interaction with kendrin, because the yeast two-hybrid analysis indicated that this fragment binds to kendrin [17]. In accordance with these results, binding of KBR-deleted DISC1 to kendrin was not observed in our immunoprecipitation assay, whereas full-length DISC1 could interact with kendrin, suggesting that KBR is indispensable for the binding of DISC1 to kendrin. However, binding between KBR and kendrin was not observed (Fig. 1). We next investigated which region or regions of DISC1 were indispensable for the interaction with kendrin using the deletion mutants of DISC1. As shown in Fig. 1, KBRC, a fragment of DISC1 containing both KBR and the carboxy-terminus, could bind to kendrin. Therefore, the carboxy-terminal region downstream of KBR is essential for the DISC1–kendrin interaction. Furthermore, the KBRC region is also required for DISC1 to target to the centrosome (Fig. 2). Previous study have indicated that the carboxy-terminal region of DISC1 from the breakpoint (i.e., BPC) is required for targeting of DISC1 to the centrosome [26]. Our results further suggest that not only the carboxy-terminal region of DISC1 but also the KBR is indispensable for DISC1 targeting to the centrosome. These results suggest that centrosomal targeting of DISC1 is required for the interaction with kendrin.

The DISC1–kendrin interaction at the centrosome regulates the microtubule network formation

The present study demonstrated that overexpression of the DISC1-binding region of kendrin perturbed the normal distribution of the stabilized microtubule network (Fig. 3). And the overexpression of DISC1 ΔKBR caused the impairment of microtubule aster formation (Fig. 3). These results suggest that the DISC1–kendrin interaction is involved in the normal microtubule network formation. In mammalian cells, two giant centrosomal proteins, kendrin and CG-NAP, have been shown to anchor the γ-tubulin complex to the centrosome, and to play critical roles in the microtubule nucleation [20]. In the carrier of the chromosomal translocation segregating with the mental diseases, the truncated mutant DISC1 protein which lacks the carboxy-terminal region would be produced, or the expression of DISC1 protein would be reduced. In the case of truncated mutant protein expression, this protein would not be able to target to the centrosome and interact with kendrin, which might induce dysfunctions of the microtubule network, such as dysfunctions of organelle transport, protein localization, cell movement and mitotic chromosome segregation. And loss of DISC1 protein expression could lead to the dysfunction of microtubules by disrupting the DISC1–kendrin interaction. In fact, several studies have reported that schizophrenia is associated with abnormality of neuronal development [27–29]. In conclusion, we herein demonstrated that the DISC1–kendrin interaction played a role in neuronal development via the microtubule organization, and our findings cast new light on the etiology of mental disorders.

Acknowledgements

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Expert Opinion

1. Introduction
2. Schizophrenia and new treatments for schizophrenia
3. PACAP and PAC1 are key susceptibility factors for major mental illnesses
4. PACAP-PAC1 signaling and mental disease
5. Expert opinion. Regulation of PACAP signaling as a schizophrenia therapy

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Central & Peripheral Nervous System

Regulation of pituitary adenylyl cyclase-activating polypeptide (PACAP, ADCYAP1: adenylyl cyclase-activating polypeptide 1) in the treatment of schizophrenia

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Background: Deficiency of pituitary adenylyl cyclase-activating polypeptide (PACAP) and its specific receptor, PAC1, causes a schizophrenia-like phenotype in mice. In addition, the relation of the PACAP and PAC1 genes to schizophrenia has been shown by single-nucleotide polymorphism association studies. Furthermore, PACAP is reported to be involved in the function of disrupted-in-schizophrenia 1. *Objective*: To summarize briefly the recent evidence relating the PACAP system and schizophrenia and discuss the application of PACAP to the treatment of schizophrenia. *Results/conclusion*: The regulation of PACAPergic signals is an interesting potential treatment for schizophrenia. Further studies of PACAP signals and the association of PACAP signals with schizophrenia should shed the light on the utility of this approach in the treatment of schizophrenia.

Keywords: Adcyap1, cAMP, DBZ, DISC1, neuropeptide, PACAP, PAC1, psychiatric disease, schizophrenia

1. Introduction

Pituitary adenylyl cyclase-activating polypeptide (PACAP) was originally isolated as a novel hypothalamic neuropeptide by Arimura's group in 1989, based on its ability to stimulate adenylyl cyclase in rat anterior pituitary cell cultures [1]. PACAP-38 and the C-terminally truncated PACAP-27 are known as biologically active forms and PACAP-27 has an amino acid sequence with 68% shared identity with vasoactive intestinal polypeptide (VIP) and 37% with secretin, indicating that PACAP is a member of the VIP/glucagon/growth hormone-releasing hormone (GHRH)/secretin superfamily. PACAP is present not only in various areas of the central nervous system, including the hypothalamus and other brain regions but also in peripheral tissues, such as testicular germ cells, pituitary gland lobes and the adrenal medulla [2,3]. PACAP has a role in various neurobiological functions, such as neurotransmission and neural plasticity, as well as having neurotrophic effects *via* three heptahelical G-protein-linked receptors, PAC1, vasoactive intestinal peptide/pituitary adenylate cyclase activating peptide receptor 1 (VPAC1) and VPAC2. The PAC1 receptor is specific for PACAP and the two other receptors, VPAC1 and VPAC2, are shared by vasoactive intestinal peptide [2,3]. Previous reports showed that PACAP- and PACAP-specific receptor- (PAC1) deficient mice exhibited prominent behavioral abnormalities, reduced anxiety-like behavior,

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and abnormal social behavior, as well as impairment of hippocampal long-term potentiation (LTP) [4-9]. These observations indicate that PACAP signaling mediated *via* the PAC1 receptor has a critical role in the development and/or functioning of neural pathways and suggests the potential clinical relevance of PACAP signaling dysfunction to neuropsychiatric disorders. Furthermore, the relationship of the PACAP and PACAP-specific receptor genes to schizophrenia was reported in a single-nucleotide polymorphisms (SNPs) association study in a Japanese population [10]. Furthermore, recent studies suggest the involvement of PACAP signals in the neural transmission of abnormalities that are seen in psychotic disorders [11], suggesting that the PACAPergic system is one of the key factors for the pathogenesis of schizophrenia.

Schizophrenia is a devastating psychiatric disorder with a lifetime prevalence of about 1% of the population worldwide, and it commonly has a chronic course. The underlying mechanisms are still largely unknown but a growing body of evidence suggests that schizophrenia is a multifactorial disorder influenced by genetic, neurodevelopmental, and social factors [12-17]. Disrupted-in-schizophrenia 1 (DISC1) has been identified as a potential susceptibility gene for major psychiatric disorders. Disruption of this gene by a balanced (1;11) (q42.1;q14.3) translocation results in a predicted C-terminal truncation of the open reading frame, and this anomaly is segregated with schizophrenia, bipolar affective disorder and recurrent major depression in a large Scottish family [18,19]. In addition, a frameshift mutation of DISC1 has been identified in an American family with schizophrenia and schizoaffective disorder [20], while the association of the single nucleotide polymorphisms of DISC1 with schizophrenia, schizoaffective disorder and bipolar disorder has also been suggested [21]. Recent accumulating studies show that DISC1 plays an important role in neural development in cooperation with binding partners, such as Nudel, lissencephaly 1 (Lis1), fasciculation and elongation protein zeta-1 (FEZ1), Kendrin, phosphodiesterase 4B (PDE4B), and so on [22-29]. In our recent studies, we reported a new DISC1 interacting partner, DISC1-binding zinc-finger protein (DBZ), and showed that PACAP signaling *via* PAC1 regulates the interaction between DISC1 and DBZ [11], suggesting that the abnormality of DISC1, DBZ or PACAP-PAC1 signaling causes immature neural developments which result in susceptibility to psychiatric diseases.

In this article, we review the involvement of PACAP signaling in mental disorders, focusing on schizophrenia, in terms of the neurotransmission and the DISC1–DBZ interaction regulated by the PACAPergic signal.

2. Schizophrenia and new treatments for schizophrenia

Schizophrenia is a chronic psychotic illness with a lifetime prevalence of about 1% of the population worldwide, which shows overt psychosis striking typically during late adolescence and causing severe lifelong disability [30]. Family studies show that the risk of schizophrenia is significantly higher in families of schizophrenia probands than in the general population or in families of other affective disorder probands [31]. Since the concordance rate in monozygotic twins is not 100%, genetic background alone does not induce schizophrenia. However, the monozygotic concordance is higher than dizygotic concordance [32]. Therefore, it is certain that genetic background plays an important role in schizophrenia. Genetic linkage studies identified several candidate loci on the genome on the following chromosomes: 1q21 – 44, 5q22 – 31, 6p24 – 22, 8p22 – 21, 10p15 – 11, 13q14 – 32, and 22q11 – 13 [30]. Subsequent studies have identified several candidate genes for schizophrenia such as DISC1, dysbindin, catechol-*O*-methyltransferase (COMT), neuregulin 1, G72, Regulator of G protein signalling 4 (RGS4), and so on [30-47].

The schizophrenic patients show three major symptoms: positive psychotic symptoms, disorganization in thought and behavior, and negative symptoms [48], showing that schizophrenia is multifunctional in origin. In addition, several functional impairments in cognitive functions are known to be symptoms of schizophrenia [49]. These impairments are used as essential diagnostic criteria in the Diagnostic and Statistical Manual IV (DSM-IV). Based on accumulating studies of the symptoms of schizophrenia, several hypotheses based on abnormal neurotransmission are supported, such as the dopamine hypothesis, which is based on hyperactive dopaminergic signal transduction, the glutamate hypothesis, which is based on hypofunction of glutamatergic signaling, and the serotonin hypothesis, which is based on hypofunction of serotonergic signaling [50-56] Furthermore, studies of brain imaging have elucidated significant changes in neural transmission in the schizophrenic brain [57-60].

New treatments for schizophrenia have been developed based on the finding that dopamine-enhancing drugs mimic psychotic symptoms. In fact, dopamine D2 receptor blockers, such as chlorpromazine, are effective for the treatment of schizophrenia, particularly for the positive psychotic symptoms. Dopamine D2 receptor blockers decrease psychotic symptoms, such as hallucinations, delusions and agitation [61-65]. However, they cause some side effects: the extrapyramidal side effects such as Parkinsonism and abnormality of the endocrine system, such as hyperprolactinemia, and they have little effect on the negative symptoms [66-67]. Thus, the 5-hydroxytryptamine 5-HT_2 receptor became the center of attention for the next target of schizophrenia therapy and serotonin-dopamine antagonists (SDA), which inhibit both dopamine D2 receptor and 5-HT_2 receptor [68]. The SDA has the advantage of reduced side effects and improvement of several negative symptoms compared with the dopamine D2 receptor inhibitor [69-74]. Now serotonin 5-HT_1A receptor partial agonists, selective

agonists for metabotropic glutamate 2/3 receptors and so on, are in the spotlight as the next targets [75-79]. It is well known that the inhibition of glutamate transmission induces positive and negative psychotic symptoms. Accordingly, selective agonists for metabotropic glutamate 2/3 receptors produced significant improvements in both positive and negative symptoms of schizophrenia compared with placebo [74]. As mentioned above, the regulation of the glutamatergic system, as well as dopaminergic and serotnergic systems, is an important target for the treatment of schizophrenia (Figure 1).

Another hypothesis is that there is an impaired development of neural networks in the brains of schizophrenia patients. Recent advances in imaging technology (such as functional MRI (fMRI) and diffusion tensor imaging (DTI)) have enabled investigators to move beyond measures of isolated regional abnormalities, and instead begin to explore the function and structure of the interconnected neural networks that are implicated in schizophrenia. The most consistent structural abnormalities found in schizophrenia include lateral and third ventricular enlargement; medial temporal lobe (hippocampal formation, subiculum, parahippocampal gyrus) volume reductions; and superior temporal gyrus volume reductions, particularly on the left, and several other abnormalities in other parts of the brain have been reported [80-82]. In addition, abnormal brain structure may be detectable via MRI prior to the onset of psychotic symptoms [83], and these anomalous late neurodevelopmental processes may interact with other environmental factors associated with the onset of psychosis (e.g., stress, substance abuse), which together have neuroprogressive sequelae that may be neurodegenerative [84,85]. These reports suggest the involvement of abnormal neurodevelopment in schizophrenia.

3. PACAP and PAC1 are key susceptibility factors for major mental illnesses

3.1 Genetic association of PACAP and PAC1 to major mental illnesses

Many studies reported that susceptibility genes may be present on chromosome 18p where the PACAP gene is located [86-91]. A possible linkage of certain psychiatric diseases with the PACAP gene Adcyap1 has been suggested. Studies in two related patients with a partial trisomy 18p revealed three copies of the PACAP gene and elevated plasma PACAP levels. These patients suffered from severe mental retardation and hematological abnormalities, although whether the former defect is a consequence of PACAP overexpression remains to be determined [92]. Fine-scale mapping of a locus for severe bipolar mood disorder on chromosome 18p11.3 suggests that Adcyap1, residing at 18p11.32, is located close to a bipolar disorder risk locus [92-94]. In addition, recent studies reported that genetic variants of the PACAP and PAC1 genes are associated with schizophrenia and that the

risk SNP of the PACAP gene could be associated with reduced hippocampal volume and poorer memory performance [10]. These reports support the association of the PACAPergic system with psychiatric disorders, especially bipolar disorder and schizophrenia.

3.2 Abnormal behaviors related to mental illnesses in PACAP-deficient mice and the effects of the defect or overexpression of PAC1 on mice

The suggestion mentioned above, which is based on data from humans, is also supported by data obtained from genetically engineered animals. PACAP-deficient mice displayed the abnormal behaviors, such as significantly increased locomotor activity with minimal habituation to the environment, explosive jumping behavior, increased exploratory behavior, and less time engaged in licking and grooming behavior in a novel environment [4]. Furthermore, PACAP-deficient mice display prepulse inhibition (PPI) deficits and impairments of PPI hippocampal LTP [4-6]. In addition, hyperactivity in PACAP knockout mice was abolished by an atypical antipsychotic drug, haloperidol (D_2 antagonist). Risperidone, a combined D2 and $5HT_{2A}$ receptor antagonist, reversed the hyperactivity and diminished PPI in PACAP KO mice to the level observed in wild type mice [10], suggesting that the abnormal behaviors in PACAP KO mice are schizophrenia-like phenotypes in rodents. In addition, the jumping behavior is suppressed by drugs that elevate extracellular serotonin, such as the selective serotonin re-uptake inhibitors (see Section 3.3) [95].
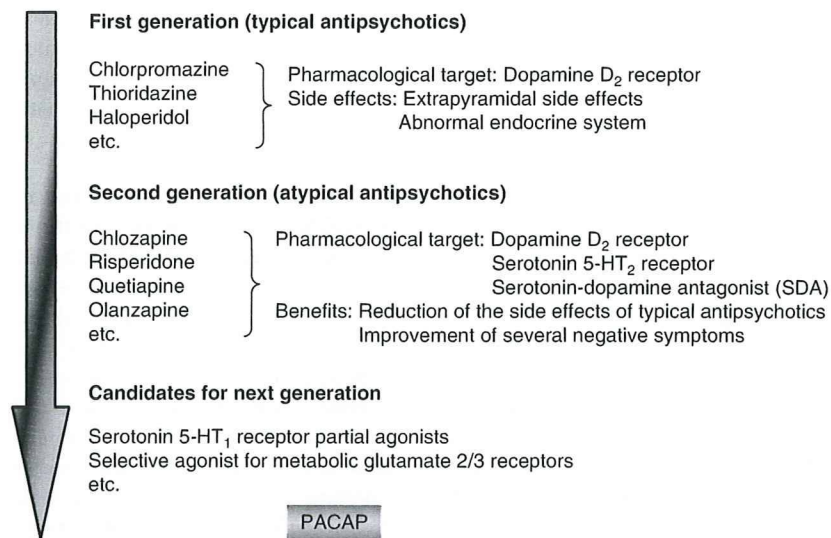
PAC1 receptor-deficient mice also exhibited an increase in locomotor activity, reduced anxiety-like behavior, and abnormal social behavior, as well as impairment of hippocampal LTP [96-98]. PAC1-overexpression displayed strikingly similar phenotypes to PAC1 knockout mice, hydrocephalus-related phenotypes [99]. Furthermore, chronic treatment with PCP, which induces positive symptoms, negative symptoms and cognitive impairments similar to those seen in patients with schizophrenia, reduced the mRNA expression of PACAP and PAC1 in the frontal cortex (Figure 2) [10].

These observations indicate that PACAP signaling, mediated via the PAC1 receptor, is associated with the pathophysiology of schizophrenia and has a critical role in the development and/or functioning of neural pathways and also indicate a potential role of PACAP signaling dysfunction in neuropsychiatric disorders.
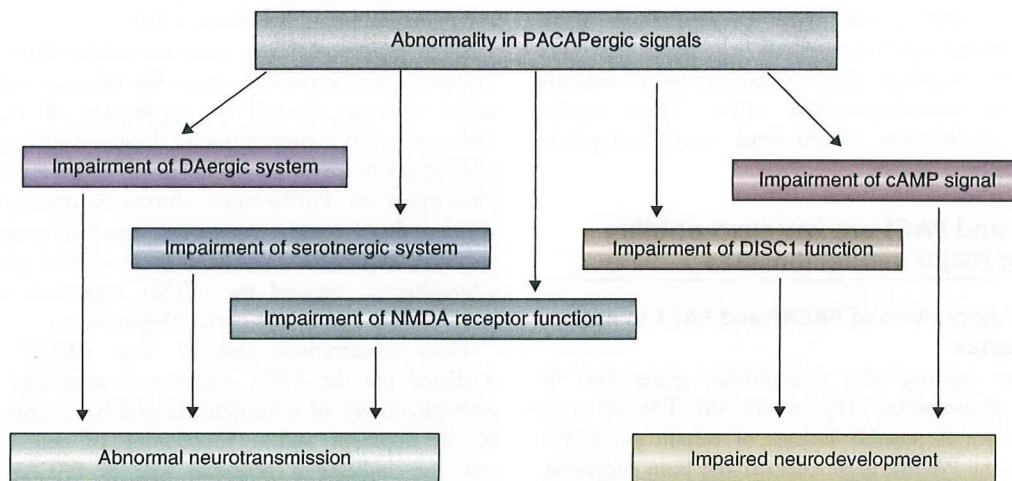
3.3 Association of the PACAP–PAC1 receptor system with other neurotransmitter systems such as the dopaminergic, glutamatergic and serotonergic systems (Figure 2)

PACAP has been shown to increase tyrosine hydroxylase (TH) protein activity and mRNA levels in vivo and in vitro [100,101]. Intracerebroventricular injection of PACAP increased dopamine release in the hypothalamus in sheep [102]. Hyperlocomotion and jumping behavior but

Regulation of pituitary adenylyl cyclase-activating polypeptide (PACAP, ADCYAP1: adenylyl cyclase-activating polypeptide 1) in the treatment of schizophrenia

First generation (typical antipsychotics)

Chlorpromazine
Thioridazine
Haloperidol
etc.

Pharmacological target: Dopamine D_2 receptor
Side effects: Extrapyramidal side effects
Abnormal endocrine system

Second generation (atypical antipsychotics)

Chlozapine
Risperidone
Quetiapine
Olanzapine
etc.

Pharmacological target: Dopamine D_2 receptor
Serotonin $5-HT_2$ receptor
Serotonin-dopamine antagonist (SDA)
Benefits: Reduction of the side effects of typical antipsychotics
Improvement of several negative symptoms

Candidates for next generation

Serotonin $5-HT_1$ receptor partial agonists
Selective agonist for metabolic glutamate 2/3 receptors
etc.

PACAP

Figure 1. Development of the treatments for schizophrenia. First, typical antipsychotics were developed as a treatment for schizophrenia. Next the 5-hydroxytryptamine ($5-HT_2$) receptor became the center of attention for schizophrenia therapy because atypical antipsychotics has the advantage of reduced side effects and improvement of several negative symptoms compared with typical antipsychotics. Now the regulation of glutamate transmission is one of the important targets of the treatment for schizophrenia. Regulation of PACAP signals might be a potential treatment for schizophrenia.

Abnormality in PACAPergic signals

Impairment of DAergic system

Impairment of serotnergic system

Impairment of NMDA receptor function

Impairment of cAMP signal

Impairment of DISC1 function

Abnormal neurotransmission

Impaired neurodevelopment

Figure 2. Impairment of PACAP signals and schizophrenia Impaired PACAP signals resulted in several abnormal phenotypes *in vivo* and *in vitro*. The abnormalities caused by the impaired PACAPergic system have close relationship to several hypotheses regarding the pathology for schizophrenia, such as abnormal neurotransmission and impaired neurodevelopment.
DISC1: Disrupted-in-schizophrenia 1.

not deficits in PPI, in PACAP deficient mice were attenuated effectively by haloperidol (a D2 antagonist) [4,5]. Furthermore, the selective serotonin re-uptake inhibitor, fluoxetine, as well as the serotonin precursor, 5-hydroxytryptophan, suppress jumping behavior. Risperidone, a combined D2 and 5-HT$_{2A}$ receptor antagonist, reversed both of these abnormalities in PACAP$^{-/-}$ mice [10]. These accumulating studies indicate the presence of an interaction between the PACAP system and the dopaminergic and serotonergic systems. On the other hand, several studies have shown that PACAP can potentiate NMDA receptor functions [103-105]. Furthermore, Mabuchi et al. showed that PACAP deficient mice do not exhibit inflammatory or neuropathic pain, and that PACAP is required for functional coupling of neuronal nitric oxide synthase to NMDA receptors in the spinal cord for chronic pain to occur [106]. These reports suggest that PACAP signals also involve the glutamatergic system via NMDA. The NMDA antagonist, MK-801, induces similar behavior to PACAP knockout [107]. Furthermore, chronic treatment with PCP, which induces positive symptoms, negative symptoms and cognitive impairments, similar to those seen in patients with schizophrenia, reduced the mRNA expression of PACAP and PAC1 in the frontal cortex [10]. These results suggest an association between the PACAP and glutamatergic system. As mentioned in Section 2, the regulation of the glutamatergic system is a potential target for treatment. The regulation of PACAP may shed light on potential schizophrenia treatments.

## 4. PACAP-PAC1 signaling and mental disease

4.1 DISC1 and schizophrenia

DISC1 has been identified as a potential susceptibility gene for major psychiatric disorders [18,19]. A balanced translocation of DISC1 segregated with schizophrenia and affective mental disorders. Therefore, it is likely that the function of the molecular complex composed of DISC1 and the binding proteins, which bind to the translocation site of DISC1, is disturbed in schizophrenic patients. Recently, using the yeast two-hybrid method, we have identified three molecules that bind to the domain of DISC1 including the translocation site, namely FEZ1, DBZ and Kendrin [11,25,26]. In addition, other authors have identified interacting partners of DISC1, such as Nudel, Lis1 and PDE4B [22-24,27-29] The function of the DISC1-FEZ1 interaction was explored by us [25]. DISC1 and FEZ1 were found to be co-localized in growth cones in association with F-actin in both SK-N-SH cells and cultured hippocampal neurons [11]. The interaction of DISC1 with FEZ1 was enhanced markedly along with neurite extension in PC12 cells by nerve growth factor (NGF) stimulation [11,108]. An association between single nucleotide polymorphisms of the FEZ1 gene and schizophrenia has also been suggested in a Japanese population [109]. In the report, we showed the importance of the DISC1-FEZ1 interaction in neural development and supported the hypothesis that impaired

brain development causes schizophrenia. We also found involvement of PACAP in neural development via the regulation of DISC1-DBZ interaction [11]. The following is a detailed description of the DISC1-DBZ interaction (see Section 4.2).

4.2 PACAP-PAC1 signaling regulates the DISC1-DBZ interaction (Figure 3)

DBZ encodes a putative 407 amino acid protein without significant homology to any other known protein and is expressed exclusively in the brain. DBZ is expressed in neurons, not in glial cells. DBZ is colocalized with DISC1 diffusely in the cytoplasm. We searched for molecules that influence the DISC1-DBZ interaction and found that PACAP has a marked influence on the endogenous DISC1-DBZ interaction. The co-immunoprecipitation of DISC1 with DBZ in lysates is reduced by 80% 1 h after treatment of PC12 cells with PACAP. Thereafter, the DISC1-DBZ interaction increases gradually, returning to the control level by 24 h after treatment. Thus, PACAP stimulation caused a transient dissociation between DISC1 and DBZ [11]. To investigate the physiological role of the DISC1-DBZ interaction, the effects of enhanced DISC1-DBZ binding on PC12 cells was examined in the PC12 cells expressing DISC1-haemagglutinin (HA) and DBZ-green fluorescent protein (GFP). After the DISC1/DBZ overexpressing cells were treated with PACAP for 48 h, the number of neurite-bearing cells was counted. Overexpression of both DBZ and DISC1, which enhances the DISC1-DBZ interaction, resulted in a significant decrease in the number of neurite-bearing PC12 cells, whereas overexpression of either DBZ or DISC1 alone did not alter the number of neurite-bearing cells significantly. No significant changes in neurite length or the number of neurites per cell were observed in either the mock- or DISC1-HA-transfected PC12 cells, irrespective of the expression of DBZ. Furthermore, we investigated the effect of the inhibition of the dissociation between DISC1 and DBZ on neurite outgrowth to confirm the role of DBZ dissociated from DISC1 under PACAP stimulation. Overexpression of the DISC1 binding domain of DBZ inhibits the neurite outgrowth of PACAP-treated PC12 cells and primary cultured neurons without any significant change in apoptosis of gene-transfected PC12 cells. These findings show that the DISC1-DBZ interaction has an important role in neurite growth and suggest that one function of DBZ is as a negative regulator of DISC1 function. Furthermore, we showed that the PAC1 inhibitor, which inhibits the effect of PACAP on neurite outgrowth, reduced the dissociation between DISC1 and DBZ caused by PACAP stimulation (Matsuzaki et al., unpublished). Thus, in the normal brain, the dissociation of the DISC1-DBZ interaction by PACAP via the PAC1 receptor should cause neurite extension, and the subsequent increase in the DISC1-DBZ interaction may inhibit neurite outgrowth and neural circuit formation (Figure 3A). In schizophrenia in DISC1 translocation

Regulation of pituitary adenylyl cyclase-activating polypeptide (PACAP, ADCYAP1: adenylyl cyclase-activating polypeptide 1) in the treatment of schizophrenia
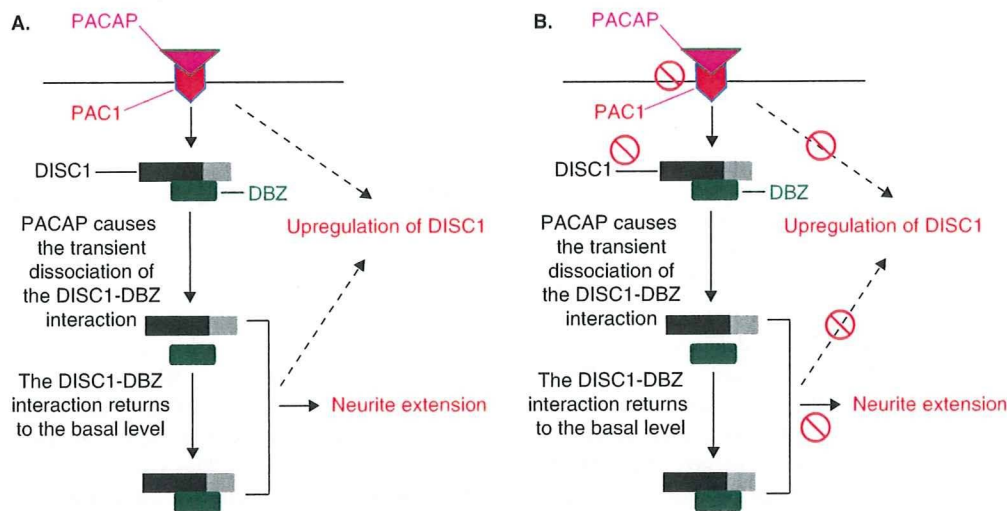
Figure 3. Regulation of DISC1 function via PACAP signal PACAP signal via PAC1 causes the transient dissociation of DISC1–DBZ interaction and results in neurite outgrowth. An abnormality of PACAP, PAC1 or DISC1 should cause the inhibition of neurite extension.

DBZ: DISC1 binding zinc-finger protein; DISC1: Disrupted-in-schizophrenia 1; PAC1: PACAP specific receptor; PACAP: Pituitary adenylyl cyclase-activating polypeptide.

carriers, DBZ cannot bind to DISC1 because of the translocation. Therefore, neurite growth by dissociation of the DISC1–DBZ interaction by PACAP is inhibited and neural circuit formation remains immature (**Figure 3B**). This event seems to occur in the granule cells of the dentate gyrus because these cells express both DBZ and PAC1 in comparisons of the distribution of neurons expressing DBZ mRNA with those expressing the PACAP receptor PAC1 mRNA (Matsuzaki *et al.* unpublished). Furthermore, PACAP in the granule cells of the dentate gyrus is supplied by the PACAP neurons in the entorhinal cortex through perforating fibers. Thus, disruption of the DISC1–DBZ interaction by abnormalities in DISC1, DBZ, or PACAP–PAC1 signaling causes immature neural circuit formation in the granule cell layer of the dentate gyrus and abnormalities in PACAP-PAC1 signaling may participate in the immature neural development of schizophrenic brains.

4.3 Molecular mechanism of upregulation of adenosine 3′,5′-monophosphate (cAMP) production by the binding of PACAP to the PAC1 receptor

PACAP stimulates cAMP and a recent paper suggests the involvement of the PAC1 receptor in the enhancement of cAMP generation [110,111]. Furthermore, patients with a trisomy 18p, who show elevated PACAP concentration in plasma, have upregulation of cAMP levels [92]. These reports suggest that the PACAP–PAC1 signal is of importance in the enhancement of cAMP. Recently, Millar *et al.* reported the regulation of cAMP level *via* the DISC1–PDE4B interaction as well as a close association of PDE4B with schizophrenia [23]. The regulation system involves the

elevation of cellular cAMP, which leads to dissociation of PDE4B, one of the inactivators of cAMP, from DISC1, and an increase in PDE4B activity. Furthermore, lower activity of PDE4B was identified in the DISC1 mutant mice, which exhibit schizophrenia-like behavior [112]. A PDE4B haplotype alters the genetic risk of schizophrenia in a Scottish population which is consistent with the known participation of this gene in biological processes associated with mental illness [113]. These findings showed that the upregulated cAMP level by binding of PACAP to PAC1 might be caused by an increase in the DISC1–PDE4B interaction and return to the basal level via the dissociation of DISC1–PDE4B interaction.

Many reports support the involvement of an abnormal cAMP level in mental disease and involvement of abnormalities in components of cAMP signaling in schizophrenia [114-117]. Lower levels of Gi were reported in schizophrenic patients [118], whereas the cAMP response to forskolin, as well as the binding of forskolin to adenylyl cyclase, were found to be elevated significantly in schizophrenic patients [119-120]. These alterations may have associated effects on PKA, a crucial component of cAMP signaling which mediates most of the actions of cAMP, including the regulation of gene expression [121]. An increase in cAMP binding to PKA was reported in schizophrenic patients [118].

As mentioned above, PACAP–PAC1 signals not only dissociate the DISC1–DBZ interaction, which cause neurite outgrowth but also increase the cAMP level. After the dissociation of the DISC1–DBZ interaction, the DISC1–PDE4B interaction might be induced and result

in the upregulation of cAMP, suggesting a strong involvement of PACAP–PAC1 signals in the pathogenesis of schizophrenia. Thus, the regulation of the PACAPergic system, especially *via* the PAC1 receptor, might be a new target for schizophrenia treatment based on the impaired neurodevelopment hypothesis (Figure 2).

5. Expert opinion. Regulation of PACAP signaling as a schizophrenia therapy

Schizophrenia is a disorder caused by the complex interplay of genetic and environmental factors, and the phenotype of the symptoms is quite variable with positive, negative and cognitive symptoms. These things make the treatment of schizophrenia difficult. However, accumulating studies have elucidated that an abnormal neural network formation and abnormal neural transmission is common to schizophrenia. Thus, it is necessary to improve these abnormalities to treat schizophrenia. As described in Sections 2 – 4, PACAP, one of the susceptibility genes of schizophrenia, is involved in both neural transmission and neural development suggesting that the regulation of PACAP might be one of the keys to the treatment of schizophrenia (Figure 2).

As mentioned before, hypofunction of PACAPergic signals is involved in schizophrenia. With hypofunction of the PACAP system, dopaminergic and serotonergic systems are activated, while the glutamergic system is inactivated. Thus, the PACAP system is present upstream of regulation of dopaminergic, serotonergic and glutamergic transmission, and regulation of the PACAP signal pathway is much more effective and selective for the treatment of schizophrenia than that of regulation of the dopamine, serotonin and glutamate systems. In addition, Shintani *et al.* showed that the alteration observed in PACAP knockout mice recovered by the addition of transient PACAP [95] and transient PACAP stimulation of dorsal root ganglion (DRG) neurons affects the NMDA receptor response by increasing the stability of the NMDA receptor via the functional coupling of neuronal nitric oxide synthase to the NMDA receptors [106]. These data suggest that the abnormal phenotype of PACAP- and PAC1-deficient mice contributes not only to the abnormal neural circuit via the impairment of PACAPergic signals but also by direct effects of PACAPergic signals on neural transmission. Thus, PACAP may be a therapeutic effector for the symptoms of schizophrenia via the abnormal transmissions. Hyper-dopaminergic signals might be ameliorated by the inhibition of PACAP signals and hypo-glutamaergic or hypo-serotonergic signals might be ameliorated by the upregulation of PACAP via the upregulation of the NMDA receptor or the upregulation of the serotonergic system. Hence, the regulation of PACAP signals may treat abnormal neural transmission observed in schizophrenic brains in multiple ways. In addition, PACAP–PAC1 signal also plays an important role in neural development and the impairments in PACAPergic signals

cause abnormal brain development (see Sections 4.2 and 4.3), whereas PACAP is thought to be one of the risk factors for schizophrenia from the standpoint of the hypothesis that abnormal neural development is one of the risks for schizophrenia.

As shown here, the regulation of PACAPergic signals as the therapy for schizophrenia should improve not only dopaminergic, serotonergic and glutamergic transmission but also nerural development. In comparison with traditional treatment strategies, PACAP therapy has this advantage. Thus, the regulation of PACAP or PACAPergic signals has promising therapeutic efficacy for schizophrenia.

Is it possible to regulate the PACAPergic signals in human? A way to regulate the PACAP signals in humans has not established yet, but several studies suggest the potential for regulating PACAP levels. A previous study showed that lithium stimulation, one of the therapies for bipolar disorder, caused the upregulation of PACAP levels [122]. This result might indicate a method for upregulation of PACAP on psychiatric diseases. On the other hand, previous studies indicate that the major isoforms of PACAP, (1 – 27) and (1 – 38), are degraded by the ubiquitous enzyme dipeptidyl peptidase IV (DPP-IV) to form PACAP(3 – 27) and (3 – 38) [123] and that DPP-IV degrade PACAP(1 – 27) to PACAP(3 – 27), to PACAP(5 – 27) and finally to PACAP(6 – 27), which has been suggested to antagonize the actions of PACAP in the pancreatic cell [124]. These findings indicate that the inhibition of DPP-IV should be effective for upregulating PACAP levels.

Lastly, we discuss the side effects of PACAPergic-based therapies for schizophrenia because we cannot ignore these. As mentioned above, PACAP–PAC1 signaling also affects DISC1 function via alteration of the DISC1–DBZ interaction [11]. We suggest that DISC1 interacts with other partners, such as FEZ1, to function at several biological levels after dissociation from DBZ. In addition, we have reported that PACAP stimulation causes the upregulation of DISC1 [11]. Thus, continuous inhibition of PACAP signaling might cause the downregulation of DISC1 function because of the downregulation of DISC1 expression. Furthermore, continuous inhibition should cause several side effects since PACAP- and PAC1-knockout mice show several abnormalities [4-6,10,96-98]. In addition, the inhibition of PACAP signaling should inhibit cAMP activity directly and indirectly via the downregulation of the DISC1–PDE4B interaction. Conversely, continuous stimulation by PACAP should affect DISC1 function and might cause the upregulation of DISC1. Kamiya *et al.* showed that overexpression of DISC1 causes the hyperformation of the centrosome [125]. Patients with trisomy 18p, whose PACAP level is elevated, show mental retardation, and PAC1-transgenic mice showed abnormal neural development [92,103]. These results suggest that continuous stimulation by PACAP causes problems in neurodevelopment via the upregulation of cAMP and DISC1 function. Thus, moderate PACAP stimulation is that key to

maintaining normal neural network systems. Summarizing these data, the pulse inhibition of PACAP might be effective in patients with mental disorders or schizophrenia via the hyper PACAPergic system and the pulse stimulation of PACAP might be effective in patients with mental disorders or schizophrenia via the hypo PACAPergic system.

In conclusion, the regulation of PACAPergic signals is an interesting potential treatment for schizophrenia. Further studies of PACAP signals and the association of PACAP signals with schizophrenia should shed light on the utility of this approach in the treatment of schizophrenia.

Declaration of interest

The authors declare no conflicts of interest and have received no payment for the preparation of this manuscript.

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RIGHTS LINK

Regulation of pituitary adenylyl cyclase-activating polypeptide (PACAP, ADCYAP1: adenylyl cyclase-activating polypeptide 1) in the treatment of schizophrenia

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RIGHTS LINK

Behavioral abnormalities and dopamine reductions in *sdy* mutant mice with a deletion in *Dtnbp1*, a susceptibility gene for schizophrenia

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ABSTRACT

Genetic susceptibility plays an important role in the pathogenesis of schizophrenia. Genetic evidence for an association between the dysbindin-1 gene (*DTNBP1*: dystrobrevin binding protein 1) and schizophrenia has been repeatedly reported in various populations worldwide. Thus, we performed behavioral analyses on homozygous *sandy* (*sdy*) mice, which lack dysbindin-1 owing to a deletion in the *Dtnbp1* gene. Our results showed that *sdy* mice were less active and spent less time in the center of an open field apparatus. Consistent with the latter observation, *sdy* mice also displayed evidence of heightened anxiety-like response and deficits in social interaction. Compared to wild-type mice, *sdy* mice displayed lower levels of dopamine, but not glutamate, in the cerebral cortex, hippocampus, and hypothalamus. These findings indicate that *sdy* mice display a number of behavioral abnormalities associated with schizophrenia and suggest that these abnormalities may be mediated by reductions in forebrain dopamine transmission.

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Schizophrenia is characterized by psychosis and profound disturbances of cognition, emotion, and social functioning. The dysbindin-1 gene (*DTNBP1*: dystrobrevin binding protein 1), have recently been identified as a susceptibility gene for schizophrenia [1,2]. In studies on postmortem brain tissue, decreased expression levels of dysbindin-1 protein [3] and mRNA [4] have been shown in patients with schizophrenia compared with controls. Chronic treatment of mice with antipsychotics did not affect the expression levels of dysbindin-1 protein and mRNA in their brains [3,5], suggesting that prior evidence of lower levels of dysbindin-1 protein and mRNA in the postmortem brains of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. These data indicate that the dysbindin-1 gene may confer susceptibility to schizophrenia through reduced expression.

Dysbindin-1 is relatively ubiquitously expressed in neuronal cell bodies in most parts of the brain and in primary dendrites of those neurons and is concentrated in axon terminals of some areas such as the hippocampal formation, striatum, substantia nigra, and cerebellum [3,6]. Previous studies reported that down-regulation of endogenous dysbindin-1 by small interfering RNA (siRNA) resulted in a reduction in the release of glutamate from primary cultured neurons [7] and an increase in the release of dopamine from PC12 cells [8]. These results suggest possible roles for the dysbindin-1 gene in glutamatergic and dopaminergic systems related to the pathophysiology of schizophrenia [9].

To investigate the functions of dysbindin-1 *in vivo*, we analyzed *sandy* (*sdy*) mutant mice, which express no dysbindin-1 protein owing to a deletion in the dysbindin-1 gene [10]. *Sdy* is a mutant mouse with diluted pigmentation that arose spontaneously in the DBA/2J inbred mouse strain and has simultaneous defects in melanosomes, lysosomes and platelet dense granules [11]. Thus, we first performed several behavioral analyses and measured dopamine and glutamate contents in several brain regions in *sdy* mice.

Materials and methods

Animals. We obtained *sdy* mice from the Jackson Laboratory (Bar Harbor, ME). *Sdy* mice have an autosomal recessive coat color

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mutation that arose spontaneously in the inbred DBA/2J strain. *Sdy* mice have a large deletion in the dysbindin-1 gene, from nucleotide 3701 of intron 5 to nucleotide 12377 of intron 7, and this deletion results in essentially total loss of dysbindin-1 [10]. Both *sdy* mice and wild-type mice derived from heterozygote crossings were used in all experiments. To minimize the influence of cage environment, they were bred under the same conditions after weaning at 3 weeks of age. The genotypes of mice were identified by their coat color and genomic PCR. Primers i6_f (5′-GCACTCAGGA GACCATGACA-3′) and i6_r (5′-GGTTGACACTCTTGCGGAAT-3′) amplified a region in intron 6, and produced 305 bp PCR products from normal DNA. Primers i5, designed for intron 5 (5′-CCTAGCCCC TCAGGAATTGT-3′), and i7, designed for intron 7 (5′-GGGAATGGG GTCTTAATGGT-3′), amplified 733 bp PCR products from mutant DNA. The genomic sequences of these PCR products were confirmed by sequence analysis. The experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Neuroscience, Japan.

Experimental design for behavioral tests. All behavioral tests were carried out as described previously [12] using male mice that were 6–9 weeks old (*sdy* mice: $n = 119$; wild-type mice: $n = 120$). We used different batches of mice for each behavioral test. Mice were housed four per cage in a temperature-controlled room under a 12 h light–dark cycle (light on at 8:00 a.m.) with ad libitum access to food and water. All behavioral tests were performed between 10:00 a.m. and 7:00 p.m. After each trial, all apparatus were cleaned with water to prevent a bias based on olfactory cues.

Open field test. Locomotor activity was measured using an open field test. Activity was recorded during the first exposure to the open field apparatus (50 × 50 × 40 cm; O'Hara & Co., Tokyo, Japan). The illumination level was 40 lux at the floor of the open field. The field was divided by software (see below) into 16 equal-sized squares containing 4 central areas and 12 peripheral areas. Time spent in the central area defined as [stay time in center (%) = time spent in central areas/objective time for analysis (15 min or 30 min)] and the number of fecal boli were recorded. Data were collected for 30 min. Data acquisition and analysis were performed automatically, using Image OF software (see Behavioral data analysis).

Elevated plus maze test. The elevated plus maze consisted of two black plastic walkways (25 × 5 cm) 34 cm above the floor intersecting at right angles with one of the walkways having 15 cm high wall. To prevent animals from falling off the apparatus, 3-mm-high ledges were provided on the open arms (O'Hara & Co., Tokyo, Japan). A mouse was placed in the central square of the maze (5 × 5 cm), facing one of the enclosed arms. The behavior was recorded during a 20 min test session, because mice entered into the open arms a few times (*sdy* mice; 2.1 ± 0.7, wild-type mice; 3.6 ± 0.8) for a 10 min test session. The illumination level was 40 lux at the central square of the maze. For data analysis, we used the following four measures: the number of entries into open arms, the total number of arm entries, the time spent on the open arms and the total distance traveled. Data acquisition and analysis were performed automatically, using Image EP software (see Behavioral data analysis).

Social interaction test. A pair of mice was placed simultaneously at opposing corners in the open field apparatus (50 × 50 × 40 cm; O'Hara & Co., Tokyo, Japan) and allowed to explore freely for 30 min. The pair of mice tested was composed of the same genotype (*sdy–sdy* pair or wild–wild pair) and had been housed in the same environmental conditions, but in different cages. The illumination level was 40 lux at the floor of the open field. Mice were familiar with the test arena by placing them singly in the apparatus, under the same light level (40 lux), for a 30 min period at least 2 days preceding the test. Total duration of contacts, the number of contacts and total distance traveled were measured. Analysis was

performed automatically using Image SI software (see Behavioral data analysis).

Behavioral data analysis. Behavioral data from the open field tests, elevated plus maze tests and social interaction tests were automatically analyzed as described previously [12]. Briefly, behaviors were monitored by a color charged-coupled device camera (Watec Co., Ltd., Yamagata, Japan) that was connected to a Macintosh computer. We used apparatuses with black-colored floors to detect behaviors of mice, because coat colors of mice were whitish (*sdy*) and dilute brown (wild-type). Images were captured at one or two frames per second. The applications used for the behavioral studies (Image OF, Image EP, Image SI, O'Hara & Co., Tokyo, Japan) were run using a Macintosh computer. These modified applications were based on the public domain NIH Image program developed at the U.S. National Institute of Mental Health.

Neurotransmitter measurements by HPLC-fluorometry. We measured the dopamine and glutamate levels in intact brain with a different batch of mice used for behavioral tests. Mice (male: 8–12 weeks old) were sacrificed by decapitation, and the decapitated heads were dropped directly into ice-cold water for 1 s to prevent degradation of neurotransmitters. Brains were removed from the calvarium and put on a chilled aluminum board. The brain was dissected into ten regions (olfactory bulb, OB; frontal pole cortex, FPC; non-frontal cerebral cortex, NF CX; cerebellum, CB; hippocampal formation, HF; striatum, ST; midbrain, MB; lower brainstem, LB; thalamus, TH; hypothalamus, HT) according to a previously reported method [13] with slight modification. Each block of brain tissue was put into a pre-weighed sampling tube. Brain tissues were homogenized in 9 volumes of 2% perchloric acid (PCA) solution (Katayama Chemical Industries Co., Ltd., Japan) including 1 mM EDTA–Na$_2$ and 1 mM Na$_2$S$_2$O$_5$ using a sonicator for 5–10 s. Homogenates were centrifuged at 10,000g for 30 min. The level of dopamine in the supernatant was determined by a fully automated HPLC system (Model HLC-725CA Catecholamine analyzer, Tosoh, Tokyo, Japan) using a diphenylethylenediamine condensation method [14]; glutamate levels were measured by a pre-label HPLC-fluorometric method [15].

Statistical analysis. Statistical analysis was conducted using SPSS 11.0J for Windows (SPSS Japan Inc., Tokyo, Japan). Data were analyzed by a two-tailed *t*-test unless otherwise noted. Fisher's exact test was used to compare *sdy* mice with wild-type mice for general health (physical characteristics, sensory/motor reflexes and the motor test). A repeated measures analysis of variance (ANOVA) was used to analyze differences in the time course of distance traveled in the open field test. For measurements of neurotransmitters, statistical significance was analyzed using the Student's *t*-test. All *p*-values reported are two-tailed. Statistical significance was defined as $p < 0.05$.

Results

General characteristics

There was no significant difference in body weight, physical characteristics (whiskers and fur), sensory-motor reflexes (eye blink, ear twitch, whisker response and righting reflex) or neuromuscular strength between *sdy* mice and wild-type mice (Supplementary Table S1). *Sdy* mice were more sensitive to 120DB auditory stimulation than wild-type mice, however, there was no significant difference in PPI between *sdy* and wild-type mice (data not shown).

Locomotor activity in a novel environment

Sdy mice showed a pronounced decrease in locomotor activity in the open field test compared with wild-type mice (Fig. 1A, geno-
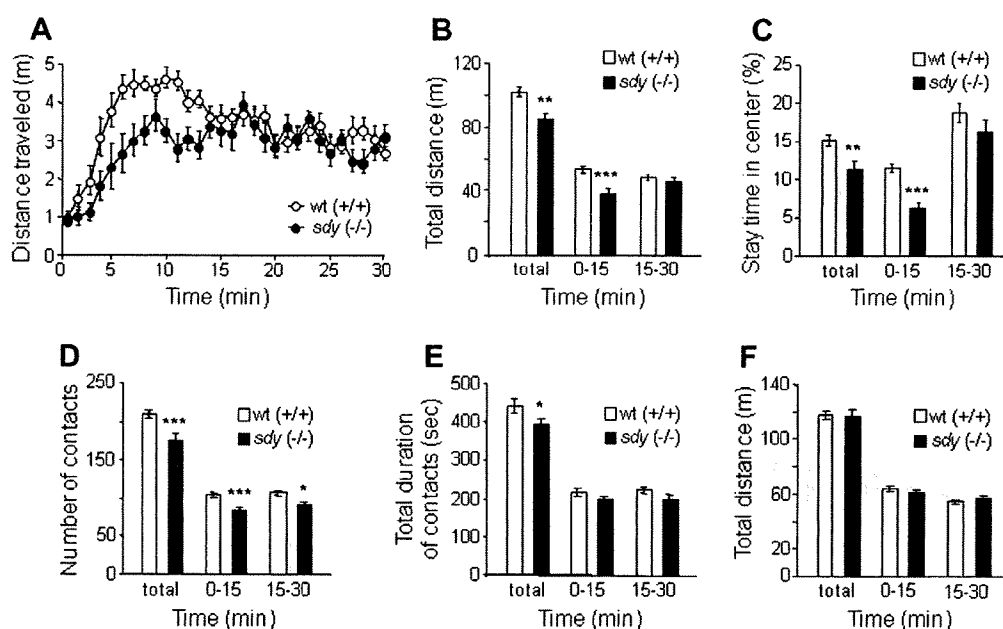
Fig. 1. Open field test with *sdy* mice (A–C). Time course of distance traveled (A), total distance traveled (B) and time spent in the central area (C), are shown (*sdy* mice: n = 18, wild-type mice: n = 21). Social interaction test in *sdy* mice (D–F). The total number of contacts (D), total duration of contacts (E) and distance traveled (F), in the social interaction test, are shown (pairs of *sdy* mice: n = 22, pairs of wild-type mice: n = 21). Data represent means ± SEM. $^*p$ < 0.05, $^{**}p$ < 0.01, $^{***}p$ < 0.001, compared with wild-type mice.

type effect, $F(1,37)$ = 12.3, p = 0.001). This hypoactivity phenotype was notable during the first half of the test period (Fig. 1B, total: $t(37)$ = 3.51, p = 0.001, 0–15 min: $t(37)$ = 3.82, p < 0.001, 15–30 min: $t(37)$ = 0.68, p = 0.50). We then compared time spent in the center of the open field apparatus between *sdy* and wild-type mice. As shown in Fig. 1C, *sdy* mice spent significantly less time in the center (total: $t(37)$ = 2.99, p = 0.005, 0–15 min: $t(37)$ = 5.26 , p < 0.001, 15–30 min: $t(37)$ = 1.19 , p = 0.24). There was no significant difference in the number of fecal boli during the open field test between *sdy* and wild-type mice (*sdy* mice: 8.0 ± 0.6, wild-type mice: 9.3 ± 1.0, $t(37)$ = 1.15 , p = 0.26).

Social interaction test

In the social interaction test, *sdy* mice showed a significant decrease in the number of social contacts compared with wild-type mice (Fig. 1D, total: $t(41)$ = 3.57, p < 0.001, 0–15 min: $t(41)$ = 3.87, p < 0.001, 15–30 min: $t(41)$ = 2.65, p = 0.012). The total duration of contacts was also decreased in *sdy* mice during a 30 min test session (Fig. 1E, total: $t(41)$ = 2.05, p = 0.047, 0–15 min: $t(41)$ = 1.49, p = 0.14, 15–30 min: $t(41)$ = 1.71, p = 0.095). The total distance traveled was not significantly different between the two genotypes (Fig. 1F, total: $t(41)$ = 0.24, p = 0.81, 0–15 min: $t(41)$ = 1.01, p = 0.32, 15–30 min: $t(41)$ = 0.62, p = 0.54).

Elevated plus maze test

In the elevated plus maze test, *sdy* mice exhibited a trend toward a reduced number of entries into the open arms during a 20 min test session compared with wild-type mice (Fig. 2A, total: $t(32)$ = 2.00, p = 0.054). During the second half of the test period, the number of entries into the open arms was significantly lower in *sdy* mice than wild-type mice (Fig. 2A, 0–10 min: $t(32)$ = 1.41, p = 0.17, 10–20 min: $t(32)$ = 2.11, p = 0.042). *Sdy* mice also showed a significant decrease in the total number of arm entries during the second half of the test period and across the entire test period compared with wild-type mice (Fig. 2B, total: $t(32)$ = 2.35, p = 0.025, 0–10 min: $t(32)$ = 2.00, p = 0.054, 10–20 min: $t(32)$ = 2.19, p = 0.036).
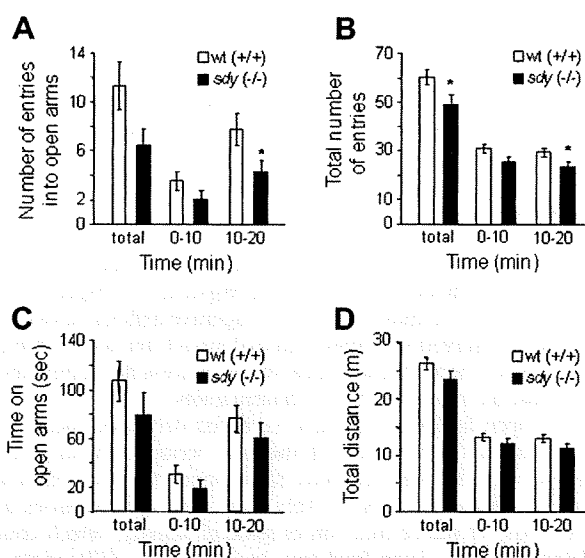

Fig. 2. Elevated plus maze test in *sdy* mice. The number of open arm entries (A), total number of arm entries (B), time on open arms (C), and distance traveled (D), are shown (*sdy* mice: n = 16, wild-type mice: n = 18). Data represent means ± SEM. $^*p$ < 0.05, compared with wild-type mice.

There were no significant differences in the amount of time spent on the open arms (Fig. 2C, total: $t(32)$ = 1.13, p = 0.27, 0–10 min: $t(32)$ = 1.18, p = 0.25, 10–20 min: $t(32)$ = 0.94, p = 0.35) or in the total distance traveled (Fig. 2D, total: $t(32)$ = 1.56, p = 0.13, 0–10 min: $t(32)$ = 1.09, p = 0.28, 10–20 min: $t(32)$ = 1.69, p = 0.10) between *sdy* mice and wild-type mice.

Dopamine and glutamate contents in the brain

Dopamine content was significantly reduced in three brain regions of *sdy* mice compared with wild-type mice: non-frontal cerebral cortex (*sdy* mice: 0.114 nmol/g, wild-type mice: 0.222 nmol/g,
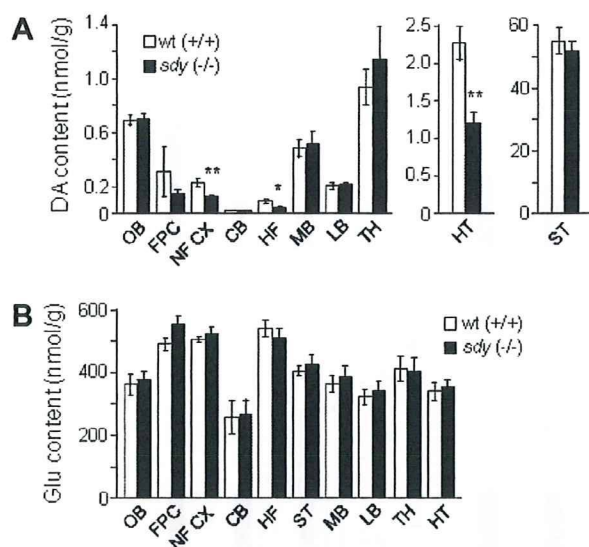
Fig. 3. Dopamine and glutamate content in the brains of *sdy* mice. Dopamine (DA) content (A) and glutamate (Glu) content (B) are shown (dopamine: $n = 4–8$, glutamate: $n = 11$). Olfactory bulb, OB; frontal pole cortex, FPC; non-frontal cerebral cortex, NF CX; cerebellum, CB; hippocampal formation, HF; striatum, ST; midbrain, MB; lower brainstem, LB; thalamus, TH; hypothalamus, HT. Data represent means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ compared with wild-type mice.

$p = 0.002$), hippocampal formation (*sdy* mice: 0.0392 nmol/g, wild-type mice: 0.0822 nmol/g, $p = 0.03$), and hypothalamus (*sdy* mice: 1.17 nmol/g, wild-type mice: 2.26 nmol/g, $p = 0.007$) (Fig. 3A). However, no significant difference in glutamate content was detected between *sdy* and wild-type mice in the brain areas examined (Fig. 3B).

Discussion

Several schizophrenia-related behaviors in rodents, such as hyperactivity, deficits in PPI, locomotor response to antipsychotics, disturbance in social interactions, and cognitive deficits, have commonly been observed in previous animal models for schizophrenia [16]. We report here that *sdy* mice, which express no dysbindin-1, show some, but not all, of these abnormalities.

In the open field test, *sdy* mice exhibited decreased locomotor activity during the first half of the test period compared with wild-type mice, and did not show habituation in a novel environment (Fig. 1A and B). It is unlikely that these abnormalities are due to a loss of motor function or general activity, which could be detected by the open field test, because of no differences in locomotor activity during the second half of the test or in neuromuscular strength between the two groups of animals. It could be caused by reduced adaptation, motivation to explore, and/or enhanced anxiety-like response in a novel environment. Indeed, *sdy* mice spent significantly less time in the center of the open field apparatus than wild-type mice (Fig. 1C), which is associated with anxiety-like response [17]. In addition, *sdy* mice showed a decrease in the number of entries into open arms and in the total number of arm entries in the elevated plus maze test (Fig. 2A and B), suggesting enhanced anxiety-related behavior in *sdy* mice. In the social interaction test, *sdy* mice showed reductions in the number of contacts and in the total duration of contacts without hypoactivity (Fig. 1D–F). The decreased social interactions may be caused largely by the proposed anxiogenic-like phenotype of the *sdy* mice and possibly by the reduced exploration. In rodents, reduced contacts with unfamiliar partners are indicative of social withdrawal, a clinical aspect of schizophrenia [16], suggesting a social with-

drawal phenotype in *sdy* mice. Previous study demonstrated that retinal melanosomes were deficient in *sdy* mice [10]. As we did not examine the visual acuity in *sdy* mice, there is a possibility that reduced visual acuity resulting from retinal defects affects multiple behavioral parameters in this study. Increased locomotor activity, which is observed in most animal models of schizophrenia, is considered to be a positive symptom of schizophrenia, like delusions and hallucinations. However, *sdy* mice showed less activity, which is unique in an animal model for schizophrenia. This phenotype could be due to a decreased motivation to explore, which might be related to the negative symptoms of schizophrenia (blunted affect, decreased motivation, and social withdrawal). Other phenotypes such as less time in the center of the open field apparatus, abnormal response in elevated plus maze, and decreased social interactions could also be related to reduced exploratory tendencies. Indeed, two recent studies show a significant association between risk haplotypes of the dysbindin-1 gene and negative symptoms in patients with schizophrenia [18,19]. These data imply that *sdy* mice, which exhibit reduced exploratory activity, heightened anxiety-like response and deficits in social interaction, could be a potential genetic model for negative symptom endophenotypes of schizophrenia.

Decreased dopamine content in three brain regions of *sdy* mice measured by HPLC-fluorometry in the present study (Fig. 3A) was consistent with dopamine reduction measured by HPLC with an electrochemical detection in the previous study [20]. Recent studies reported that reduced dysbindin-1 protein by *DTNBP1* siRNA transfection increased surface expression of dopamine D2 receptor (DRD2) and blocked dopamine-induced internalization of DRD2 in SH-SY5Y cells [21], and that dopamine release was increased by siRNA-mediated silencing of dysbindin-1 protein in PC12 cells [8]. These results suggest that the lack of dysbindin-1 causes an imbalance of the dopaminergic system. As DRD2 mutant mice show decreased activity [22,23], decreased locomotor activity in *sdy* mice could be due to the abnormal regulation of dopaminergic system by lack of dysbindin-1 protein. As glutamate content in *sdy* mouse brain was not altered, behavioral abnormalities in *sdy* mice could be related to the dopaminergic system rather than the glutamatergic system.

This new genetic mouse model could shed light on the etiology of schizophrenia and lead us to new hypotheses, novel diagnostic tools, and more effective therapies for the disorder.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.016.

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Induction of Amyloid β Accumulation by ER Calcium Disruption and Resultant Upregulation of Angiogenic Factors in ARPE19 Cells

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Purpose. To investigate the intracellular mechanisms that induce amyloid β (Aβ) accumulation and angiogenesis in the human retinal pigment epithelial cell line ARPE19.

Methods. The authors used two endoplasmic reticulum (ER) stress-inducing reagents, thapsigargin (TG), which inhibits the sarcoplasmic/endoplasmic calcium $(Ca)^{2+}$-ATPase, and tunicamycin (TM), which inhibits N-linked glycosylation. The expression pattern of Aβ-precursor protein (APP) splice variants was investigated by reverse transcription (RT)-PCR. Cellular expressions of both a series of Aβ metabolism-related factors and angiogenic factors were evaluated by real-time RT-PCR and Western blot (VEGF). Expression of caspase-4 was examined by real-time RT-PCR and Western blot to evaluate the effect of the ER stressor. Intracellular Ca elevation by TG was evaluated by Ca^{2+} imaging experiments. Dimethyl sulfoxide and staurosporine were used as a nonreagent control and as an apoptosis-inducing reagent through mitochondria not ER, respectively.

Results. TG-treated ARPE19 cells increased the mRNA expression of Aβ production-inducing *APP* splice variants and reduced that of neprilysin, a catabolic enzyme for Aβ. TG-treated ARPE19 cells produced increases in *VEGF, TNF-α, TACE* mRNA, and VEGF protein expressions and a decrease in *PEDF* mRNA expression. TG-treated ARPE19 cells induced the expression of active more than TM-treated casepase-4. The intracellular Ca concentration was elevated in only TG-treated ARPE19 cells.

Conclusions. TG-treated ARPE19 cells showed both Aβ accumulation-inducible and angiogenic factor mRNA expression patterns. This study suggests the possibility that ER stress through ER calcium disruption may induce the expression not only of Aβ deposit-promoting factors but also angiogenic factors in the retinal pigment epithelium. (*Invest Ophthalmol Vis Sci.* 2008;49:2376–2383) DOI:10.1167/iovs.07-1067

A ge-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the elderly.[1] Although our understanding of molecular events presaging AMD has grown in the past decade, the pathogenesis of AMD remains poorly understood. AMD is classified as dry or nonexudative AMD and as wet or exudative AMD. The most severe complication in wet or exudative AMD is the development of choroidal neovascularization (CNV), which originates in choroidal blood vessels that grow through Bruch membrane into the sub-retinal pigment epithelium (RPE) or the subretinal space, or both. The clinical hallmark of AMD is the appearance of drusen,[2] localized deposits lying between the basement membrane of the RPE and Bruch membrane.

Recently, new evidence has indicated that, in AMD, substructural elements within drusen contain amyloid β (Aβ),[3–5] which is a major component of senile plaques and cerebrovascular deposits found in the brains of patients with Alzheimer disease (AD). Aβ accumulation has been reported to increase the expression of an angiogenic growth factor, vascular endothelial growth factor (VEGF), which plays an important role in conditions that involve ocular angiogenesis, including CNV,[6–9] and to decrease the expression of the potent antiangiogenic factor[10,11] pigment epithelium-derived factor (PEDF), secreted by retinal pigment epithelial cells.[12] These results suggest that Aβ is related to the pathogenesis of AMD. However, the mechanisms that induce the accumulation of Aβ in the RPE of AMD patients have not been determined.

Aβ is known to be a physiological peptide, the steady state level of which is maintained by a metabolic balance between synthesis and degradation, and is constitutively secreted from cells.[13,14] In the amyloidogenic pathway, Aβ is produced by the sequential proteolytic processing of Aβ-precursor protein (APP) by the β site APP cleaving enzyme (BACE) and a presenilin complex.[15–17] Under physiological conditions, Aβ is degraded by peptidases, such as neprilysin[18] and endothelin-converting enzyme (ECE),[19] immediately after production.[20]

Endoplasmic reticulum (ER) stress, which leads to the accumulation of unfolded protein, results in ER dysfunction and subsequent cell death.[21] Neuro 2a cells expressing presenilin2-splice variants, which is expressed in human brains in sporadic AD, or the dominant-negative form of Ire1 are vulnerable to ER stress and to increased Aβ production.[22] Therefore, ER stress plays an important role in Aβ accumulation. We used two ER-stress inducers, thapsigargin (TG) and tunicamycin (TM), in this study. TG, a highly lipophilic sesquiterpene lactone, is broadly used as a selective inhibitor of the sarcoplasmic reticulum calcium-ATPase, which pumps Ca^{2+} from the cytosol into the lumen of ER in mammalian cells. TG-mediated irreversible inhibition of ER Ca^{2+} ATPases can also cause the induction of Ca^{2+} leakage from the ER to the cytoplasm, further facilitating the depletion of Ca^{2+} within the ER, and can result in increases in cytoplasmic Ca^{2+} levels.[23] Long-term elevations of intracellular Ca induced ER stress from abnormal accumulations of folding protein.[24,25] TM is the glucosamine-containing