of primers used for amplification are listed in Supplementary Table 1. Detailed information on amplification conditions is available upon request. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

2.4. SNP genotyping

All SNPs were genotyped using the TaqMan 5′-exonuclease assay (Supplementary Table 2), as described previously (Watanabe et al., 2006).

2.5. Statistical analysis

Deviation from the Hardy–Weinberg equilibrium (HWE) was tested using the χ^2 test for goodness-of-fit. LD blocks defined in accordance with Gabriel's criteria (Gabriel et al., 2002) and haplotype frequencies were determined using Haploview v4.1. The allele, genotype and haplotype frequencies of the patients and control subjects were compared using the χ^2 test or Fisher's exact test. Permutation tests were performed to calculate corrected p values for multiple testing using Haploview v4.1.

We imputed the genotype distributions of 59 SNPs in *DRD3* (chr3:115307882..115402406) using the observed SNPs and the HapMap database (release#24, Han Chinese in Beijing [CHB] + JPT), using MACH 1.0 (Li and Abecasis, 2006). We adopted imputed SNPs with an Rsq (which estimates the squared correlation between imputed and true genotypes) greater than 0.3 as recommended (Li and Abecasis, 2006).

Power calculation was performed using Genetic Power Calculator (Purcell et al., 2003). Power was estimated with an α of 0.05, assuming a disease prevalence of 0.01 and the risk allele frequencies to be the values observed in control subjects.

2.6. Meta-analysis

To assess whether the haplotype T–T–T–G for SNPs rs7631540–rs1486012–rs2134655–rs963468 confers protection against schizophrenia, we performed an updated meta-analysis combining the results of three previous studies (Costas et al., 2009; Domínguez et al., 2007; Talkowski et al., 2006) and our current study, as described previously (Watanabe et al., 2007). First, we explored whether heterogeneity was present using Q statistics. Second, a fixed effects model meta-analysis was performed within groups of homogeneous odds ratios (ORs). The significance of the pooled OR was determined using a Z-test. Third, publication bias was assessed using a linear regression analysis to measure funnel plot asymmetry (Egger et al., 1997).

3. Results

Twelve SNPs were selected as tagging SNPs for *DRD3* from the HapMap database. We also included rs963468, as described above. By resequencing the exon regions of *DRD3*, we detected four SNPs: rs6280 (Ser9Gly), rs3732783 (Ala17Ala), rs3732791 (His359His), and g.−6664T>G. Three of these SNPs had previously been reported: rs6280 (Ser9Gly) in exon 2, rs3732783 (Ala17Ala) in exon 2 and rs3732791 (His359His) in exon 7. The SNP g.−6664T>G in exon 1 (GenBank accession no. NG_008842.1; position 5146) was previously unidentified.

A total of 16 SNPs (12 tagging SNPs, rs963468 and three SNPs detected) were genotyped in the first population (Table 1). Their order and physical locations are shown in Fig. 1A. The genotype distributions of all SNPs did not deviate significantly from the HWE in both groups, with the exception of rs17605608 in patients ($p=0.033$). None of the genotype or allele frequencies of the SNPs examined differed significantly between patients and control subjects. In *DRD3*, five LD blocks were defined (Fig. 1B). There were no significant associations between common haplotypes of these LD blocks and schizophrenia (Table 2).

Table 1
Genotype and allele frequencies of 16 SNPs in the first population.

SNP #	dbSNP ID	Allele[a]	Patients						Controls						p	
			n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	Genotype	Allele
1	rs9288990	C/T	594	0.143	267	274	53	0.320	597	0.694	255	267	75	0.349	0.126	0.129
2	rs10934251	A/G	593	0.437	446	134	13	0.135	593	0.216	449	130	14	0.133	0.948	0.904
3	rs7631540	C/T	595	0.975	218	284	93	0.395	596	0.570	201	296	99	0.414	0.570	0.333
4	rs1486012	A/T	595	0.623	174	301	120	0.455	596	0.944	156	297	143	0.489	0.221	0.092
5	rs3732790	T/A	593	0.228	262	274	57	0.327	596	0.366	278	265	53	0.311	0.683	0.405
6	rs3732791	G/A	595	0.820	584	11	0	0.009	597	0.724	580	17	0	0.014	0.255	0.258
7	rs2134655	C/T	595	0.944	287	253	55	0.305	596	0.962	277	259	60	0.318	0.793	0.496
8	rs963468	G/A	593	0.882	257	268	68	0.341	593	0.485	260	271	62	0.333	0.856	0.696
9	rs9880168	A/G	595	0.366	439	141	15	0.144	597	0.211	431	157	9	0.147	0.297	0.842
10	rs2630350	C/T	594	0.299	500	92	2	0.081	598	0.971	485	107	6	0.100	0.191[c]	0.111
11	rs167771	A/G	595	0.829	397	179	19	0.182	597	0.322	374	202	21	0.204	0.338	0.174
12	rs3732783	T/C	594	0.723	577	17	0	0.014	598	0.647	576	22	0	0.018	0.428	0.432
13	rs6280	T/C	594	0.510	301	239	54	0.292	595	0.815	306	243	46	0.282	0.700	0.569
14	g.−6664T>G	T/G	595	0.820	584	11	0	0.009	597	0.756	582	15	0	0.013	0.433	0.435
15	rs17605608	G/A	595	0.033	364	191	40	0.228	596	0.791	357	210	29	0.225	0.257	0.866
16	rs16822440	C/T	595	0.585	513	78	4	0.072	598	0.259	501	95	2	0.083	0.343[c]	0.337

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.
[a] Major/minor allele.
[b] Genotypes, major and minor alleles are denoted by 1 and 2, respectively.
[c] Calculated using Fisher's exact test.

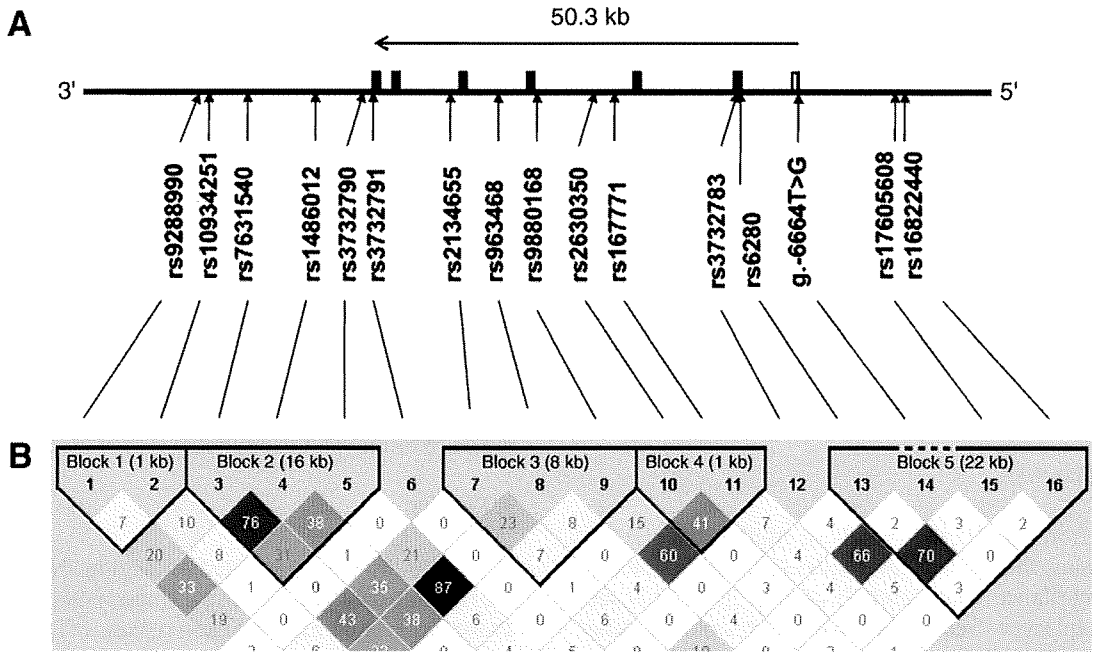


Fig. 1. Genomic structure and linkage disequilibrium (LD) of *DRD3*. (A) Genomic structure of *DRD3* and the locations of the single nucleotide polymorphisms (SNPs) analyzed in the present study. *DRD3* has seven exons (rectangles) and spans approximately 50.3 kb. Black and white rectangles represent coding and untranslated regions, respectively. The horizontal arrow and vertical arrows indicate the transcriptional orientation and locations of SNPs, respectively. (B) LD between markers of *DRD3*. A block is defined in accordance with Gabriel's criteria using Haploview v4.1. Each box represents the r^2 value corresponding to each pair-wise SNP.

Out of the 59 SNPs included for imputation analysis, we adopted 58 imputed SNPs with an Rsq greater than 0.3 (Supplementary Table 3). We found no significant associations between any of these imputed SNPs and schizophrenia after correction for multiple comparisons.

To assess whether the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 confers protection against schizophrenia (Costas et al., 2009), we conducted haplotype analyses of these SNPs (Table 3). Although the haplotype T–T–T–G was less frequent in patients than in control subjects (26.5% vs. 28.6%), this difference did not reach statistical significance ($p=0.261$).

To further test for such a haplotype association, we genotyped these four SNPs in the second population (Table 4). In the patient group, the genotype distributions of rs7631540 and rs963468 significantly deviated from the HWE ($p=0.046$ and 0.043, respectively). There were no significant associations between any of the four SNPs examined and schizophrenia in the second population. We also could not confirm an association of the haplotype T–T–T–G with schizophrenia, even in our large sample (Table 5).

We then performed an updated meta-analysis of the haplotype T–T–T–G (Table 6). The total sample sizes for the patients and control subjects from five independent populations were 3585 and 3966, respectively. We did not observe significant heterogeneity among ORs ($Q=8.22$, $df=4$, $p=0.084$). Our updated meta-analysis failed to provide

Table 2
Haplotype analyses of LD blocks.

Haplotype	Patients	Controls	*p*
Block 1 (SNP #1–2)			0.290[a]
CA	0.546	0.517	0.166
TA	0.320	0.349	0.125
CG	0.135	0.133	0.924
Block 2 (SNP #3–4–5)			0.346[a]
TTT	0.395	0.412	0.386
CAA	0.323	0.301	0.265
CAT	0.223	0.210	0.433
CTT	0.055	0.068	0.200
Block 3 (SNP #7–8–9)			0.882[a]
CAA	0.341	0.334	0.722
TGA	0.305	0.318	0.491
CGA	0.211	0.202	0.595
CGG	0.144	0.146	0.856
Block 4 (SNP #10–11)			0.265[a]
CA	0.818	0.796	0.177
CG	0.101	0.105	0.784
TG	0.081	0.099	0.116
Block 5 (SNP #13–15–16)			0.637[a]
TGC	0.634	0.635	0.927
CAC	0.225	0.223	0.907
TGT	0.071	0.081	0.346
CGC	0.066	0.057	0.335

LD, linkage disequilibrium; SNP, single nucleotide polymorphism.
[a] Global *p* values.

Table 3
Haplotype analyses of the SNPs rs7631540–rs1486012–rs2134655–rs963468 in the first population.

Haplotype	Patients	Controls	*p*[a]
C–A–C–A	0.332	0.318	0.461
T–T–T–G	0.265	0.286	0.261
C–A–C–G	0.181	0.171	0.525
T–T–C–G	0.126	0.123	0.807
C–T–C–G	0.047	0.056	0.353
C–A–T–G	0.033	0.022	0.119

SNP, single nucleotide polymorphism.
[a] Global $p=0.438$.

Table 4
Genotype and allele frequencies of four SNPs in the second population.

SNP #	dbSNP ID	Allele[a]	Patients						Controls						p	
			n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	n	HWE	1/1[b]	1/2[b]	2/2[b]	MAF	Genotype	Allele
3	rs7631540	C/T	2080	0.046	722	969	389	0.420	2177	0.206	731	1036	410	0.426	0.727	0.555
4	rs1486012	A/T	2079	0.211	567	1010	502	0.484	2213	0.984	560	1106	547	0.497	0.342	0.240
7	rs2134655	C/T	2089	0.209	971	888	230	0.323	2195	0.928	999	965	231	0.325	0.613	0.811
8	rs963468	G/A	2093	0.043	955	887	251	0.332	2197	0.558	997	957	243	0.328	0.577	0.736

SNP, single nucleotide polymorphism; HWE, Hardy–Weinberg equilibrium; MAF, minor allele frequency.
[a] Major/minor allele.
[b] Genotypes, major and minor alleles are denoted by 1 and 2, respectively.

sufficient evidence for the existence of a protective haplotype (pooled OR = 0.93, 95% CI = 0.87–1.00, $Z = 1.90$, $p = 0.058$). A linear regression analysis showed significant funnel plot asymmetry ($t = -4.48$, $p = 0.021$; Supplementary Fig. 1).

4. Discussion

We carried out a moderate-scale case–control association study using 16 genotyped SNPs (12 tagging SNPs from the HapMap database, rs963468 and three SNPs detected through resequencing) and 58 imputed SNPs that are not directly genotyped. However, we could not obtain supportive evidence for an association between *DRD3* and schizophrenia in the Japanese population. Almost all previous studies of the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. Specifically, the most extensively investigated SNP is the Ser9Gly polymorphism. Our study is in line with two recent large meta-analyses reporting no association of this polymorphism with schizophrenia (Allen et al., 2008; Ma et al., 2008). Interestingly, several studies have reported associations of the Ser9Gly polymorphism with promising endophenotypes for schizophrenia, including the intensity of eye movement (Rybakowski et al., 2001), executive functions (Bombin et al., 2008; Szekeres et al., 2004), event-related P300 potentials (Mulert et al., 2006) and prepulse inhibition of the acoustic startle reflex (Roussos et al., 2008), whereas other studies failed to find these associations (Rybakowski et al., 2005; Tsai et al., 2003). Taken together, these findings indicate that the Ser9Gly polymorphism does not contribute to genetic susceptibility to schizophrenia, but may have effects on the endophenotypes for schizophrenia.

Table 5
Haplotype analyses of the SNPs rs7631540–rs1486012–rs2134655–rs963468 in the second population.

Haplotype	Patients	Controls	p[a]
C–A–C–A	0.306	0.299	0.454
T–T–T–G	0.278	0.276	0.793
C–A–C–G	0.172	0.161	0.178
T–T–C–G	0.128	0.130	0.815
C–T–C–G	0.050	0.058	0.087
C–A–T–G	0.032	0.035	0.374
T–T–C–A	0.009	0.012	0.241

SNP, single nucleotide polymorphism.
[a] Global $p = 0.337$.

Two recent studies identified common haplotypes of *DRD3* associated with schizophrenia using different sets of tagging SNPs based on LD (Domínguez et al., 2007; Talkowski et al., 2006). The haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was significantly less frequent in patients with schizophrenia than in control subjects (25% vs. 31%) in the Galician population (Domínguez et al., 2007). This was not replicated in the Catalonian population (23% vs. 26%) (Costas et al., 2009). In the Catalonian population, the protective haplotype reported by Domínguez et al. (2007) was always associated with the haplotype A–T–G–A for the SNPs rs10934254–rs2134655–rs324030–rs324029, and vice versa (Costas et al., 2009). This haplotype was less frequent in patients with schizophrenia than in control subjects (26% vs. 31%) in a U.S. Caucasian population (Talkowski et al., 2006). A meta-analysis combining the results of these three previous studies showed that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was less frequent in patients with schizophrenia than in control subjects (Costas et al., 2009). Our moderate- and large-scale case–control studies did not confirm this finding in Japanese populations (27% vs. 29% in the first population; 28% vs. 28% in the second population).

To assess the collective evidence across individual studies, an updated meta-analysis with a total sample size of 7551 was performed. The results suggested that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may not confer protection against schizophrenia (pooled OR = 0.93, 95% CI = 0.87–1.00). However, considering a limited number of studies and the existence of publication bias, the findings of our meta-analysis should be interpreted with caution. There is the possibility that this haplotype may be regarded as a protective haplotype in Caucasian populations, but not in Asian populations. The frequencies of the haplotype T–T–T–G in control subjects ranged from 26% to 31% among ethnic groups (Supplementary Table 4). The third most common haplotype among Spanish populations is T–T–C–G, whereas, among Japanese populations, the third most common haplotype is C–A–C–G. Three major haplotypes accounted for approximately 0.9 of the total chromosomes in Spanish populations, but less than 0.8 in Japanese populations. These differences in haplotype structures among ethnic groups may account for the inconsistent results between Costas et al.'s and our meta-analyses. To draw any conclusion, further studies using larger samples are required in various ethnic populations.

We recognize some limitations of the present study. First, the sample size of the first population constitutes one of the

Table 6
Meta-analysis of the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468.

Study	Ethnicity	Patients		Controls		T–T–T–G vs. the others	
		n	T–T–T–G	*n*	T–T–T–G	OR	95% CI
Talkowski et al. (2006)	U.S. Caucasian	331	0.26	274	0.31	0.78	0.61–1.00
Domínguez et al. (2007)	Spanish	260	0.25	354	0.31	0.73	0.56–0.94
Costas et al. (2009)	Spanish	273	0.23	512	0.26	0.86	0.67–1.10
Current study (I)	Japanese	595	0.27	598	0.29	0.91	0.76–1.08
Current study (II)	Japanese	2126	0.28	2228	0.28	1.00	0.91–1.10
Pooled[a]		3585		3966		0.93	0.87–1.00

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval.
[a] $Q=8.22$, $df=4$, $p=0.084$ for heterogeneity.

largest samples examined for an association of *DRD3* with schizophrenia, but may not have sufficient power to detect associations between schizophrenia and SNPs with low minor allele frequencies and small effects. A power calculation showed that, when the genotypic relative risk was set to 1.69 for homozygous risk allele carriers under the multiplicative model of inheritance, the power was 0.12–0.90. Second, our subjects were not assessed using a standardized structured interview. However, the diagnosis of schizophrenia was assigned on the basis of all available sources of information. To the best of our knowledge, there were no control subjects who were likely to develop schizophrenia at their present stage of life. Thus, it is unlikely that our failure to find a significant association is attributable to misdiagnosis.

In conclusion, we obtained no supportive evidence for an association between *DRD3* and schizophrenia in our Japanese subjects. The findings of our updated meta-analysis also suggest that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may not confer protection against schizophrenia. However, to draw a definitive conclusion, further studies using larger samples and sufficient markers should be carried out in various ethnic populations.

Role of funding source

Funding for this study was provided by a Grant from the Niigata Medical Association (to A.N.), a Grant from the Research Group For Schizophrenia, Japan (to N.K.), a Grant for the Promotion of Niigata University Research Projects (to N.K.), and the Tsukada Memorial Grant for Niigata University Medical Research (to T.S.1). The funding sources had no involvement in the study design; collection, analysis and interpretation of the data; or writing of the report and the decision to submit it for publication.

Contributors

Author Nunokawa designed the study, conducted resequencing and undertook statistical analyses. Author Watanabe designed the study, performed the TaqMan assays and undertook statistical analyses. Author Kaneko conducted resequencing. Author Sugai designed the study. Author Yazaki performed the TaqMan assays. Authors Arinami, Ujike, Inada, Iwata, Kunugi, Sasaki, Itokawa, Ozaki and Hashimoto managed sample collection. Author Someya supervised the study. All authors contributed to and have approved the final manuscript.

Conflict of interest

None of the authors have a conflict of interest to declare.

Acknowledgements

The authors thank the patients, their families and the healthy volunteers for their participation; Mr. H. Kusano and Ms. N. Yamazaki for excellent technical assistance.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.schres.2009.10.016.

References

Allen, N.C., Bagade, S., McQueen, M.B., Ioannidis, J.P.A., Kavvoura, F.K., Khoury, M.J., Tanzi, R.E., Bertram, L., 2008. Systematic meta-analyses and field synopsis of genetic association studies in schizophrenia: the SzGene database. Nat. Genet. 40 (7), 827–834.

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

Bombin, I., Arango, C., Mayoral, M., Castro-Fornieles, J., Gonzalez-Pinto, A., Gonzalez-Gomez, C., Moreno, D., Parellada, M., Baeza, I., Graell, M., Otero, S., Saiz, P.A., Patiño-Garcia, A., 2008. DRD3, but not COMT or DRD2, genotype affects executive functions in healthy and first-episode psychosis adolescents. Am. J. Med. Genet. B Neuropsychiatr. Genet. 147B (6), 873–879.

Brzustowicz, L.M., Hodgkinson, K.A., Chow, E.W.C., Honer, W.G., Bassett, A.S., 2000. Location of a major susceptibility locus for familial schizophrenia on chromosome 1q21–q22. Science 288 (5466), 678–682.

Costas, J., Carrera, N., Domínguez, E., Vilella, E., Martorell, L., Valero, J., Gutiérrez-Zotes, A., Labad, A., Carracedo, A., 2009. A common haplotype of *DRD3* affected by recent positive selection is associated with protection from schizophrenia. Hum. Genet. 124 (6), 607–613.

Crocq, M.A., Mant, R., Asherson, P., Williams, J., Hode, Y., Mayerova, A., Collier, D., Lannfelt, L., Sokoloff, P., Schwartz, J.C., Gill, M., Macher, J.P., McGuffin, P., Owen, M.J., 1992. Association between schizophrenia and homozygosity at the dopamine D3 receptor gene. J. Med. Genet. 29 (12), 858–860.

de Bakker, P.I., Yelensky, R., Pe'er, I., Gabriel, S.B., Daly, M.J., Altshuler, D., 2005. Efficiency and power in genetic association studies. Nat. Genet. 37 (11), 1217–1223.

Domínguez, E., Loza, M.I., Padín, F., Gesteira, A., Paz, E., Páramo, M., Brenlla, J., Pumar, E., Iglesias, F., Cibeira, A., Castro, M., Caruncho, H., Carracedo, A., Costas, J., 2007. Extensive linkage disequilibrium mapping at HTR2A and DRD3 for schizophrenia susceptibility genes in the Galician population. Schizophr. Res. 90 (1–3), 123–129.

Egger, M., Smith, D.G., Schneider, M., Minder, C., 1997. Bias in meta-analysis detected by a simple, graphical test. BMJ 315 (7109), 629–634.

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

Gurevich, E.V., Bordelon, Y., Shapiro, R.M., Arnold, S.E., Gur, R.E., Joyce, J.N., 1997. Mesolimbic dopamine D_3 receptors and use of antipsychotics in patients with schizophrenia. A postmortem study. Arch. Gen. Psychiatry 54 (3), 225–232.

Ilani, T., Ben-Shachar, D., Strous, R.D., Mazor, M., Sheinkman, A., Kotler, M., Fuchs, S., 2001. A peripheral marker for schizophrenia: increased levels of D_3 dopamine receptor mRNA in blood lymphocytes. Proc. Natl. Acad. Sci. U. S. A. 98 (2), 625–628.

Ishiguro, H., Okuyama, Y., Toru, M., Arinami, T., 2000. Mutation and association analysis of the 5′ region of the dopamine D3 receptor gene in schizophrenia patients: identification of the Ala38Thr polymorphism and suggested association between DRD3 haplotypes and schizophrenia. Mol. Psychiatry 5 (4), 433–438.

Kaneko, N., Muratake, T., Kuwabara, H., Kurosaki, T., Takei, M., Ohtsuki, T., Arinami, T., Tsuji, S., Someya, T., 2007. Autosomal linkage analysis of a Japanese single multiplex schizophrenia pedigree reveals two candidate loci on chromosomes 4q and 3q. Am. J. Med. Genet. B Neuropsychiatr. Genet. 144B (6), 735–742.

Kennedy, J.L., Billett, E.A., Macciardi, F.M., Verga, M., Parsons, T.J., Meltzer, H.Y., Lieberman, J., Buchanan, J.A., 1995. Association study of dopamine D3 receptor gene and schizophrenia. Am. J. Med. Genet. 60 (6), 558–562.

Li, Y., Abecasis, G.R., 2006. Mach 1.0: rapid haplotype reconstruction and missing genotype inference. Am. J. Hum. Genet. S79, 2290.

Lundstrom, K., Turpin, M.P., 1996. Proposed schizophrenia-related gene polymorphism: expression of the Ser9Gly mutant human dopamine D_3 receptor with the Semliki Forest virus system. Biochem. Biophys. Res. Commun. 225 (3), 1068–1072.

Ma, G., He, Z., Fang, W., Tang, W., Huang, K., Li, Z., He, G., Xu, Y., Feng, G., Zheng, T., Zhou, J., He, L., Shi, Y., 2008. The Ser9Gly polymorphism of the dopamine D3 receptor gene and risk of schizophrenia: an association study and a large meta-analysis. Schizophr. Res. 101 (1–3), 26–35.

Meador-Woodruff, J.H., Haroutunian, V., Powchik, P., Davidson, M., Davis, K.L., Watson, S.J., 1997. Dopamine receptor transcript expression in striatum and prefrontal and occipital cortex. Focal abnormalities in orbitofrontal cortex in schizophrenia. Arch. Gen. Psychiatry 54 (12), 1089–1095.

Mulert, C., Juckel, G., Giegling, I., Pogarell, O., Leicht, G., Karch, S., Mavrogiorgou, P., Möller, H.J., Hegerl, U., Rujescu, D., 2006. A Ser9Gly polymorphism in the dopamine D3 receptor gene (DRD3) and event-related P300 potentials. Neuropsychopharmacology 31 (6), 1335–1344.

Nunokawa, A., Watanabe, Y., Muratake, T., Kaneko, N., Koizumi, M., Someya, T., 2007. No associations exist between five functional polymorphisms in the catechol-O-methyltransferase gene and schizophrenia in a Japanese population. Neurosci. Res. 58 (3), 291–296.

Purcell, S., Cherny, S.S., Sham, P.C., 2003. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics 19 (1), 149–150.

Roussos, P., Giakoumaki, S.G., Bitsios, P., 2008. The dopamine D_3 receptor Ser9Gly polymorphism modulates prepulse inhibition of the acoustic startle reflex. Biol. Psychiatry 64 (3), 235–240.

Rybakowski, J.K., Borkowska, A., Czerski, P.M., Hauser, J., 2001. Dopamine D3 receptor (DRD3) gene polymorphism is associated with the intensity of eye movement disturbances in schizophrenic patients and healthy subjects. Mol. Psychiatry 6 (6), 718–724.

Rybakowski, J.K., Borkowska, A., Czerski, P.M., Kapelski, P., Dmitrzak-Weglarz, M., Hauser, J., 2005. An association study of dopamine receptors polymorphisms and the Wisconsin Card Sorting Test in schizophrenia. J. Neural Transm. 112 (11), 1575–1582.

Schmauss, C., Haroutunian, V., Davis, K.L., Davidson, M., 1993. Selective loss of dopamine D_3-type receptor mRNA expression in parietal and motor cortices of patients with chronic schizophrenia. Proc. Natl. Acad. Sci .U. S. A. 90 (19), 8942–8946.

Schwartz, J.C., Diaz, J., Pilon, C., Sokoloff, P., 2000. Possible implications of the dopamine D_3 receptor in schizophrenia and in antipsychotic drug actions. Brain Res. Brain Res. Rev. 31 (2–3), 277–287.

Shaikh, S., Collier, D.A., Sham, P.C., Ball, D., Aitchison, K., Vallada, H., Smith, I., Gill, M., Kerwin, R.W., 1996. Allelic association between a Ser-9-Gly polymorphism in the dopamine D3 receptor gene and schizophrenia. Hum. Genet. 97 (6), 714–719.

Sokoloff, P., Giros, B., Martres, M.P., Bouthenet, M.L., Schwartz, J.C., 1990. Molecular cloning and characterization of a novel dopamine receptor (D_3) as a target for neuroleptics. Nature 347 (6289), 146–151.

Szekeres, G., Kéri, S., Juhász, A., Rimanóczy, Á., Szendi, I., Czimmer, C., Janka, Z., 2004. Role of dopamine D3 receptor (DRD3) and dopamine transporter (DAT) polymorphism in cognitive dysfunctions and therapeutic response to atypical antipsychotics in patients with schizophrenia. Am. J. Med. Genet. B Neuropsychiatr. Genet. 124B (1), 1–5.

Talkowski, M.E., Mansour, H., Chowdari, K.V., Wood, J., Butler, A., Varma, P.G., Prasad, S., Semwal, P., Bhatia, T., Deshpande, S., Devlin, B., Thelma, B.K., Nimgaonkar, V.L., 2006. Novel, replicated associations between dopamine D3 receptor gene polymorphisms and schizophrenia in two independent samples. Biol. Psychiatry 60 (6), 570–577.

Tanaka, T., Igarashi, S., Onodera, O., Tanaka, H., Takahashi, M., Maeda, M., Kameda, K., Tsuji, S., Ihda, S., 1996. Association study between schizophrenia and dopamine D3 receptor gene polymorphism. Am. J. Med. Genet. 67 (4), 366–368.

Tsai, S.J., Yu, Y.W., Chen, T.J., Chen, M.C., Hong, C.J., 2003. Association analysis for dopamine D3 receptor, dopamine D4 receptor and dopamine transporter genetic polymorphisms and P300 event-related potentials for normal young females. Psychiatr. Genet. 13 (1), 51–53.

Utsunomiya, K., Shinkai, T., De Luca, V., Hwang, R., Sakata, S., Fukunaka, Y., Chen, H.I., Ohmori, O., Nakamura, J., 2008. Genetic association between the dopamine D3 gene polymorphism (Ser9Gly) and schizophrenia in Japanese populations: evidence from a case-control study and meta-analysis. Neurosci. Lett. 444 (2), 161–165.

Vogel, M., Pfeifer, S., Schaub, R.T., Grabe, H.J., Barnow, S., Freyberger, H.J., Cascorbi, I., 2004. Decreased levels of dopamine D_3 receptor mRNA in schizophrenic and bipolar patients. Neuropsychobiology 50 (4), 305–310.

Watanabe, Y., Muratake, T., Kaneko, N., Nunokawa, A., Someya, T., 2006. No association between the *brain-derived neurotrophic factor* gene and schizophrenia in a Japanese population. Schizophr. Res. 84 (1), 29–35.

Watanabe, Y., Nunokawa, A., Kaneko, N., Someya, T., 2007. Meta-analysis of case–control association studies between the C270T polymorphism of the *brain-derived neurotrophic factor* gene and schizophrenia. Schizophr. Res. 95 (1–3), 250–252.

Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2009 | 110 | 1567–1574 doi: 10.1111/j.1471-4159.2009.06257.x

Proteomic analysis reveals novel binding partners of dysbindin, a schizophrenia-related protein

Takao Hikita,*,† Shinichiro Taya,*,† Yasutaka Fujino,*,† Setsuko Taneichi-Kuroda,* Kanae Ohta,* Daisuke Tsuboi,*,† Tomoyasu Shinoda,* Keisuke Kuroda,*,† Yusuke Funahashi,*,† Junko Uraguchi-Asaki,*,† Ryota Hashimoto†,‡,§ and Kozo Kaibuchi*,†

*Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan

†Japan Science and Technology Agency, CREST, Kawaguchi, Japan

‡The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Osaka University Graduate School of Medicine, Suita, Japan

§Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Japan

Abstract

Schizophrenia is a complex mental disorder with fairly high level of heritability. Dystrobrevin binding protein 1, a gene encoding dysbindin protein, is a susceptibility gene for schizophrenia that was identified by family-based association analysis. Recent studies revealed that dysbindin is involved in the exocytosis and/or formation of synaptic vesicles. However, the molecular function of dysbindin in synaptic transmission is largely unknown. To investigate the signaling pathway in which dysbindin is involved, we isolated dysbindin-interacting molecules from rat brain lysate by combining ammonium sulfate precipitation and dysbindin-affinity column chromatography, and identified dysbindin-interacting proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and liquid chromatography-tandem mass spectrometry. Proteins involved in protein localization process, including Munc18-1, were identified as dysbindin-interacting proteins. Munc18-1 was co-immunoprecipitated with dysbindin from rat brain lysate, and directly interacted with dysbindin *in vitro*. In primary cultured rat hippocampal neurons, a part of dysbindin was co-localized with Munc18-1 at pre-synaptic terminals. Our result suggests a role for dysbindin in synaptic vesicle exocytosis via interaction with Munc18-1.

Keywords: dysbindin, Munc18-1, proteomics, schizophrenia, susceptibility gene.

J. Neurochem. (2009) **110**, 1567–1574.

Schizophrenia is a complex genetic disorder with fairly high level of heritability (Cardno and Gottesman 2000). Several genes were identified as putative susceptibility genes for schizophrenia, including Disrupted-in-schizophrenia 1 (Millar *et al.* 2000; Blackwood *et al.* 2001; Craddock *et al.* 2005), Neuregulin 1 (Stefansson *et al.* 2002), G72 (Chumakov *et al.* 2002), Catechol-O-methyltransferase (Egan *et al.* 2001; Bilder *et al.* 2002; Shifman *et al.* 2002), and others (Craddock *et al.* 2005; Harrison and Weinberger 2005). Although the causes of schizophrenia remain unclear, it is now widely accepted that it is a neurodevelopmental and neurodegenerative disorder involving disconnectivity and disorder of the synapses.

The dystrobrevin binding protein 1 (*DTNBP1*) gene, encoding dysbindin protein, is one of the susceptibility genes for schizophrenia. An association between *DTNBP1* and schizophrenia was identified by multipoint linkage analysis of 270 Irish high-density pedigrees (Straub *et al.* 1995), and confirmed by several independent samples (Wang

Received March 4, 2009; revised manuscript received May 13, 2009; accepted June 16, 2009.

Address correspondence and reprint requests to Kozo Kaibuchi, MD., PhD, Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, 65 Tsurumai, Nagoya, Aichi, 466-8550, Japan.
E-mail: kaibuchi@med.nagoya-u.ac.jp

Abbreviations used: AP-3, Adaptor-related protein complex 3; BLOC-1, Biogenesis of lysosome-related organelles complex 1; CHC, Clathrin heavy chain; *DTNBP1*, Dystrobrevin binding protein 1; GST, Glutathione-*S*-transferase; LC/MS/MS, Liquid chromatography-tandem mass spectrometry; LRO, Lysosome-related organelle; MALDI TOF-MS, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PIP5KIIβ, Phosphatidylinositol-4-phosphate 5-kinase type II beta; V-ATPase, Vacuolar ATP synthase subunit.

et al. 1995; Morris *et al.* 2008). In patients with schizophrenia, the expression level of dysbindin is reduced in presynapses of the hippocampus (Talbot *et al.* 2004; Weickert *et al.* 2008) and prefrontal cortex (Weickert *et al.* 2004).

A mouse mutant of the *DTNBP1* homologue, *sandy* (*sdy*), was identified as a model of Hermansky-Pudlak syndrome, a genetically heterogeneous disorder characterized by oculocutaneous albinism, prolonged bleeding, and pulmonary fibrosis caused by abnormal vesicle trafficking to lysosome-related organelles (LROs), such as melanosomes and platelet-dense granules (Li *et al.* 2003). *Sdy* mice show morphological abnormalities of the actin cytoskeleton in the growth cone of cultured hippocampal neurons (Kubota *et al.* 2009), abnormal neurosecretion and vesicular morphology in neuroendocrine cells and hippocampal synapses (Chen *et al.* 2008), and increased dopamine turnover in the brain (Murotani *et al.* 2007). Behavioral analysis revealed that *sdy* mice display several behaviors related to schizophrenia (Hattori *et al.* 2008; Takao *et al.* 2008).

Dysbindin was first identified as an interacting molecule of dystrobrevin, a member of the dystrophin protein complex (Benson *et al.* 2001). In primary neurons, down-regulation of dysbindin by siRNA decreased the levels of synaptosomal-associated protein, 25 kDa and synapsin I, and reduced the release of glutamate, suggesting that decreased dysbindin may decrease the exocytosis of glutamate-containing synaptic vesicles (Numakawa *et al.* 2004). In the rat pheochromocytoma cell line (PC12), knockdown of dysbindin by small interfering RNA increased dopamine release (Kumamoto *et al.* 2006). Recent studies revealed that dysbindin is a component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). BLOC-1 is involved in the biogenesis of LROs, including melanosomes and platelet-dense granules (Dell'Angelica 2004). BLOC-1 is required to transport selective cargo proteins from the endosome to LROs (Setty *et al.* 2007). These reports suggest multiple functions of dysbindin, including intracellular protein transport toward LROs and regulation of exocytosis of synaptic vesicles. Although a number of dysbindin-interacting proteins have been reported (Rodriguez-Fernandez and Dell'angelica 2009; Guo *et al.* 2009), the molecular function of dysbindin in synaptic transmission is largely unknown.

To investigate the functions of dysbindin, we identified dysbindin-interacting molecules by affinity column chromatography, followed by matrix-assisted laser desorption time-of-flight mass spectrometry and liquid chromatography-tandem mass spectrometry (LC/MS/MS) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proteins involved in the protein localization process, including Munc18-1 and the adaptor-related protein complex 3 (AP-3) complex, were identified as dysbindin-interacting molecules, suggesting a role for dysbindin in membrane transport process, including neurotransmission.

Materials and methods

Materials and chemicals

The cDNA fragment encoding dysbindin was amplified by PCR from the human fetal brain cDNA library. The full-length dysbindin (1–352aa) was inserted into pGEX-4T-2 (GE Healthcare Biosciences, Buckinghamshire, UK) and pMAL-c2 (New England Laboratories, Beverly, MA, USA). The full-length cDNA of Munc18-1 was kindly provided by Dr. T. Südhof (University of Texas Southwestern Medical Center, Dallas, TX, USA). The cDNA encoding full-length Munc18-1 (1–594aa) was inserted into pRSET-C1 (Invitrogen, Carlsbad, CA, USA). Antibodies against AP-3 δ, p47A, AP-3 σ, Munc18-1 (mouse monoclonal), clathrin heavy chain (CHC) (Transduction Laboratories, Lexington, KY, USA), phosphatidylinositol-4-phosphate 5-kinase type II beta (PIP5KIIβ) (Abgent, San Diego, CA), USA, Munc18-1 (rabbit polyclonal) and synaptophysin (Sigma-Aldrich, St. Louis, MO, USA) were purchased. The rabbit polyclonal antibody against dysbindin was raised by immunizing the rabbit with glutathione-*S*-transferase (GST)-dysbindin, and affinity-purified using maltose-binding protein-dysbindin as a ligand. Trypsin for mass spectrometry was purchased from Promega Co. (Madison, WI, USA).

Mass spectrometry

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) analysis was performed as previously reported (Taya *et al.* 2007). Briefly, the 500 mM eluates from the affinity column were separated by 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Proteins were stained using a gold-colloidal staining kit (Bio-Rad Laboratories, Hercules, CA, USA). Spectrometry and theoretical peptide masses from the proteins were registered in the National Center for Biotechnology Information database.

For LC/MS/MS analysis, the proteins in the eluates were digested by trypsin for 16 h at 37°C after reduced alkylation, demineralization, and concentration. Nanoelectrospray tandem mass analysis was performed using Finnigan LTQ/Orbitrap mass spectrometry (Thermo Finnigan Co., San Jose, CA, USA) combined with a Paradigm MS4 HPLC system (Michrom BioResources Inc., Auburn, CA, USA). Samples were injected into the Paradigm MS4 HPLC System equipped with a Magic C18AQ column of 0.075 mm in diameter and 50 mm in length (Michrom BioResources Inc.). Reversed phase chromatograph separation was performed with solvent A (2% acetonitrile with 0.1% formic acid) and solvent B (90% acetonitrile with 0.1% formic acid) in a linear gradient (0 min, 5% B; 50 min, 30% B) at an estimated flow rate of 300 nL/min. The mass spectrometer was equipped with a XYZ interface (AMR Inc., Tokyo, Japan). Ionization was performed by a 20-mm-diameter PicoTip (New Objective Inc., Woburn, MA, USA) with a capillary voltage of 2.5 kV and temperature of 200°C. The precursor ion scan was carried out using a 400–1500 mass to charge (m/z) prior to MS/MS analysis.

Protein identification

Data from multiple MS/MS spectra were submitted to the program MASCOT (Matrix Science Inc., Boston, MA, USA) for the MS/MS

ion search. Search parameters used for MASCOT database search were as follows: Variable modifications – Carbamidomethyl (C) and Oxidation (M); Mass values – Monoisotopic; Protein mass – Unrestricted; Peptide mass tolerance – ±10 ppm; Fragment mass tolerance – ±0.8 Da; Max missed cleavages; Instrument type: ESI-FTICR.

Immunoprecipitation assay

The rat brain P2 fraction was solubilized by the addition of lysis buffer (20 mM Tris–HCl, pH 8.0; 1 mM EDTA; 50 mM NaCl; 1% NP-40, Sigma-Aldrich, St Louis, MO, USA) and then clarified by centrifugation at 100,000 *g* for 20 min. The soluble supernatants were incubated with control rabbit IgG or rabbit anti-dysbindin antibody for 2 h. The immunocomplexes were precipitated with Protein G-Sepharose 4B (GE Healthcare Biosciences). The immunocomplexes were washed thrice with lysis buffer, the bound proteins were eluted with the addition of sodium dodecyl sulfate sample buffer, and then subjected to immunoblot analyses with specific antibodies. To detect dysbindin, the Rabbit IgG TrueBlot™ Set (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions.

In vitro binding assay

Glutathione-*S*-transferase or His fusion proteins were expressed in *Escherichia coli* BL21 (DE3) cells and purified according to the manufacturer's instructions. His-Munc18-1 or His-RhoGDI (200 pmol) was mixed with glutathione–Sepharose 4B beads coated with 100 pmol of either GST or GST-dysbindin in buffer A [20 mM Tris–HCl, pH 8.0; 1 mM EDTA; 1 mM dithiothreitol; 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonate (CHAPS)]. The bound His-protein was co-eluted with GST fusion proteins by the addition of sample buffer containing sodium dodecyl sulfate. Portions of the eluates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by the immunoblot analysis with the anti-His antibody.

Preparation and culture of rat hippocampal neurons

Hippocampal neurons were prepared from E18 rat embryos using papain (Inagaki *et al.* 2001). Neurons were seeded on poly-D-lysine-coated cover slips and cultured in Neurobasal medium (Invitrogen) supplemented with a B-27 supplement (Invitrogen) and 1 mM glutamine.

Immunofluorescence analysis

Hippocampal neurons were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 min and treated with phosphate-buffered saline containing 0.3% TritonX-100 for 10 min. Neurons were incubated overnight with primary antibodies, washed, and incubated for 1 h with secondary antibodies. Immunofluorescence analyses were examined with a laser scanning confocal microscope (Model LSM510, Zeiss, Oberkochen, Germany). Triple staining of rat hippocampal neurons was performed using Molecular Probes' Zenon Labeling Kits (Invitrogen) according to the manufacturer's instructions. Briefly, anti-dysbindin and anti-Munc18-1 (both rabbit polyclonal antibody) were labeled using Zenon alexa-fluor 488 and 555, respectively. Post-fixed samples were stained using anti-synaptophysin antibody (mouse monoclonal antibody) followed with anti-mouse alexa-647 secondary antibody to visualize presynaptic terminals.

Results

Identification of dysbindin-interacting molecules

To isolate dysbindin-interacting molecules, we performed affinity column chromatography. P5-8 rat brain cytosol (cytosol) or membrane extract fraction (P2 extract), both of which were concentrated with ammonium sulfate precipitation, was used as starting materials (Figure S1a). Ammonium sulfate precipitation of rat brain lysate is essential, because precipitation of proteins using ammonium sulfate concentrates and delipidates the samples, and dramatically improves the efficiency of the affinity column chromatography.

Cytosol or P2 extract was loaded onto the affinity column coated with GST-dysbindin or the negative control GST purified from *E. coli* (Figure S1b). The affinity columns were washed with TED buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA) and the interacting proteins were then sequentially eluted with TED buffer (20 mM Tris-HCl, pH 8.0; 1 mM EDTA) containing 50 mM NaCl, 500 mM NaCl or 10 mM glutathione (Figure S1b). From the P2 extract-loaded affinity columns, proteins with molecular masses of 210, 150, 130, 120, 70, 45, and 35 kDa were detected by silver staining in the 500 mM NaCl eluate from the GST-dysbindin affinity column, but not the GST affinity column (Fig. 1a). Specific bands of similar molecular size were detected in the eluates of the cytosol-loaded affinity column; however, intensity of these bands were weaker than the bands detected from eluates from P2 extract-loaded affinity column. Furthermore, a number of non-specific bands were observed in the eluates of the cytosol-loaded column (Fig. 1a). This result led us to focus on the analysis of the eluate from the P2 extract-loaded affinity column. Candidates for dysbindin-interacting proteins were identified by MALDI TOF-MS and LC/MS/MS analyses.

A wide range of molecules interacted specifically with GST-dysbindin, especially proteins involved in protein localization (Fig. 1b and Table S1). By MALDI TOF-MS analyses, proteins with molecular masses of ~210, 150, 70, 45, and 35 were identified as rat AP-3 delta subunit (AP-3 δ), AP-3 β subunit, Munc18-1, AP-3 μ subunit, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase, glycogen synthase kinase-3-beta, vacuolar ATP synthase subunit C (V-ATPase C), V-ATPase D, glyceraldehyde-3-phosphate dehydrogenase. By LC/MS/MS, several proteins involved in the protein localization process, including Munc18-1, AP-3 subunits, CHC, V-ATPase E, PIP5K type-2 alpha and beta, ADP-ribosylation factor 1 and ADP-ribosylation factor GTPase activating protein with SH3 domain, and ankyrin repeat and PH domain 1 were identified (Table S1). The AP-3 complex is a member of the adaptor protein complex family that is involved in clathrin-mediated membrane budding (Robinson and Bonifacino 2001). The AP-3 complex is composed of four subunits, δ, β, μ, and σ. Two isoforms, the ubiquitously

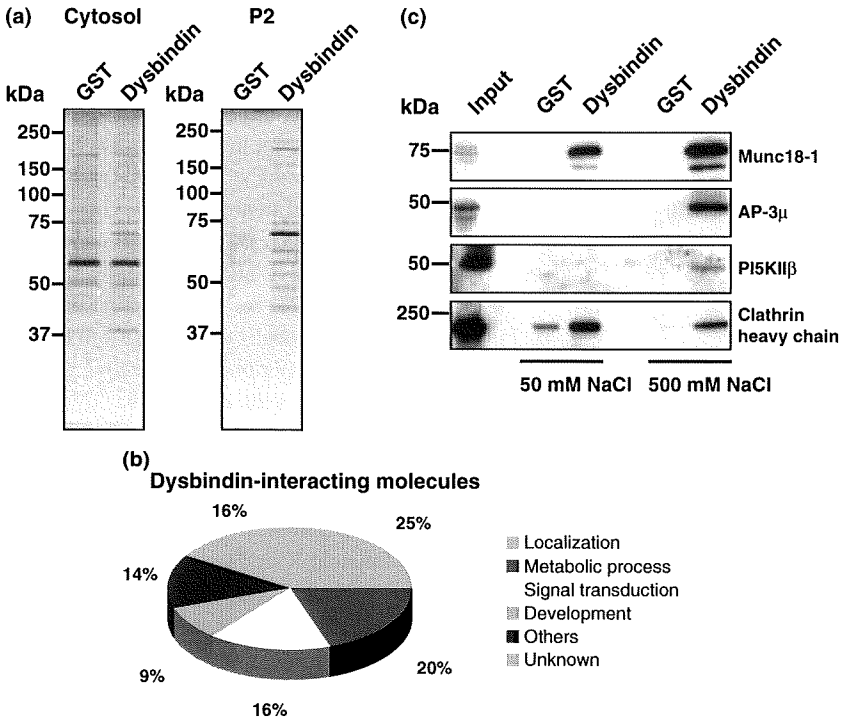


Fig. 1 Identification of the dysbindin-interacting molecules. (a) The 500 mM NaCl eluates of affinity columns loaded with cytosol or P2 extract were analyzed by SDS–PAGE, followed by silver staining. (b) Summary of dysbindin-interacting molecules. Proteins identified by MALDI-TOF-MS and LC/MS/MS were classified into six categories according to gene ontology: localization; metabolic; signal transduction; cell communication; development; others; and unknown function. (c) Validation of MS results. The 50 mM and 500 mM NaCl eluates of the P2 extract-loaded affinity column were subjected to western blotting using specific antibodies against the indicated proteins.

expressed AP-3a and the neuron-specific AP-3b, have been reported (Odorizzi *et al.* 1998). All subunits of both AP-3 complexes, AP-3a and AP-3b, were identified by LC/MS/MS. The previous study shows that the AP-3 complex interacts with BLOC-1, which includes dysbindin (Di Pietro *et al.* 2006). Munc18-1 is a member of the Sec1/Munc18 protein family (SM protein) that interacts with t-soluble N-ethylmaleimide sensitive factor attachment proteins receptor syntaxin1A (Hata *et al.* 1993). Munc18-1 is a neuron-specific protein and is essential for the exocytosis of synaptic vesicles (Südhof 2004). 2′,3′-Cyclic-nucleotide 3′-phosphodiesterase is distributed in cells of oligodendrocyte lineage and is used as a marker of myelin-forming cells (Nave and Trapp 2008). Glycogen synthase kinase-3-beta is a serine/threonine protein kinase that is involved in the regulation of metabolism, the cytoskeleton, and gene expression (Cohen and Frame 2001). V-ATPase C, D, and E are subunits of the multisubunit ATP-driven proton pump that functions in vesicular acidification (Forgac 2007). PIP5K is a phosphoinositide kinase, which produces the versatile phospholipid phosphatidylinositol 4,5-bisphosphate), and its interaction with the AP-3 complex has been previously reported (Loijens and Anderson 1996; Kanaho *et al.* 2007). Many of these proteins are involved in protein localization, suggesting a role for dysbindin in the membrane transport process.

Validation of dysbindin-interacting molecules

To confirm the identities of the candidate dysbindin-interacting proteins, we performed immunoblotting using specific antibodies (Fig. 1c). The μ subunit of the AP-3 complex, Munc18-1, and PIP5KIIβ were strongly detected in 500 mM NaCl eluates of GST-dysbindin-immobilized affinity column, while CHC was mainly detected in the 50 mM NaCl eluates (Fig. 1c). This result suggests that the AP-3 complex, Munc18-1, and PIP5KIIβ strongly interact with dysbindin. To confirm the interaction between dysbindin and the affinity-purified molecules under physiological conditions, we performed immunoprecipitation of endogenous dysbindin from rat brain P2 lysate (Fig. 2). When endoge-

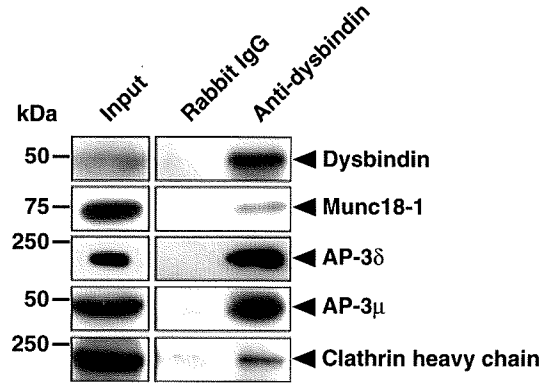


Fig. 2 *In vivo* complex formation with dysbindin and identified molecules. The P2 fraction from C57B6 mice brain homogenate that was dissolved in buffer A containing 50 mM NaCl and 1% NP-40 was used in an immunoprecipitation assay with antibodies against dysbindin. Pre-immune rabbit IgG was used as a negative control. Bound proteins and assay input were analyzed by immunoblotting with antibodies against the indicated proteins. Aliquots of the original samples (10% input) and eluates (30%) were subjected to SDS–PAGE.

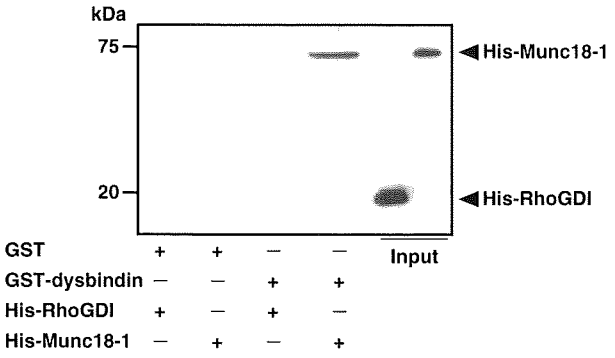


Fig. 3 *In vitro* complex formation with dysbindin and Munc18-1. An *in vitro* binding assay was performed using purified recombinant proteins. Beads immobilized with GST alone or GST-dysbindin was incubated with His-Munc18-1 or His-RhoGDI as a negative control. Bound proteins were analyzed by immunoblotting with antibodies against the His tag.

nous dysbindin was immunoprecipitated using the anti-dysbindin antibody, the μ and δ subunits of the AP-3 complex, Munc18-1, and CHC were identified from the immunoprecipitate (Fig. 2). This result suggests that dysbindin forms a complex with the AP-3 complex, Munc18-1, and CHC *in vivo*.

Dysbindin is localized at pre- and post-synapses *in vivo* (Talbot *et al.* 2004), exists in the synaptic vesicle fraction (Taneichi-Kuroda *et al.* 2009), and is involved in the exocytosis of neurotransmitters (Numakawa *et al.* 2004; Kumamoto *et al.* 2006). *Sandy* mice, which contain a mutation of the dysbindin homologue, show mild defects in both the formation and the exocytosis of synaptic vesicles (Chen *et al.* 2008). However, how dysbindin regulates the exocytosis of synaptic vesicles is still unclear. These reports led us to focus on the interaction between dysbindin and Munc18-1. To confirm whether dysbindin directly interacts with Munc18-1, we performed an *in vitro* binding assay using purified recombinant proteins. GST- or GST-dysbindin-immobilized beads were incubated with His-Munc18-1 or His-RhoGDI, a negative control protein. His-Munc18-1, but not the control His-RhoGDI, interacted with GST-dysbindin (Fig. 3). This result indicates that there is a direct interaction between dysbindin and Munc18-1.

Co-localization of dysbindin and Munc18-1 in hippocampal neurons

A previous report shows that dysbindin is localized at both pre- and post-synapses in the hippocampus (Talbot *et al.* 2006). To examine whether dysbindin and Munc18-1 are co-localized at pre-synaptic terminals, we performed triple staining of DIV21 hippocampal neurons, using anti-dysbindin, anti-Munc18-1 and anti-synaptophysin antibodies. We found that dysbindin, Munc18-1 and synaptophysin were partially co-localized in DIV21 rat hippocampal neurons (Fig. 4). Furthermore, the co-localization of dysbindin and Munc18-1 was observed at the periphery of MAP-2 positive processes (Figure S2). These results suggest that dysbindin was co-localized with Munc18-1 at pre-synaptic terminal. However, the large part of dysbindin was not co-localized with the immunosignal of Munc18-1 (Fig. 4). The AP-3 complex is largely co-localized with dysbindin in both pre- and post-synapses (Taneichi-Kuroda *et al.* 2009). These results suggest that a limited population of dysbindin interacts with Munc18-1 *in vivo*.

Discussion

To clarify the molecular function of dysbindin protein, we designed this study to identify the dysbindin-interacting proteins by affinity column chromatography, followed by MALDI TOF-MS and LC/MS/MS. The dysbindin-interacting molecules were strongly detected in the P2 extract, a fraction that is enriched with membrane-bound proteins (Figure S1 and Fig. 1). About 25% of the dysbindin-interacting proteins, isolated from the P2 extract, were involved in the protein localization process (Fig. 1b and Table S1). These results strongly support previous studies suggesting a role for dysbindin in the membrane transport process, especially in membrane fusion. Notably, several proteins detected from the P2 extract-loaded column, including Munc18-1 and subunits of the AP-3 complex, were detected in an eluate form the cytosol-loaded column (data not shown).

We identified Munc18-1 as a novel dysbindin-interacting molecule. Mutation of the Munc18-1 gene in which amino acid replacement and destabilization of Munc18-1 protein

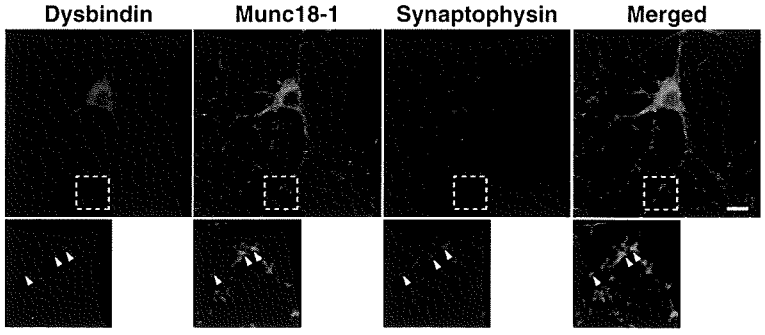


Fig. 4 Co-localization of dysbindin with Munc18-1 at pre-synaptic terminals. Dysbindin (red), Munc18-1 (green) and synaptophysin (blue) were visualized by triple staining method in DIV21 primary-cultured rat hippocampal neurons. Arrowheads indicate the co-localization of dysbindin, Munc18-1 and synaptophysin. Scale bar, 20 μm.

occurs causes Early infantile epileptic encephalopathy with suppression-burst (Saitsu *et al.* 2008). Munc18-1 KO mice show defects in synaptic vesicle fusion in the CNS (Verhage *et al.* 2000). Munc18-1 interacts with Syntaxin1A, a t-SNARE that forms the SNARE complex with VAMP2 and SNAP25 (Toonen and Verhage 2007). A recent *in vitro* study showed that Munc18-1 positively regulates a SNARE complex-mediated membrane fusion process (Shen *et al.* 2007). Although the role of Munc18-1 in exocytosis is controversial, these reports suggest that Munc18-1 plays a critical role in synaptic vesicle exocytosis. In our study, dysbindin interacted with Munc18-1 both *in vitro* and *in vivo*. We found that a part of dysbindin was co-localized with Munc18-1 at pre-synaptic terminals. Although the functional meaning of this interaction is still unclear, dysbindin deficiency down-regulates the kinetics of neurotransmitter release (Chen *et al.* 2008). We speculate that dysbindin positively regulates exocytosis via its interaction between Munc18-1.

The AP-3 complex is a member of the adaptor protein complex family that is involved in the biogenesis of LROs and synaptic vesicle formation (Newell-Litwa *et al.* 2007). The AP-3 complex interacts functionally, physically, and genetically with BLOC-1, which includes dysbindin. BLOC-1 binds to the AP-3 complex in fibroblasts (Di Pietro *et al.* 2006) and co-exists on the synaptic-like microvesicles in PC12 cells (Salazar *et al.* 2005). A deficiency in BLOC-1 results in the altered targeting of several AP-3 cargo proteins, including phosphatidylinositol 4-kinase type II alpha and vesicle-associated membrane protein 7 (Salazar *et al.* 2006). Meanwhile, in melanocytes, the AP-3 complex and BLOC-1 are reported to mediate the transport of different proteins. In our study, the AP-3 complex was the sole adaptor protein identified as a dysbindin-interacting molecule. Recently, we found that dysbindin directly interacts with the μ subunit of the AP-3 complex via the Yxxϕ motif (tyrosine motif, where ϕ is a hydrophobic amino acid) of dysbindin (Taneichi-Kuroda *et al.* 2009). The Yxxϕ motif is reported to function as a sorting signal for lysosomal membrane proteins such as lysosomal-associated membrane protein 1. These results suggest that BLOC-1 mediates budding of specific cargo proteins, including phosphatidylinositol-5-phosphate 4-kinase typeII alpha, via an interaction with the AP-3 complex.

In this study, no subunit of BLOC-1 was detected. We confirmed by western blotting that snapin, a subunit of BLOC-1, exists in the P2 extract (data not shown). By immunoprecipitation of endogenous dysbindin, snapin was co-precipitated with dysbindin (data not shown). We speculate that subunits of BLOC-1 interact tightly, thus GST-dysbindin could not interact with the endogenous BLOC-1. Beta dystrobrevin was detected by LC/MS/MS analysis of the 500 mM NaCl eluate of P2-loaded column, but with very low MASCOT score (data not shown).

We speculate that there are at least two populations of dysbindin. A large population of dysbindin forms BLOC-1 and interacts with the AP-3 complex, regulating the protein transport at the site of budding. A limited population of dysbindin functions as an adaptor protein that connects the BLOC-1/AP-3-mediated synaptic vesicle to the membrane-bound SNARE complex via Munc18-1, and then positively regulates synaptic vesicle exocytosis.

Acknowledgements

We thank Dr. T. Südhof for providing Munc18-1 cDNA. We also thank Ms. Yuko Yamashita, Satoko Suzuki, and Ai Nimura for their technical assistance; and Ms. Takako Ishii for secretarial assistance. This research was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), a Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science, the MEXT 21st Century Center of Excellence Program, a Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labour and Welfare, a Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the MEXT (17025021), the Japan Health Sciences Foundation (Research on Health Sciences Focusing on Drug Innovation), the Core Research for Evolutional Science and Technology, a Grant-in-Aid for Scientific Research on Priority Areas-Integrative Brain Research (Shien) – from MEXT in Japan, a Grant-in-Aid from the Neuroinformatics Japan Center, RIKEN, and a Grant-in-Aid from the Institute for Bioinformatics Research and Development of Japan Science and Technology Agency.

Supporting Information

Additional Supporting information may be found in the online version of this article.

Figure S1. Preparation of cytosol and P2 extract for affinity columns.

Figure S2. Localization of dysbindin and Munc18-1 at periphery of dendrites.

Table S1. List of dysbindin-interacting molecules.

Appendix S1. Supplemental methods.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

References

Benson M. A., Newey S. E., Martin-Rendon E., Hawkes R. and Blake D. J. (2001) Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. *J. Biol. Chem.* **276**, 24232–24241.

Bilder R. M., Volavka J., Czobor P. *et al.* (2002) Neurocognitive correlates of the COMT Val(158)Met polymorphism in chronic schizophrenia. *Biol. Psychiatry* **52**, 701–707.

Blackwood D. H., Fordyce A., Walker M. T., St Clair D. M., Porteous D. J. and Muir W. J. (2001) Schizophrenia and affective disorders–cosegregation with a translocation at chromosome 1q42 that directly disrupts brain-expressed genes: clinical and P300 findings in a family. *Am. J. Hum. Genet.* **69**, 428–433.

Cardno A. G. and Gottesman II (2000) Twin studies of schizophrenia: from bow-and-arrow concordances to star wars Mx and functional genomics. *Am. J. Med. Genet.* **97**, 12–17.

Chen X. W., Feng Y. Q., Hao C. J. *et al.* (2008) DTNBP1, a schizophrenia susceptibility gene, affects kinetics of transmitter release. *J. Cell Biol.* **181**, 791–801.

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

Cohen P. and Frame S. (2001) The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2**, 769–776.

Craddock N., O'Donovan M. C. and Owen M. J. (2005) The genetics of schizophrenia and bipolar disorder: dissecting psychosis. *J. Med. Genet.* **42**, 193–204.

Dell'Angelica E. C. (2004) The building BLOC(k)s of lysosomes and related organelles. *Curr. Opin. Cell Biol.* **16**, 458–464.

Di Pietro S. M., Falcon-Perez J. M., Tenza D., Setty S. R., Marks M. S., Raposo G. and Dell'Angelica E. C. (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. *Mol. Biol. Cell* **17**, 4027–4038.

Egan M. F., Goldberg T. E., Kolachana B. S., Callicott J. H., Mazzanti C. M., Straub R. E., Goldman D. and Weinberger D. R. (2001) Effect of COMT Val108/158 Met genotype on frontal lobe function and risk for schizophrenia. *Proc. Natl. Acad. Sci. USA* **98**, 6917–6922.

Forgac M. (2007) Vacuolar ATPases: rotary proton pumps in physiology and pathophysiology. *Nat. Rev. Mol. Cell Biol.* **8**, 917–929.

Guo A. Y., Sun J., Riley B. P., Thiselton D. L., Kendler K. S. and Zhao Z. (2009) The dystrobrevin-binding protein 1 gene: features and networks. *Mol. Psychiatry* **14**, 18–29.

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

Hata Y., Slaughter C. A. and Südhof T. C. (1993) Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. *Nature* **366**, 347–351.

Hattori S., Murotani T., Matsuzaki S. *et al.* (2008) Behavioral abnormalities and dopamine reductions in sdy mutant mice with a deletion in Dtnbp1, a susceptibility gene for schizophrenia. *Biochem. Biophys. Res. Commun.* **373**, 298–302.

Inagaki N., Chihara K., Arimura N., Menager C., Kawano Y., Matsuo N., Nishimura T., Amano M. and Kaibuchi K. (2001) CRMP-2 induces axons in cultured hippocampal neurons. *Nat. Neurosci.* **4**, 781–782.

Kanaho Y., Kobayashi-Nakano A. and Yokozeki T. (2007) The phosphoinositide kinase PIP5K that produces the versatile signaling phospholipid PI4,5P(2). *Biol. Pharm. Bull.* **30**, 1605–1609.

Kubota K., Kumamoto N., Matsuzaki S. *et al.* (2009) Dysbindin engages in c-Jun N-terminal kinase activity and cytoskeletal organization. *Biochem. Biophys. Res. Commun.* **379**, 191–195.

Kumamoto N., Matsuzaki S., Inoue K., Hattori T., Shimizu S., Hashimoto R., Yamatodani A., Katayama T. and Tohyama M. (2006) Hyperactivation of midbrain dopaminergic system in schizophrenia could be attributed to the down-regulation of dysbindin. *Biochem. Biophys. Res. Commun.* **345**, 904–909.

Li W., Zhang Q., Oiso N. *et al.* (2003) Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat. Genet.* **35**, 84–89.

Loijens J. C. and Anderson R. A. (1996) Type I phosphatidylinositol-4-phosphate 5-kinases are distinct members of this novel lipid kinase family. *J. Biol. Chem.* **271**, 32937–32943.

Millar J. K., Wilson-Annan J. C., Anderson S. *et al.* (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Hum. Mol. Genet.* **9**, 1415–1423.

Morris D. W., Murphy K., Kenny N. *et al.* (2008) Dysbindin (DTNBP1) and the biogenesis of lysosome-related organelles complex 1 (BLOC-1): main and epistatic gene effects are potential contributors to schizophrenia susceptibility. *Biol. Psychiatry* **63**, 24–31.

Murotani T., Ishizuka T., Hattori S., Hashimoto R., Matsuzaki S. and Yamatodani A. (2007) High dopamine turnover in the brains of Sandy mice. *Neurosci. Lett.* **421**, 47–51.

Nave K. A. and Trapp B. D. (2008) Axon-glial signaling and the glial support of axon function. *Annu. Rev. Neurosci.* **31**, 535–561.

Newell-Litwa K., Seong E., Burmeister M. and Faundez V. (2007) Neuronal and non-neuronal functions of the AP-3 sorting machinery. *J. Cell Sci.* **120**, 531–541.

Numakawa T., Yagasaki Y., Ishimoto T. *et al.* (2004) Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. *Hum. Mol. Genet.* **13**, 2699–2708.

Odorizzi G., Cowles C. R. and Emr S. D. (1998) The AP-3 complex: a coat of many colours. *Trends Cell Biol.* **8**, 282–288.

Robinson M. S. and Bonifacino J. S. (2001) Adaptor-related proteins. *Curr. Opin. Cell Biol.* **13**, 444–453.

Rodriguez-Fernandez I. A. and Dell'angelica E. C. (2009) A data-mining approach to rank candidate protein-binding partners-The case of biogenesis of lysosome-related organelles complex-1 (BLOC-1). *J. Inherit. Metab. Dis.* **32**, 190–203.

Saitsu H., Kato M., Mizuguchi T. *et al.* (2008) De novo mutations in the gene encoding STXBP1 (MUNC18-1) cause early infantile epileptic encephalopathy. *Nat. Genet.* **40**, 782–788.

Salazar G., Craige B., Wainer B. H., Guo J., De Camilli P. and Faundez V. (2005) Phosphatidylinositol-4-kinase type II alpha is a component of adaptor protein-3-derived vesicles. *Mol. Biol. Cell* **16**, 3692–3704.

Salazar G., Craige B., Styers M. L. *et al.* (2006) BLOC-1 complex deficiency alters the targeting of adaptor protein complex-3 cargoes. *Mol. Biol. Cell* **17**, 4014–4026.

Setty S. R., Tenza D., Truschel S. T. *et al.* (2007) BLOC-1 is required for cargo-specific sorting from vacuolar early endosomes toward lysosome-related organelles. *Mol. Biol. Cell* **18**, 768–\780.

Shen J., Tareste D. C., Paumet F., Rothman J. E. and Melia T. J. (2007) Selective activation of cognate SNAREpins by Sec1/Munc18 proteins. *Cell* **128**, 183–195.

Shifman S., Bronstein M., Sternfeld M. *et al.* (2002) A highly significant association between a COMT haplotype and schizophrenia. *Am. J. Hum. Genet.* **71**, 1296–1302.

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

Straub R. E., MacLean C. J., O'Neill F. A. *et al.* (1995) A potential vulnerability locus for schizophrenia on chromosome 6p24-22: evidence for genetic heterogeneity. *Nat. Genet.* **11**, 287–293.

Südhof T. C. (2004) The synaptic vesicle cycle. *Annu. Rev. Neurosci.* **27**, 509–547.

Takao K., Toyama K., Nakanishi K., Hattori S., Takamura H., Takeda M., Miyakawa T. and Hashimoto R. (2008) Impaired long-term memory retention and working memory in sdy mutant mice with a deletion in Dtnbp1, a susceptibility gene for schizophrenia. *Mol. Brain* **1**, 11.

Talbot K., Eidem W. L., Tinsley C. L. *et al.* (2004) Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. *J. Clin. Invest.* **113**, 1353–1363.

Talbot K., Cho D. S., Ong W. Y. *et al.* (2006) Dysbindin-1 is a synaptic and microtubular protein that binds brain snapin. *Hum. Mol. Genet.* **15**, 3041–3054.

Taneichi-Kuroda S., Taya S., Hikita T., Fujino Y. and Kaibuchi K. (2009) Direct interaction of Dysbindin with the AP-3 complex via its μ subunit. *Neurochem. Int.* **54**, 431–438.

Taya S., Shinoda T., Tsuboi D. *et al.* (2007) DISC1 regulates the transport of the NUDEL/LIS1/14-3-3epsilon complex through kinesin-1. *J. Neurosci.* **27**, 15–26.

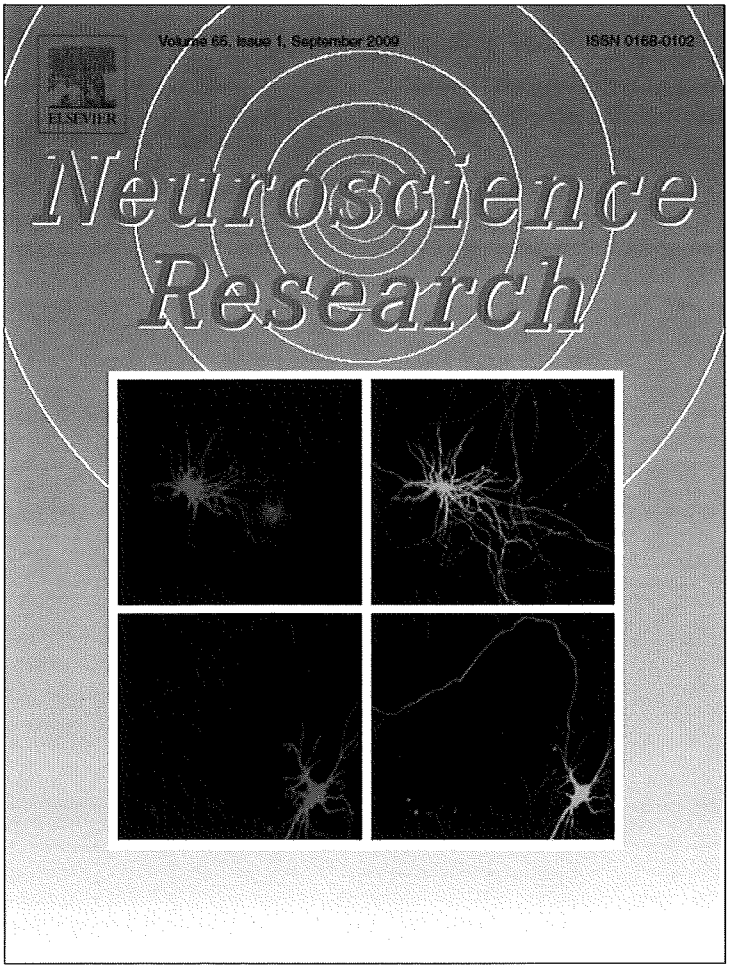
Toonen R. F. and Verhage M. (2007) Munc18-1 in secretion: lonely Munc joins SNARE team and takes control. *Trends Neurosci.* **30**, 564–572.

Verhage M., Maia A. S., Plomp J. J. *et al.* (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. *Science* **287**, 864–869.

Wang S., Sun C. E., Walczak C. A., Ziegle J. S., Kipps B. R., Goldin L. R. and Diehl S. R. (1995) Evidence for a susceptibility locus for schizophrenia on chromosome 6pter-p22. *Nat. Genet.* **10**, 41–46.

Weickert C. S., Straub R. E., McClintock B. W., Matsumoto M., Hashimoto R., Hyde T. M., Herman M. M., Weinberger D. R. and Kleinman J. E. (2004) Human dysbindin (DTNBP1) gene expression in normal brain and in schizophrenic prefrontal cortex and midbrain. *Arch. Gen. Psychiatry* **61**, 544–555.

Weickert C. S., Rothmond D. A., Hyde T. M., Kleinman J. E. and Straub R. E. (2008) Reduced DTNBP1 (dysbindin-1) mRNA in the hippocampal formation of schizophrenia patients. *Schizophr. Res.* **98**, 105–110.



Neuroscience Research 65 (2009) 113–115

Contents lists available at ScienceDirect

Neuroscience Research

journal homepage: www.elsevier.com/locate/neures

Rapid communication

Association analysis between schizophrenia and the AP-3 complex genes

Ryota Hashimoto [a,b,c,d,*], Kazutaka Ohi [b,d], Takeya Okada [c], Yuka Yasuda [a,b,d], Hidenaga Yamamori [b,e], Hiroaki Hori [c], Takao Hikita [d,f], Shinichiro Taya [d,f], Osamu Saitoh [g], Asako Kosuga [h], Masahiko Tatsumi [i], Kunitoshi Kamijima [h], Kozo Kaibuchi [d,f], Masatoshi Takeda [a,b], Hiroshi Kunugi [c]

[a] The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan
[b] Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[c] Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
[d] Japan Science and Technology Agency, CREST, Kawaguchi, Saitama, Japan
[e] Department of Molecular Neuropsychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan
[f] Department of Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Aichi, Japan
[g] Department of Psychiatry, National Center Hospital, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan
[h] Department of Psychiatry, Showa University School of Medicine, Shinagawaku, Tokyo, Japan
[i] Yokohama Shinryo Clinic, Yokohama, Kanagawa, Japan

ARTICLE INFO

Article history:
Received 1 April 2009
Received in revised form 16 May 2009
Accepted 19 May 2009
Available online 27 May 2009

Keywords:
Schizophrenia
AP3M1
Adaptor protein (AP)-3 complex
Dysbindin
Single nucleotide polymorphism

ABSTRACT

A susceptibility gene for schizophrenia, dysbindin, is a component of BLOC-1, which interacts with the adaptor protein (AP)-3 complex. As a direct interaction between dysbindin and AP-3 complex was reported, we examined a possible association between 16 SNPs in the AP3 complex genes and schizophrenia using 432 cases and 656 controls. Nominal association between rs6688 in the AP3M1 gene and schizophrenia ($\chi^2 = 6.33$, $P = 0.012$, odds ratio = 0.80) was no longer positive after correction for multiple testing (corrected $P = 0.192$). The present results suggest that AP3 complex genes might not play a major role in the pathogenesis of schizophrenia in this population.

1. Introduction

Schizophrenia is a complex genetic disorder characterized by profound disturbances of cognition, emotion and social functioning. It affects approximately 1% of the general population worldwide. Dysbindin-1 gene (*DTNBP1*: dystrobrevin binding protein 1) has been identified as a susceptibility gene for schizophrenia (Harrison and Weinberger, 2005). Postmortem brain studies have indicated reduced expression of dysbindin in hippocampus and prefrontal cortices of patients with schizophrenia (Talbot et al., 2004; Weickert et al., 2004). Long-term treatment of mice with antipsychotics did not alter the expression levels of dysbindin in the frontal cortex and hippocampus (Chiba et al., 2006; Talbot et al., 2004), suggesting that the prior evidence of decreased expression of DTNBP1 in the postmortem brains of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. Dysbindin was also associated with human cognition such as general cognitive ability and memory performance, which is deeply impaired in schizophrenia (Burdick et al., 2006; Hashimoto et al., 2009). Mice lacking dysbindin, sandy mice, also showed abnormal behaviors including memory disturbance (Hattori et al., 2008; Takao et al., 2008). Although there was a large number of evidence for association between schizophrenia and dysbindin including postmortem brain, cognition, and animal model findings in addition to genetic findings, recent large scale genome wide association study did not support the association between schizophrenia and this gene (O'Donovan et al., 2008).

Dysbindin is one of the essential components of the biogenesis of lysosome-related organelles complex 1 (BLOC-1) (Li et al., 2003). BLOC-1 interacts physically and functionally with the adaptor protein (AP)-3 complex, which consists of four subunits (β, δ, σ and μ) (Newell-Litwa et al., 2007). AP-3 complex is essential for vesicle or protein sorting. The AP-3 complex is involved in the formation of lysosome-related organelles and a subset of synaptic vesicles by sorting of specific cargo proteins such as tyrosinase-related

* Corresponding author at: The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Osaka University Graduate School of Medicine, D3, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3074; fax: +81 6 6879 3059.
E-mail address: hashimor@psy.med.osaka-u.ac.jp (R. Hashimoto).

0168-0102/$ – see front matter © 2009 Elsevier Ireland Ltd and the Japan Neuroscience Society. All rights reserved.
doi:10.1016/j.neures.2009.05.008

Table 1
Allele and genotype frequencies of SNPs in the AP3M1 gene between the patients with schizophrenia and controls.

Gene	Marker		Location	M/m	SCZ			CON			Genotypic p-value (df = 2)	SCZ MAF	CON	Allelic p-value (df = 1)	OR
	db SNP number	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m					
AP3M1 (reverse strand)	rs6688	24432831	Exon 10, 3′UTR	C/T	**0.391**	**0.466**	**0.143**	**0.323**	**0.493**	**0.184**	**0.041**	**0.376**	**0.430**	**0.012**	**0.797**
	rs3812639	24462835	5′-region	A/C	0.481	0.436	0.083	0.469	0.421	0.110	0.353	0.301	0.320	0.342	0.913

SCZ: schizophrenia, CON: controls, m: minor allele, M: major allele, MAF: minor allele frequency, OR: odds ratio.
[a] db SNP build 129.

protein1, melanosomal membrane protein, lysosomal-associated membrane proteins, and zinc transporter 3 (Newell-Litwa et al., 2007). As abnormal neurotransmission in synapse is considered to be involved in the pathophysiology of schizophrenia (Stephan et al., 2006), AP-3 complex genes could be candidate genes for schizophrenia. Moreover, we reported direct interaction of dysbindin with the AP-3 complex via its μ subunit (Taneichi-Kuroda et al., 2009). Thus, we attempted to examine the possible association of the AP3M1 gene (μ subunit of the AP-3 complex) with schizophrenia.

There were 432 patients with schizophrenia [55.3% male, with a mean age of 44.6 years (SD 14.0)] and 656 healthy controls [41.2% male, with a mean age of 44.5years (SD 16.5)]. All the subjects were biologically unrelated Japanese. Patients were recruited at the National Center Hospital of Neurology and Psychiatry or the Department of Psychiatry, Showa University School of Medicine. Healthy controls were recruited from local advertisements, including hospital and institutional staffs. Consensus diagnosis was made for each patient by at least two trained psychiatrists, according to the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV), based on unstructured clinical interviews. No subject with additional axis I diagnoses, such as major depression, bipolar disorder and schizoaffective disorder, was included in this study. Controls, including the hospital and institutional staffs, were recruited through local advertisements. Psychiatrically healthy controls were evaluated to exclude individuals who had current or past contact with psychiatric services using unstructured interviews. After a description of the study, written informed consent was obtained from every subject. The study protocol was approved by institutional ethics committees and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Venous blood was drawn from subjects and genomic DNA was extracted from whole blood according to standard procedures. Single nucleotide polymorphisms (SNPs) in the AP3 complex genes (AP3M1, AP3M2, AP3D1, AP3B1, AP3B2) were genotyped using the TaqMan 5′-exonuclease allelic discrimination assay, as described previously (Hashimoto et al., 2006, 2007). Sixteen SNPs with more than 0.15 of minor allele frequency were selected from the public database (HAPMAP: http://www.hapmap.org/index.html.ja) in order to cover the each gene (10–50 KB per SNP). Average distance to cover one gene was 22.4 KB per SNP. Primers and probes for detection of the SNPs are available upon request. Statistical analysis of genetic association study was performed using SNPAlyse (DYNACOM, Yokohama, Japan). The presence of Hardy–Weinberg equilibrium was examined by using the χ^2-test for goodness of fit. Allele and genotype distributions between patients and controls were analyzed by the χ^2-test for independence. Statistical significance was defined as $P < 0.05$. Bonferroni correction was applied for multiple testing. We performed power calculations using the Power Calculator for Two Stage Association Studies (http://www.sph.umich.edu/csg/abecasis/CaTS/; Skol et al., 2006). Power (>0.80) was calculated under prevalence of 0.01, the allele frequency in patients at 0.376 (rs6688) and an alpha level of 0.05 using a multiplicative model, assuming varying degrees of the odds ratio.

We have recently demonstrated a direct interaction of dysbindin, a susceptibility gene for schizophrenia, with the AP-3 complex via its μ subunit (Taneichi-Kuroda et al., 2009). Therefore, in the present study, we examined the possible association between genetic variants in the AP3M1 gene, the μ subunit of the AP-3 complex, and schizophrenia. A significant difference in allele and genotype frequencies in the rs6688 of the AP3M1 gene was observed, while there was no difference between allele or genotype frequency of the rs3812639 (Table 1). The major allele of the rs6688 of the AP3M1 gene was in excess in patients with schizophrenia ($\chi^2 = 6.33$, $P = 0.012$: odds ratio = 0.80, 95% confidence interval 0.67–0.95; Table 1). We further tested the association between other subunits of the AP-3 complex (AP3M2, AP3D1, AP3B1, and AP3B2) and schizophrenia (Table 2). There was no significant association between schizophrenia and the other AP-3 complex genes, except for the weak association with the rs7726585 in the AP3B1 gene ($P = 0.043$, Table 2). However, these associations did not survive after correction for multiple testing (rs6688: corrected $P = 0.192$, rs7726585: corrected $P = 0.688$, Bonferroni correction: total 16 examined SNPs). The genotype distributions of all examined SNPs in the AP-3 complex genes were in Hardy–Weinberg equilibrium for both controls and patients with schizophrenia (data not shown).

We did not detect the association between schizophrenia and the AP-3 complex genes in our sample of 432 patients and 565 healthy controls in a Japanese population. As the AP3M1 gene is a direct binding partner with dysbindin, a susceptibility gene for schizophrenia, and it co-localizes with dysbindin at the presynapse (Taneichi-Kuroda et al., 2009), the interaction between AP-3 complex and dysbindin could play an important role in the pathophysiology of schizophrenia. Several neuronal function of dysbindin have been reported such as neurotransmission (Hattori et al., 2008; Iizuka et al., 2007; Kumamoto et al., 2006; Murotani et al., 2007; Numakawa et al., 2004), cellular signaling (Benson et al., 2001; Kubota et al., 2009; Numakawa et al., 2004) and neuronal survival (Numakawa et al., 2004). Among these dysbindin functions, interaction between AP-3 complex and dysbindin might be related to the neurotransmission. Overexpression of dysbindin increases glutamate release, whereas the depletion of dysbindin decreases glutamate release in primary cortical neurons (Numakawa et al., 2004). As AP-3 complex is known to be essential for the sorting and exocytosis of synaptic vesicles (Danglot and Galli, 2007; Newell-Litwa et al., 2007; Scheuber et al., 2006), impaired glutamate release by dysbindin could be mediated by the association with the AP-3 complex.

There were several limitations in our study. Lack of structured interview in patients and controls could influence the results. Our method of SNP selection was not a gene based method such as selection of Tagging SNPs. Our subjects of 432 cases and 656 controls had sufficient power (>0.80) to detect an effect at odds ratio of 1.29 or larger (0.77 or less, as minor allele frequency is less

Table 2
Allele and genotype frequencies of SNPs in other AP-3 complex genes between the patients with schizophrenia and controls.

Gene	Marker		Location	M/m	SCZ			CON			Genotypic *p*-value (*df* = 2)	SCZ	CON	Allelic *p*-value (*df* = 1)	OR
	db SNP number	Position[a]			M/M	M/m	m/m	M/M	M/m	m/m		MAF			
AP3M2 (forward strand)	rs1050275	12348926	Exon 9, 3′UTR	T/C	0.483	0.450	0.068	0.524	0.390	0.086	0.122	0.293	0.281	0.562	1.058
	rs4737038	12331728	Intron1	A/G	0.324	0.457	0.219	0.289	0.531	0.180	0.056	0.448	0.446	0.929	1.008
AP3D1 (reverse strand)	rs2072306	2049019	Intron 29	T/C	0.329	0.452	0.219	0.354	0.474	0.172	0.156	0.445	0.409	0.098	1.158
	rs2159213	2076102	Intron 4	C/T	0.378	0.473	0.149	0.361	0.467	0.172	0.568	0.385	0.406	0.343	0.918
	rs2238612	2089694	Intron 1	C/T	0.392	0.466	0.143	0.378	0.448	0.174	0.391	0.375	0.398	0.286	0.908
	rs2238608	2085330	Intron 1	A/G	0.457	0.457	0.087	0.503	0.433	0.064	0.196	0.315	0.280	0.088	1.180
AP3B1 (reverse strand)	rs402883	27939101	Intron 22	C/G	0.590	0.363	0.047	0.552	0.379	0.070	0.235	0.229	0.259	0.113	0.849
	rs252761	27975081	Intron 22	G/T	0.405	0.455	0.140	0.416	0.432	0.152	0.741	0.368	0.368	0.999	1.000
	rs6453373	28019386	Exon 16, T585A	T/A	0.276	0.499	0.226	0.262	0.478	0.260	0.410	0.525	0.499	0.246	1.108
	rs4704487	28076568	Intron 7	C/T	0.489	0.398	0.113	0.459	0.409	0.131	0.544	0.312	0.336	0.253	0.897
	rs7726585	28141154	Intron 2	A/G	0.413	0.454	0.133	0.363	0.468	0.170	0.133	**0.360**	**0.404**	**0.043**	**0.831**
AP3B2 (reverse strand)	rs2278355	451510	Intron 22	C/T	0.279	0.485	0.236	0.308	0.468	0.225	0.607	0.479	0.459	0.364	1.084
	rs11638815	472636	Intron 4	A/C	0.656	0.306	0.038	0.704	0.262	0.034	0.253	0.191	0.165	0.121	1.195
	rs4779050	489092	Intron 1	G/T	0.259	0.468	0.273	0.290	0.468	0.243	0.414	0.507	0.477	0.171	1.129

SCZ: schizophrenia, CON: controls, m: minor allele, M: major allele, MAF: minor allele frequency, OR: odds ratio.
[a] db SNP build 129.

in the patient population); however, the odds ratio of the rs6688 was 0.80. These limitations and application of strict correction for multiple testing, Bonferroni correction, could lead to the type II error. Therefore, it is necessary to carry out further investigations to confirm our findings in other samples with much larger sample size, SNPs with better gene coverage, and subjects with structured interviews.

Acknowledgement

This work was supported in part by Grants-in-Aid from the Japanese Ministry of Health, Labor and Welfare (H18-kokoro-005, H19-kokoro-002).

References

Benson, M.A., Newey, S.E., Martin-Rendon, E., Hawkes, R., et al., 2001. Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. J. Biol. Chem. 276, 24232–24241.
Burdick, K.E., Lencz, T., Funke, B., Finn, C.T., et al., 2006. Genetic variation in DTNBP1 influences general cognitive ability. Hum. Mol. Genet. 15, 1563–1568.
Chiba, S., Hashimoto, R., Hattori, S., Yohda, M., et al., 2006. Effect of antipsychotic drugs on DISC1 and dysbindin expression in mouse frontal cortex and hippocampus. J. Neural Transm. 113, 1337–1346.
Danglot, L., Galli, T., 2007. What is the function of neuronal AP-3? Biol. Cell. 99, 349–361.
Harrison, P.J., Weinberger, D.R., 2005. Schizophrenia genes, gene expression, and neuropathology: on the matter of their convergence. Mol. Psychiatry 10, 40–68.
Hashimoto, R., Numakawa, T., Ohnishi, T., Kumamaru, E., et al., 2006. Impact of the DISC1 Ser704Cys polymorphism on risk for major depression, brain morphology and ERK signaling. Hum. Mol. Genet. 15, 3024–3033.
Hashimoto, R., Hashimoto, H., Shintani, N., Chiba, S., et al., 2007. Pituitary adenylate cyclase-activating polypeptide is associated with schizophrenia. Mol. Psychiatry 12, 1026–1032.
Hashimoto, R., Noguchi, H., Hori, H., Ohi, K., et al., 2009. The association analysis between the dysbindin gene (DTNBP1) and cognitive functions in Japanese subjects. Psychiatry Clin. Neurosci, in press.
Hattori, S., Murotani, T., Matsuzaki, S., Ishizuka, T., et al., 2008. Behavioral abnormalities and dopamine reductions in sdy mutant mice with a deletion in Dtnbp1, a susceptibility gene for schizophrenia. Biochem. Biophys. Res. Commun. 373, 298–302.
Iizuka, Y., Sei, Y., Weinberger, D.R., Straub, R.E., 2007. Evidence that the BLOC-1 protein dysbindin modulates dopamine D2 receptor internalization and signaling but not D1 internalization. J. Neurosci. 27, 12390–12395.
Kubota, K., Kumamoto, N., Matsuzaki, S., Hashimoto, R., et al., 2009. Dysbindin engages in c-Jun N-terminal kinase activity and cytoskeletal organization. Biochem. Biophys. Res. Commun. 379, 191–195.
Kumamoto, N., Matsuzaki, S., Inoue, K., Hattori, T., et al., 2006. Hyperactivation of midbrain dopaminergic system in schizophrenia could be attributed to the down-regulation of dysbindin. Biochem. Biophys. Res. Commun. 345, 904–909.
Li, W., Zhang, Q., Oiso, N., Novak, E.K., et al., 2003. Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). Nat. Genet. 35, 84–89.
Murotani, T., Ishizuka, T., Hattori, S., Hashimoto, R., et al., 2007. High dopamine turnover in the brains of Sandy mice. Neurosci. Lett. 421, 47–51.
Newell-Litwa, K., Seong, E., Burmeister, M., Faundez, V., 2007. Neuronal and non-neuronal functions of the AP-3 sorting machinery. J. Cell Sci. 120, 531–541.
Numakawa, T., Yagasaki, Y., Ishimoto, T., Okada, T., et al., 2004. Evidence of novel neuronal functions of dysbindin, a susceptibility gene for schizophrenia. Hum. Mol. Genet. 13, 2699–2708.
O'Donovan, M.C., Craddock, N., Norton, N., Williams, H., et al., 2008. Identification of loci associated with schizophrenia by genome-wide association and follow-up. Nat. Genet. 40, 1053–1055.
Scheuber, A., Rudge, R., Danglot, L., Raposo, G., et al., 2006. Loss of AP-3 function affects spontaneous and evoked release at hippocampal mossy fiber synapses. Proc. Natl. Acad. Sci. USA 103, 16562–16567.
Skol, A.D., Scott, L.J., Abecasis, G.R., Boehnke, M., 2006. Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. Nat. Genet. 38, 209–213.
Stephan, K.E., Baldeweg, T., Friston, K.J., 2006. Synaptic plasticity and dysconnection in schizophrenia. Biol. Psychiatry 59, 929–939.
Takao, K., Toyama, K., Nakanishi, K., Hattori, S., et al., 2008. Impaired long-term memory retention and working memory in sdy mutant mice with a deletion in Dtnbp1, a susceptibility gene for schizophrenia. Mol. Brain 1, 11.
Talbot, K., Eidem, W.L., Tinsley, C.L., Benson, M.A., et al., 2004. Dysbindin-1 is reduced in intrinsic, glutamatergic terminals of the hippocampal formation in schizophrenia. J. Clin. Invest. 113, 1353–1363.
Taneichi-Kuroda, S., Taya, S., Hikita, T., Fujinoa, Y., et al., 2009. Direct interaction of Dysbindin with the AP-3 complex via its μ subunit. Neurochem. Int. 54 (7), 431–438.
Weickert, C.S., Straub, R.E., McClintock, B.W., Matsumoto, M., et al., 2004. Human dysbindin (DTNBP1) gene expression in normal brain and in schizophrenic prefrontal cortex and midbrain. Arch. Gen. Psychiatry 61, 544–555.

Journal of Neurochemistry JNC

JOURNAL OF NEUROCHEMISTRY | 2009 | 110 | 595–602 doi: 10.1111/j.1471-4159.2009.06168.x

Depression-like behavior in the forced swimming test in PACAP-deficient mice: amelioration by the atypical antipsychotic risperidone

Hitoshi Hashimoto,*,†,[1] Ryota Hashimoto,†,‡,§,[1] Norihito Shintani,*,[1] Kazuhiro Tanaka,*,[1] Akiko Yamamoto,*,[1] Michiyoshi Hatanaka,* Xiaohong Guo,* Yoshiko Morita,* Mamoru Tanida,¶ Katsuya Nagai,¶ Masatoshi Takeda†,‡ and Akemichi Baba*

*Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan

†The Osaka-Hamamatsu Joint Research Center for Child Mental Development, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

‡Department of Psychiatry, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

§Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan

¶Institute for Protein Research, Osaka University, Suita, Osaka, Japan

Abstract

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a neuropeptide with pleiotropic functions. We report here that PACAP-deficient (PACAP−/−) mice showed increased immobility in a forced swimming test, which was reduced by the antidepressant desipramine, to a similar extent as in wild-type mice. The atypical antipsychotic risperidone and the selective serotonin (5-HT)$_2$ antagonist ritanserin normalized the depression-like behavior in PACAP−/− mice. The 5-HT$_2$ agonist (±)-2,5-dimethoxy-4-iodoamphetamine-induced 5-HT syndrome was exaggerated in PACAP−/− mice, which suggests a 5-HT$_2$-receptor-dependent mechanism in the depression-like behavior. The circadian rhythm of plasma corticosterone and body core temperature was significantly flattened in the mutants. mRNA expression of glucocorticoid receptor was reduced in the mutant hippocampus. The present results suggest that alterations in PACAP signaling might contribute to the pathogenesis of certain depressive conditions amenable to atypical antipsychotic drugs.

Keywords: atypical antipsychotic risperidone, depression, hypothalamus–pituitary–adrenal axis, pituitary adenylate cyclase-activating polypeptide-deficient mice, schizophrenia, serotonin receptor.

J. Neurochem. (2009) **110**, 595–602.

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a pleiotropic neuropeptide acting as a neurotransmitter, neuromodulator or neurotrophic factor (Miyata *et al.* 1989; Vaudry *et al.* 2000; Hashimoto *et al.* 2006). To understand the functions of PACAP signaling *in vivo*, we developed mice that lack the PACAP gene (PACAP−/−) (Hashimoto *et al.* 2001). These mice exhibit marked phenotypes, including behavioral abnormalities, which propose a previously uncharacterized role for PACAP in the regulation of psychomotor functions, and suggest a role for altered PACAP-mediated signaling pathways in certain psychiatric disorders (Hashimoto *et al.* 2001; Tanaka *et al.* 2006). Genetic linkage studies, followed by fine-scale mapping in a 331-kb region in a bipolar disorder candidate region, have suggested that the PACAP gene, which resides at 18p11.32 (chr18:0.895-0.900 Mb), is located close to a bipolar disorder risk locus (McInnes *et al.* 2001). More directly, Hashimoto *et al.* (2007) have provided evidence for a

Received March 16, 2009; accepted April 28, 2009.

Address correspondence and reprint requests to Akemichi Baba, Laboratory of Molecular Neuropharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: baba@phs.osaka-u.ac.jp

[1]HH, RH, NS, KT and AY contributed equally to this work.

Abbreviations used: 5-HT, serotonin; DISC1, disrupted in schizophrenia 1; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine; FST, forced swimming test; HPA, hypothalamus–pituitary–adrenal; PACAP, pituitary adenylate cyclase-activating polypeptide; PCP, phencyclidine.

possible association between PACAP signaling, mediated by its specific receptor PAC_1, and schizophrenia. In addition, Hattori *et al.* (2007) have shown that PACAP increases the expression of the product of disrupted in schizophrenia 1 (DISC1), which is recognized as a leading candidate risk gene for schizophrenia. PACAP also markedly but transiently decreases the association between the DISC1 protein and the DISC1-interacting protein DBZ, which may be one of the molecular pathways that underlies the pathogenesis of schizophrenia.

Disrupted in schizophrenia 1 was first described in a Scottish family as a novel gene that was disrupted by a (1;11)(q42.1;q14.3) translocation that segregated with major psychiatric disorders (Millar *et al.* 2000). Despite the origin of the name 'DISC1', this Scottish family might be atypical due to the wide spectrum of disorders (Millar *et al.* 2000). Among the 29 subjects with the translocation, there were 10 with recurrent major depression and seven with schizophrenia (Blackwood *et al.* 2001). Genes, including those encoding dopamine D_1 and D_2 receptors, serotonin receptor (5-HT) 2A, catechol-*O*-methyl transferase, brain-derived neurotrophic factor and reelin, have been suggested to be associated with schizophrenia and other psychiatric disorders (Abdolmaleky *et al.* 2005). In considering the emerging picture that major psychiatric disorders might share, at least in part, common genetic etiologies, it is plausible to assume that PACAP may also be a risk factor for major mental illnesses, other than schizophrenia.

Major depression is a common and highly prevalent mental disorder with symptoms that include deficits in a range of cognitive, psychomotor, and emotional processes. Although genetic susceptibility is considered to be involved in major depression, this disorder is heterogeneous, and gene–environment interactions, as well as epigenetic factors are implicated in its pathophysiology (Wong and Licinio 2001; Kato 2007; Mill and Petronis 2007). Despite recent advances in our understanding of the molecular basis of psychiatric disorders, efforts in drug discovery have been relatively unsuccessful (Agid *et al.* 2007).

In the present study, we therefore examined the impact of the deletion of PACAP in mice on depression-like behavior and its responsiveness to antidepressant and antipsychotic drugs, as well as on circadian rhythm of plasma corticosterone and body core temperature, and mRNA expression of glucocorticoid receptor.

Materials and methods

Animals

All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University, Japan. Generation of PACAP−/− mice by a gene targeting technique has been reported previously (Hashimoto *et al.* 2001). The null mutation was backcrossed onto the genetic background of Crlj:CD1 (Institute of Cancer Research, Charles River, Tokyo, Japan) at least 10 times. All wild-type control and PACAP−/− mice used were obtained from the intercross of animals heterozygous for the mutant PACAP gene, and experiments were conducted with naïve male mice of 2–4 months of age. Mice were housed in a temperature (23 ± 1°C) and light-controlled room with a 12-h light/12-h dark cycle (lights on from 08:00 to 20:00 h), and allowed free access to water and food, except during behavioral testing.

Forced swimming test

Forced swimming test (FST) was performed as described (Porsolt *et al.* 1977; Matsuda *et al.* 1995), with minor modifications. Mice were forced to swim individually in a vertical glass cylinder (height 30 cm, diameter 18.5 cm) filled with water maintained at 24–26°C to a depth of 13 cm. After testing in the water, mice were removed and allowed to dry in a heated enclosure. Duration of immobility (making only minimal movements to keep the head above water or floating), swimming (movement of all four legs with body aligned horizontally in the water), and climbing (movement of all four legs with body aligned vertically in the water) were measured from videotapes by a trained blind observer. The total duration of each of the three behavioral parameters, immobility, swimming and climbing, was separately recorded by different observation sessions. To examine the robustness of this method, we compared the immobility time separately measured and that calculated by subtracting total time by the sum of swimming and climbing times. Figure S1 shows time of immobility determined by the latter, which was principally the same as that determined by the former method shown in Fig. 1(a). The overall difference between them was < 5%.

Desipramine (Research Biochemicals, Natick, MA, USA) and risperidone (Sigma–Aldrich, St Louis, MO, USA) were dissolved in saline, while haloperidol (Sigma–Aldrich) was dissolved in saline containing 0.1% acetic acid. Ritanserin (Sigma–Aldrich) was dissolved in a few drops of hydrochloric acid, the volume was made up with distilled water, and the pH was adjusted. All drugs were administered intraperitoneally in a volume of 10 mL/kg body weight, 30 min before the behavioral test. PACAP38 (Peptide Institute, Osaka, Japan) was dissolved in Ringer's solution and injected intracerebroventricularly 30 min before the behavioral test, by the following method: mice were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and a guide cannula was implanted in the lateral ventricle (0.4 mm posterior and 1.0 mm lateral to the bregma; 2.3 mm ventral from the dura) using stereotaxic apparatus (Narishige, Tokyo, Japan). After at least 7 days for recovery, mice were injected with 20 pmol PACAP38 (1 μL/min, total volume 2 μL), which seems to be in the physiological range (Fang *et al.* 1995). For controls, mice were administered Ringer's solution. At the end of the experiments, the successful administration was verified by infusion of Evans Blue (Wako Pure Chemical, Osaka, Japan).

5-HT_2 agonist-induced head twitch and ear scratch responses

Mice were individually placed in the observation cages (19 × 10 × 11 cm) for a 30-min habituation period. They were then intraperitoneally injected with either saline or (±)-2,5-dimethoxy-4-iodoamphetamine (DOI) (Sigma–Aldrich) and videotaped for

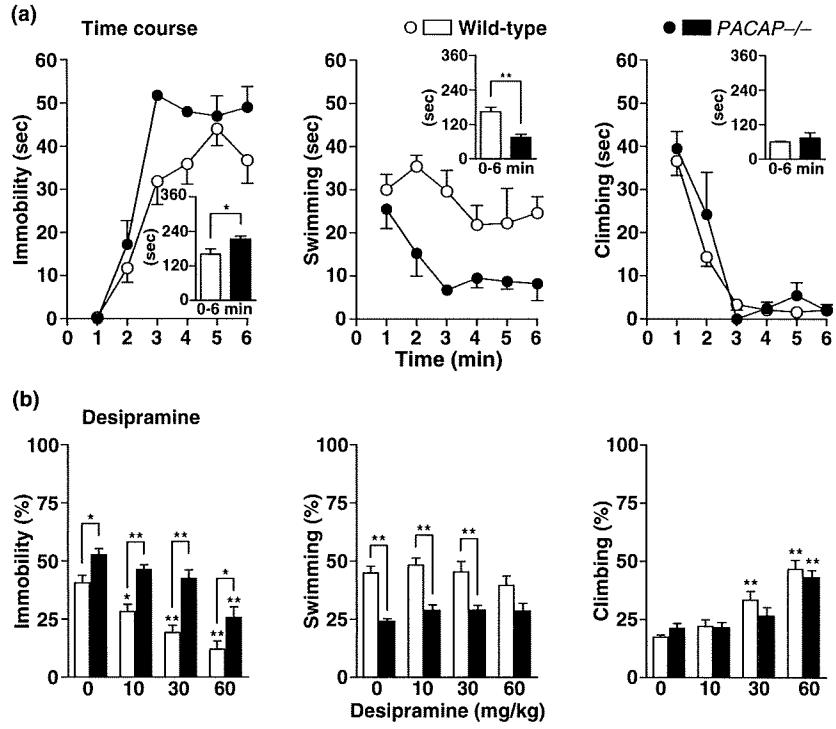


Fig. 1 Duration of immobility, swimming and climbing behavior in FST, and the effect of desipramine on FST behavior in PACAP-/- mice. PACAP-/- (closed circles and bars) and wild-type (open circles and bars) mice were subjected to FST and the duration of immobility (left), swimming (center), and climbing (right) were determined. (a) Time course of behavior duration (n = 4–8 per group). The insets shows the total duration for 6 min. (b) Desipramine-induced decrease in immobility and increase in climbing behavior (n = 9–22 per group). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle-treated mice of the same genotype unless otherwise indicated.

30 min. Scoring began immediately after injection from videotapes by a trained blind observer. The head twitch response is a distinctive paroxysmal head-twitching behavior that is easily distinguished from head bobbing, lateral movements of the head, or grooming. The ear scratch response is a rapid scratching movement of the head, neck, or lateral area by either hindlimb.

Circadian corticosterone levels, dexamethasone suppression test, and adrenocorticosteroid receptor mRNA levels

Animals maintained in a 12-h light (100 lux)/12-h dark cycle were killed at each time-point indicated, and trunk blood was collected and mixed with one-tenth volume of 38 g/L citric acid. Plasma was then prepared by centrifugation at 2000 g for 10 min. Corticosterone levels were determined with a radioimmunoassay kit (rat RIA [^{125}I] System; GE Healthcare, Tokyo, Japan).

Dexamethasone (0.1 mg/kg body weight; Sigma–Aldrich) was injected intraperitoneally at 14:00 h (zeitgeber time 6:00), and 4 h later, the plasma corticosterone levels were determined as mentioned above.

mRNA expression of glucocorticoid and mineralocorticoid receptors was determined in the hippocampus by quantitative real-time reverse transcriptase-PCR. Total RNA was extracted from the hippocampus using guanidine thiocyanate–acid phenol and reverse-transcribed using Moloney murine leukemia virus Rnase H^{-} reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as described previously (Hashimoto *et al.* 1993). Real-time PCR was performed using Dynamo SYBR Green qPCR kit (Finnzymes, Espoo, Finland), and the following primers for mouse glucocorticoid receptor: 5′-ATG CCGCTATCGAAAATGTC-3′ (sense) and 5′-AGCAGTGACA CCAGGGTAGG-3′ (antisense); mineralocorticoid receptor: 5′-ACA ATTCCAAGCCTGACACC-3′ (sense) and 5′-CAACTCAAGGC AAACGATGA-3′ (antisense); and GAPDH: 5′-CTCATGACCA CAGTCCATGC-3′ (sense) and 5′-CACATTGGGGGTAGGAA CAC-3′ (antisense). GAPDH was amplified and used as an internal control.

Telemetry-recording of core temperature

To measure core temperature, the Telemetry System (Star Medical, Tokyo, Japan) was used as described previously (Tanida *et al.* 2007). Seven days before temperature measurement, a capsule containing a temperature sensor, battery and transmitter was implanted into the abdominal cavity under pentobarbital anesthesia. The output signals were converted from analog to digital and stored on a personal computer.

Statistical analysis

Statistically significant differences were assessed by ANOVA, followed by *post hoc* Dunnett's test or t-test, where applicable. All values were expressed as the mean ± SEM. Statistical significance was defined as $p < 0.05$.

Results

Increased immobility behavior in FST in PACAP-/- mice

Forced swimming test (Porsolt *et al.* 1977) is arguably the most reliable model available with strong predictive ability that provides a broad spectrum of antidepressant effects (Petit-Demouliere *et al.* 2005). In this test, PACAP-/- mice displayed a significantly increased time of immobility and a reduced time of swimming compared with those in wild-type mice (Fig. 1a). The time of climbing, however, did not differ

