseki Hospital), and a university hospital (Nagoya University Hospital).

The eligibility criteria were as follows:

(1) attending at one of the three hospitals consecutively
(2) 20 years of age or older
(3) ability to understand the questionnaire written in Japanese.

Procedure

We explained our research design and methods to pregnant women at maternity programs or outpatient care. In these three hospitals, every outpatient equally receives an orientation for birth hospitalization during the second trimester at outpatient care or maternity program. We matched the timing of the invitation with the timing of the orientation during the second trimester which every patient participates. At the same time, participants received a set of agreement documents and questionnaires. Each woman was asked to participate in the study voluntarily and to answer all of the questions according to the predefined schedule. If she agreed to participate in the study, she was requested to return the two sealed envelopes that contained the anonymous questionnaire and the signed agreement separately. This was to guarantee privacy. We considered a voluntarily returned envelope consent to participate in this research.

Measurements

We assessed depressive symptoms in participants as well as their social background (i.e. years of schooling, demographic parameters). Depressive symptoms were evaluated by EPDS at about 1 month after childbirth.

EPDS is a 10-item self-report screening tool for postnatal depression. Each item is scored on a 4-point scale ranging from 0 to 3. Total scores can range from 0 to 30. The English version of

EPDS has good internal consistency (Cronbach's alpha = 0.87) and reliability (split half reliability = 0.88) [10].

EPDS was translated into Japanese by Okano et al. in 1996 and confirmed that the retranslated English version was the equivalent to the original English version [21]. The validity and reliability of this Japanese version of EPDS were also examined against 115 non-pregnant women and 47 women at 1 month postpartum by Okano et al [21]. It had good internal consistency (Cronbach's alpha = 0.78) and test–retest reliability (Spearman correlation = 0.92) [21]. The validity was examined against a diagnosis of major depressive disorder from the semi-structured interview-based Research Diagnostic Criteria (RDC) [22] as external criteria. The total score of the women who have postpartum depression (N = 4) was higher than that of the non-depressive postpartum women (N = 43) [21] and the cut-off point of ≥9 showed good sensitivity (75%) and specificity (93%) [21].

This is the standardized Japanese version and no other Japanese version of EPDS is used in Japan. In this study, we used this Japanese version of EPDS and the cut-off point of ≥9 in accordance with the previous study [21].

Data analysis

We randomly divided all participants who completed all items of EPDS into two sample sets. The first sample set was used for exploratory factor analysis, and the second sample set for confirmatory factor analysis.

Exploratory Factor Analysis (EFA)

The number of factors was determined by scree plot. An EFA with maximum-likelihood extraction was undertaken on the full 10-item EPDS. Oblique rotation using the promax rotation was performed due to an expectation that factors would be correlated.

Confirmatory Factor Analysis (CFA)

We chose the model identified in EFA and the models reported in the original English version of EPDS as follows:

(1) Tuohy & McVey/King; three-factor [11,15]
(2) Astbury et al. /Matthey/Phillips et al.; two-factor [13,16,17]
(3) Ross et al.; three-factor [14]
(4) Jomeen et al.; three-factor [12]
(5) Swalm et al.; two-factor [18]
(6) Model identified in the EFA; three-factor

As recommended for structural equation modeling applications [23,24], we used the goodness-of-fit index (GFI) [25], adjusted goodness-of-fit index (AGFI) [23], comparative fit index (CFI) [26], and root mean square error of approximation (RMSEA) [27]. A good fit is defined as a GFI greater than 0.95, an AGFI greater than 0.90, a CFI greater than 0.97 and an RMSEA less than 0.05. An acceptable fit is defined as a GFI greater than 0.90, an AGFI greater than 0.85, a CFI greater than 0.95 and an RMSEA less than 0.08 [25] [23] [26] [27]. Data were analyzed using SPSS version 20.0 and Amos 19.0 (IBM Japan, Tokyo, Japan).

Results

Characteristics of participants

812 participants agreed to participate in this study. The mean age of the participants was 32.1 years (range: 20 to 45 years, Standard Deviation (S.D.) 4.5 years, interquartile range (IQR) 29–35). Average years of schooling were 14.4 years (range: 9 to 18 years, S.D. = 1.6 years, IQR 14–16). In terms of parity, 67.0% of participants were nulliparous, 24.4% of participants were primiparous, 7.9% of participants had given birth two times, and 0.6% of participants had given birth three times (range: 0 to 3 children, S.D. = 0.7, IQR 0–1). 690 out of the 812 women completed all items of EPDS. The non-response rate is 75 of 812 and the non-valid response rate is 51 of 812.

EPDS scores

The median postpartum EPDS score was 3 (range: 0–22, S.D. = 4.53, IQR 1–7). Approximately 18.4% of women scored 9–22 and were considered at high risk of postpartum depression. The median infant age was 31.7 days (range: 16–64, S.D. = 6.9 days, IQR 30–34).

Factor analysis

690 participants who completed all items of EPDS were divided randomly into two groups. The first sample set of 345 participants was used for EFA, and the second sample set of 345 participants was used for CFA.

EFA

The dataset was found suitable for factor analysis (the Kaiser-Meyer-Olkin index = 0.886). The Cronbach's alpha for the 10-item EPDS was 0.856, indicating the test has good instrument

Table 2. Factor analysis of the Japanese version Edinburgh Postnatal Depression Scale.

Items of the EPDS	Factor 1	Factor 2	Factor 3
1. I have been able to laugh and see the funny side of things.	−.034	1.055	−.075
2. I have looked forward with enjoyment to things.	.026	.599	.135
3. I have blamed myself unnecessarily when things went wrong.	.684	.067	.026
4. I have been anxious or worried for no good reason.	.755	.023	−.072
5. I have felt scared or panicky for not very good reason.	.803	−.078	−.044
6. Things have been getting on top of me.	.238	.140	.141
7. I have been so unhappy that I have had difficulty sleeping.	.011	.028	.741
8. I have felt sad or miserable.	.352	.065	.497
9. I have been so unhappy that I have been crying.	−.112	.000	.824
10. The thought of harming myself has occurred to me.	.255	−.086	.267

(N = 690, maximum-likelihood estimation, promax rotation).
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Table 3. Goodness-of-fit of the models.

First author, Published year	Factor structure			Goodness-of-fit of the models			
	Factor 1	Factor 2	Factor 3	GFI	AGFI	CFI	RMSEA
1. Tuohy & McVey, 2008/King, 2012	Non-specific depressive symptoms:7, 8, 9, 10	Anhedonia:1, 2	Anxiety symptoms:3, 4, 5	0.965	0.934	0.958	0.065
2. Astbury, 1994/Matthey, 2008/Phillips, 2009	Depression:1, 2, 6, 7, 8, 9, 10	Anxiety:3, 4, 5	-	0.870	0.796	0.852	0.136
3. Ross, 2003	Depression:1, 2, 8, 9	Anxiety:3, 4, 5	Suicide:10	0.896	0.790	0.881	0.152
4. Jomeen, 2005	Depression:1, 2, 6, 7, 8, 9	Anxiety:3, 4, 5	Suicide:10	0.883	0.810	0.868	0.132
5. Swalm, 2010	Anhedonia:1, 2	Anxiety:3, 4, 5	-	0.992	0.970	0.995	0.05
6. Model identified in the EFA in this study	Anxiety:3, 4, 5	Anhedonia:1, 2	Depression:7, 8, 9	0.954	0.902	0.962	0.092

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internal reliability. The scree test indicated a three-factor solution which accounted for 64.4% of the variance. The anhedonia, anxiety and depression factors appeared consistent with factors identified in our studies. A coefficient level of 0.45 or above was chosen to indicate significant item factor loading.

The first factor, which explained 43.4% of the total variance, included EPDS items 3, 4, 5 with factor loadings >0.45 (listed in Table 2). Items 3, 4 and 5 were found to have the highest factor loadings (all>0.65), consistent with previous findings that identified this factor as an "anxiety" subscale within EPDS. The second factor explained 12.1% of the total variance, and included items 1 and 2 with factor loadings>0.45. Items 1 and 2 had the highest factor loadings (>0.55), consistent with previous findings that identified this factor as an "anhedonia" subscale within EPDS. The third factor explained 8.8% of the total variance, and included items 7, 8 and 9 with factor loadings >0.45. Items 7 and 9 had the highest factor loadings (>0.7), consistent with previous findings that identified this factor as a "depression" subscale within EPDS.

CFA

The goodness-of-fit for the data represented by GFI, AGFI, CFI and RMSEA are shown in Table 3. In the models of Tuohy & McVey and King, GFI and AGFI showed good fit while CFI and RMSEA showed acceptable fit. In the models of Astbury et al. and Matthey and Phillips et al., GFI, AGFI, CFI and RMSEA showed unsatisfactory fit. In the model of Ross et al., GFI, AGFI, CFI and RMSEA showed unsatisfactory fit. In the model of Jomeen et al., GFI, AGFI, CFI and RMSEA showed unsatisfactory fit. In the model of Swalm et al., GFI, AGFI, CFI and RMSEA showed good fit. In the model identified in EFA, GFI and AGFI showed good fit. CFI and RMSEA showed acceptable fit. We therefore concluded that there were four acceptable models: those of Tuohy & McVey, King, Swalm et al., and as identified in EFA. We also concluded that the models of Astbury et al., Matthey and Phillips et al., Ross et al. and Jomeen et al. were unsatisfactory.

Correlations between factors in the models of an acceptable fit or a good fit were as follows. In the models of Tuohy & McVey and King, correlation between "anxiety" and "depression" was 0.84, correlation between "depression" and "anhedonia" was 0.64, and correlation between "anhedonia" and "anxiety" was 0.60. In the model of Swalm et al., correlation between "anhedonia" and "anxiety" was 0.60. In the model identified in EFA, correlation between "anxiety" and "depression" was 0.85, correlation between "depression" and "anhedonia" was 0.66, and correlation between "anhedonia" and "anxiety" was 0.60.

Discussion

This is the first study demonstrating the factor structure of the Japanese version of EPDS using a large sample of postpartum women. The model of EFA reported by Tuohy & McVey, King and Swalm et al., was consistent with our model in the present study of the Japanese version of EPDS. The model consists of common factors, an anxiety factor (items 3, 4 and 5) and an anhedonia factor (items 1 and 2). Thus, our findings suggest that factor structure of EPDS in Japan is basically the same as already reported in Western countries, although there was variance between studies on some items of EPDS.

No previous papers have reported the factor structure of the Japanese version of EPDS, however there are some studies about the symptoms of PPD in Japan. Tamaki et al. showed that women with PPD have strong anxiety symptoms by the State-Trait Anxiety Inventory Trait test [28]. Sato Y et al. revealed that the

prevalence of anxiety symptoms was higher than that of depressive symptoms after childbirth [29]. These results also suggest that anxiety symptoms are important to understand the symptomatic character of PPD in Japan.

In the other Asian countries, there are a few studies of the factor structure of EPDS. Small et al. analyzed the factor structure of EPDS in Vietnam, Turkey and Philippines [30].Small et al. pointed out that Item 6 loaded less consistently in the different countries, however they also suggest that EPDS have two or three factors which consists of anxiety and depression. Lau Y et al. showed that EPDS in China consists of the three factors as depression factor(items 6, 7, 8. 9 and 10), anxiety factor(items 3,4 and 5) and anhedonia factor(items 1 and 2) [31]. These results are very similar to the factor structure of EPDS in Japan by our study.

The depression factor

The depression factor varies across studies. Tuohy & McVey and King suggested items 7, 8, 9, and 10, Swalm suggested no depression factor, and the EFA in our study showed items 7, 8, and 9. Though the best fit was the model of Swalm et al., we proposed the EFA model because the model of Swalm excluded half of all EPDS items. Cross-cultural studies are needed to examine whether a common depression factor exists or not.

The anhedonia factor

All of the acceptable models show the anhedonia factor (items 1 and 2), which is reverse scoring. As reverse scoring items tend to be in the same cluster [32], we must take into account that reverse scoring items has been found to affect factor analysis.

The anxiety factor

The anxiety factor (items 3, 4 and 5) was shown in many countries, such as Brazil [33,34], China [31], and the Netherlands [19]. Considering the existence of a common anxiety factor across different countries and cultures, the importance of anxiety symptoms for PPD has been revealed. In fact, it is reported that about 10% of the women experiencing postpartum depression have anxiety symptoms [35].

The utility of the anxiety factor (items 3, 4 and 5) is discussed in some studies as follows. Some studies have suggested that items 3, 4 and 5 can measure anxiety disorder [18,36], and other studies suggested that items 3, 4 and 5 are enough for PPD screening [14] [37]. Although the utility of the anxiety factor (item 3, 4 and 5) varies by study, as mentioned before, there is some possibility of common utility of the anxiety factor around the world.

Correlation between factors

The correlations between "anxiety" and "depression" were found to be high in the models of acceptable fit to the data. These results suggested that there was a very close relationship between depression and anxiety, as previously reported [38,39], and showed that it was important to focus on anxiety symptoms in PPD screening and care.

Limitations

There are some types of study bias in this study. First, there is a self-selection bias. They participated in this study voluntarily. This also means that they pay more attention to their mental health. Second, there are losses to follow up. Women with depression are hard to reply the questionnaire. Third, there is a membership bias. We cannot affirm that a standard population in Japan is shown in these participants from three characteristic hospitals, a general hospital, an obstetrical and gynecological hospital, and a university hospital. The patients at the university hospital tended to have pregnancy complications, but these participants accounted for a small percentage of all participants (N = 42, 5.2%). Fourth, there is a non-response bias, however the non-response rate is 75 of 812 and the non-valid response rate is 51 of 812. We consider that these rates are not so high and the result is not affected.

Furthermore, there is a problem with this study design. We did not ask participants for their nationality, citizenship or ethnicity. However, Japan is considered to be highly homogenous in terms of population, therefore we consider this problem does not affect the result of the study.

Conclusions

We examined factor structure of the Japanese version of EPDS in a large sample size of postpartum women in Japan. As a result, "anxiety", "depression" and "anhedonia" factors exist for EPDS, as already reported in Western countries. Our findings suggest that the factor structure of EPDS is mostly common across countries and cultures.

Ethics Statement

This study protocol has been approved by the Ethics Committee of the Nagoya University Graduate School of Medicine.

Author Contributions

Conceived and designed the experiments: SM SG AK TM NO. Performed the experiments: CK YN SK MM TS AT HO NB TM. Analyzed the data: CK MA NO. Contributed reagents/materials/analysis tools: CK YN SK MM TS AT HO NB TM. Wrote the paper: CK TO BA YN SK MA NO.

References

1. O'hara MW, Swain AM (1996) Rates and risk of postpartum depression—a meta-analysis. International Review of Psychiatry 8: 37–54.
2. Beck CT (2001) Predictors of postpartum depression: an update. Nurs Res 50: 275–285.
3. Ishikawa N, Goto S, Murase S, Kanai A, Masuda T, et al. (2011) Prospective study of maternal depressive symptomatology among Japanese women. J Psychosom Res 71: 264–269.
4. Weissman MM, Jensen P (2002) What research suggests for depressed women with children. J Clin Psychiatry 63: 641–647.
5. Lindahl V, Pearson JL, Colpe L (2005) Prevalence of suicidality during pregnancy and the postpartum. Arch Womens Ment Health 8: 77–87.
6. Beck CT (1998) The effects of postpartum depression on child development: a meta-analysis. Arch Psychiatr Nurs 12: 12–20.
7. Luoma I, Tamminen T, Kaukonen P, Laippala P, Puura K, et al. (2001) Longitudinal study of maternal depressive symptoms and child well-being. J Am Acad Child Adolesc Psychiatry 40: 1367–1374.
8. Murray L, Fiori-Cowley A, Hooper R, Cooper P (1996) The impact of postnatal depression and associated adversity on early mother-infant interactions and later infant outcome. Child Dev 67: 2512–2526.
9. Buist A (1998) Childhood abuse, postpartum depression and parenting difficulties: a literature review of associations. Aust N Z J Psychiatry 32: 370–378.
10. Cox JL, Holden JM, Sagovsky R (1987) Detection of postnatal depression. Development of the 10-item Edinburgh Postnatal Depression Scale. Br J Psychiatry 150: 782–786.
11. Tuohy A, McVey C (2008) Subscales measuring symptoms of non-specific depression, anhedonia, and anxiety in the Edinburgh Postnatal Depression Scale. Br J Clin Psychol 47: 153–169.
12. Jomeen J, Martin CR (2007) Replicability and stability of the multidimensional model of the Edinburgh Postnatal Depression Scale in late pregnancy. J Psychiatr Ment Health Nurs 14: 319–324.
13. Phillips J, Charles M, Sharpe L, Matthey S (2009) Validation of the subscales of the Edinburgh Postnatal Depression Scale in a sample of women with unsettled infants. J Affect Disord 118: 101–112.
14. Ross LE, Gilbert Evans SE, Sellers EM, Romach MK (2003) Measurement issues in postpartum depression part 1: anxiety as a feature of postpartum depression. Arch Womens Ment Health 6: 51–57.