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

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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.

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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

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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

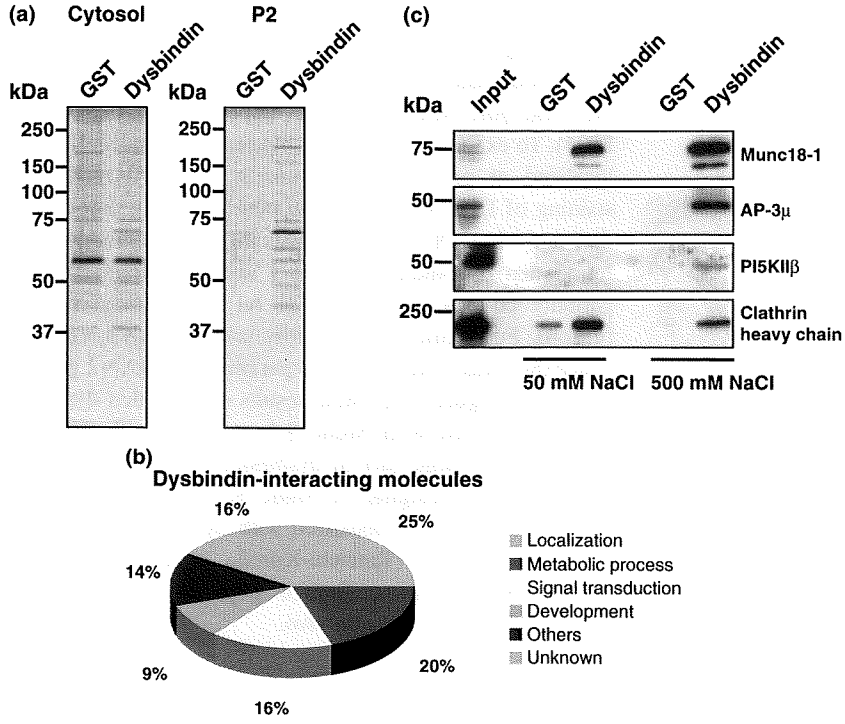

Fig. 1 Identification of the dysbindin-interacting molecules. (a) The 500 mM NaCl eluates of affinity columns loaded with cytosol or P2 extract were analyzed by SDS–PAGE, followed by silver staining. (b) Summary of dysbindin-interacting molecules. Proteins identified by MALDI-TOF-MS and LC/MS/MS were 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-

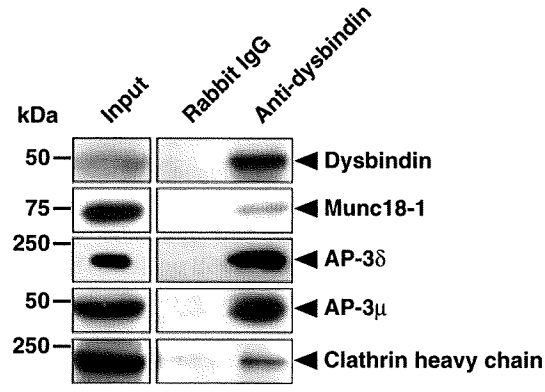

Fig. 2 *In vivo* complex formation with dysbindin and identified molecules. The P2 fraction from C57B6 mice brain homogenate that was dissolved in buffer A containing 50 mM NaCl and 1% NP-40 was used in an immunoprecipitation assay with antibodies against dysbindin. Pre-immune rabbit IgG was used as 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.

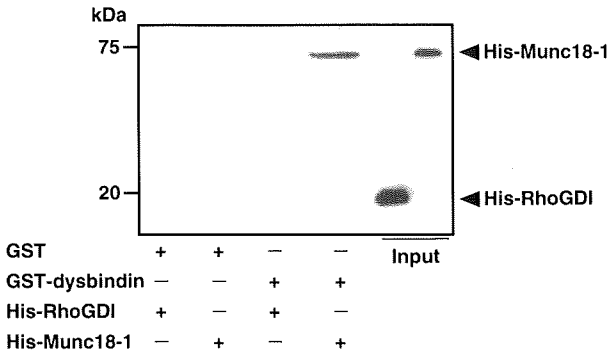

Fig. 3 *In vitro* complex formation with dysbindin and Munc18-1. An *in vitro* binding assay was performed using purified recombinant proteins. Beads immobilized with GST alone or GST-dysbindin was incubated with His-Munc18-1 or His-RhoGDI as a negative control. Bound proteins were analyzed by immunoblotting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

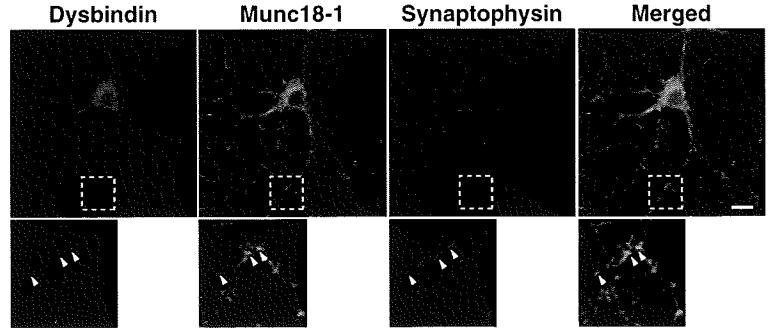

Fig. 4 Co-localization of dysbindin with Munc18-1 at pre-synaptic terminals. Dysbindin (red), Munc18-1 (green) and synaptophysin (blue) were visualized by triple staining method in DIV21 primary-cultured rat hippocampal neurons. Arrowheads indicate the co-localization of dysbindin, Munc18-1 and synaptophysin.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

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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.

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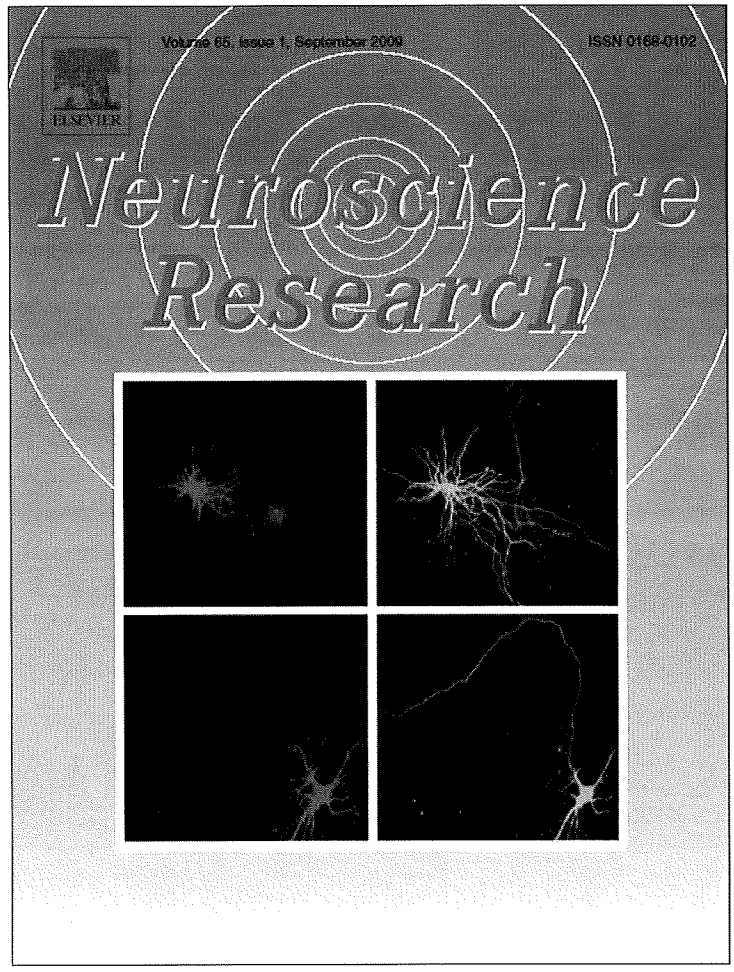

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Rapid communication

Association analysis between schizophrenia and the AP-3 complex genes

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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

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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

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

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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 $(5\text{-HT})_2$ 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.

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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

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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

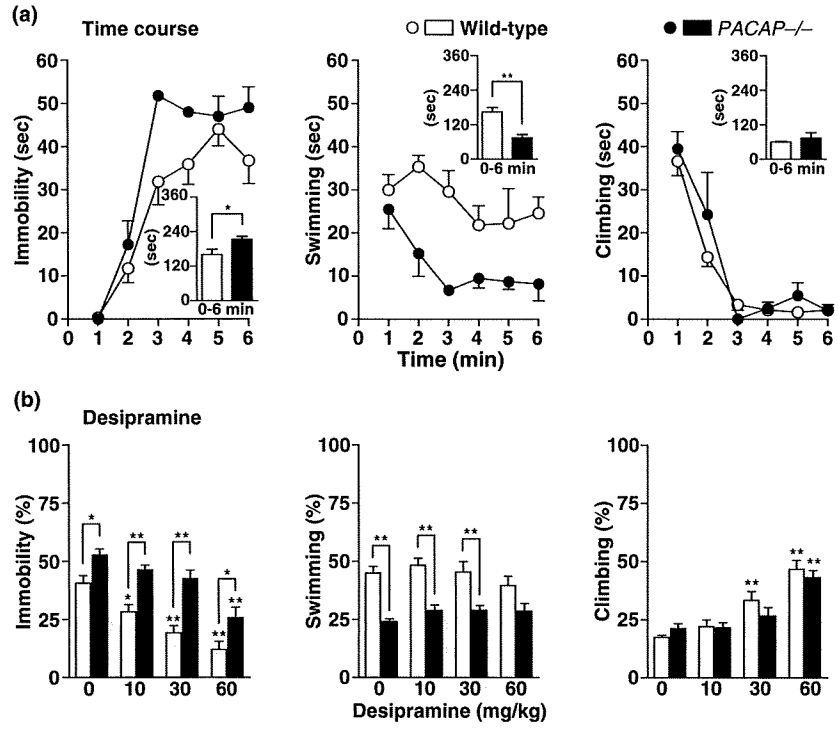

Fig. 1 Duration of immobility, swimming and climbing behavior in FST, and the effect of desipramine on FST behavior in PACAP-/- mice. PACAP-/- (closed circles and bars) and wild-type (open circles and bars) mice were subjected to FST and the duration of immobility (left), swimming (center), and climbing (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

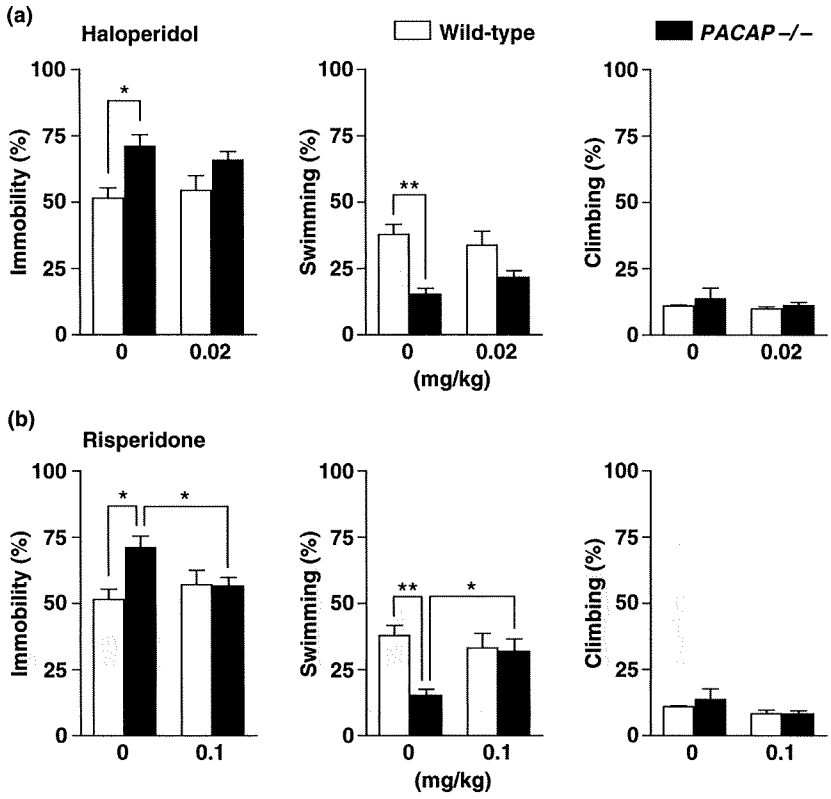

Fig. 2 Behavioral effects of the antipsychotic haloperidol and risperidone in FST in PACAP−/− mice. PACAP−/− (closed bars) and wild-type (open bars) mice (n = 4–9 per group) were treated intraperitoneally with haloperidol (a) and risperidone (b) at the indicated doses, or vehicle, and duration of immobility (left), swimming (center), and climbing (right) in FST was determined. Values for control are common between (a) and (b). $^{*}p < 0.05$, $^{**}p < 0.01$.

significantly between the mutant and wild-type mice (Fig. 1a).

Desipramine is an antidepressant known to decrease immobility time in FST (Petit-Demouliere *et al.* 2005), and indeed it decreased the immobility time and increased the climbing time in wild-type mice (Fig. 1b). In PACAP−/− mice, desipramine decreased the immobility and increased the climbing behavior to a similar extent as in wild-type mice (Fig. 1b).

Amelioration of depression-like behavior in FST by risperidone, ritanserin, and intracerebroventricular PACAP

We examined the effect of the antipsychotic drugs on FST behavior in PACAP−/− mice. When the typical antipsychotic haloperidol was administered, the immobility did not significantly change in either wild-type or PACAP−/− mice (Fig. 2a). Higher doses of haloperidol, however, induced catalepsy in both mutant and wild-type mice (data not shown). In contrast, the atypical antipsychotic risperidone decreased the immobility time and increased the swimming time in PACAP−/− mice, but had no effect on wild-type mice (Fig. 2b). The selective 5-HT_2 antagonist ritanserin also completely ameliorated the depression-like behavior in PACAP−/− mice (Fig. 3a). To further address the mechanism underpinning increased immobility behavior in PACAP−/− mice, animals were injected intracerebroventricularly with PACAP38 and subjected to the test. As shown in Fig. 3(b), PACAP38 injection resulted in a tendency to reduced immobility time and increased swimming time in PACAP−/− mice.

Increased head twitch and ear scratch responses induced by DOI in PACAP−/− mice

To examine the possible alteration in the 5-HT_2 receptor function in PACAP−/− mice, we measured the 5-HT_2 agonist DOI-induced head twitch response and ear scratch response that are mediated via activation of 5-HT_{2A} receptors (Darmani *et al.* 1996). As shown in Fig. 4(a and b), DOI increased head twitch and ear scratch responses in both wild-type and PACAP−/− mice. However, both responses were significantly higher in PACAP−/− mice compared with wild-type mice (head twitch, $p < 0.01$; ear scratch, $p < 0.01$; two-way ANOVA).

Impaired circadian corticosterone rhythm and body temperature rhythm in PACAP−/− mice, and reduced mRNA expression of glucocorticoid receptor in the PACAP−/− hippocampus

As altered activity of the hypothalamus–pituitary–adrenal (HPA) axis is one of the most commonly observed neuroendocrine abnormalities associated with depressive symptoms (Thomson and Craighead 2008), we examined the plasma corticosterone levels and its circadian rhythm in PACAP−/− mice (Fig. 4c). The typical circadian rhythm in corticosterone levels was observed in wild-type mice, in

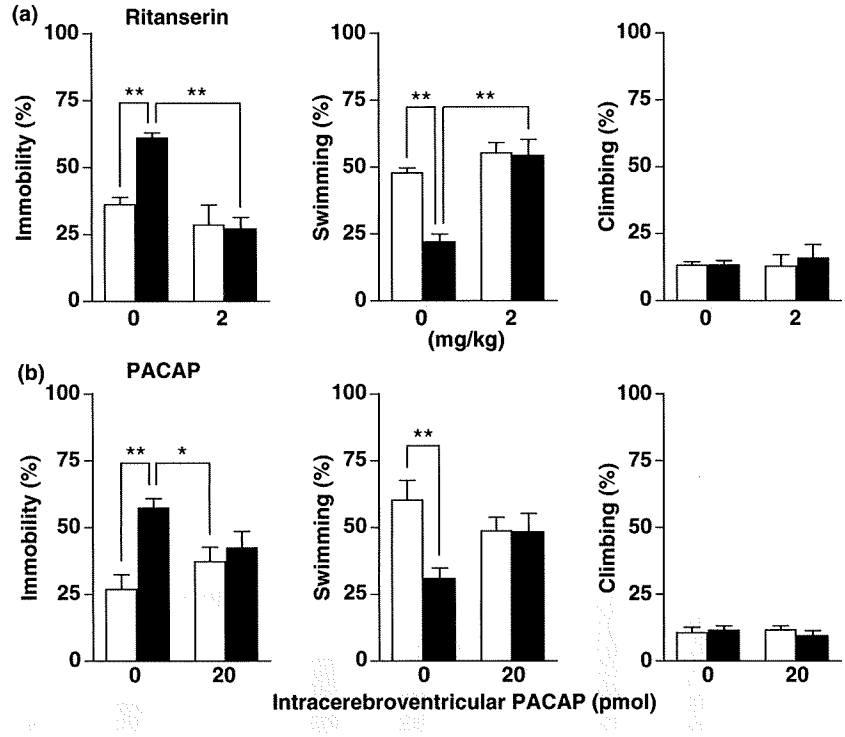

Fig. 3 Amelioration of depression-like behavior in FST by the 5-HT_2 antagonist ritanserin and intracerebroventricular PACAP. PACAP−/− (closed bars) and wild-type (open bars) mice were injected intraperitoneally with ritanserin (a; $n = 4–5$ per group) and intracerebroventricularly with PACAP38 (b; $n = 10–15$ per group) at the indicated doses, or vehicle, and duration of immobility (left), swimming (center), and climbing (right) in FST was determined. $^*p < 0.05$, $^{**}p < 0.01$.

which the levels peaked at zeitgeber time 12. In contrast, corticosterone levels in PACAP−/− mice showed no significant differences due to the time of day sampled. The dexamethasone-induced suppression in corticosterone levels was normally seen in mutant mice compared with wild-type mice (Fig. 4d). The hippocampal mRNA level of glucocorticoid receptor was significantly reduced in the mutants, while that of mineralocorticoid receptor was the same between the two groups (Fig. 4e). In addition, telemetry recording of core temperature revealed that circadian rhythm of body temperature seen in wild-type mice was virtually absent in PACAP−/− mice (Fig. 4f). In wild-type mice, body temperature was high during the dark phase, whereas, in PACAP−/− mice, this rise in body temperature was not observed.

Discussion

The aim of this study was to examine if PACAP deficiency in mice results in increased depression-like behavior, given that PACAP may share the same pathway with DISC1 (Hattori *et al.* 2007), one of the most probable risk factors for major psychiatric disorders, including both schizophrenia and depression. In the present study, PACAP−/− mice exhibited depression-like behavior in the FST, impaired circadian corticosterone rhythm, reduced expression of glucocorticoid receptor mRNA in the hippocampus, and flattened and low body temperature. All these phenotypes are considered to be related to clinical features of depression (Porsolt *et al.* 1977; van Londen *et al.* 2001; Petit-Demouliere *et al.* 2005; Ridder *et al.* 2005; Thomson and Craighead 2008). A limitation in the present study is that we could not provide data from other behavioral tests related to depression. Although we performed a tail suspension test in PACAP−/− mice, we could not obtain a determinate result because of some problem conducting this test in PACAP−/− mice.

Risperidone is a combined dopamine D_2 and 5-HT_2 receptor antagonist, while ritanserin, the predecessor of risperidone, is a selective 5-HT_2 antagonist. Pharmacological study with these drugs suggests that 5-HT_2 receptor signaling is relevant to the depression-like behavior in the FST in PACAP−/− mice. Exaggerated DOI-induced 5-HT syndrome in PACAP−/− mice suggests altered 5-HT_2 receptor signaling. It has been demonstrated that enhancement of 5-HT neurotransmission mediates swimming behavior in FST, whereas enhancement of norepinephrine neurotransmission increases climbing behavior (Page *et al.* 1999). Indeed, we observed that desipramine, a selective norepinephrine reuptake inhibitor, increased climbing behavior, but not swimming behavior, to a similar extent in wild-type and PACAP−/− mice. However, risperidone and ritanserin normalized immobility behavior solely via an increase in swimming behavior in PACAP−/− mice, without any effect in wild-type mice. These observations may suggest the 5-HT-dependent mechanisms for reduced swimming time in the FST in PACAP−/− mice. In line with this possibility, the 5-HT metabolite 5-hydroxyindoleacetic acid was slightly decreased in the cortex and striatum of the PACAP−/− mouse brain (Hashimoto *et al.* 2001) and the hypothermic response to 5-HT_{1A} agonists was significantly reduced in

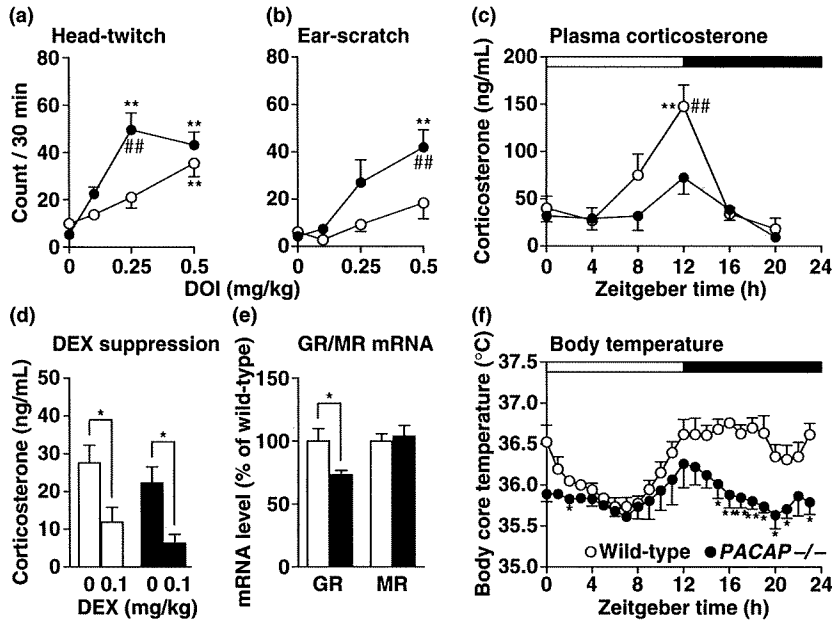

Fig. 4 The 5-HT_2 agonist DOI-induced head twitch and ear scratch responses, circadian and dexamethasone-suppressed corticosterone levels, glucocorticoid and mineralocorticoid receptor mRNA levels, as well as circadian body temperature rhythm in PACAP–/– mice. (a, b) PACAP–/– (closed circles) and wild-type (open circles) mice (n = 7–10 per group) were treated intraperitoneally with DOI at the indicated doses, and the head twitch (a) and ear scratch (b) responses for 30 min were counted. $^{**}p < 0.01$ compared with vehicle-treated mice of the same genotype, $^{\#\#}p < 0.01$ compared with the wild-type mice at the same dose. (c) PACAP–/– (closed circles) and wild-type (open circles) mice (n = 4 per group) were maintained in a 12-h light (100 lux)/12-h dark cycle, and plasma corticosterone levels were determined at the indicated times. Bar above the graph indicates the light/dark conditions (open, light; close, dark). $^{**}p < 0.01$ compared with zeitgeber time 0 of the same genotype; $^{\#\#}p < 0.01$ compared with the PACAP–/– mice at the same time. (d) PACAP–/– (closed bars) and wild-type (open bars) mice (n = 4–7 per group) were treated intraperitoneally with dexamethasone (DEX) at zeitgeber time 6 and the plasma corticosterone levels were determined at zeitgeber time 10, $^{*}p < 0.05$. (e) mRNA expression of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) was determined in hippocampus by quantitative real-time reverse transcriptase PCR in PACAP–/– (closed bars) and wild-type (open bars) mice (n = 5 per group), $^{*}p < 0.05$. (f) Body core temperature in PACAP–/– (closed circles) and wild-type (open circles) mice (n = 3–4 per group) were recorded using a telemetry-recording system. Bar above the graph indicates the light/dark conditions (open, light; close, dark). $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the wild-type mice at the same time.

these mice (Tanaka *et al.* 2006), although we have not yet been able to find any change in 5-HT receptor expression in the PACAP–/– mouse brain. Taking these observations into consideration, it is suggested that PACAP deficiency leads to certain depressive conditions, but not a typical 'depression-like' profile, that might be due to perturbed 5-HT signaling, and therefore, is responsive to atypical antipsychotics and 5-HT_2 receptor antagonism.

As increasing evidence has suggested that PACAP plays diverse roles in mammalian neurogenesis and patterning (Waschek *et al.* 1998; Wu *et al.* 2006), we conducted a rescue experiment to determine whether the behavioral defect is due to an early developmental impairment, or a consequence of the absence of the PACAP pathway in otherwise normal behavior. The observation that intracerebroventricular injection of PACAP resulted in a tendency to reduced depression-like behavior in PACAP–/– mice implies a direct causal link between PACAP deficiency and depression-like behavior.

In PACAP–/– mice, the circadian plasma corticosterone level was flattened, although overall corticosterone secretion was lower, instead of higher, than wild-type mice. This observation is inconsistent with a previous study by Hamelink *et al.* (2002) that showed that the diurnal rhythm of plasma corticosterone was not altered in their PACAP–/– mouse line developed separately from our colony. Although the reason for this discrepancy remains unclear, it may in part be attributable to different genetic backgrounds (a mixed genetic background of C57/BL6 and 129 vs. CD1).

Altered activity of the HPA axis and cortisol secretion are commonly associated with depression (Thomson and Craighead 2008). In addition, altered glucocorticoid receptor signaling has been implicated in depression-like bahvaior (Boyle *et al.* 2005). Mice with forebrain-specific disruption of glucocorticoid receptors show increased depression-like behavior, such as increased immobility in the FST. In the present study, glucocorticoid receptor mRNA levels were reduced in the PACAP–/– hippocampi, however, the dexamethasone-induced suppression in corticosterone levels was normally seen. The flattened circadian corticosterone level seen in our mutant mice might be consistent with studies

demonstrating depressed individuals exhibit a relatively flat and unresponsive pattern of cortisol secretion (Young *et al.* 1994; Burke *et al.* 2005). As a large body of evidence indicates that the 5-HT system and the HPA axis have complex inter-relationships that may central to the pathophysiology of depression (Maes *et al.* 1995; Porter *et al.* 2004), further studies are needed to address the mechanisms through which altered PACAP-signaling influences the 5-HT–corticosteroid interaction relevant to depression-like behavior. Such scrutiny may lead to dissection of cause and effect between HPA axis activation, glucocorticoid receptor down-regulation, and altered 5-HT systems.

Circadian rhythms of body temperature are regulated by the biological clock in the hypothalamic suprachiasmatic nucleus and, in some depressed patients, a weak 24-h periodicity of body temperature has been shown (van Londen *et al.* 2001). PACAP is co-stored with glutamate, an essential modulator of light entrainment, in a subset of retinal ganglion cells and in the retinohypothalamic tract, and relays photic information from the eyes to the suprachiasmatic nucleus (Hannibal *et al.* 2000). Recently, we have shown that PACAP−/− mice exhibit circadian defects characterized by an attenuated-phase advanced response to light stimulation at the late night/predawn period (Kawaguchi *et al.* 2003). Taking these results together, PACAP involvement in adjustment of the biological clock might underlie the development of depressive symptoms.

It has been known that the non-competitive NMDA receptor antagonist phencyclidine (PCP) reproduces a schizophrenia-like psychosis including both positive and negative symptoms. In mice, PCP-induced enhancement of immobility in FST is attenuated by the atypical antipsychotics, but not by the typical antipsychotics (Mouri *et al.* 2007). Given that the deficiency of PACAP leads to a hypofunction of NMDA receptor-mediated neurotransmission (Mabuchi *et al.* 2004; Ohnishi *et al.* 2008), the responsiveness to the atypical antipsychotics in FST common to the PCP model and PACAP−/− mice might be reasonably explained.

We have previously demonstrated that the novelty-induced hyperactivity and pre-pulse inhibition deficits in PACAP−/− mice are ameliorated by amphetamine, a psychostimulant widely prescribed to treat attention-deficit hyperactivity disorder (Tanaka *et al.* 2006). This result and the findings that the PACAP gene might be associated with schizophrenia (Hashimoto *et al.* 2007) suggest that PACAP is involved in endophenotypes, such as impairment of neurophysiology of mental and cognitive processes, rather than being associated with specific psychiatric disorders. Therefore, PACAP is implicated as a pre-disposing risk factor for neuropsychiatric disorders. This situation is similar to DISC1, as the locus of which is implicated as a risk factor for neuropsychiatric disorders, including schizophrenia, depression and autistic spectrum disorders (Millar *et al.* 2000; Blackwood *et al.* 2001; Kilpinen *et al.* 2008).

The pathophysiology of mental disorders can be a combination of subtle alterations of major signaling pathways. Products of promising risk genes can influence multiple pathways, and consequently act as strong predisposing factors. PACAP is known to have pleiotropic actions, e.g. modulation of various signaling systems such as dopamine (Takei *et al.* 1998), 5-HT (Hashimoto *et al.* 2001), and NMDA receptor-mediated signaling (Mabuchi *et al.* 2004; Ohnishi *et al.* 2008), as well as neurotrophic and neuroendocrine actions (Vaudry *et al.* 2000). Therefore, it is plausible that PACAP is part of a common genetic etiology shared by multiple mental disorders, and that PACAP signaling may be a target candidate for new therapies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Duration of immobility behavior in FST in PACAP−/− mice.

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