



## Dysbindin engages in c-Jun N-terminal kinase activity and cytoskeletal organization

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### ABSTRACT

A number of reports have provided genetic evidence for an association between the DTNBP1 gene (coding dysbindin) and schizophrenia. In addition, sandy mice, which harbor a deletion in the DTNBP1 gene and lack dysbindin, display behavioral abnormalities suggestive of an association with schizophrenia. However, the mechanism by which the loss of dysbindin induces schizophrenia-like behaviors remains unclear. Here, we report that small interfering RNA-mediated knockdown of dysbindin resulted in the aberrant organization of actin cytoskeleton in SH-SY5Y cells. Furthermore, we show that morphological abnormalities of the actin cytoskeleton were similarly observed in growth cones of cultured hippocampal neurons derived from sandy mice. Moreover, we report a significant correlation between dysbindin expression level and the phosphorylation level of c-Jun N-terminal kinase (JNK), which is implicated in the regulation of cytoskeletal organization. These findings suggest that dysbindin plays a key role in coordinating JNK signaling and actin cytoskeleton required for neural development.

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Schizophrenia is a heritable mental disease that devastates about 1% of the population worldwide, affecting their perception, emotion, and judgment [1]. The DTNBP1 gene (coding dysbindin; dystrobrevin binding protein 1) was identified as a candidate for involvement in schizophrenia [2]. In studies of postmortem brain tissue, patients with schizophrenia had lower dysbindin expression than controls [3–5]. Moreover, long-term treatment with typical or atypical antipsychotics did not alter the mRNA expression levels or protein levels of dysbindin in the mouse frontal cortex and hippocampus [4,6]. Together these findings suggest that decreased dysbindin levels may confer susceptibility to schizophrenia. Sandy (*sd*) mice that express no dysbindin, owing to a deletion of the DTNBP1 gene showed behavioral abnormalities such as reduced activity, heightened anxiety-like response, and deficits in social interaction, memory, and learning [7–9], which could be endophenotypes of schizophrenia. *sd* mice also displayed

lower levels of dopamine, but not glutamate, in the cerebral cortex, hippocampus, and hypothalamus [8,10].

Dysbindin is known to be widely distributed in the brain and located presynaptically and postsynaptically in the central nervous system [11]. The downregulation of endogenous dysbindin by small interfering RNA (siRNA) reportedly inhibited the release of glutamate from hippocampal cultured neurons and increased the release of dopamine from PC12 cells [12,13]. In addition, *sd* mice reportedly exhibit defective synaptic structure and function in the hippocampal CA1 neurons [14]. However, the molecular mechanism underlying the effects of dysbindin on synaptogenesis remains elusive. On the other hand, an increasing number of studies have shown that cytoskeletal organization is essential for the dynamics of synaptogenesis [15,16]. Therefore, to examine the effects of low dysbindin levels on cytoskeletal organization, we performed an immunocytochemical analysis using SH-SY5Y cells, which have been used as an *in vitro* model to study neural development. Furthermore, to confirm the influences of dysbindin knockdown *in vivo*, we analyzed *sd* mice similarly. In addition, we investigated whether alterations in dysbindin expression affect c-Jun N-terminal kinase (JNK) activity, which has been known to phosphorylate many cytoskeletal proteins and regulate neural development [17–20].

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## Materials and methods

**Mice.** The *sdv* and control DBA/2J mice were originally obtained from the Jackson Laboratory and bred in the Institute of Experimental Animal Sciences, Osaka University Graduate School of Medicine. All the experiments were performed in accordance with our institutional guidelines after obtaining the permission of the Laboratory Animal Committee.

**Cell culture.** The SH-SY5Y cell line was obtained from the European Collection of Cell Culture (Wiltshire, UK) and maintained according to the manufacturer's protocol. Hippocampal neurons were cultured from E15 mice embryos using the Nerve-cell Culture System (SUMITOMO BAKELITE, Tokyo, Japan). Neurons ( $2.1 \times 10^5$ ) in MEM (Invitrogen) containing 10% fetal horse serum and antibiotics (50 U/ml penicillin G and 50 µg/ml streptomycin) were plated onto 3.5 cm poly-L-lysine-coated dish. After 4 h of incubation, medium was changed to Neurobasal Medium containing 2% B27 (Invitrogen) and antibiotics.

**Plasmid.** FLAG-human *dysbindin* (AF394226) was cloned into pcDNA 3.1/Zeo (+) vector (Invitrogen). Transfection into cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

**RNA interference.** We used 5'-AAGUGACAAGUCAAGAGAA-3' siRNA, the sequence of which is corresponding to nucleotides 175–197 of human *dysbindin* mRNA. Scrambled siRNA 5'-UUCUCUUGACUUGUCACUU-3' was used as a negative control. Both sense and antisense strands with two base overhangs were synthesized by NIPPON-EGT (Toyama, Japan) in desalted form. siRNA transfection was performed with Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

**Immunocytochemistry.** Plasmid-transfected cells were fixed with cold 95% ethanol for 7 min at  $-20^\circ\text{C}$  and subjected to blocking in 2% BSA/PBS for 10 min. After incubating with an anti-FLAG antibody (1:150, Sigma–Aldrich, St. Louis, USA) overnight, cells were incubated with an Alexa 488 conjugated anti-rabbit secondary antibody (1:500, Invitrogen) for 2 h. siRNA-transfected cells were fixed with cold methanol for 10 min at  $-20^\circ\text{C}$  and incubated in PBS containing 5% BSA and 0.3% Triton X-100 for 30 min. The cells were then incubated with an anti- $\beta$ -tubulin antibody (1:500, Sigma–Aldrich) at  $4^\circ\text{C}$  overnight followed by an Alexa 568 conjugated anti-mouse secondary antibody (1:500, Invitrogen) for 2 h. For detection of actin filament, cells were fixed with 2.5% paraformaldehyde/PBS for 20 min, subjected to permeabilization with 0.1% Triton X-100/PBS for 3 min and then incubated with the Alexa Fluor 568 phalloidin staining solution (5 U/ml, Invitrogen) in PBS containing 1% BSA for 20 min. The coverslips were mounted onto the slides using VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Peterborough, England). Fluorescence images were acquired using a digital camera DP70 connected with a stereomicroscope (Carl Zeiss, Oberkochen, Germany). Hippocampal cultured neurons at stage 3 were similarly subjected to immunocytochemistry. Fluorescence images were acquired using a confocal laser scanning microscope (LSM-510 UV/META, Carl Zeiss).

**Western blot analysis.** siRNA-transfected cells were lysed in RIPA buffer containing 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF and Protease Inhibitor Cocktail (Roche Diagnostics, Basel, Switzerland), incubated for 20 min at  $4^\circ\text{C}$  and centrifuged at  $17,000g$  for 20 min at  $4^\circ\text{C}$ . Proteins (3 µg) were separated on SDS–PAGE and electrotransferred onto Immobilon-P Transfer Membranes (MILLIPORE, Billerica, USA). Membranes were incubated in PBS containing 5% skim milk and 0.05% Tween 20 for 1 h and blotted with primary antibodies at  $4^\circ\text{C}$  overnight. An anti-dysbindin antibody (1:1000), anti-phospho-JNK antibody (1:1000, Cell Signaling Technology, Danvers, USA), anti-JNK antibody (1:1000, Cell Signaling Technology) and anti-GAPDH antibody (1:5000, Abcam, Cambridge, USA) were used as primary

antibodies. The membranes were incubated with an anti-mouse or anti-rabbit HRP-linked secondary antibody (1:2000, Cell Signaling Technology) for 1 h. Mouse monoclonal anti-dysbindin antibody was produced using the GST fused human dysbindin as antigen. High titer clones to dysbindin were selected by ELISA using the dysbindin protein and the immunoreactivity of the clones were checked by Western blot analysis. For analyses of mice, hemisphere of E16 embryo was homogenized in RIPA buffer and similarly subjected to Western blot analysis.

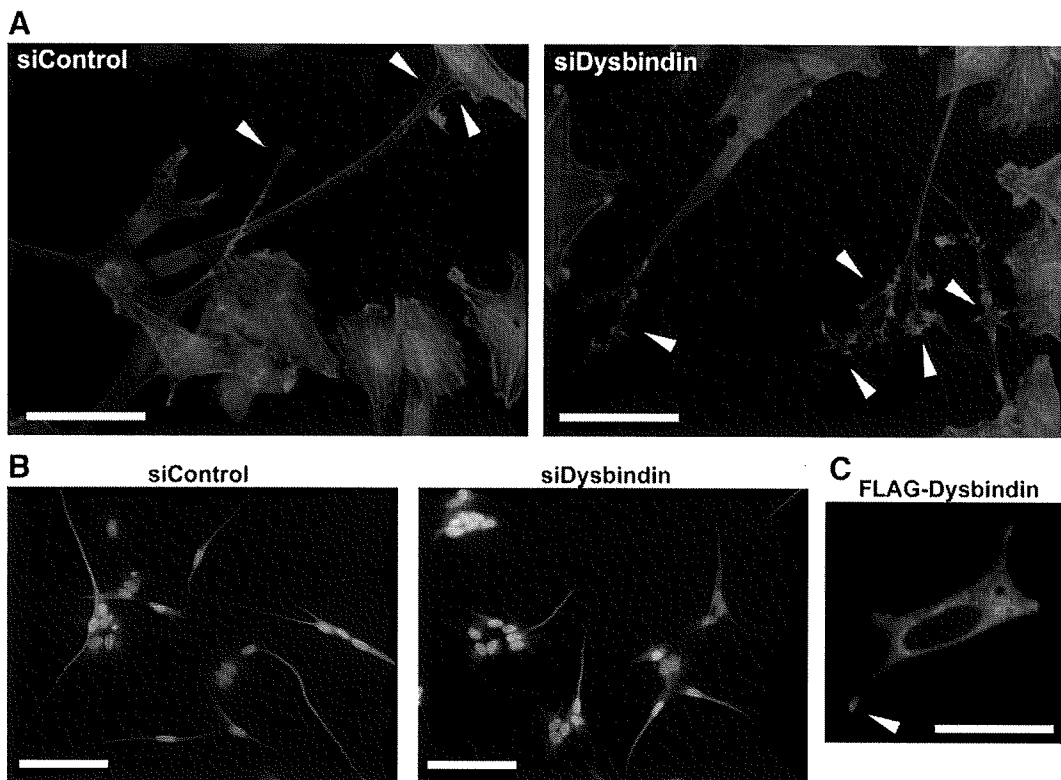
## Results

### *Portions of dysbindin were localized to the tips of protrusions, and dysbindin knockdown influenced the organization of actin cytoskeleton in SH-SY5Y cells*

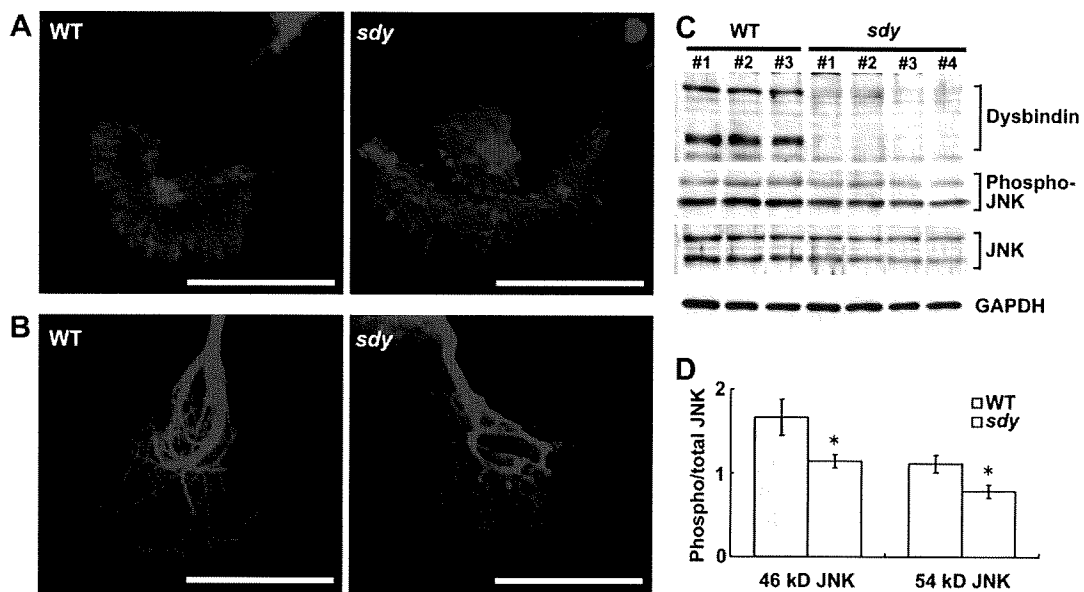
Dysbindin was previously shown to exist in axon terminals in the hippocampus and to decline in patients with schizophrenia [4,11]. However, dysbindin's function in axon terminals in the hippocampus remains unclear. Thus, to explore the role of dysbindin in neurite formation, we attempted to downregulate it in differentiating SH-SY5Y cells. To clarify how dysbindin operates at the tip of a protrusion, we used an RNA interference method to investigate whether or not alterations in dysbindin expression could influence the morphology of the terminal region of protrusions. Retinoic acid treatment gives rise to the differentiation of SH-SY5Y cells and induces neurite outgrowth [21,22]. Control or dysbindin siRNA-transfected SH-SY5Y cells were incubated with retinoic acid to differentiate and then were analyzed in cytoskeletal organization by visualizing actin filament with rhodamine-phalloidin. Interestingly, the organization of actin cytoskeleton at the tips of neurites of differentiating SH-SY5Y cells was dramatically disrupted by the dysbindin knockdown (Fig. 1A). In addition, immunocytochemical analysis with anti- $\beta$ -tubulin antibody was performed to compare the lengths of neurites of dysbindin knockdown cells to those of controls. As shown in Fig. 1B, the  $\beta$ -tubulin-positive neurites of dysbindin knockdown cells were apparently shorter than those of the controls. We confirmed that dysbindin with FLAG-tag was expressed in the cell body as well as at the tips of protrusions of SH-SY5Y cells (Fig. 1C). These results suggest that dysbindin knockdown significantly affects the organization of actin cytoskeleton, bringing about the inhibition of neurite outgrowth in differentiating SH-SY5Y cells.

### *The derangement of cytoskeletal organization was observed in growth cones of hippocampal cultured neurons derived from *sdv* mice*

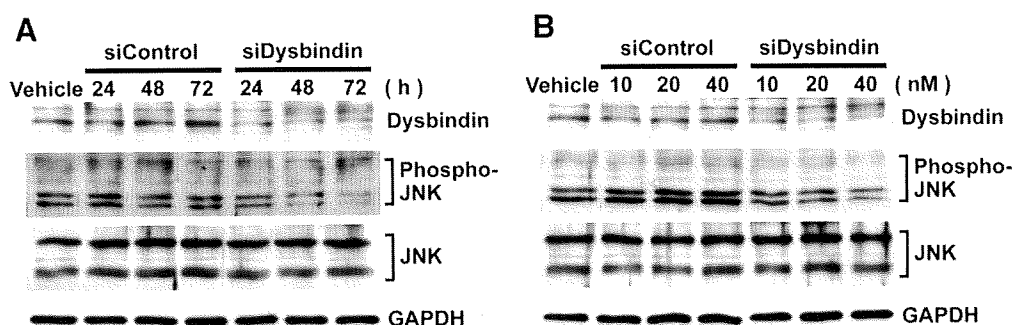
To confirm the effects of dysbindin knockdown on actin cytoskeleton, we analyzed hippocampal cultured neurons derived from *sdv* mice, which lack dysbindin. The growth cone consists mainly of actin-based structures and can be divided into three distinct regions: the peripheral domain, the central domain, and the transition zone [15]. The peripheral domain has linear actin bundles comprising filopodia and mesh-like gels comprising lamellipodia. The central domain, which is rich in microtubules, has hardly any actin superstructures. In the transition zone located between the peripheral domain and the central domain, transverse bundles of actin filaments are observed. To explore the effects of dysbindin knockdown on actin cytoskeleton in growth cones, hippocampal neurons prepared from E15 mice embryos were cultured, fixed at stage 3, and stained with rhodamine-phalloidin. In neurons derived from wild-type mice, most of the growth cones had a characteristic shape (Fig. 2A). In contrast, the growth cones of neurons derived from *sdv* mice showed significant changes in actin-based



**Fig. 1.** Dysbindin is involved in neurite morphogenesis. (A) The effects of dysbindin knockdown on actin cytoskeleton. SH-SY5Y cells were transfected with control (siControl) or dysbindin siRNA (siDysbindin), followed by incubation with retinoic acid for 60 h. Actin filament was visualized by rhodamine-phalloidin (red) and DAPI (blue). Bar = 50  $\mu$ m. (B) The effects of dysbindin knockdown on neurite length. SH-SY5Y cells were transfected with control (siControl) or dysbindin (siDysbindin) siRNA, followed by incubation with retinoic acid for 60 h. The cells were then immunostained using anti- $\beta$ -tubulin antibody (red) followed by Alexa 568-labeled secondary antibody and DAPI (blue). Bar = 100  $\mu$ m. (C) Localization of dysbindin in cell body and at the tips of protrusions. SH-SY5Y cells were transfected with FLAG-tagged dysbindin. They were cultured for 24 h and stained with anti-FLAG antibody, followed by Alexa 488-labeled secondary antibody. Bar = 25  $\mu$ m. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)



**Fig. 2.** Dysbindin is involved in the regulation of growth cone morphology. (A) Actin cytoskeleton within the growth cone. Embryonic hippocampi were dissociated from E15 wild-type (WT) or *sdY* mice (*sdY*) and cultured. They were stained with rhodamine-phalloidin to visualize actin filament at stage 3. Bar = 20  $\mu$ m. (B) Microtubule cytoskeleton within the growth cone. Embryonic hippocampal neurons derived from E15 wild-type (WT) or *sdY* mice (*sdY*) were cultured. The neurons were stained with anti- $\beta$ -tubulin antibody at stage 3, followed by Alexa 568-labeled secondary antibody. Bar = 20  $\mu$ m. (C) JNK activity in the brains of wild-type (WT) or *sdY* mice (*sdY*). Lysates homogenized from the hemisphere of E16 embryos were immunoblotted with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody. (D) Quantitated data. Relative ratios of 46 kDa phospho/total JNK and 54 kDa phospho/total JNK in the brains of wild-type (WT) or *sdY* mice (*sdY*) were analyzed using NIH ImageJ software and represented graphically. Statistical comparisons were performed using the unpaired Student's *t*-test. Data represent means  $\pm$  SD. \**P* < 0.05 versus control.



**Fig. 3.** The JNK phosphorylation level was susceptible to the dysbindin expression level. (A) Time-course dependent effects of dysbindin siRNA on the expression level of dysbindin and phosphorylated JNK. SH-SY5Y cells were transfected with control (siControl) or dysbindin siRNA (siDysbindin), incubated for the indicated times, and subjected to immunoblotting with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody. (B) Dose-dependent effects of dysbindin siRNA on the dysbindin and phosphorylated JNK expression levels. SH-SY5Y cells were transfected with the indicated volumes of control (siControl) or dysbindin siRNA (siDysbindin) for 48 h and harvested. Lysates were immunoblotted with anti-dysbindin antibody, anti-phosphorylated JNK antibody, anti-JNK antibody, and anti-GAPDH antibody.

structures. In those neurons, transverse bundles of actin filament in the transition zone disappeared, and then the central domain became difficult to discern. Additionally, in the peripheral domain, the palm-like shapes consisting of filopodia and lamellipodia were perturbed. On the other hand, microtubules are known to organize into bundles in the neurites, whereas upon entering the central domain of growth cones, they diverge from each other and collaborate with the actin cytoskeleton to contribute growth cone motility and axon elongation [23]. Then, we analyzed microtubule stabilization by staining  $\beta$ -tubulin with a specific antibody. The results revealed that microtubules in the growth cones of neurons derived from *sdv* mice were affected slightly, whereas the controls were unaffected (Fig. 2B). Our findings suggest that hippocampal neurons in *sdv* mice may tend to show the morphological disorder of growth cones.

*JNK activity was suggested to be attenuated in the brains of *sdv* mice embryos*

Recent works have shown that activated JNK might play a role in axon formation [20,24]. In hippocampal cultured neurons, JNK has been known to be predominately distributed with cytoskeleton-associated structures such as growth cones [25]. Interestingly, we previously performed pathway analysis with the dysbindin binding proteins, interactions of which were detected by yeast two-hybrid screening, and then found that JNK signaling is a candidate pathway for involvement in dysbindin function (personal communication). Thus, to examine whether or not JNK activity could be altered in the brains of *sdv* mice compared to those of the wild type, a hemisphere from each of seven E16 embryos was homogenized in lysis buffer and subjected to Western blotting analysis. As previously reported [7], we confirmed that the expression of both 50 kDa and 40 kDa dysbindin are abolished in *sdv* mice. Intriguingly, the phosphorylated JNK was lower in the brain lysate derived from *sdv* mice compared to that from the wild type, indicating that dysbindin might regulate the organization of actin cytoskeleton via modulating JNK activity (Fig. 2C and D).

*The phosphorylation level of JNK was reduced in proportion with the dysbindin expression level in SH-SY5Y cells*

To determine whether or not a reduction in JNK activity was due specifically to the loss of dysbindin, we compared the JNK phosphorylation level with the dysbindin expression level in control or dysbindin siRNA-treated SH-SY5Y cells by Western blot analysis. Though two splice variants of dysbindin exist in DBA/2J

mice (Fig. 2C), SH-SY5Y cells predominantly express 50 kDa dysbindin in our experiments. The downregulation of dysbindin was observed in a time-dependent manner in dysbindin siRNA-treated cells, and then phosphorylated JNK was similarly decreased in proportion to the dysbindin expression level (Fig. 3A). In addition, we performed dose-response analysis with each siRNA and found that the expression level of phosphorylated JNK is highly sensitive to that of dysbindin (Fig. 3B). Our data unequivocally demonstrate that there is a significant correlation between dysbindin level and JNK activity.

## Discussion

Recent studies have suggested that dysfunction in neurodevelopment and neurotransmission is important for the etiology of schizophrenia [26,27]. For example, DISC1, a candidate gene for susceptibility to schizophrenia, has been known to be part of the NUDEL/LIS1/14-3-3 $\epsilon$  complex and to regulate the transport of the protein complex into axons, leading to neuronal migration and axon elongation [28,29]. On the other hand, it has been reported that dysbindin might influence the exocytotic glutamate release and the dopaminergic system via modulation of SNAP25 and synapsin 1 expression [12,13]. Additionally, dysbindin has been suggested to regulate cell surface levels of DRD2 (dopamine D2 receptor) and the strength of the DRD2-mediated  $G_i$  signaling pathway [30]. Although a common molecular mechanism under these observations has long been unexplained, morphological abnormalities in developing neurons, which may bring about subsequent dysfunction of synapses, shall be one of the probable causes of susceptibility to schizophrenia.

In this study, we show that dysbindin is required for the normal arrangement of actin cytoskeleton, especially at neurite tips, in differentiating SH-SY5Y cells. Furthermore, we found that the morphological abnormalities are observed in growth cones of cultured hippocampal neurons derived from *sdv* mice, which lack dysbindin. In developing neurons, growth cones are involved in axon elongation and migration [31]. Therefore, the morphological dysfunction of growth cones by a loss of dysbindin may result in an insufficiency of neural circuit formation and synaptogenesis.

Recently, an increasing number of reports have strongly suggested that JNK is relevant to cytoskeletal function [25]. Moreover, it has been demonstrated that phosphorylated JNK was enriched in axons and necessary for proper axon development [20,25]. The JNK family consists of three isoforms: JNK1, JNK2, and JNK3 [32]. Mice devoid of both JNK1 and JNK2 suffer from multiple abnormalities during development of the central nervous system [33]. In addi-

tion, the mutation of JNK3 gene reportedly results in the severe encephalopathy phenotype in children [34]. Interestingly, the present study revealed that the phosphorylation level of JNK is altered by the expression level of dysbindin, raising the possibility that dysbindin functions as a mediator of the JNK signaling pathway, at least at neurite ends, where dysbindin is colocalized with JNK.

Hence, we speculate that a loss of dysbindin results in the failure of normal axon guidance by the disruption of growth cones during the embryonic stage and evokes aberrations in the neurosecretory system in adulthood. However, it remains unclear how dysbindin regulates JNK phosphorylation and how the morphological changes of growth cones in developing neurons contribute to the pathogenic mechanism of schizophrenia. Further analyses are needed to obtain the precise molecular function of dysbindin.

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## Major depression: what caused the crisis?

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In July, 2008, a 67-year-old woman with refractory depression was referred to our institute. In 2006, she had a thymectomy for thymoma. In January, 2008, after experiencing family discord, she lost her appetite, and her bodyweight decreased by 5 kg in 1 month. She became pessimistic and self-recriminating and made several suicide attempts. CT showed no evidence of a recurrence of the thymoma. Neurological examination showed only slight muscle weakness of her limbs, but the cause of her anorexia remained unclear despite further in-hospital examinations such as gastrointestinal tract endoscopies and systemic contrast-enhanced CT. She was diagnosed as having depression and was transferred to a regional psychiatric hospital, where she was treated sequentially with sertraline, paroxetine, clomipramine, and nortriptyline, and augmentation lithium. These treatments were ineffective, and her body weight decreased from 60 kg to 33.5 kg. She was then transferred to us.

Her depressed mood, decreased interests, hypogeusia, anorexia, insomnia, anxious restlessness, decreased energy and fatigue, guilty feelings, poor concentration, and suicidal ideation persisted. She fulfilled the DSM-IV diagnostic criteria for major depressive disorder. Her total score on the 17-item Hamilton Depression Rating Scale (total-HDRS) was 40. She could walk and had no ocular and bulbar symptoms, but neurological examinations showed mild proximal muscle weakness and atrophy of her limbs. Blood test results indicated hypoalbuminaemia (albumin 3.4 g/dL); other investigations including CT chest, tumour markers, electroencephalography, and brain MRI were normal. Ten sessions of electroconvulsive therapy (ECT) were done in September, but the depressive symptoms persisted. She stopped taking medication, other than quetiapine as required (prescribed by us), but at the end of October, she suddenly developed impaired consciousness with a reduced respiratory rate. A blood

gas analysis showed carbon dioxide narcosis, and she was immediately placed on a ventilator. No evidence of pulmonary disease was found, and a diagnosis of myasthenic crisis was made on the basis of a high acetylcholine receptor antibody (AChR-Ab) titre (120 nmol/L), waning on the Harvey-Masland test, and a history of thymoma. She was treated with plasmapheresis and immunoadsorption followed by prednisolone treatment (maximum dose, 50 mg/day). Her respiratory function subsequently improved, and she was extubated. In December, she was treated with pyridostigmine (180 mg/day). As the anti-AChR-Ab titre decreased, total-HDRS score improved substantially without antidepressant therapy (figure). Her long-lasting depressive symptoms improved completely, and her bodyweight recovered to 40 kg; she was discharged in July, 2009. When last seen in September, 2009, both her depression and myasthenia gravis were in remission.

The pathology of depressive symptoms associated with myasthenia gravis, including the hypothalamo-pituitary-adrenal axis dysfunction resulting from chronic stress and central cholinergic deficit, is controversial and remains to be elucidated.<sup>1</sup> Although some patients with major depressive disorder complicated with myasthenia gravis improve after ECT,<sup>2</sup> the potential to misdiagnose myasthenia gravis as depression has been highlighted.<sup>3</sup> 20% of people with myasthenia gravis are initially diagnosed as having a psychiatric disorder,<sup>4</sup> and improvements in depressive symptoms associated with improvements in myasthenia gravis have been reported.<sup>1,5</sup> Whether depressive symptoms in individual cases are attributable to myasthenia gravis or major depressive disorder should be investigated. Since the AChR-Ab titre and the depressive symptoms improved over time in our case, we concluded that the patient's depressive symptoms could predominantly be attributed to myasthenia gravis. When managing treatment-resistant depressive patients, the medical history must be sufficiently considered.

### Contributors

All the authors participated in the management of the patient. NO wrote the Case Report. Written consent to publish was obtained.

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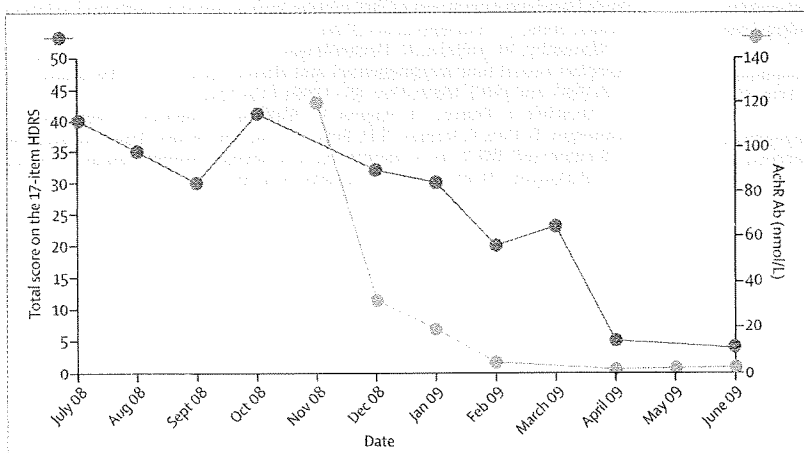
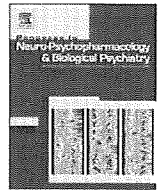


Figure: Changes in AChR-Ab titres and total-HDRS



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# Progress in Neuro-Psychopharmacology & Biological Psychiatry

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## Association analysis of *GRM2* and *HTR2A* with methamphetamine-induced psychosis and schizophrenia in the Japanese population

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### ABSTRACT

**Background:** Abnormalities in glutamergic neural transmission have been suggested to be involved in the pathogenesis of schizophrenia. A recent study reported that alterations in the 5-HT<sub>2A</sub>–mGluR2 complex may be involved in neural transmission in the schizophrenic cortex. In addition, methamphetamine-induced psychosis is thought to be similar to schizophrenia. Therefore, we conducted a case-control study with Japanese samples (738 schizophrenia patients, 196 methamphetamine-induced psychosis patients, and 802 controls) to evaluate the association and interaction between *GRM2*, *HTR2A* and schizophrenia.

**Methods:** We selected three 'tagging SNPs' in *GRM2*, and two biologically functional SNPs in *HTR2A* (T102C and A1438G), for the association analysis.

**Results:** We detected a significant association between methamphetamine-induced psychosis and *GRM2* in a haplotype-wise analysis, but not *HTR2A*. We did not detect an association between *GRM2* or *HTR2A* and schizophrenia. In addition, no interactions of *GRM2* and *HTR2A* were found in methamphetamine-induced psychosis or schizophrenia. We did not detect any novel polymorphisms in *GRM2* when we performed a mutation search using methamphetamine-induced psychosis samples.

**Conclusion:** Our results suggested that *GRM2* may play a role in the pathophysiology of methamphetamine-induced psychosis but not schizophrenia in the Japanese population. A replication study using larger samples or samples of other populations will be required for conclusive results.

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### 1. Introduction

The glutamate hypothesis for the pathophysiology of schizophrenia is well-known (Weinberger, 2007). A recent clinical study also showed that LY379268, an agonist of the metabotropic glutamate 2/3

receptor (mGluR2/3), which belongs to group II mGluR, regulates glutamate neurotransmission through a presynaptic negative regulatory mechanism (Patil et al., 2007). LY379268 also has been shown to have an effect on psychotic symptoms in schizophrenia that is almost equivalent to the effect with olanzapine (Patil et al., 2007).

Recently, the hyperactivity of mGluR3 knockout mice (induced by amphetamine) was shown to be a reverse abnormal behavior mediated by LY379268 (Woolley et al., 2008). However, LY379268 did not correct the abnormal behavior of these mGluR2 knockout mice (Woolley et al., 2008). This result might show that mGluR2 is a more important therapeutic target than mGluR3 for the antipsychotic effect of LY379268 (Woolley et al., 2008).

Another recent animal study showed that mGluR2 and serotonin 2A receptor (5-HT<sub>2A</sub>) form complexes that mediate alterations in cellular response in the brain, and that these alterations were reversed by

**Abbreviations:** mGluR2/3, metabotropic glutamate 2/3 receptor; 5-HT<sub>2A</sub>, serotonin 2A receptor; LSD, lysergic acid diethylamide; *HTR2A*, 5-HT<sub>2A</sub> gene; *GRM2*, mGluR2 gene; METH, methamphetamine; SD, standard deviation; JGIDA, Japanese Genetics Initiative for Drug Abuse; LD, linkage disequilibrium; MAFs, minor allele frequencies; dHPLC, denaturing high performance liquid chromatography; HW, Hardy–Weinberg equilibrium; MDR, multifactor dimensionality reduction; CD–CV hypothesis, common disease–common variants hypothesis; *GRM3*, mGluR3 gene.

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mGluR2 antagonist (Gonzalez-Maeso et al., 2008). This was supported by evidence from a postmortem study using schizophrenia patients untreated by antipsychotics, who showed increased 5-HT<sub>2A</sub> and decreased mGluR2 in the cortex compared with age and gender match control samples (Gonzalez-Maeso et al., 2008). These findings suggest that abnormality of mGluR2 and 5-HT<sub>2A</sub> complexes might be involved in the pathophysiology for schizophrenia (Gonzalez-Maeso et al., 2008; Snyder, 2008).

Several genetic studies have reported an association between the 5-HT<sub>2A</sub> gene (*HTR2A*) and schizophrenia (Abdolmaleky et al., 2004; Baritaki et al., 2004; Golimbet et al., 2007; Inayama et al., 1996). However, other studies showed no association (Basile et al., 2001; Dominguez et al., 2007; Ertugrul et al., 2004; Pae et al., 2005; Sanders et al., 2008; Zhang et al., 2004). Moreover, only one genetic study detected no association between the mGluR2 gene (*GRM2*) and Japanese schizophrenia (Joo et al., 2001). Several genome-wide association studies (GWASs) reported that *HTR2A* and *GRM2* were not associated with schizophrenia (Holmans et al., 2009; Kirov et al., 2009; Moskvina et al., 2009; O'Donovan et al., 2008; O'Donovan et al., 2009; Purcell et al., 2009; Stefansson et al., 2009) or substance dependence (Chen et al., 2009). However, since schizophrenia is a complex disease, it seemed to us that evaluation of gene-gene interactions of *HTR2A* and *GRM2* in relation to the pathophysiology of schizophrenia was necessary.

LY379268 significantly inhibited hyperlocomotion in mice induced by methamphetamine (METH) (Satow et al., 2008). This animal model is considered to reflect the positive symptoms of schizophrenia. The symptoms of METH-induced psychosis are similar to those of paranoid type schizophrenia (Sato et al., 1992), which may indicate that METH-induced psychosis and schizophrenia have common susceptibility genes (Bousman et al., 2009). In support of this hypothesis, we reported that the V-act murine thymoma viral oncogene homologue 1 (*AKT1*) gene was associated with METH-induced psychosis (Ikeda et al., 2006) and schizophrenia (Ikeda et al., 2004) in the Japanese population. Furthermore, we performed an association analysis of these genes with methamphetamine (METH)-induced psychosis, since METH-induced psychosis is similar to schizophrenia (Sato et al., 1983).

*GRM2* (OMIM \*604099, 5 exons in this genomic region spanning 10.466 kb) and *HTR2A* (OMIM \*182135, 3 exons in this genomic region spanning 63.463 kb) are located on 3p and 13q, respectively. The locations of these genomic regions were shown to be in a susceptibility region for schizophrenia (Badner and Gershon, 2002; Hovatta et al., 1998; Lewis et al., 2003; Maziade et al., 2001; Pulver et al., 1995). Therefore, we conducted a case-control study using Japanese schizophrenia and METH-induced psychosis samples.

## 2. Materials and methods

### 2.1. Subjects

The subjects were 738 schizophrenia patients (395 males and 343 females; mean age  $\pm$  standard deviation (SD) 41.2  $\pm$  13.8 years), 196 METH-induced psychosis and METH-dependence patients (163 males and 33 females; mean age  $\pm$  SD 37.0  $\pm$  10.8 years) and 802 healthy controls (351 males and 451 females; 37.6  $\pm$  14.3 years). All the patients examined in this study suffered not only from METH-induced psychosis but also METH dependence. Consensus diagnoses of methamphetamine psychosis were made by two trained psychiatrists according to the ICD-10-DCR criteria (F15.2 and F15.5) on the basis of interviews and medical records. The patients with methamphetamine psychosis in the present study usually showed predominant positive symptoms such as delusion and hallucination. We excluded cases in which the predominant symptoms were of the negative and/or disorganized type in order to maintain the homogeneity of the patient group. The patients were categorized by prognosis into two types, a

transient type and a prolonged type, based on the duration of the psychotic state after METH discontinuance. The transient type of patient was defined as a patient whose symptoms improved within 1 month after METH discontinuance and the start of treatment with antipsychotic, and the prolonged type was defined as a patient whose psychosis continued for more than 1 month after METH discontinuance and the start of treatment with an antipsychotic. In this study, there were 112 patients (56.9%) with the transient type and 85 patients (43.1%) with the prolonged type patients of METH psychosis. Cannabinoids were the most frequency abused drugs (31.4%), followed by cocaine (9.09%), LSD (9.09%), opioids (7.69%), and hypnotics (7.69%). Subjects with METH-use disorder were excluded if they had a clinical diagnosis of psychotic disorder, mood disorder, anxiety disorder, or eating disorder. More detailed characterizations of these subjects have been published elsewhere (Kishi et al., 2008, 2009b).

All healthy controls were also psychiatrically screened based on unstructured interviews including current and past psychiatric history. None had severe medical complications such as cirrhosis, renal failure, heart failure or other Axis-I disorders according to DSM-IV. No structured methods were used to assess psychiatric symptoms in the controls, which included hospital staff and medical students. Written informed consent was obtained from each subject. This study was approved by the ethics committees at Fujita Health University and Nagoya University Graduate School of Medicine, and by each participating member of the Institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

### 2.2. SNP selection and linkage disequilibrium (LD) evaluation

We first consulted the HapMap database (release#23.a.phase2, Mar 2008, www.hapmap.org, population: Japanese Tokyo: minor allele frequencies (MAFs) of more than 0.05) and included 4 SNPs covering *GRM2* (5'-flanking regions including about 6.3 kb from the initial exon and about 1 kb downstream (3') from the last exon: HapMap database contig number chr17: 51711684, 51730152). Then three 'tagging SNPs' were selected with the criteria of an  $r^2$  threshold greater than 0.8 in 'pair-wise tagging only' mode using the 'Tagger' program (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger), an implement of the HAPLOVIEW software program (Barrett et al., 2005), for the following association analysis. *HTR2A* has been reported to have two biologically functional SNPs (T102C: rs6313, A1438G: rs6311) (Myers et al., 2007; Spurlock et al., 1998). According to the HapMap database, LD in these two SNPs in *HTR2A* was  $r^2 = 0.770$ ; therefore, we performed an association analysis for these SNPs in this study.

### 2.3. SNP genotyping

We used TaqMan assays (ABI: Applied Biosystems, Inc., Foster City, CA,) for all SNPs. One allelic probe was labeled with FAM dye and the other with fluorescent VIC dye. The plates were heated for 2 min at 50 °C and 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 58 °C for 1 min. Please refer to ABI for the primer sequence. Detailed information, including primer sequences and reaction conditions, can be seen in our previous papers (Kishi et al., 2009b,d; Tsunoka et al., 2009).

### 2.4. Mutation screening

We detected significant association between *GRM2* and METH-induced psychosis. Therefore, we performed mutation screening with *GRM2* divided into 17 parts (promoter region, all exons including branch site) using 32 METH-induced psychosis patients (16 males and 16 females) and the primer extension method. Denaturing high performance liquid chromatography (dHPLC) analysis was carried out



198 to detect mutation. DNA sequencing was then performed using a  
199 3100-Avant Genetic Analyzer (Applied Biosystems, CA). Primers were  
200 designed to cover the coding regions, the splice sites and approxi-  
201 mately 1.0 kb of the 5'UTR and 500 bp of the 3'UTR of *GRM2*, using the  
202 Primer 3 primer design program ([http://www.broad.mit.edu/cgi-bin/  
203 primer/primer3.www.cgi](http://www.broad.mit.edu/cgi-bin/primer/primer3.www.cgi)) (Rozen and Skaletsky, 2000). A more  
204 detailed description of the methods can be seen in a previous paper  
205 (Suzuki et al., 2003). Detailed information, including primer sequence,  
206 is available on request.

### 207 2.5. Statistical analysis

208 Genotype deviation from the Hardy–Weinberg equilibrium  
209 (HWE) was evaluated by chi-square test (SAS/Genetics, release 8.2,  
210 SAS Japan Inc., Tokyo, Japan). Marker-trait association analysis was  
211 used to evaluate allele- and genotype-wise association with the chi-  
212 square test (SAS/Genetics, release 8.2, SAS Japan Inc., Tokyo, Japan).  
213 The distribution of patient characteristics in the schizophrenia group,  
214 METH-induced psychosis group and healthy control group was ana-  
215 lyzed using a *t* test or a chi-square test. We found significant differences  
216 in gender distribution among these groups ( $P_{\text{schizophrenia}} \leq 0.001$  and  
217  $P_{\text{METH-induced psychosis}} \leq 0.001$ ), however, there was no difference in age  
218 among them ( $P_{\text{schizophrenia}} = 0.238$  and  $P_{\text{METH-induced psychosis}} = 0.765$ ).  
219 We therefore performed logistic regression analysis to compare the  
220 phenotype of each of the examined SNPs genotypes to adjust for possible  
221 confounding. The phenotype (each disorder or control) was the  
222 dependent variable, and gender, age at the time of recruitment and  
223 each examined SNP genotype were set as the independent variables. The  
224 statistical package JMP for windows was used for logistic regression  
225 analysis (JMP 5.0. 1J, SAS Japan Inc., Tokyo, Japan). Haplotype-wise  
226 association analysis was evaluated with a likelihood ratio test using the  
227 COCAPHASE2.403 program (Dudbridge, 2003). This software uses the  
228 EM algorithm to estimate the haplotype frequencies of unphased  
229 genotype data and standard unconditional logistic regression analysis,  
230 applying the likelihood ratio test under a log-linear model to compare  
231 haplotype frequencies between cases and controls. In order to avoid  
232 misleading results caused by rare haplotypes, all haplotypes with a  
233 frequency less than or equal to 5% in both the cases and the controls were  
234 declared rare and clumped together for a test of the null hypothesis,  
235 using the command line option 'rare 0.05.' This analysis adjusted for age  
236 and gender. To control inflation of the type I error rate, we used  
237 Bonferroni's correction. Power calculation was performed using a

238 genetic power calculator (Purcell et al., 2003). We set each item in  
239 each value in the Genetic Power Calculator as follows: prevalence: 0.01  
240 in schizophrenia and METH-induced psychosis, User-defined: 0.01 (5  
241 SNPs examined in this study. Bonferroni's correction was used to control  
242 inflation of the type I error rate).  
243

The significance level for all statistical tests was 0.05.  
244

### 244 3. Results

245 The LD structure in *GRM2* from the HapMap database can be seen  
246 in our previous paper (Tsunoka et al., 2009). Genotype frequencies of  
247 all SNPs were in HWE (Table 1). In addition, we added twenty-five  
248 randomly selected samples that were genotyped again as a measure of  
249 genotyping quality control, and the genotype consistency rates for all  
250 four SNPs were 100% (Tsunoka et al., 2009). We detected a significant  
251 association between *GRM2* and METH-induced psychosis in the allele/  
252 genotype-wise analysis with the chi-square test but not with logistic  
253 regression adjusted for age and gender (Tables 1 and 2). In addition,  
254 we found an association between *GRM2* and METH-induced psychosis  
255 in the haplotype-wise analysis adjusting age and gender (Tables 3).  
256 However, *HTR2A* was not associated with schizophrenia or METH-  
257 induced psychosis (Tables 1–3). Although we performed mutation  
258 screening for *GRM2* using METH-induced psychosis samples, we did  
259 not detect any novel polymorphisms in *GRM2* in the METH-induced  
260 psychosis samples.

261 To evaluate the interactions with each SNP in these genes, we  
262 analyzed the gene–gene interactions with the use of the Multifactor  
263 Dimensionality Reduction (MDR) method (Hahn et al., 2003). In this  
264 study, each of the genotype variables in one dimension were assessed  
265 to determine test accuracy (defined as mean sensitivity and  
266 specificity) in terms of predicting delivery type using 10-fold cross-  
267 validation for each disorder and control. MDR analysis was performed  
268 using MDR software (v 1.0.0; <http://www.epistasis.org/>). In this  
269 analysis, however, no interactions were found in METH-induced  
270 psychosis and schizophrenia (data not shown).

271 In the power analysis, we obtained more than 80% power for the  
272 detection of association when we set the genotype relative risk at  
273 1.45–1.90 and 1.32–1.60 in METH-induced psychosis and schizophre-  
274 nia, respectively, for *GRM2*, and at 1.45–1.47 and 1.27–1.32 in METH-  
275 induced psychosis and schizophrenia, respectively, for *HTR2A* under a  
276 multiplicative model of inheritance.

11.1 **Table 1**

11.2 Association analysis of single markers in *HTR2A* and *GRM2* with schizophrenia and methamphetamine-induced psychosis.

Gene	SNP ID	Phenotype <sup>a</sup>	MAFs <sup>b</sup>	N	Genotype distribution <sup>c</sup>				P-value <sup>e</sup>		Corrected P-value <sup>ef</sup>	
					M/M	M/m	m/m	HWE <sup>d</sup>	Genotype	Allele	Genotype	Allele
<i>HTR2A</i>	rs6311	Controls	0.440	802	262	374	166	0.128				
		Schizophrenia	0.409	738	264	344	130	0.328	0.225	0.0828		
	–1438A/G	METH-induced psychosis	0.459	196	58	96	42	0.846	0.708	0.497		
		Controls	0.485	802	220	386	196	0.301				
<i>GRM2</i>	rs6313	Schizophrenia	0.5	738	182	374	182	0.713	0.440	0.407		
		METH-induced psychosis	0.492	196	52	95	49	0.671	0.965	0.795		
	102T/C	Controls	0.0468	802	731	67	4	0.0751				
		Schizophrenia	0.0420	738	676	62	0	0.234	0.158	0.523		
<i>GRM2</i>	rs3821829	METH-induced psychosis	0.0408	196	181	14	1	0.219	0.856	0.613		
		Controls	0.333	802	346	378	78	0.0834				
	C>T	Schizophrenia	0.308	738	354	314	70	0.976	0.150	0.132		
		METH-induced psychosis	0.258	196	106	79	11	0.453	<b>0.0126</b>	<b>0.00413</b>	0.0630	<b>0.0207</b>
<i>GRM2</i>	rs12487957	Controls	0.376	802	300	401	101	0.0632				
		Schizophrenia	0.360	738	299	347	92	0.574	0.435	0.352		
	T>A	METH-induced psychosis	0.281	196	100	82	14	0.612	<b>0.00116</b>	<b>0.000414</b>	<b>0.00580</b>	<b>0.00207</b>
		Controls	0.281	802	300	401	101	0.0632				

11.20 <sup>a</sup> SCZ: schizophrenia METH psychosis: methamphetamine-induced psychosis.

11.21 <sup>b</sup> MAFs: minor allele frequencies.

11.22 <sup>c</sup> M: major allele, m: minor allele.

11.23 <sup>d</sup> Hardy–Weinberg equilibrium.

11.24 <sup>e</sup> Bold numbers represent significant P-value.

11.25 <sup>f</sup> Calculated by Bonferroni's correction.

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**Table 2**  
Logistic regression analysis of single markers in *HTR2A* and *GRM2* with schizophrenia and methamphetamine-induced psychosis.

Gene	SNP ID	Genotype	Schizophrenia			METH-induced psychosis <sup>a</sup>		
			P-value	OR <sup>b</sup>	95% CI <sup>c</sup>	P-value	OR <sup>b</sup>	95% CI <sup>c</sup>
<i>HTR2A</i>	rs6311	AG	0.853	1.03	0.340–2.22	0.924	0.836	0.760–1.40
	–1438A/G	GG	0.978	1.23	0.618–1.55	0.579	0.291	0.839–1.81
	rs6313	TC	1.02	0.965	0.646–1.60	0.940	0.817	0.716–1.31
	102T/C	CC	1.07	0.961	0.633–1.83	0.801	0.826	0.676–1.37
	rs3821829	CT	1.42	0.889	0.160–7.45	0.702	0.703	0.539–1.22
<i>GRM2</i>	C>T	TT	0.486	0.909	0.0288–30.2	0.659	0.709	0.557–1.44
	rs12487957	TC	1.02	1.23	0.556–1.81	0.956	0.241	0.869–1.74
	T>C	CC	2.21	1.19	0.923–5.91	0.0912	0.506	0.717–1.98
	rs4687771	TA	1.14	1.04	0.649–1.95	0.648	0.797	0.754–1.45
	T>A	AA	2.01	1.27	0.910–4.82	0.0986	0.314	0.802–2.01

Reference genotypes are common genotype. Adjustment for age and gender.

<sup>a</sup> METH-induced psychosis: methamphetamine-induced psychosis.

<sup>b</sup> OR: odds ratio.

<sup>c</sup> CI: confidential interval.

#### 4. Discussion

In the single marker association study, we detected a significant association between *GRM2* and METH-induced psychosis with chi-square test. However, this association may have been due to biased samples, which is unmatched for age. We therefore performed a logistic regression analysis to compare the phenotypes of each of the examined SNPs genotypes, using several clinical factors as other independent variables to adjust for possible confounding. Although we did not detect an association between the three tagging SNP genotypes in *GRM2* and METH-induced psychosis with logistic regression analysis, we found an association between *GRM2* and METH-induced psychosis in the haplotype-wise analysis adjusting for age and gender. Our results therefore suggest that *GRM2* plays a role in the pathophysiology of METH-induced psychosis in the Japanese population. We did not detect novel polymorphisms, although we performed a mutation search for *GRM2* (promoter region, all exons including branch site) using METH-induced psychosis samples.

We designed the study design based on the common disease–common variants hypothesis (CD–CV hypothesis) (Chakravarti, 1999). A recent study has shown associations between common diseases such as schizophrenia and rare variants (Weickert et al., 2008). If the genetic background of METH-induced psychosis is described by the common disease–rare variants hypothesis, further investigation, such as medical resequencing using larger samples, will be required. Moreover, mGluR2/3 agonist has been observed to have certain antipsychotic effects (Patil

et al., 2007), and the mGluR3 gene (*GRM3*) has been considered a good candidate gene for the pathogenesis of METH-induced psychosis. Further investigations will be necessary to analyze gene–gene interactions between *GRM2* and *GRM3* in METH-induced psychosis.

It has also been suggested that alterations in mGluR2 and the 5-HT<sub>2A</sub> complex might be involved in the pathophysiology of schizophrenia. Because 5-HT<sub>2A</sub> receptors are one of the major pharmacological therapeutic targets of atypical antipsychotics, the pharmacogenomics of psychotic disorders (response to antipsychotics) will also need to be investigated in the future.

In this study, we found an association between *GRM2* and METH psychosis but not schizophrenia in the Japanese population. METH psychosis has long been considered a pharmacologic model of schizophrenia (Snyder, 1973; Ujike, 2002). To date, several genes have been reported to have an association with METH psychosis (Ikeda et al., 2006; Kishi et al., 2009a,c; Kishimoto et al., 2008a,b; Kotaka et al., 2009; Morita et al., 2008; Otani et al., 2008; Ujike et al., 2009). However, only a few of these genes have been found to be associated with Japanese schizophrenia (Ikeda et al., 2006; Kishimoto et al., 2008a). One of the reasons for the inconsistent results among these studies is considered to be the difference in sample size among the studies of these disorders. A replication study using larger samples or samples of other populations will be required for conclusive results (Bousman et al., 2009).

A few points of caution should be mentioned with respect to our results. First, the positive association may be due to biased samples,

**Table 3**  
All markers haplotype-wise analysis of *HTR2A* and *GRM2*.

Gene	Marker	Phenotype <sup>a</sup>	Haplotype frequency	OR <sup>b</sup>	95% CI <sup>c</sup>	Individual haplotype P-value <sup>b</sup>	Phenotype <sup>a</sup>	Global P-value <sup>b</sup>	Corrected global P-value <sup>b,c</sup>
<i>HTR2A</i>	rs6311–rs6313	A–T	Control	0.0778					
		Schizophrenia	0.100	1.37	0.908–2.06	0.177			
		METH-induced psychosis	0.0830	1.39	0.750–2.58	0.327			
	G–T	Control	0.467				Schizophrenia	0.298	
		Schizophrenia	0.430	1.00	1.00–1.00	0.212			
		METH-induced psychosis	0.465	1.01	0.698–1.71	0.468			
G–C	Control	0.455				METH-induced psychosis	0.589		
	Schizophrenia	0.470	1.11	0.825–1.45	0.653				
	METH-induced psychosis	0.452	1.02	0.498–1.89	0.922				
<i>GRM2</i>	C–C–A	Control	0.673						
		Schizophrenia	0.659	1.00	1.00–1.00	0.424	Schizophrenia	0.424	
		METH-induced psychosis	0.746	1.00	1.00–1.00	<b>0.00822</b>			
	C–T–T	Control	0.327						
		Schizophrenia	0.341	1.07	0.909–1.26	0.424	METH-induced psychosis	<b>0.00746</b>	<b>0.0149</b>
		METH-induced psychosis	0.254	0.686	0.518–0.908	<b>0.00822</b>			

<sup>a</sup> SCZ: schizophrenia METH psychosis: methamphetamine-induced psychosis.

<sup>b</sup> Bold numbers represent significant P-value.

<sup>c</sup> Calculated by Bonferroni correction.

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328 such as unmatched gender samples, or small sample size. On average,  
329 the METH-induced psychosis patients were much younger than the  
330 controls. We therefore performed a logistic regression analysis to  
331 compare the phenotypes of each of the examined SNPs genotypes,  
332 using several clinical factors as other independent variables to adjust  
333 for possible confounding. Our control samples for 3SNPs in *GRM2*  
334 were within a limit that satisfies HWE. The positive association with  
335 METH-induced psychosis could be due to type I error, possibly  
336 because of population stratification. However, another recent study  
337 confirmed that there is no population stratification in our control  
338 samples (Ikeda et al., 2009). In addition, we added twenty-five randomly  
339 selected samples that were genotyped again as a measure of genotyping  
340 quality control, and the genotype consistency rates for all four SNPs were  
341 100% (Tsunoka et al., 2009). Second, we did not include a mutation scan  
342 to detect rare variants with functional effects for schizophrenia.  
343 However, Joo et al. reported no association of *GRM2* with Japanese  
344 schizophrenia after mutation screening for *GRM2* (Joo et al., 2001). In  
345 addition, it is difficult to evaluate the association of rare variants, unless  
346 statistical power is obtained. To overcome these limitations, a replication  
347 study using larger samples or samples of other populations will be  
348 required for conclusive results (Bousman et al., 2009).

## 349 5. Conclusion

350 In conclusion, our results suggest that *GRM2* may play a major role  
351 in the pathophysiology of METH-induced psychosis but not schizo-  
352 phrenia in the Japanese population. However, an interaction between  
353 mGluR2 and 5-HT2A seen in an animal study was not detected with  
354 these genes levels.

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## Failure to find an association between *myosin heavy chain 9, non-muscle (MYH9)* and schizophrenia: A three-stage case–control association study

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### ABSTRACT

Several genome-wide linkage studies have suggested linkage between markers on the long arm of chromosome 22 and schizophrenia. It has also been reported that 22q11.2 deletions increase the risk of schizophrenia. Therefore, 22q is a candidate region for schizophrenia. To search for genetic susceptibility loci for schizophrenia on 22q, we conducted a three-stage case–control association study in Japanese individuals. In the first stage, we examined 13 microsatellite markers on 22q in 766 individuals (340 patients with schizophrenia and 426 control individuals) and found a potential association of AFM262VH5 (D22S283) with schizophrenia. In the second stage, we performed fine mapping of the *myosin heavy chain 9, non-muscle (MYH9)* gene, where AFM262VH5 is located, using 25 tagging single nucleotide polymorphisms (SNPs). We obtained potential associations between three SNPs in *MYH9* and schizophrenia in 1193 individuals (595 patients and 598 controls), which included the individuals analyzed in the first stage. In the third stage, however, we could not replicate these associations in 4694 independent individuals (2288 patients and 2406 controls). Our results suggest that *MYH9* does not confer increased susceptibility to schizophrenia in the Japanese population, although we could not exclude possible contributions of other genes on 22q to the pathogenesis of schizophrenia.

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### 1. Introduction

Several genome-wide linkage studies have suggested linkage between markers on the long arm of chromosome 22 and schizophrenia (Blouin et al., 1998; DeLisi et al., 2002; Faraone et al., 2006; Williams et al., 2003). Two meta-analyses provided

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supportive evidence for susceptibility loci for schizophrenia on 22q (Badner and Gershon, 2002; Lewis et al., 2003), whereas a multicenter study and the most recent meta-analysis conducted both failed to find linkage of 22q to schizophrenia (Mowry et al., 2004; Ng et al., 2009). There is a higher incidence of schizophrenia among patients with velocardiofacial syndrome (Murphy et al., 1999; Shprintzen et al., 1992), which is associated with a hemizygous interstitial deletion of 22q11.2. It has been reported that interstitial deletion of 22q11.2 increases the risk of schizophrenia (Arinami, 2006; Karayiorgou et al., 1995), and this was confirmed by recent genome-wide surveys of rare copy number variants (International Schizophrenia Consortium, 2008; Xu et al., 2008). In addition, there are some interesting candidate genes for schizophrenia in this region including *proline dehydrogenase 1 (PRODH)* (Liu et al., 2002), *catechol-O-methyltransferase (COMT)* (Shifman et al., 2002) and *zinc finger, DHHC-type containing 8 (ZDHC8)* (Mukai et al., 2004). Therefore, 22q is a candidate region for schizophrenia, although the results of previous studies are not necessarily consistent.

To search for genetic susceptibility loci for schizophrenia on 22q, we conducted a three-stage case–control association study in Japanese individuals. In the first stage, we examined 13 microsatellite markers on 22q in 766 individuals (340 patients with schizophrenia and 426 control individuals) and found a potential association of AFM262VH5 (D22S283) with schizophrenia. In the second stage, we performed a fine mapping of the *myosin heavy chain 9, non-muscle (MYH9)* gene, where AFM262VH5 is located, using 25 tagging single nucleotide polymorphisms (SNPs) in 1193 individuals (595 patients and 598 controls), which included the individuals analyzed in the first stage. In the third stage, potential associations obtained in the second stage were further assessed in 4694 independent individuals (2288 patients and 2406 controls).

## 2. Materials and methods

### 2.1. Subjects

The present study was approved by the Ethics Committee of each participating institute, and written informed consent was obtained from each participant. All participants were unrelated Japanese individuals.

The screening population in the first stage consisted of 340 patients with schizophrenia (180 men and 160 women; mean age, 41.8 [SD 14.9] years) and 426 control individuals (219 men and 207 women; mean age, 38.3 [SD 10.4] years). The expanded screening population in the second stage consisted of 595 patients with schizophrenia (313 men and 282 women; mean age, 40.2 [SD 14.1] years) and 598 control individuals (311 men and 287 women; mean age, 38.1 [SD 10.5] years). The expanded screening population included the screening population. The confirmatory population in the third stage consisted of 2288 patients with schizophrenia (1213 men and 1075 women; mean age, 46.5 [SD 14.4] years) and 2406 control individuals (1270 men and 1136 women; mean age, 45.9 [SD 13.9] years), and this population did not overlap with the expanded screening population.

We conducted a psychiatric assessment of every participant, as described previously (Watanabe et al., 2006). In brief, the patients were diagnosed according to the *Diagnostic and*

*Statistical Manual of Mental Disorders Fourth Edition (DSM-IV)* criteria by at least two experienced psychiatrists, on the basis of all available sources of information, including unstructured interviews, clinical observations and medical records. The control individuals were mentally healthy subjects with no self-reported history of psychiatric disorders; they showed good social and occupational skills, but were not assessed using a structured psychiatric interview.

### 2.2. Genotyping

Initially, we screened 13 microsatellite markers on 22q with an average inter-marker interval of 2.63 Mb. All microsatellite markers were genotyped using an ABI 377 genetic analyzer (Applied Biosystems, Foster City, CA) with the GeneScan program v2.1 (Applied Biosystems), as described previously (Kaneko et al., 2007). The sequences of primers used for amplification are available upon request.

Next, we examined 25 tagging SNPs for *MYH9*, covering gene region and the 5' and 3' flanking regions (chr22:34996074...35125125). These tagging SNPs were selected from the HapMap database (release #22, population: Japanese in Tokyo [JPT], minor allele frequency [MAF]: more than 0.05). We applied the criterion of an  $r^2$  threshold greater than 0.8 in 'aggressive tagging: use 2- and 3-marker haplotype' mode using the 'Tagger' program (de Bakker et al., 2005), as implemented in Haploview v3.32 (Barrett et al., 2005). All SNPs were genotyped using the TaqMan 5'-exonuclease assay, as described previously (Watanabe et al., 2006). The sequences of probes used for the TaqMan assay are available upon request.

### 2.3. Statistical analysis

Deviation from the Hardy–Weinberg equilibrium (HWE) of microsatellite markers was tested using the GENEPOP v4.0.9 program (Rousset, 2008). The allele frequencies of microsatellite markers between patients and control individuals were compared using CLUMP v2.3 (Sham and Curtis, 1995). The number of simulations was 10,000 in each test, and the TI statistic was adopted.

Deviations from the HWE for any of the SNPs were tested using the likelihood ratio test. Linkage disequilibrium (LD) blocks defined in accordance with Gabriel's criteria (Gabriel et al., 2002) and haplotype frequencies were determined using Haploview v4.01. The genotype, allele and haplotype frequencies of SNPs in patients and control subjects were compared using  $\chi^2$  test or Fisher's exact test. A probability level of  $p < 0.05$  was considered to indicate statistical significance.

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

## 3. Results

Initially, we examined 13 microsatellite markers on chromosome 22q in the screening population (Table 1). However, the alleles of AFM268YG1 (D22S1170) could not be precisely assigned. Mean heterozygosity for 12 markers was 0.747. The genotype distribution of no marker deviated significantly from the HWE in either group. We observed a potential association of

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**Table 1**  
Case-control association study of 13 microsatellite markers on 22q in the screening population.

Marker	Patients		Controls		Heterozygosity	Allelic <i>p</i>
	<i>n</i>	HWE	<i>n</i>	HWE		
AFM217XF4 (D22S420)	338	0.445	424	0.186	0.730	0.593
AFMA037ZD1 (D22S539)	337	0.503	424	0.107	0.529	0.144
AFM309WD5 (D22S1174)	325	0.585	398	0.638	0.822	0.675
AFM183XE9 (D22S315)	338	0.110	426	0.525	0.821	0.704
AFMA298YB5 (D22S1154)	338	0.157	417	0.305	0.520	0.907
AFMB294ZC1 (D22S1163)	335	0.651	423	0.533	0.718	0.192
AFM225XF6 (D22S280)	337	0.872	423	0.635	0.806	0.803
AFM168XA1 (D22S277)	339	0.253	426	0.697	0.866	0.608
AFM262VH5 (D22S283)	332	0.077	417	0.296	0.795	0.047
AFM261XD9 (D22S423)	339	0.722	425	0.888	0.787	0.318
AFM164TH8 (D22S274)	337	0.319	424	0.172	0.823	0.728
AFM268YG1 (D22S1170)		NA		NA	NA	NA
AFMB337ZH9 (D22S1169)	338	0.751	424	0.758	0.748	0.674

HWE, Hardy–Weinberg equilibrium; NA, not analyzed.

AFM262VH5 (D22S283) with schizophrenia (allelic  $p = 0.047$ ), suggesting that there may be susceptibility loci for schizophrenia near this marker.

Because AFM262VH5 is located in intron 1 of *MYH9*, we investigated 25 tagging SNPs for *MYH9* in the expanded screening population (Table 2). The genotype distribution of no SNP deviated significantly from the HWE in either group. We found potential associations of rs1557538 (SNP#11) in intron 11, rs5756154 (SNP#15) in intron 5, and rs739096 (SNP#17) in intron 2 with schizophrenia (allelic  $p = 0.021$ , 0.023 and 0.020, respectively). In *MYH9*, five LD blocks were

defined (Table 3). The haplotype 2–1–2 of block 4, which contained the minor allele of rs5756154, the major allele of rs11704382 and the minor allele of rs739096, was potentially associated with schizophrenia ( $p = 0.024$ ).

To confirm the potential associations of rs1557538, rs5756154 and rs739096 with schizophrenia, we examined these SNPs in the confirmatory population (Table 4). However, we were unable to replicate these associations in the confirmatory population or a combined population comprising the expanded screening and confirmatory populations. Because rs5756154 (SNP#15) and rs739096 (SNP#17) were in LD, we

**Table 2**  
Genotype and allele frequencies of 25 tagging SNPs in *MYH9* in the expanded screening population.

SNP #	db SNP ID	Allele <sup>a</sup>	Patients					Controls					<i>p</i>	
			<i>n</i>	1/1 <sup>b</sup>	1/2 <sup>b</sup>	2/2 <sup>b</sup>	MAF	<i>n</i>	1/1 <sup>b</sup>	1/2 <sup>b</sup>	2/2 <sup>b</sup>	MAF	Genotype	Allele
1	rs4821475	T/C	594	282	249	63	0.316	598	294	252	52	0.298	0.520	0.341
2	rs767855	C/T	593	498	92	3	0.083	598	500	94	4	0.085	0.983 <sup>c</sup>	0.815
3	rs11703176	A/C	593	252	264	77	0.352	591	249	271	71	0.349	0.840	0.877
4	rs735854	C/T	595	354	214	27	0.225	596	348	218	30	0.233	0.885	0.642
5	rs5756129	C/T	592	311	226	55	0.284	595	309	239	47	0.280	0.610	0.831
6	rs5756130	C/T	595	470	119	6	0.110	597	474	118	5	0.107	0.961 <sup>c</sup>	0.821
7	rs2239788	A/G	593	537	55	1	0.048	598	542	55	1	0.048	1.000 <sup>c</sup>	0.963
8	rs5756133	T/A	595	443	139	13	0.139	594	465	120	9	0.116	0.265	0.100
9	rs2239781	T/C	593	233	270	90	0.379	597	239	270	88	0.373	0.958	0.767
10	rs3830104	T/C	593	430	149	14	0.149	597	425	154	18	0.159	0.741	0.504
11	rs1557538	A/G	594	349	217	28	0.230	596	391	182	23	0.191	0.051	0.021
12	rs9610489	C/T	595	280	261	54	0.310	598	286	256	56	0.308	0.932	0.899
13	rs2239784	C/T	594	428	152	14	0.152	598	429	159	10	0.150	0.666	0.900
14	rs1005570	G/A	595	482	109	4	0.098	598	482	110	6	0.102	0.889 <sup>c</sup>	0.764
15	rs5756154	C/T	594	420	165	9	0.154	595	458	129	8	0.122	0.047	0.023
16	rs11704382	C/A	595	473	117	5	0.107	597	470	120	7	0.112	0.845 <sup>c</sup>	0.667
17	rs739096	G/C	592	426	157	9	0.148	597	467	122	8	0.116	0.043	0.020
18	rs11089788	C/A	592	538	53	1	0.046	595	555	38	2	0.035	0.205 <sup>c</sup>	0.170
19	rs9306310	G/A	595	518	76	1	0.066	598	533	63	2	0.056	0.431 <sup>c</sup>	0.330
20	rs933224	T/C	595	406	170	19	0.175	598	412	163	23	0.175	0.754	0.998
21	rs6000262	A/G	595	396	181	18	0.182	597	407	165	25	0.180	0.363	0.885
22	rs2294356	C/A	595	458	125	12	0.125	598	454	130	14	0.132	0.877	0.615
23	rs5756168	T/C	595	499	91	5	0.085	597	516	77	4	0.071	0.431 <sup>c</sup>	0.213
24	rs9610498	G/A	592	527	64	1	0.056	598	543	51	4	0.049	0.174 <sup>c</sup>	0.483
25	rs11703137	G/A	595	303	247	45	0.283	598	330	223	45	0.262	0.306	0.238

SNP, single nucleotide polymorphism; *MYH9*, myosin, heavy chain 9, non-muscle; MAF, minor allele frequency.

<sup>a</sup> Major/minor alleles.

<sup>b</sup> Genotypes, major and minor alleles are denoted by 1 and 2, respectively.

<sup>c</sup> Calculated using Fisher's exact test.

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**Table 3**  
Haplotype analyses of five LD blocks in *MYH9* in the expanded screening population.

Haplotype	Patients	Controls	<i>p</i>
Block 1 (SNP #2–3–4)			0.863 <sup>a</sup>
1–1–1	0.648	0.650	0.908
1–2–2	0.142	0.148	0.683
1–2–1	0.128	0.118	0.430
2–2–2	0.082	0.085	0.828
Block 2 (SNP #5–6)			0.121 <sup>a</sup>
1–1	0.606	0.613	0.736
2–1	0.284	0.280	0.831
1–2	0.110	0.107	0.826
Block 3 (SNP #8–9)			0.252 <sup>a</sup>
1–1	0.619	0.625	0.740
1–2	0.243	0.258	0.381
2–2	0.137	0.115	0.117
Block 4 (SNP #15–16–17)			0.073 <sup>a</sup>
1–1–1	0.738	0.766	0.112
2–1–2	0.146	0.115	0.024
1–2–1	0.107	0.112	0.665
Block 5 (SNP #21–22–23–24–25)			0.376 <sup>a</sup>
1–1–1–1–1	0.718	0.732	0.443
2–2–1–1–2	0.122	0.128	0.653
1–1–2–1–2	0.084	0.068	0.153
2–1–1–2–2	0.055	0.046	0.335

LD, linkage disequilibrium; *MYH9*, myosin, heavy chain 9, non-muscle; SNP, single nucleotide polymorphism.

Major and minor alleles are denoted by 1 and 2, respectively.

<sup>a</sup> Global *p* values.

performed haplotype analyses of these SNPs (Table 5). In the expanded screening population, the haplotype 1–1, which was constructed from the major alleles of rs5756154 and rs739096, was significantly less frequent in patients than in control individuals ( $p=0.018$ ). By contrast, the haplotype 2–2, which was constructed from the minor alleles of these SNPs, was significantly more frequent in patients than in control individuals ( $p=0.024$ ). However, these associations could not be replicated in either the confirmatory or combined populations.

#### 4. Discussion

Our three-stage case-control association study failed to find an association between *MYH9* within the 22q region and

schizophrenia in the Japanese population. In the first stage, we examined 13 microsatellite markers on 22q to pinpoint genes for association analysis. There was a potential association of the marker AFM262VH5 in *MYH9* with schizophrenia. *MYH9* encodes the heavy chain of non-muscle myosin IIA (NMHC II-A), one of three NMHC II isoforms (A, B and C). The biological functions of NMHC II-A in the brain are poorly understood. Blebbistatin, which inhibits both NMHC II-A and -B, altered the structure of dendritic spines and decreased excitatory synaptic transmission (Ryu et al., 2006). Inhibition of NMHC II-B most likely underlay the morphological and functional abnormalities of spines caused by blebbistatin because *NMHC II-B* mRNA is predominantly expressed in the human brain among the three *NMHC* isoforms (Golomb et al., 2004), and because RNAi of *NMHC II-B* altered the structure of dendritic spines similarly to blebbistatin (Ryu et al., 2006). However, it could not be excluded that NMHC II-A may be implicated in regulation of the structure and function of spines. Interestingly, it has been reported that dendritic spine density is decreased in the brains of patients with schizophrenia (Glantz and Lewis, 2000; Rosoklija et al., 2000). Although further investigation will be needed, the role of NMHC II-A in the development of dendritic spines has possible relevance to schizophrenia.

Application of corrections for multiple testing decreases the probability of type I error (false positive), but increases that of type II error (false negative). Although the sample size of the expanded screening population was moderate, the power was only 0.12–0.49 when the genotypic relative risk was set at 1.4 for homozygous risk allele carriers under the multiplicative model of inheritance. To avoid inflation of the type II error probability, we did not apply corrections for multiple testing. Replication is essential for establishing the credibility of genetic associations (NCI-NHGRI Working Group on Replication in Association Studies, 2007). Therefore, possible associations observed in the moderate-scale population were further assessed in the large-scale independent population. However, we were unable to replicate these associations. The nominally significant associations in the first and second stages were most likely the results of type I error. It is unlikely that the negative results in the third stage were caused by type II errors because the power was more than 0.8 in the confirmatory population. There is another possible explanation for the discrepancy between the results in the

**Table 4**  
Genotype and allele frequencies of three SNPs in *MYH9* in the confirmatory and combined populations.

db SNP ID	Patients					Controls					<i>p</i>	
	<i>n</i>	1/1 <sup>a</sup>	1/2 <sup>a</sup>	2/2 <sup>a</sup>	MAF	<i>n</i>	1/1 <sup>a</sup>	1/2 <sup>a</sup>	2/2 <sup>a</sup>	MAF	Genotype	Allele
<i>rs1557538</i>												
Confirmatory	2233	1370	762	101	0.216	2375	1471	790	114	0.214	0.776	0.858
Combined	2827	1719	979	129	0.219	2971	1862	972	137	0.210	0.301	0.233
<i>rs5756154</i>												
Confirmatory	2257	1658	555	44	0.142	2359	1728	585	46	0.144	0.987	0.886
Combined	2851	2078	720	53	0.145	2954	2186	714	54	0.139	0.624	0.377
<i>rs739096</i>												
Confirmatory	2268	1706	521	41	0.133	2380	1783	555	42	0.134	0.957	0.853
Combined	2860	2132	678	50	0.136	2977	2250	677	50	0.131	0.659	0.381

SNP, single nucleotide polymorphism; *MYH9*, myosin, heavy chain 9, non-muscle; MAF, minor allele frequency.

<sup>a</sup> Genotypes, major and minor alleles are denoted by 1 and 2, respectively.

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**Table 5**

Haplotype analyses for the SNPs rs5756154–rs739096 in *MYH9* in the screening, confirmatory and combined populations.

Haplotype	Patients	Controls	<i>p</i>
Expanded screening population			0.023 <sup>a</sup>
1–1	0.844	0.878	0.018
2–2	0.146	0.115	0.024
Confirmatory population			0.518 <sup>a</sup>
1–1	0.838	0.842	0.627
2–2	0.114	0.119	0.413
Combined population			0.619 <sup>a</sup>
1–1	0.840	0.849	0.149
2–2	0.120	0.118	0.735

Major and minor alleles are denoted by 1 and 2, respectively.

<sup>a</sup> Global *p* values.

second stage and those in the third stage. Allelic heterogeneity may exist for *MYH9*. In this case, it would be difficult to provide convincing evidence for an association. It might be noteworthy that a recent genome-wide association study (GWAS) of major depressive disorder suggested that there may be allelic heterogeneity for *glutamate receptor, metabotropic 7 (GRM7)* (Muglia et al., 2008). Nevertheless, our results could not show sufficient evidence for an association of *MYH9* with schizophrenia in the Japanese population.

The genetic variants of *MYH9* have previously been tested for associations with schizophrenia. An initial study showed significantly distorted transmission of AFM262VH5 in 23 families multiply affected with schizophrenia (Vallada et al., 1995). However, subsequent case–control studies failed to replicate this association (Kitao et al., 2000; Williams et al., 1997). Our three-stage case–control study could not provide sufficient evidence for an association of *MYH9* with schizophrenia. It is noteworthy that although there were no significant associations between seven SNPs in *MYH9* and schizophrenia, four SNPs (rs3752463 in intron 9, rs1557540 in intron 3, rs713839 in intron 3, and rs739097 in intron 1) in *MYH9* were associated with a subgroup of schizophrenia patients without deficits in sustained attention (Liu et al., 2008). Considering these findings together, *MYH9* does not contribute to genetic susceptibility to schizophrenia, but may have effects on the neuropsychological endophenotypes for schizophrenia.

We recognize some limitations of the present study. First, we screened only 12 microsatellite markers with an average inter-marker interval of 2.87 Mb. Therefore, our results cannot exclude possible contributions of other genes on 22q to the pathogenesis of schizophrenia. Recent studies using large samples (more than 1000 cases and 1000 controls) have indicated that genes on 22q including *protein interacting with PRKCA 1 (PICK1)*, *claudin 5 (CLDN5)*, *DiGeorge syndrome critical region gene 2 (DGCR2)*, *armadillo repeat gene deletes in velocardiofacial syndrome (ARVCF)* and *COMT* are not associated with schizophrenia (Ishiguro et al., 2007, 2008a,b; Okochi et al., 2009; Sanders et al., 2008). However, to draw a definitive conclusion, further studies using large samples and sufficient markers should be carried out in various ethnic populations. Several GWAS of schizophrenia have been published (International Schizophrenia Consortium, 2009; Kirov et al., 2009; Lencz et al., 2007; Mah et al., 2006; Need et al., 2009; O'Donovan et al., 2008; Shi et al., 2009; Shifman et al., 2008; Stefansson et

al., 2009; Sullivan et al., 2008). Interestingly, a polymorphism in intron 1 of *myosin XVIII B (MYO18B)* on 22q12.1 was most significantly associated with schizophrenia in a large-scale GWAS (International Schizophrenia Consortium, 2009). A meta-analysis of GWAS for schizophrenia, which is currently being conducted (Psychiatric GWAS Consortium Coordinating Committee, 2009), will be useful for the process of narrowing down the region for fine mapping on 22q. Second, our individuals were not assessed using a standardized structured interview. However, the diagnosis of schizophrenia was made on the basis of all available sources of information. To the best of our knowledge, there were no control individuals who were likely to develop schizophrenia at their present stage of life. Thus, it is unlikely that our failure to find a significant association is attributable to misdiagnosis. Despite these caveats, our results suggest that *MYH9* does not confer increased susceptibility to schizophrenia in the Japanese population.

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#### Contributors

Authors Amagane and Watanabe designed the study and performed genotyping. Authors Kaneko and Nunokawa undertook statistical analyses. Author Muratake designed the study. Author Ishiguro performed genotyping. Authors Arinami, Ujike, Inada, Iwata, Kunugi, Sasaki, Hashimoto, Itokawa, and Ozaki managed sample collection. Author Someya supervised the study. All authors contributed to and have approved the final manuscript.

#### Conflict of interest

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

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## Letter to the Editor

**No association between DAO and schizophrenia in a Japanese patient population: A multicenter replication study**

Dear Editors

D-serine, a co-agonist that enhances N-methyl-D-aspartate (NMDA) glutamate receptors, is hypothesized to be involved in the pathophysiology of schizophrenia because treatment with D-serine improves some schizophrenic symptoms (Tsai et al., 1998). Thus, genes that are related to D-serine metabolism have arisen as candidate genes that may play a role in schizophrenia. Genomic case-control studies of one such candidate gene, D-amino acid-oxidase (*DAO*; 12q24), have shown relatively consistent positive results in which *DAO* is a susceptibility locus for patients with schizophrenia (Chumakov et al., 2002; Corvin et al., 2007; Schumacher et al., 2004; Wood et al., 2007). A previous well-explained report about the role of *DAO* in schizophrenia (Verrall et al., 2009) and two representative meta-analyses showed an association between *DAO* and schizophrenia, specifically with the SNP, rs4623951 (Allen et al., 2008; Shi et al., 2008). Previously, we performed stage 1 genomic case-control studies (340 schizophrenia patients, 340 healthy controls) for genes (*PHGDH*, *SHMT1*, *SRR*, *DAO*) related to D-serine synthesis/degradation. Our results from a haplotype case-control analysis showed that only three SNPs (rs3825251, rs3918347, and rs4964770) in *DAO* which are in strong linkage disequilibrium (LD) and show an association with schizophrenia, even after correction for multiple testing (Ohnuma et al., 2009). Some negative results have also been reported regarding a possible association between *DAO* and schizophrenia (Fallin et al., 2005; Jonsson et al., 2009; Liu et al., 2006; Shinkai et al., 2007; Vilella et al., 2008; Yamada et al., 2005). Thus, we performed a stage 2 replication study to clarify the conclusions of a possible role for *DAO* in schizophrenia. We reinvestigated the association between the above *DAO* haplotype (rs3825251–rs3918347–rs4964770) and schizophrenia using adequate statistical power.

The case-control genetic association studies were performed using 1656 unrelated Japanese patients with schizophrenia (863 males, 793 females; mean age 45.2 years, S.D.  $\pm$  15.4) who met the DSM-IV diagnosis of schizophrenia. We also used 1842 unrelated healthy controls (784 males, 1058 females; mean age 47.1 years, S.D.  $\pm$  19.1). Patients and controls were recruited from four geographic regions of Japan: Osaka, Aichi, Saitama, and Tokyo. The criteria for

enrolling both patients and controls have been previously described (Ohnuma et al., 2009). Of these participants, 340 patients with schizophrenia and 340 healthy controls had been analyzed in our previous stage 1 study (Ohnuma et al., 2009); the remaining patients and controls were new to the present study. The mean age of the schizophrenic group was significantly different from that of the control group (Student's *t*-test,  $t = 3.27$ ,  $P = 0.001$ ), and the gender distribution of the two groups was significantly different ( $\chi^2 = 31.9$ ,  $P < 0.001$ ). The Ethics Committee of the Juntendo University School of Medicine approved this study. All participants gave their written informed consent prior to participating in the study.

Genomic DNA was extracted from peripheral white blood cells using a QIAamp<sup>®</sup> DNA Blood Maxi kit (Qiagen, Courtaboeuf, France). Three SNPs (rs3825251, rs3918347, and rs4964770) were typed using TaqMan<sup>®</sup> technology as previously described in detail (Ohnuma et al., 2009). SNP information, including position and distance between SNPs, has also been described (Ohnuma et al., 2009).

For the case-control association study, genetic statistical analysis, Hardy–Weinberg Equilibrium (HWE) testing, differences in genotypic/allelic frequencies, LD, and case-control haplotype analysis were all done using SNPalyze V7.0 Pro (Ohnuma et al., 2009). All *P*-values reported are two-tailed and were considered statistically significant when  $< 0.05$ . We performed power calculations using the Power Calculator for Two Stage Association Studies (<http://www.sph.umich.edu/csg/abecasis/CaTS/>). Power was calculated under the prevalence of 0.01 using an additive or a multiplicative model, which was based on allelic frequencies of the associated markers that ranged from 0.49 (rs3918347) to 0.50 (rs4964770). The odds ratio ranged from 1.152 (rs3918347) to 1.166 (rs4964770) for the SNPs investigated in our current study (Ohnuma et al., 2009), with an alpha level of 0.05. Results of power analysis showed that the power ranged from 80% (rs3918347) to 90% (rs4964770).

The three SNPs in *DAO* were genotyped in 1656 patients with schizophrenia and in 1842 controls. No deviation from HWE in either patients or controls was observed, and no single SNP showed a significant association between their allelic or genotypic frequencies and schizophrenia (Supplementary Table 1). Further investigation of the three SNPs showed a strong LD (Table 1), but case-control haplotype association analysis (minor haplotypes with frequencies less than 3% in either group were omitted) using windows of two or three SNPs failed to show a significant association with Japanese schizophrenia (Table 1).

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