Table 2
Cortical regions showing significant between-group differences in oscillatory activity power changes.

ERD/ERS	Location	Talairach (*x*, *y*, *z*)	BA	ERD/ERS at t_{maxima}				*t* value
				CIP	nPE	SCZ	HC	
Alpha ERD	Right SFG	21, 18, 52 19, 20, 52[b]	8	−4.31 ± 2.50 ***−5.32 ± 1.80***	−0.28 ± 2.11 ***−5.90***[c]	−5.41 ± 3.01	−0.74 ± 2.37	−4.389[c] −4.56[b,c]
Alpha ERS	Right PreC	4, −54, 55	7	6.54 ± 6.13 ***6.15 ± 7.38***	−2.85 ± 5.70 ***2.97*** ***P = 0.012***			4.03[c]
Alpha ERS	Left PPC	−27, −54, 27	7	4.83 ± 4.25 ***3.96 ± 4.93***	−3.96 ± 6.23 ***3.28*** ***P = 0.007***			4.254[c]
Alpha ERS	Left ITG	−57, −29, −12 −37, −10, −23[b]	37 20[b]	0.13 ± 5.19 ***1.16 ± 5.18***	−9.56 ± 6.72 ***4.18***[c]	1.47 ± 6.36	−8.04 ± 6.71	4.14 3.90[b]
Theta ERS	Left ITG	−41, −8, −24	20			3.15 ± 7.52	−6.17 ± 4.69	3.95[c]
Alpha2 ERD	Right Mid-preF	15, 24, 25	9			−9.16 ± 7.05	2.38 ± 5.16	−4.94[c]

[a]Values in boldface and italic refer to an exploratory analysis excluding low-performing patients with CIP. ERD, event-related desynchronization; ERS, event-related synchronization; BA, Brodmann area; CIP, chronic interictal psychosis; nPE, nonpsychotic epilepsy; SCZ, schizophrenia; HC, healthy controls; SFG, superior frontal gyrus; PreC, precuneus; PPC, posterior parietal cortex; ITG, inferior temporal gyrus; Mid-preF, mid-prefrontal.
[b] Comparison between patients with SCZ and HC.
[c] Significant difference for $P < 0.001$ (uncorrected).

band. The source localization of these activities was similar to those observed in CIP relative to patients with nPE, specifically the right DLPFC and left inferior temporal lobe for alpha activity and the left inferior temporal cortex for the theta band (Fig. 5). The difference in mean values at t_{maxima} of alpha and theta ERD/ERS sources is given in Table 2. Like the full alpha band, the alpha2 subband consistently showed statistically relevant ERD in the right DLPFC (value at t_{maxima}: schizophrenia −9.0 ± 4.2%; healthy controls −0.4 ± 3.2%; $t = -6.04$, $P < 0.001$). However, the alpha2 subband showed an additional ERD source in the right medial prefrontal cortex in patients with schizophrenia.

A comparison between patients with CIP and schizophrenia showed no clear source-power changes in any frequency band, with t_{maxima} in the statistical maps for the alpha band ($t = 3.45$, $P < 0.005$) located over the left inferior parietal cortex, and for the theta band ($t = 2.92$, $P < 0.01$) over the medial-parietal cortex.

3.4. Correlation analysis

To examine the potential influence of medication on source-power changes in patients with CIP and schizophrenia, alpha and theta ERD/ERS mean peak values were correlated with chlorpromazine equivalents and antiepileptic drug (AED) plasma levels. All correlations were two-tailed and controlled for task performance as well as IQ. No statistically significant correlations were identified.

4. Discussion

In the present study, we compared the MEG oscillatory response based on ERD/ERS during a visual-object WM task in patients with CIP, nPE, and schizophrenia and healthy controls to determine whether patients with CIP and primary schizophrenia share WM-related neural abnormalities. Our main findings are: (1) patients with CIP have WM deficits involving a frontotemporal network, (2) the neurophysiological basis for these deficits is primarily changes in the alpha frequency band, and (3) patients with CIP and schizophrenia compare to each other with respect to the WM-compromising brain regions, namely, the right DLPFC and the left temporal cortex, although patients with schizophrenia manifested wider activation in prefrontal areas. In addition, our results showed that the two groups perform at equal levels on a visual-object WM task as far as answer correctness and reaction time are concerned.

4.1. Event-related desynchonization pattern

In this study, frontal lobe WM dysfunction in CIP as well as in schizophrenia manifested as alpha ERD, particularly in the right DLPFC, when compared with patients with nPE and healthy controls (Fig. 5). Given that alpha suppression or desynchronization (i.e., ERD) is considered an electrophysiological correlate of cortical activation involving processing of sensory or cognitive information [30], the prefrontal cortex source-power changes, namely, alpha ERD, seen in our study most likely reflect hyperactivation in both psychotic groups. It is generally accepted that the DLPFC plays a crucial role in WM processing and that its function is impaired in schizophrenia [57]. However, whether its impairment is the result of hyperactivation or hypoactivation is still controversial [58]. Recent analyses have revealed that patients with schizophrenia can show patterns of both hyperactivation and hypoactivation depending on task demands and behavioral performance. Patients with low performance show primarily hypofrontality, whereas high-performing subjects exhibit prefrontal hyperactivation [59]. In this context, it is interesting that the patients with schizophrenia in our study evidenced DLPFC hyperactivation related to WM performance while at the same time performing at par with healthy controls in behavioral accuracy. In addition, high-performing patients with CIP also manifested DLPFC hyperactivation. The fact that the DLPFC activation was detected in the right hemisphere is in line with recent reports proposing that object memory storage is more likely to produce activation in the right prefrontal cortex than storage of other materials (e.g., verbal, visuospatial) [57].

In addition to convergent functional abnormalities in the right DLPFC in the two psychotic groups, we found an alpha2 subband ERD source in the medial prefrontal cortex of patients with schizophrenia, which was not observed when patients with CIP and nPE were compared. This observation is consistent with findings from MRI studies indicating significantly higher medial prefrontal activation [59], particularly in the right side [60] in patients with schizophrenia. As the medial prefrontal cortex is functionally associated with focused attention [35], a function known to be impaired in schizophrenia [16], it is conceivable that the schizophrenic hyperactivation in this area is a reflection of a compensatory mechanism or increased attentional effort in performing

successfully on the WM task. Given that the same pattern of alpha ERD in the medial prefrontal cortex nearly reached statistical significance in CIP when compared with nPE ($P < 0.005$), the possibility that an increase in sample size could also result in significantly different activation in this area for patients with CIP cannot be ruled out.

4.2. Event-related synchronization pattern

The averaged power changes in oscillatory activity in the two psychotic groups revealed that these patients have notable alpha ERS in posterior brain regions, namely, the parieto-occipital and posterior temporal cortex, which was not seen in patients with nPE and healthy subjects (Fig. 4). It is well known that alpha activity typically enhances or synchronizes in the visual (occipital) cortex when subjects are in a relaxed state with their eyes closed, and this phenomenon has therefore been regarded as reflecting mental rest or cortical idling [30]. Thus, alpha ERS in patients with psychosis when engaged in a cognitive task is an intriguing finding. It is important, however, to point out that posterior alpha ERS in eyes-open condition can also occur during motor or cognitive tasks, possibly representing deactivated cortical areas or an inhibited cortical network [61]. In this context, active inhibition in neural networks may be of great importance in optimizing task demands and controlling excitatory processes in cortical areas directly involved in the performance of the task, by blocking, for instance, processing of task-irrelevant sensory or cognitive information [30,36,61]. Nevertheless, the statistical group analysis revealed significant alpha ERS only over the left inferior temporal cortex in the psychotic groups versus their nonpsychotic counterparts (Fig. 5), with the parieto-occipital alpha ERS in patients with CIP being related to poor task performance (Table 2), as discussed below. Interestingly, patients with schizophrenia, and even their nonaffected twins, have been reported to have excessive EEG alpha ERS in cortical areas responsible for processing task-specific visual information during WM retention, correlating in magnitude with memory load [62]. These findings are consistent with the assumption of increased alpha ERS in the posterior-inferior temporal cortex reflecting regional cortical hypoactivation in our study, which most likely indicates WM capacity limitations or inefficiency of cognitive mechanisms subserving active maintenance of information in WM in patients with CIP and schizophrenia [62].

In the schizophrenia group, ERS in the left inferior temporal cortex involved not only the alpha, but also the theta frequency band (Table 2). This finding is in keeping with evidence of alpha and theta oscillations having similar physiological reactivity in WM tasks [32,34]. Similar to our approach, Ince et al. [37] applied MEG and a spectro-temporospatial analysis during the retention phase of a WM task and found that power changes in the theta and alpha bands allowed for successful discrimination between patients with schizophrenia and healthy controls. These authors also noted a left temporal source for the theta ERS, similar to our findings. However, alpha ERS in their study was not distributed over the temporal cortex but over parieto-occipital areas. The discrepancy in the findings may in part be explained by differences in WM storage material, as the aforementioned authors examined verbal WM, whereas our study used a visual nonspatial WM paradigm.

Unlike DLPFC dysfunction, which is largely unexplored in patients with CIP, temporal lobe abnormalities, especially in the left hemisphere, are well linked to both schizophrenia and CIP [10,12,18–25]. Most of the supporting data stem from functional MRI studies in patients with schizophrenia. On the other hand, findings for CIP come from structural MRI, neuropsychological, and neuropathological studies, with very few cognitive functional neuroimaging investigations. Our results of left temporal hypoactivation and a frontotemporal dysfunction during WM in patients with CIP are strengthened by findings from existing functional neuroimaging studies on psychoses in epilepsy, indicating left temporal hypoperfusion using SPECT [23,24] and impaired metabolism in frontal and temporal areas via PET [25]. In addition, correlations between neuropsychological assessments and measures of microstructural integrity in the left temporal lobe as well as in frontotemporal regions in patients with CIP have been reported and provide further support of our findings [19,26]. The ERS sources in patients with CIP and schizophrenia were not visualized in the exact same area within the left inferior temporal cortex, the neural activity in patients with CIP occurring in a more posterior region (Brodmann area 37) compared with that in patients with schizophrenia (Brodmann area 20). However, both areas of the inferior temporal cortex are known to be involved in visual processing of the ventral stream, which is associated with the representation of object features [57]. The finding of left temporal abnormalities in both patients with CIP and those with schizophrenia described herein is thus consistent with dysfunction in cortical regions engaged specifically in performance of the type of task used, namely, visual-object WM.

Alpha ERS in the right precuneus and left posterior parietal cortex clearly distinguished patients with CIP from patients with nPE, yet it was entirely absent in patients with schizophrenia. Interestingly, the ERS in parietal areas was associated with low performance on the task rather than with WM dysfunction itself, as the power changes in oscillatory activity were not significantly different from those of patients with nPE once low-performing patients were excluded (Table 2). Local hypoactivation in the parietal cortex may hence reflect disengagement from the task due to reduced attention in patients with CIP. This would be in agreement with evidence implicating the precuneus and posterior parietal cortex in several modalities of attention such as object-selective attention [63], which is critical to WM, especially for visual object information.

4.3. ERD/ERS sources and external clinical parameters

We tested the potentially confounding effect of medication as most of our patients with psychosis were taking antipsychotic drugs, with the patients with schizophrenia receiving higher doses than the patients with CIP. Patients with CIP were on regular AED treatment. We found no correlation between ERD/ERS sources and chlorpromazine equivalents or AED plasma levels in either psychotic group. This indicates that medication did not influence our findings. The fact that ERD and ERS were calculated as power changes during the memory retention (target) period with respect to the baseline period in the MEG time-series data lends further support to this view, given that medication would equally affect the baseline and the memorization periods. Therefore, normalization with respect to baseline is expected to remove any additive effect.

4.4. Limitations

Some methodological issues in the present study need to be considered. First is sample size. Our CIP sample was relatively small. This is not uncommon in CIP neuroimaging research, and may be the direct result of the difficulty in recruiting patients with CIP and a single brain pathology owing to the relative rarity of the disorder [1,2,6,8]. Nevertheless, we matched patients with CIP for age, gender, type of focal epilepsy, and side of epileptic focus with the nPE group, and patients with low IQ and gross organic lesions were excluded. Second is medication artifacts. Most patients were stably medicated. Based on the results of the correlation analysis of chlorpromazine equivalents and AED plasma levels and the fact that we subtracted time-series data of the baseline period from

those of the memorization (target) period, it is unlikely that pharmacological agents influenced our findings. Third is task dependency. Because we assessed visual-object WM with a fixed memory set of five digits, we cannot rule out that areas engaged in other storage (e.g., verbal or visuospatial) or greater task demands show cortical activation patterns that distinguish CIP from primary schizophrenia if a different paradigm or variation in WM loads is employed. Fourth is diagnostic bias. Patients in the CIP and schizophrenia groups were assessed with different measures of psychotic symptoms, namely, the BPRS for patients with CIP and the PANSS for patients with schizophrenia, which was the result of how the subjects had been ascertained. These two groups can therefore not be directly compared in terms of severity of psychiatric symptoms. Although measures of equivalence between BPRS and PANSS scores in response to antipsychotic treatment were used [64], it is likely that patients with CIP and those with schizophrenia had comparable levels of psychopathology. Regardless, our study was not designed to assess brain activity related to psychopathology itself but to cognitive deficits, in particular WM dysfunction.

4.5. Summary

In this study we found a similar MEG pattern of right DLPFC and left temporal functional abnormalities in patients with CIP and those with schizophrenia during performance of Sternberg's visual WM task, prompting us to consider that similar pathophysiological mechanisms may operate in these two groups. Consistent with this idea, recent studies [13,16] have demonstrated that the profile of neuropsychological impairment in CIP closely resembles that of primary schizophrenia, especially regarding temporal and prefrontal function. Structural MRI of patients with CIP and schizophrenia has further revealed that they share a similar topographic pattern of deficits in temporal and extratemporal cortical gray matter [14]. Together, these data suggest that the DLPFC and temporal cortex are regions closely associated with cognitive dysfunction in both patients with CIP and those with primary schizophrenia, which argues against the concept of CIP as a nosological entity different from schizophrenia. To our knowledge, this is the first neuroimaging study applying MEG for detection of functional cognitive abnormalities in patients with CIP. Larger functional studies on CIP using MEG or different neuroimaging modalities may help elucidate the pathophysiology of chronic psychosis in patients with epilepsy.

Acknowledgments

We thank Christoph Lossin for critical reading of this article, and Dr. Yuka Yasuda, Dr. Hidenaga Yamamori, Dr. Kazutaka Ohi, and Dr. Motoyuki Fukumoto for their contribution in the collection of clinical data of patients with schizophrenia.

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Dysbindin Regulates the Transcriptional Level of Myristoylated Alanine-Rich Protein Kinase C Substrate *via* the Interaction with NF-YB in Mice Brain

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Abstract

Background: An accumulating body of evidence suggests that Dtnbp1 (Dysbindin) is a key susceptibility gene for schizophrenia. Using the yeast-two-hybrid screening system, we examined the candidate proteins interacting with Dysbindin and revealed one of these candidates to be the transcription factor NF-YB.

Methods: We employed an immunoprecipitation (IP) assay to demonstrate the Dysbindin-NF-YB interaction. DNA chips were used to screen for altered expression of genes in cells in which Dysbindin or NF-YB was down regulated, while Chromatin IP and Reporter assays were used to confirm the involvement of these genes in transcription of Myristoylated alanine-rich protein kinase C substrate (MARCKS). The sdy mutant mice with a deletion in Dysbindin, which exhibit behavioral abnormalities, and wild-type DBA2J mice were used to investigate MARCKS expression.

Results: We revealed an interaction between Dysbindin and NF-YB. DNA chips showed that MARCKS expression was increased in both Dysbindin knockdown cells and NF-YB knockdown cells, and Chromatin IP revealed interaction of these proteins at the MARCKS promoter region. Reporter assay results suggested functional involvement of the interaction between Dysbindin and NF-YB in MARCKS transcription levels, *via* the CCAAT motif which is a NF-YB binding sequence. MARCKS expression was increased in sdy mutant mice when compared to wild-type mice.

Conclusions: These findings suggest that abnormal expression of MARCKS *via* dysfunction of Dysbindin might cause impairment of neural transmission and abnormal synaptogenesis. Our results should provide new insights into the mechanisms of neuronal development and the pathogenesis of schizophrenia.

Citation: Okuda H, Kuwahara R, Matsuzaki S, Miyata S, Kumamoto N, et al. (2010) Dysbindin Regulates the Transcriptional Level of Myristoylated Alanine-Rich Protein Kinase C Substrate *via* the Interaction with NF-YB in Mice Brain. PLoS ONE 5(1): e8773. doi:10.1371/journal.pone.0008773

Editor: Kenji Hashimoto, Chiba University Center for Forensic Mental Health, Japan

Received November 4, 2009; **Accepted** December 9, 2009; **Published** January 19, 2010

Funding: This work was supported by grants from the Osaka Medical Research Foundation For Incurable Diseases (Shinsuke Matsuzaki) and the Twenty-First Century Center of Excellence (COE) program and the Global COE program (Hiroaki Okuda, Ryusuke Kuwahara, Shinsuke Matsuzaki, Shingo Miyata, Natsuko Kumamoto, Tsuyoshi Hattori, Shoko Shimizu, Kohei Yamada, Taiichi Katayama and Masaya Tohyama). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Schizophrenia is a common and devastating psychiatric disorder. Lack of patient compliance, due to undesirable side effects and efficacy restricted to positive symptoms, highlights the need to develop novel therapeutics. The etiology of the disease remains unknown, but in recent years a convergence of genetic, pharmacological, and neuroanatomical findings suggest that neural transmission [1–4] and synapse formation [5–11] are involved in schizophrenia. Recent studies suggest that disturbances of Dysbindin (dystrobrevin-binding protein 1; DTNBP1) are involved in this abnormal neural transmission.

The cause of schizophrenia is thought to involve the combined effects of multiple gene components. Genetic linkage and association studies have identified potential susceptibility genes such as Dysbindin [12,13], Neuregulin [14,15], Catechol-*O*-methyltransferase [16–18] and RG4 [19–22]. In particular, it has been reported that chromosome 6p is one of the highest susceptibility regions in linkage studies of schizophrenia [23,24]. Among them, genetic variants in a gene 6p22.3 expressing Dysbindin, which is identified as a protein interacting with dystrobevins [25], have been shown to be strongly associated with schizophrenia [12].

In studies on postmortem brain tissue, decreased levels of Dysbindin protein [26] and mRNA [27] have been shown in

patients with schizophrenia compared with controls. Chronic treatment of mice with antipsychotics did not affect the expression levels of Dysbindin protein and mRNA in their brains [26,28], suggesting that evidence of lower levels of Dysbindin protein and mRNA in the postmortem brains of schizophrenics is not likely to be a simple artifact of antemortem drug treatment. In addition, previous reports have shown that diverse high-risk single nucleotide polymorphisms (SNPs) and haplotypes could influence Dysbindin mRNA expression [27,29]. These data indicate that the Dysbindin gene may confer susceptibility to schizophrenia through reduced Dysbindin expression.

Several lines of evidence suggest that Dysbindin may be associated with brain function. SNPs in *Dysbindin* have been associated with intermediate cognitive phenotypes related to schizophrenia such as IQ and working and episodic memory, and a Dysbindin haplotype has been associated with higher educational attainment [30,31]. In addition, several reports suggest the involvement of Dysbindin in cognitive functions [32–34]. These findings strongly suggest the importance of Dysbindin in brain function. At the cellular level, Dysbindin is located at both pre- and post-synaptic terminals [26,35], and is thought to be involved in postsynaptic density (PSD) function and the trafficking of receptors (NMDA, GABAergic, and nicotinic). Over-expression of Dysbindin increases glutamate release from pyramidal neurons in cell culture, possibly because of its role in vesicular trafficking [36]. Decreases in Dysbindin mRNA and protein levels have been reported in regions previously implicated in schizophrenia: the prefrontal cortex, midbrain, and hippocampus [26,27]. However, the molecular mechanisms of how decreases in Dysbindin expression may contribute to vulnerability to schizophrenia remain unknown.

Thus, we examined the interacting partners of Dysbindin using yeast two-hybrid analysis in order to help elucidate the function of Dysbindin. These interacting-protein data suggest that Dysbindin is involved in such processes as neurotransmission, cell signaling, the cytoskeleton and transcription. (Matsuzaki S *et al.* in submission). In addition, our previous reports suggest the following; (1) decreased expression of Dysbindin might increase dopamine release in the brain resulting in the observed abnormal behavior in sdy mice (Dysbindin KO mice) [37,38], (2) Dysbindin is likely involved in dopaminergic or glutamatergic transmission [36,39], (3) Dysbindin is likely involved in neurotransmission by binding with the BLOC1 complex, and with transcription by binding with transcription-related genes (Matsuzaki S *et al.* in submission), (4) the expression level of Dysbindin may affect the expression of SNAP25 [36,39], (5) Dysbindin may play a key role in coordinating JNK signaling and actin cytoskeleton required for neural development [40]. These findings suggest that Dysbindin may influence neurotransmission and neural development *via* interaction with other factors or by regulation of transcription.

In a previous paper, we identified several Dysbindin interacting partners including the transcription factor, nuclear transcription factor Y beta (NF-YB) (Matsuzaki S *et al.* in submission). NF-YB belongs to a family of CCAAT-binding transcription factors, which are important for the basal transcription of a class of regulatory genes and are involved in cellular reactions [41–44]. Subsequently, in this study, we examined the functional involvement of Dysbindin in transcription *via* its interaction with NF-YB. As a result, we showed that the NF-YB/Dysbindin complex regulates the transcription of MARCKS *via* interaction with certain CCAAT sequences, and abnormal NF-YB/Dysbindin interaction could cause alterations such as impaired neural transmission and abnormal development of neurons.

Results

Dysbindin Exists within the Nucleus in Addition to the Cytoplasm

We examined the existence of Dysbindin in the nucleus, because Dysbindin should exist within the nucleus to play a functional role in transcriptional regulation. We used an overexpression vector for Dysbindin tagged with –FLAG or –V5 to check the intracellular localization of Dysbindin. The fractionation study using Dysbindin-FLAG-overexpressing HEK293 cells shows that Dysbindin exists mainly in the cytosol while a small amount exists in the nucleus (Figure 1A), and Dysbindin-V5 showed the same results (data not shown). These results are in accordance with a previous report [45]. Morphologically, Dysbindin is localized mainly in the cytoplasm with a perinuclear high density region in HEK293 cells and SY5Y cells; however, a faint immunoreaction was also seen within the nucleus (Figure 1B -a and -b). Furthermore, pretreatment with leptomycine-B (LPB), which inhibits export from the nucleus to the cytoplasm, caused a slight Dysbindin increase in cells, which then showed nuclear localization of Dysbindin (Figure 1B -c and -d). These findings suggest that Dysbindin protein is shuttled between the nucleus and the cytoplasm.

Dysbindin Binds to the Transcription Factor NF-YB

Using yeast two-hybrid screening, we identified several transcriptional factors as candidates that may interact with Dysbindin. We selected NF-YB, one of the candidates, and confirmed a Dysbindin-NF-YB interaction by immunoprecipitation assay using HEK293T cells which express NF-YB endogenously (Figure 1A). HEK293T cells were transfected with expression vectors for Dysbindin-V5, and cell lysates were subjected to immunoprecipitation with anti-V5 or anti-NF-YB antibodies, followed by Western blot analysis with a reciprocal antibody. NF-YB was detected in the immunoprecipitates with an anti-V5 body, comparing to the immunoprecipitates with control IgG (Figure 2A), while Dysbindin-V5 was detected in the immunoprecipitates with an anti-NF-YB antibody, comparing to control IgG (data not shown). Thus, Dysbindin and NF-YB are physiologically associated with each other in transfected mammalian cells.

To further our research, we produced a specific anti-Dysbindin antibody with high titer. The antibody detects endogenous Dysbindin in cell and mouse brain samples, though it did not detect any bands corresponding to Dysbindin from the lysates of

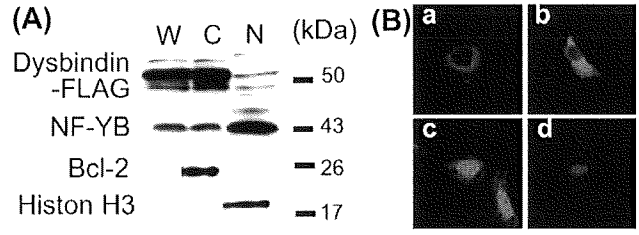

Figure 1. The nuclear localization of Dysbindin. (A) HEK293 cells overexpressing Dysbindin-FLAG were separated into nuclear and cytosolic fractions. Anti-Bcl2 antibody was used for the cytosolic fraction marker and anti-Histone H3 antibody was used for the nuclear fraction marker. W: Whole cell lysates, N: Nuclear Fraction, C: Cytosolic fraction. Dysbindin-FLAG was slightly present in the nuclear fraction. (B) Dysbindin-GFP was overexpressed in HEK293 cells (a and c) or in SH-SY5Y cells (b and d). Dysbindin was usually localized in the cytoplasm and slightly in the nucleus (a and b). After treatment with LMB, a potent inhibitor of CRM1-dependent nuclear export, Dysbindin-GFP accumulated in the nucleus (c and d).
doi:10.1371/journal.pone.0008773.g001

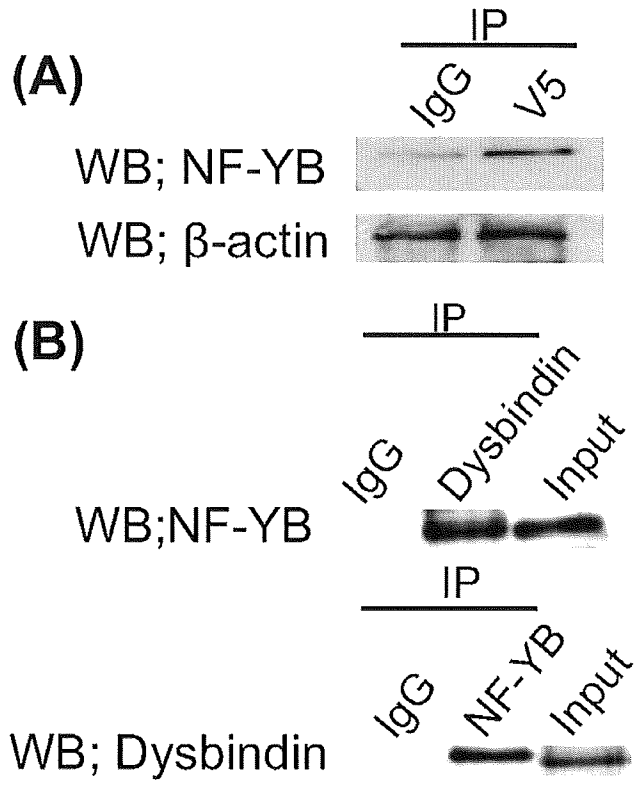

Figure 2. The interaction between Dysbindin and NF-YB. (A) HEK293 cells were transfected with Dysbindin-V5. Immunoprecipitates (IP) of lysates of HEK293 cells expressing Dysbindin-V5 obtained by antibodies to tag proteins (V5) (2nd lane), or nonspecific rabbit IgG (IgG) (1st lane) were subjected to Western blot with anti- NF-YB antibody (upper panel). Dilutions of the lysate (5%, HEK293 cells) were subjected to Western blot with anti-β-actin antibody (lower panel). (**B**) Immunoprecipitates (IP) of lysates of SH-SY5Y cells obtained by antibodies to Dysbindin (upper panel 2nd lane), NF-YB (lower panel 2nd lane), or nonspecific rabbit IgG (IgG) (1st lane of both panels) were subjected to Western blot with anti- NF-YB antibody (upper panel) or Dysbindin antibody (lower panel). Dilutions of the lysate (5%, HEK293 cells) were subjected to Western blot with anti-NF-YB antibody (3rd lane of upper panel) or Dysbindin antibody (3rd lane of lower panel).
doi:10.1371/journal.pone.0008773.g002

Dysbindin knockout mouse brain [40]. The existence of endogenous Dysbindin and endogenous NF-YB in lysates from SH-SY5Y cells was confirmed by Western Blot (Figure 2B, 3rd lane of both panels). Immunoprecipitation using the lysates with antibodies for Dysbindin and NF-YB and subsequent Western blot revealed the interaction of endogenous Dysbindin with endogenous NF-YB (Figure 2B, 2nd lane of both panels), and this binding was also confirmed using adult mouse brain lysates (data not shown).

Downregulation of Dysbindin Causes Upregulation in Expression Levels of Myristoylated Alanine-Rich Protein Kinase C Substrate (MARCKS)

As shown above, we had revealed an interaction between Dysbindin and NF-YB. This result suggests that Dysbindin may be functionally involved in transcription of some genes regulated by NF-YB. We screened for genes displaying altered expression by means of a DNA chip, using RNA extracts from the Dysbindin or NF-YB knockdown human neural cell line, SH-SY5Y. The expression of either *Dysbindin* or *NF-YB* was decreased by the corresponding siRNA for each gene, and the effects of siRNA on Dysbindin or NF-YB were confirmed by Western blot analysis (Figure S1). The genes showing increased expression in the Dysbindin knockdown cells, as well as in the NF-YB knockdown cells, are listed in Table 1A, while those showing decreased expression are listed in Table 1B. Next, using the DANASIS 2.0 system or sequencing of the promoter region, we screened for genes having the CCAAT sequence in the promoter region, because NF-YB is known to bind with high specificity to the CCAAT motif in the promoter region of a variety of genes (Table 1, gene names shown in red). We then focused on three genes; Myristoylated alanine-rich protein kinase C substrate (*MARCKS*) [46–48], Phospholipase C beta 4 (*PLCB4*) [49] and Synaptotagmin 1 (*SYT1*) [50], because an accumulating number of reports point to the involvement of impaired neural transmission in the schizo-

Table 1. The list of genes altered by Dysbindin as well as NF-YB.

(A) Upregulated genes

Dysbindin		NF-YB		
2 h	**24 h**	**2 h**	**24 h**	**Gene name**
1.343	1.393	1.234	1.409	"Chaperonin containing TCP1, subunit 4 (delta)"
1.344	1.325	1.232	1.352	BCL2-associated athanogene
1.296	1.406	1.485	1.394	***Thymine-DNA glycosylase***
1.315	1.476	1.261	1.295	***Myristoylated alanine-rich protein kinase C substrate***
1.355	1.559	1.430	1.434	Homer homolog 3 (Drosophila)
1.411	1.400	1.368	1.224	Hypothetical protein MGC2749
1.238	2.037	1.368	1.259	***Secretogranin II (chromograninC)***

(B) Decreased genes

Dysbindin		NF-YB		
2 h	**24 h**	**2 h**	**24 h**	**Gene name**
0.768	0.701	0.762	0.642	***Brain protein 44-like***
0.747	0.649	0.757	0.677	***Jun dimerization protein 2***
0.827	0.643	0.805	0.803	Kinesin family member 3A
0.734	0.790	0.764	0.601	Sarcosine dehydrogenase
0.814	0.698	0.762	0.722	***Phospholipase C, beta 4"***
0.761	0.645	0.670	0.699	***Synaptotagmin I***
0.815	0.518	0.741	0.780	B cell RAG associated protein
0.729	0.634	0.790	0.796	Hypothetical protein FLJ39370
0.763	0.631	0.760	0.668	SEC63-like (S. cerevisiae)
0.813	0.776	0.824	0.811	***ADP-ribosylation-like factor 6 interacting protein 5***
0.732	0.588	0.608	0.749	***Prothymosin, alpha (gene sequence 28)"***
0.693	0.645	0.769	0.787	***Homeodomain interacting protein kinase 3***
0.710	0.711	0.744	0.618	Similar to AV028368 protein
0.772	0.759	0.762	0.682	***Tropomyosin 4***
0.833	0.651	0.819	0.753	Lactate dehydrogenase A

(A) The genes upregulated by the knockdown of Dysbindin that were in common with those upregulated by the knockdown of NF-YB are listed. The genes showed by bold and italic format have the CCAAT motif.
(B) The genes downregulated by the knockdown of Dysbindin that were in common with those downregulated by the knockdown of NF-YB are listed. The genes showed by bold and italic format have the CCAAT motif.
doi:10.1371/journal.pone.0008773.t001

phrenia pathology. In addition, we considered the involvement of the genes in psychiatric diseases and we narrow down to MARCKS [51] and SYT1 [52]. Interestingly, a previous report suggests the alteration of SYT1 in schizophrenia patients [52]. The paper shows increase of SYT1 mRNA in younger schizophrenia patients group, while it shows decrease of SYT1 mRNA in older schizophrenia patients. These results suggest the complicated and multiple regulation of SYT1 transcriptional regulation. Thus, we examined the functional involvement of the Dysbindin-NF-YB interaction in *MARCKS* transcription.

To confirm the involvement of the knockdown of Dysbindin or NF-YB in the upregulation of MARCKS, we performed Western blot analysis using Dysbindin or NF-YB knockdown SH-SY5Ycells. Comparing the expression level of the MARCKS protein with that of control cells, Dysbindin knockdown cells showed upregulation of MARCKS protein (Figure 3A). To confirm the effect of Dysbindin on MARCKS *in vivo*, we examined the expression of MARCKS protein in the hippocampus with advancing age of the Dysbindin knockout mice, comparing with that found in wild-type mice. In the wild-type mice, a peak in MARCKS protein expression in the hippocampus was identified at postnatal day 15 and 20 (Figure 3B), and then decreased markedly over time. However, such a decrease was not observed in the Dysbindin knockout mice, where large amounts of Dysbindin protein were still expressed in the hippocampii of older mice (Figure 3B). These findings suggest that downregulation of Dysbindin may enhance transcription of the *MARCKS* gene, resulting in the upregulation of MARCKS protein.

We performed chromatin IP analysis using SH-SY5Y cells over-expressing Dysbindin-Flag, to explore the possibility that the Dysbindin-NF-YB complex could affect the transcription of *MARCKS via* interaction with the promoter region of *MARCKS*. The cells were stimulated by retinoic acid to induce *MARCKS*, and were collected as the samples for chromatin IP. PCR products from the chromatin IPs suggest that Dysbindin and NF-YB simultaneously interact with the promoter region of *MARCKS*, but control IgG experiments did not show this result (Figure 3C). These findings indicate that the Dysbindin-NF-YB complex interacts with the promoter region of the *MARCKS* gene resulting in inhibition of *MARCKS* transcription.

The Transcriptional Level of the *MARCKS* Gene Is Regulated by Dysbindin *via* the NF-YB Binding Motif, CCAAT-2

As shown in Figure 4A, the 5′−UTR region of the *MARCKS* gene has two kinds of CCAAT sequences; one CCAAT motif located between UTR −1152 and −700 and the other located between UTR −700 and −614. In this study, we tentatively named the former CCAAT sequence "CCAAT-1" and the latter "CCAAT-2." It is well known that NF-YB binds to the CCAAT motif to regulate transcription of target genes. Thus, we examined whether CCAAT motifs are essential to the regulation of *MARCKS* transcription by means of a luciferase assay, using the following five vectors containing shorter RNA probes; UTR(1152)-Luc, UTR (953)-Luc, UTR(700)-Luc, UTR(614)-Luc, and UTR(462)-Luc (Figure 4A). These constructs were transiently transfected into SH-SY5Y cells which express Dysbindin and NF-YB endogenously, and luciferase activity in each cell line was measured 24 hours after stimulation with retinoic acid. As baseline, we used luciferase activity detected in the SH-SY5Y cells expressing the UTR(1152)-Luc after retinoic acid stimulation (Figure 4A). In the cells transfected with UTR (953)-Luc containing both CCAAT sequences and UTR(700)-Luc containing the CCAAT-1 sequence but lacking the CCAAT-2 sequence, luciferase activity remained at baseline level after stimulation with retinoic acid (Figure 4A). However, luciferase activity was markedly increased in the cells expressing UTR(614)-Luc after retinoic acid stimulation (Figure 4A). These results suggest that the CCAAT-2 motif plays an important role in inhibition of *MARCKS* transcription. Furthermore, the SH-SY5Y cells transfected with UTR(462)-Luc lacking CCAAT-1, CCAAT-2 and the Sp1 region showed very low luciferase activity (Figure 4A), indicating that Sp1 is indispensable for *MARCKS* transcription.

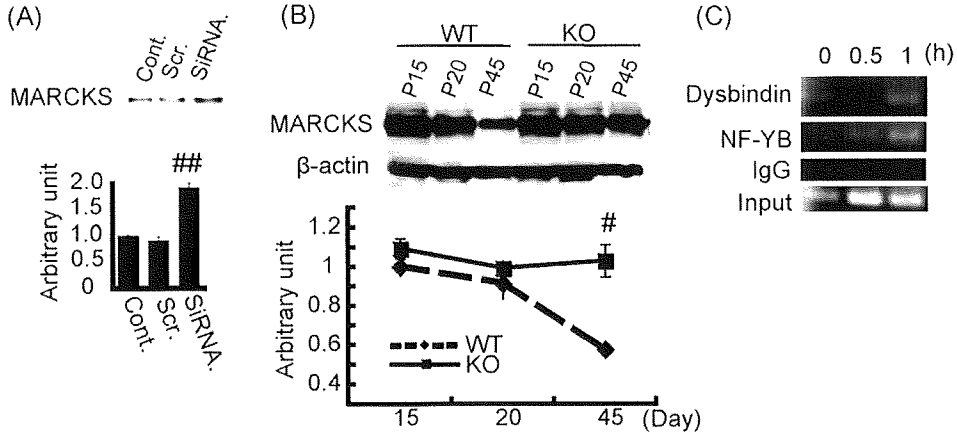

Figure 3. The effects of Dysbindin on MARCKS expression levels. (A) SH-SY5Y cells were transfected with scrambled siRNA or siRNA for Dysbindin. Cell lysate of non-treated cells (Cont.), scrambled RNAi-transfected cells (Scr.) and RNAi for Dysbindin-transfected cells (siRNA) were subjected to Western blot with anti-MARCKS antibody. Columns and vertical bars denote the means ± SEM (triplicate independent experiments). Dysbindin knockdown cells exhibited significant reduction of MARCKS expression compared with control cells ($P<0.001$, Student's t-test). (B) Hippocampus lysates were collected from wild-type mice or Dysbindin KO mice at P15, P20 and P45. The lysates were subjected to Western blot with anti-MARCKS antibody. Graphs and vertical bars denote the means ± SEM (triplicate independent experiments). At P45, Wild-type mice showed significant decreased MARCKS expression, while Dysbindin KO mice showed a maintained MARCKS expression. These data were confirmed by triplicate independent experiments ($P<0.01$, Student's t-test). (**C**)Chromatin IP (ChIP) was performed using SH-SY5Y cells under the stimulation of retinoic acid. The promoter region of *MARCKS* was detected both in the IPs of anti-Dysbindin antibody (1st panel) and those of anti-NF-YB antibody (2nd panel), but not in the IPs of IgG (3rd panel).
doi:10.1371/journal.pone.0008773.g003

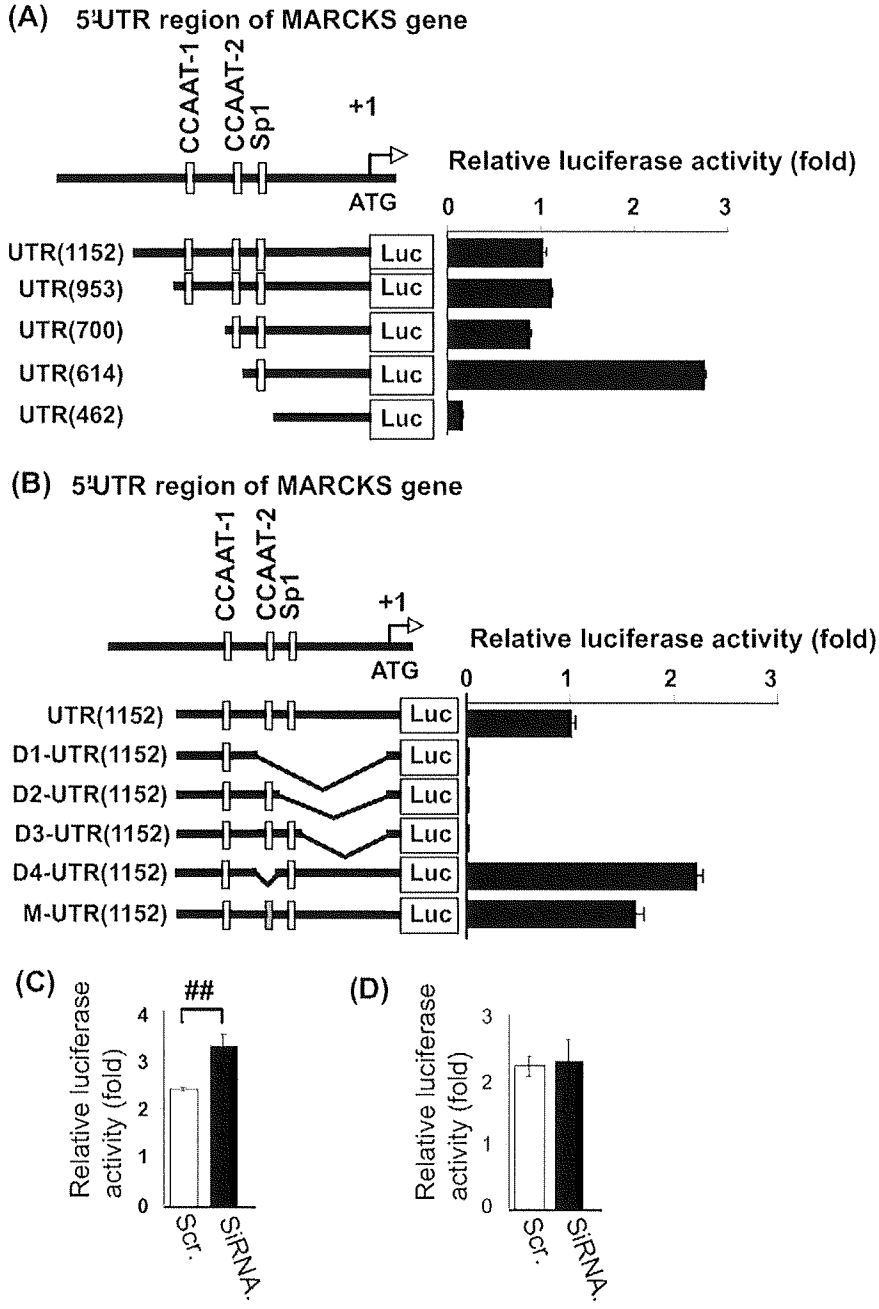

Figure 4. Dysbindin regulates the transcription of MARCKS via the CCAAT2 sequence. (A) The following five vectors were used for luciferase assay, containing shorter DNA probes; UTR(1152)-Luc, UTR (953)-Luc, UTR(700)-Luc, UTR(614)-Luc, and UTR(462)-Luc, were transfected into SH-SY5Y cells and Luciferase activity was measured. UTR(614), which lacks CCAAT1, showed increased luciferase activity. The luciferase activity of UTR(1152) was used as control. Columns and vertical bars denote the means ± SEM (triplicate independent experiments). (B) UTR(1152)-Luc vector and deleted or point mutation of UTR(1152)-Luc vectors, [D1-UTR(1152)-Luc], [D2-UTR(1152)-Luc], [D3-UTR(1152)-Luc] [D4-UTR(1152)-Luc] and [M-UTR(1152)-Luc], were transfected into SH-SY5Y cells and Luciferase activity was measured. [D4-UTR(1152)-Luc], which lacks CCAAT2, and [M-UTR(1152)-Luc], which has a point mutation in the CCAAT2 sequence, showed increased luciferase activity. The luciferase activity of UTR(1152) was used as the control. Columns and vertical bars denote the means ± SEM (triplicate independent experiments). (C and D) Scrambled RNAi-transfected SH-SY5Y cells and Dysbindin RNAi-transfected SH-SY5Y cells were transfected with the UTR(1152)-Luc vector (C) or D4-UTR(1152)-Luc (D) and Luciferase activity was measured. UTR(1152)-Luc vector-expressing cells showed the effect of Dysbindin expression levels on luciferase activity, but D4-UTR(1152)-Luc expressing cells did not. Columns and vertical bars denote the means ± SEM (triplicate independent experiments; $P<0.001$, Student's t-test).
doi:10.1371/journal.pone.0008773.g004

To confirm that the CCAAT-2 region is important in regulation of *MARCKS* transcription, we prepared several probes for the luciferase assay; D1-UTR(1152)-Luc which lacks the CCAAT-2 motif and its downstream region including Sp1 from UTR(1152)-Luc, D2-UTR(1152)-Luc which lacks the Sp1 region and downstream sequence from UTR(1152)-Luc, D3-UTR(1152)-Luc which lacks only sequence downstream of the Sp1 region, D4-UTR(1152)-Luc which lacks only the CCAAT-2 motif from

UTR(1152)-Luc, and M-UTR(1152)-Luc which has a point mutation in the CCAAT-2 motif (Figure 4B). Luciferase activity was detected from the SH-SY5Y cells transfected with each probe, and luciferase activity detected in the cells transfected with UTR(1152)-Luc was used as the baseline value (Figure 4B). Cells transfected with M-UTR(1152)-Luc and those transfected with D4-UTR(1152)-Luc exhibited marked increases in luciferase activity (Figure 4B), showing that the CCAAT-2 motif plays a key role in inhibition of *MARCKS* transcription. Furthermore, cells expressing D1-UTR(1152)-Luc, D2-UTR(1152)-Luc or D3-UTR(1152)-Luc exhibited no luciferase activity. These findings suggest that the sequence downstream of the Sp1 region, as well as the Sp1 region itself, is indispensable for *MARCKS* transcription.

To confirm the involvement of Dysbindin in the altered *MARCKS* transcription levels *via* the CCAAT-2 motif, we compared the luciferase activity of UTR(1152)-Luc detected in Dysbindin knockdown cells with that of control cells. As shown in Figure 4C, knockdown of Dysbindin resulted in upregulation of luciferase activity in the UTR(1152)-Luc transfected cells. However, the effect of knockdown of Dysbindin on luciferase activity was not observed in the D1-UTR(1152)-Luc transfected cells (Figure 4D). These results suggest that Dysbindin regulates *MARCKS* transcription *via* the CCAAT2 motif; the NF-YB binding site. On the other hand, since negligible levels of luciferase activity were observed in cells transfected with any of the probes lacking the sequence downstream of the Sp1 region, the sequence downstream of Sp1 appears to be essential for *MARCKS* transcription (Figure 4A and 4B).

Discussion

Numerous reports support the role of Dysbindin in the etiology of schizophrenia [13,30,37,53–60]. Previous studies have reported a decrease in Dysbindin expression in the brains of schizophrenic patients both at the mRNA and protein levels [26,27]. However, the functional involvement of Dysbindin in the neural system is not yet well elucidated. In this study, we examined involvement of Dysbindin in neural transmission and neural formation *via* transcriptional regulation, because abnormalities in these neural processes are very important in the pathogenesis of schizophrenia.

Regulation of *MARCKS* Transcription by the Dysbindin/NF-YB Interaction

As a result of the yeast-two-hybrid assay and immunoprecipitation assay, we revealed an interaction between NF-YB and Dysbindin (Figure 1 and 2). In addition, we showed the binding of NF-YB and Dysbindin to the *MARCKS* promoter region (Figure 3C). These findings suggest involvement of this complex in transcriptional regulation of MARCKS. As shown in Figure 4, we found two CCAAT sequence motifs at the 5′−UTR of the *MARCKS* gene. Previous reports show that members of the NF-Y family including NF-YB bind to CCAAT sequences and can regulate transcription of a number of genes. Our results suggest that one of the CCAAT sequences, CCAAT-2, is important for *MARCKS* transcriptional regulation. On the other hand, our luciferase assay results suggest that both the Sp1 region and the sequence downstream of Sp1 are indispensable for *MARCKS* transcription (Figure 4A and 4B).

Dysbindin Knockdown Increases MARCKS Protein Levels *In Vivo* and *In Vitro*

In accordance with the enhanced *MARCKS* transcription mediated by the knockdown of Dysbindin, Dysbindin knockdown cells show increased MARCKS levels (Figure 3A). Next, we examined the expression level of MARCKS in Dysbindin knockout mice. As shown in Figure 3b, in the wild-type mouse brain the peak in MARCKS expression is at postnatal day 15; thereafter decreasing markedly with advancing age until only low levels of MARCKS expression are seen in adults (P45). Comparable alternations in MARCKS expression were also observed in another mouse line, ICR (data not shown). These findings support the hypothesis that MARCKS plays an important role in brain development. However, in the Dysbindin knockout mice, there is no effect on MARCKS expression during the developmental stage, when MARCKS is abundantly expressed in wild-type mice. During this stage, *MARCKS* transcription may be regulated by multiple molecules, which compensate for the lack of Dysbindin. With increasing age of the mouse, MARCKS expression decreases gradually to a low level of expression in adults (Figure 3b). In contrast, a decrease in MARCKS expression was not observed in Dysbindin knockout mice (Figure 3b) and even in adult mice brains, a high level of expression of MARCKS was detected. These findings show that Dysbindin likely plays a major role in regulation of MARCKS expression in the adult brain, in contrast to in the developmental stage. Therefore, considering the results in Dysbindin knockout mice, it is likely that MARCKS is expressed at high levels in schizophrenic brains, compared with age-matched control brains.

MARCKS and Neural Transmission

It has been shown that MARCKS impacts on neurotransmission *via* F-actin and on vesicular transport *via* synaptic vesicles [46–48]. Furthermore, many reports indicate that dopaminergic transmission is increased in the brains of schizophrenics [1–4]. Dopamine D2 antagonists are an effective treatment in schizophrenia, and dopamine-enhancing drugs mimic psychotic symptoms of schizophrenia. In the schizophrenic brain, the expression of Dysbindin is decreased, resulting in an increase in MARCKS protein expression, which impacts on neurotransmission. Furthermore, we found that decreases in Dysbindin levels upregulate dopamine release [39]. Therefore, the enhanced dopaminergic transmission produced by the lower expression level of Dysbindin may be partially attributable to activation of MARCKS. Thus, the impairment of neural transmission in the schizophrenic brain may be caused by alterations of MARCKS expression levels *via* changes in Dysbindin.

Dysbindin May Regulate Neural Formation *via* Alteration of MARCKS Levels

Many studies support the hypothesis that schizophrenia is a neurodevelopmental disease. Disrupted-In Schizophrenia 1 (DISC1) is a gene disrupted by a (1;1) (q42.1; q14.3) translocation that segregates with major psychiatric disorders, including schizophrenia in a Scottish family [61,62]. Previously, we examined the physiological role of the molecular complex composed of DISC1 and its interacting partners, Fasciculation and elongation protein zeta 1 (Fez1) [63] and DISC1-Binding Zinc finger protein (DBZ)[64]. Both the DISC1-Fez1 interaction and the DISC1-DBZ interaction are involved in neurite extension. These reports suggest that abnormalities in the schizophrenia susceptibility genes, such as DISC1, likely cause an impairment of brain development resulting in schizophrenia. In addition, several reports suggest that the PKC signal is involved in psychiatric disorders, as well as other signals such as ERK, which play important roles in neural development. In addition, we previously showed the importance of Dysbindin for growth cone formation [40]. These previous reports suggest that abnormal neural formation could cause psychiatric disorders and that Dysbindin

may be one of the important factors in normal neural development. In this study, we demonstrate the transcriptional regulation of MARCKS *via* Dysbindin and the upregulation of MARCKS by downregulation of Dysbindin. Since MARCKS is involved not only in neural transmission[48] but also in neural developmental processes such as synaptogenesis and maintaining spine morphology [46,47], these results suggest that dysfunction of Dysbindin likely causes the upregulation of MARCKS and may induce abnormal development of the nervous system *via* alterations of MARCKS levels.

Thus, in this paper, we report the following findings; (1) Dysbindin interacts with NF-YB, (2) NF-YB and Dysbindin bind to the promoter region of MARCKS, (3) one of the CCAAT sequences is likely essential for the transcriptional regulation of MARCKS and (4) the downregulation of Dysbindin upregulates the expression of MARCKS *in vitro* and *in vivo*. On the other hand, we previously showed that Dysbindin knockout mice exhibit schizophrenia-like behavior and abnormalities of the dopaminergic system. These phenotypes may be at least partly attributable to over-activation of MARCKS *via* a decrease in Dysbindin levels.

In conclusion, these results may help shed some light on the causes of schizophrenia, and indicate that the transcriptional regulation of Dysbindin may contribute to schizophrenia. Further studies of Dysbindin and its association with MARCKS and with schizophrenia may reveal novel treatment targets for schizophrenia.

Materials and Methods

Antibodies

Monoclonal anti-Dysbindin antibody was produced. Briefly, GST-fused human Dysbindin was used as antigen and the Dysbindin protein for ELISA was made by thrombin digestion of GST-Dysbindin. High-titer clones for Dysbindin were selected by ELISA using the Dysbindin protein and the immunoreactivity of the clones was checked by Western blot. Antibodies of anti-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag (Sigma-Aldrich, St Louis, MO), anti-V5 (Invitrogen), anti-β-actin (Chemicon International, Temecula, CA), anti-NF-YB (Santa Cruz Biotechnology), anti-MARCKS (Upstate), HRP-conjugated anti-mouse and Rabbit IgG (Cell Signaling Technology, Beverly, MA), and mouse normal IgG (Sigma-Aldrich) were purchased commercially.

Plasmids

We previously constructed the pEGFP-C1 expression vector (Clontech) carrying the full-length human *Dysbindin* cDNA (-GFP is tagged to N-terminal) [22]. The human Dysbindin-V5 (-V5 is tagged to C-terminal), Dysbindin-FLAG (-FLAG is tagged to N-terminal) and NF-YB moieties were amplified from a human brain cDNA library using PCR and subcloned into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA). Dysbindin and NF-YB were amplified using rTaq DNA polymerase (Takara Bio Inc., Kyoto, Japan) with the following primer set: Dysbindin-V5, 5′−CTCGAGTTACGTAGAATCGAGACCGAGGAGAGGG-TTAGGGATAGGCTTACCAGAGTCGCTGTCCTCACC−3′-(forward) and 5′−GGTACCGCCACCATGCTGGAGACCCT-TCGCGA−3′ (reverse); NF-YB, 5′−GCTAGCGCCACCAT-GACAATGGATGGTGACAGTTCT−3′ (forward) and 5′−G-ATATCTGAAAACTGAATTTGCTGAAC−3′ (reverse). The amplified fragments were TA cloned into the pGEM-T vector (Promega Corp.).

pMARCKS-Luc(−1152) was generated by subcloning promoters into pGL3-(R2.2) Basic (Promega). We generated 5′ deletion constructs of pMARCKS-Luc(−1152) and an internal deletion construct of the region −700∼−1. Other deletion constructs of the region (−231∼−150) and point mutation constructs of pMARCKS-Luc/dl(−204∼−187), were generated by inserting double-stranded oligonucleotides (Figure 2B and 2D). The plasmid pMARCKS-Luc(−736/mt) was generated by site-directed mutagenesis, which changed the same nucleotides as those of mutant 5.

Cell Culture

Human neuroblastoma SH-SY5Y cells were obtained from the Human Science Research Resources Bank (HSRRB). These cells were maintained in tissue culture dishes (Nalge Nunc, Rochester, NY, USA) in 50% minimal essential medium (Invitrogen) /50% F-12 (Invitrogen) containing 15% heat-inactivated fetal bovine serum (Invitrogen) at 37°C in an atmosphere of 95% air /5% CO_2.

Animals

sdy mice (Dysbindin KO mice) and wild-type littermates were provided by the Takeda lab, Department of Psychiatry, Osaka University Graduate School of Medicine. The mice were deeply anesthetized with sodium pentobarbital. Brains (hippocampus) were dissected from each aged mouse. All animal experiments were carried out in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Osaka University.

Immunocytochemistry

SY5Y cells were grown on poly-l-lysine-coated four-well chamber dishes at a density of 3×10^4 cells/cm^2. The cells were fixed in 2% paraformaldehyde in 0.1 M PBS, permeabilized, and blocked with 0.02 M PBS containing 0.3% Triton X-100, 3% BSA and 10% goat serum for 30 min at room temperature, and then incubated with antibodies specific for the individual protein. Confocal microscopy was performed using a Carl Zeiss LSM-510 confocal microscope.

Fractionation Assay

Cells were collected after washing with ice-cold PBS. Cells and brains were homogenized in Tris buffer (20 mM Tris–HCl, pH 7.8, 1 mM EDTA, 150 mM NaCl and protease inhibitor cocktail (Roche)). After homogenization, the homogenized proteins were lysed by the addition of 0.5% NP-40 for 30 min on ice and centrifuged at 500 ×*g* for 10 min to collect the nuclear pellet. The supernatant was collected as the cytosolic fraction.

Immunoprecipitation (IP)

After washing cells with ice-cold PBS, cells were collected and resuspended in 1 mL lysis buffer (20 mM Tris–HCl, pH 7.8, 0.2% NP-40, 1 mM EDTA, 150 mM NaCl and protease inhibitor cocktail (Roche)). Cells were frozen in dry ice/EtOH and stored at −80°C. Cell lysates were incubated on ice for 30 min and then centrifuged for 5 min at 13,600×*g*. After centrifugation, the supernatants were precleared with protein Sepharose G beads and IP was carried out in lysis buffer with antibody/protein G Sepharose beads for 1 h at 4°C. After washing in lysis buffer, immunoprecipitated proteins were immunoblotted.

Immunoblotting

Aliquots of whole cell lysates or IP lysates separated by SDS-PAGE were blotted onto an Immobilon-P membrane (Millipore), and then incubated with antibodies specific for individual protein. Proteins were detected by ECL plus Western Blotting Detection

System (GE Healthcare), followed by exposure to X-ray films according to the manufacturer's protocol.

Knockdown Experiment Using Small Interfering RNA (siRNA)

Stealth siRNA against Dysbindin (5′−CCAAAGUACUCUGCUGGAUUAGAAU−3′ and 5′−GCUCCCAGCUUUAAUCGCAGACUUA−3′), NF-YB 5′−UACUGAGGACAGCAUGAAUGAUCAU−3′ , and negative control duplexes (scrambled siRNA for Dysbindin, 5′−CCATGATCTCGTCGTTAGAAAGAAA−3′ and 5′−GCTACCGTTATTAGCACAGCCCTTA−3′ ; and scrambled siRNA for NF-YB, 5′−UACGGAACAACGAGUGUAUAUGCAU−3′) were provided by Invitrogen Corp. SY5Y cells were transfected with 100 pM of each siRNA and scrambled siRNA using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's instructions.

RNA Extracts and Microarray

Total RNA was extracted from cells using RNeasy columns (Qiagen) according to the manufacturer's instructions. Five hundred nanograms of total RNA from control and experimental cells was separately amplified and labeled with either Cy3- or Cy5-labeled CTP (Perkin Elmer) with an Agilent low input linear amplification kit (Agilent Technologies) according to manufacturer's instructions. After labeling and cleanup, amplified RNA was quantified by UV-vis spectroscopy. One microgram each of Cy3- and Cy5-labeled targets were combined and hybridized with a Whole Human Genome Oligo Microarray Kit (G4112F)according to the manufacturer's instructions. Three biological replicates were used at each time point with one of the replicates being a dye reversal of the other two. Microarrays were imaged on a Hitachi image scanner and data analyzed with GeneSpring 6 (Silicon Genetics).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP analysis was performed using a Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. Briefly, protein−DNA complexes were crosslinked with 1% formaldehyde (10 min at room temperature) and cells were harvested. DNA was sonicated to lengths of 500−1000 bp. Antibodies specific for individual protein were used for immunoprecipitating protein-DNA complexes overnight at 4°C. PCR was performed with individual specific primer sets for the MARCKS promoter: the proximal CCAAT region, 5′−GGTTTGCTCTTTGATGCTCTTGAT−3′ and 5′−ACTTTCGGGTGGGGTGTAA−3′

Reporter Assay

Reporter plasmids were transfected into cells using Lipofectamine 2000 (Invitrogen) together with phRG-TK (Renilla reporter for internal control) which monitored transfection efficiency. Luciferase activities were assayed using the Dual Luciferase Assay System (Promega). All assays were performed three times in duplicate and values are shown as means ± SD.

Supporting Information

Figure S1 The preparation of mRNAs for microarray analysis. (A-(a) and B-(a)) To prepare RNAs for microarrays analysis, we transfected the siRNA for Dysbindin, NF-YB, or scrambled as a control. The effect of each RNAi was confirmed by Western blot using the antibody for Dysbindin or NF-YB. (A-(b) and B-(b)) The columns and vertical bars denote the means ± SEM (triplicate independent experiments; $P<0.001$, Student's t-test). Dysbindin or NF-YB was knocked-down significantly by transfection of the siRNA for Dysbindin or NF-YB, compared with the control.
Found at: doi:10.1371/journal.pone.0008773.s001 (1.10 MB EPS)

Acknowledgments

We thank Ms. Arakawa, Ms. Moriya, and Ms. Ohashi for preparing our experiments.

Author Contributions

Conceived and designed the experiments: HO SM SM MT. Performed the experiments: HO RK SM. Analyzed the data: HO RK SM SM NK TH RH TK MT. Contributed reagents/materials/analysis tools: SM NK SS KY KK RH MT TK. Wrote the paper: HO RK SM MT.

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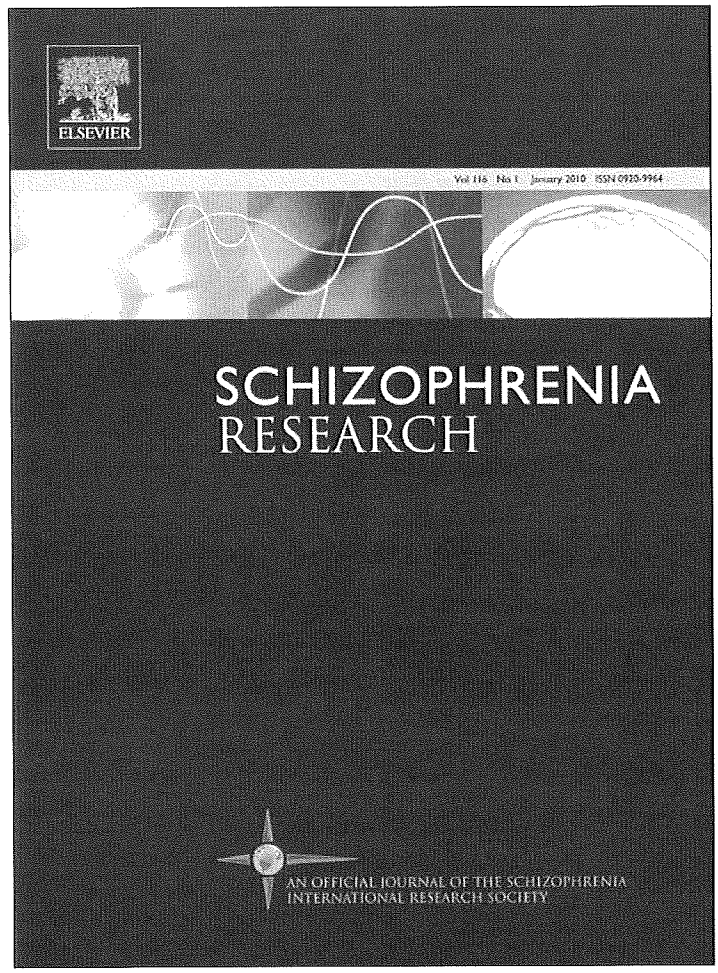

Schizophrenia Research 116 (2010) 61–67

Contents lists available at ScienceDirect

Schizophrenia Research

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

The *dopamine D3 receptor* (*DRD3*) gene and risk of schizophrenia: Case–control studies and an updated meta-analysis

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 15 October 2009
Accepted 17 October 2009
Available online 7 November 2009

Keywords:
DRD3
Resequencing
Schizophrenia
Tagging SNP

ABSTRACT

The dopamine D3 receptor (DRD3) has been suggested to be involved in the pathophysiology of schizophrenia. *DRD3* has been tested for an association with schizophrenia, but with conflicting results. A recent meta-analysis suggested that the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 may confer protection against schizophrenia. However, almost all previous studies of the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. To assess whether *DRD3* is implicated in vulnerability to schizophrenia, we conducted case–control association studies and performed an updated meta-analysis. In the first population (595 patients and 598 controls), we examined 16 genotyped single nucleotide polymorphisms (SNPs), including tagging SNPs selected from the HapMap database and SNPs detected through resequencing, as well as 58 imputed SNPs that are not directly genotyped. To confirm the results obtained, we genotyped the SNPs rs7631540–rs1486012–rs2134655–rs963468 in a second, independent population (2126 patients and 2228 controls). We also performed an updated meta-analysis of the haplotype, combining the results obtained in five populations, with a total sample size of 7551. No supportive evidence was obtained for an association between *DRD3* and schizophrenia in our Japanese subjects. Our updated meta-analysis also failed to confirm the existence of a protective haplotype. To draw a definitive conclusion, further studies using larger samples and sufficient markers should be carried out in various ethnic populations.

1. Introduction

The dopamine D3 receptor (DRD3) has been suggested to be involved in the pathophysiology of schizophrenia (for a

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doi:10.1016/j.schres.2009.10.016

review, Schwartz et al., 2000). DRD3 has relatively strong affinity for both first- and second-generation antipsychotics (Sokoloff et al., 1990). Postmortem studies have revealed changes in the mRNA and protein levels of DRD3 in the brains of patients with schizophrenia (Gurevich et al., 1997; Meador-Woodruff et al., 1997; Schmauss et al., 1993). Altered levels of *DRD3* mRNA in blood lymphocytes of patients with schizophrenia have also been reported (Ilani et al., 2001; Vogel et al., 2004). *DRD3* is located on 3q13.3 where some linkage analyses have suggested a region of susceptibility to schizophrenia (Brzustowicz et al., 2000; Kaneko et al., 2007). Therefore, *DRD3* is a promising functional and positional candidate gene for schizophrenia.

More than 60 studies have tested an association between *DRD3* and schizophrenia (Allen et al., 2008). The most extensively investigated *DRD3* polymorphism is Ser9Gly (rs6280) in exon 2 resulting in a serine to glycine substitution at codon 9. This polymorphism has been reported to be associated with altered dopamine binding affinity, suggesting that the Ser9Gly polymorphism may be functional (Lundstrom and Turpin, 1996). An initial study reported an association between homozygosity of this polymorphism and schizophrenia (Crocq et al., 1992). Some studies showed an association of the Ser allele with schizophrenia (Ishiguro et al., 2000; Shaikh et al., 1996), whereas others reported that the Gly allele was over-represented in patients with schizophrenia (Kennedy et al., 1995; Utsunomiya et al., 2008). However, two recent large meta-analyses did not provide evidence for an association between the Ser9Gly polymorphism and schizophrenia (Allen et al., 2008; Ma et al., 2008). Therefore, if *DRD3* is implicated in genetic susceptibility to schizophrenia, this cannot be wholly accounted for by the Ser9Gly polymorphism. This view has been supported by two studies using tagging single nucleotide polymorphisms (SNPs) based on linkage disequilibrium (LD) (Domínguez et al., 2007; Talkowski et al., 2006). A recent meta-analysis showed that the second most common haplotype (T–T–T–G) for the SNPs rs7631540–rs1486012–rs2134655–rs963468 was less frequent in patients with schizophrenia than in control subjects, suggesting that this haplotype may confer protection against schizophrenia (Costas et al., 2009).

Almost all previous studies on the association between *DRD3* and schizophrenia have been performed using a relatively small sample size and a limited number of markers. Here, we tried to increase the power by increasing the sample size and testing more markers, including tagging SNPs selected from the HapMap database and SNPs detected through resequencing of whole exon regions of *DRD3*. First, we conducted a moderate-scale case–control association study (595 patients and 598 controls) using 16 genotyped SNPs and 58 imputed SNPs that have not been directly genotyped. Second, we carried out an independent large-scale case–control association study (2126 patients and 2228 controls) to confirm the results of the first study, specifically to test the association of the haplotype T–T–T–G for the SNPs rs7631540–rs1486012–rs2134655–rs963468 with schizophrenia. Third, we performed an updated meta-analysis of this haplotype to assess the collective evidence across individual studies.

2. Materials and methods

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

2.1. Subjects

The first population consisted of 595 patients with schizophrenia (313 men and 282 women; mean age, 40.2 [SD 14.1] years) and 598 control subjects (311 men and 287 women; mean age, 38.1 [SD 10.5] years). These subjects partially overlapped with those in the report of Tanaka et al. (1996). Case and control groups were matched for sex ($p = 0.836$). Although the mean age of the patients was significantly higher than that of the control subjects ($p = 0.004$), the difference in mean age between the groups was relatively small (2.1 years). The second population consisted of 2126 patients with schizophrenia (1137 men and 989 women; mean age, 47.3 [SD 14.3] years) and 2228 control subjects (1189 men and 1039 women; mean age, 46.6 [SD 13.9] years). Case and control groups were matched for sex ($p = 0.940$) and age ($p = 0.083$).

We conducted a psychiatric assessment of every participant, as described previously (Nunokawa et al., 2007). 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 subjects 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.

The subjects for resequencing of exon regions were six patients with schizophrenia from a Japanese single multiplex schizophrenia pedigree. In this pedigree, our previous linkage analysis revealed that 3q is one of the candidate regions for schizophrenia (Kaneko et al., 2007). These patients were diagnosed according to the DSM-IV criteria by two experienced psychiatrists, on the basis of all available sources of information, including direct interviews using the Structured Clinical Interview for DSM-IV Axis I disorders and Axis II disorders, medical records, and information from reliable relatives and psychiatric professionals.

2.2. Tagging SNP selection

Tagging SNPs for *DRD3*, covering gene region and the 5′ and 3′ flanking regions (chr3:115307882..115402406), 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 the *'aggressive tagging: use 2- and 3-marker haplotype'* mode using the 'Tagger' program (de Bakker et al., 2005), as implemented in Haploview v4.0 (Barrett et al., 2005); rs6280 (Ser9Gly) was forced to be selected as a tagging SNP. To confirm the existence of a common protective haplotype (Costas et al., 2009), we also included rs963468.

2.3. Resequencing of exon regions

All seven exons of *DRD3* were screened for polymorphisms using direct sequencing of PCR products. The sequences

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

2.4. SNP genotyping

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

2.5. Statistical analysis

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

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

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

2.6. Meta-analysis

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

3. Results

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

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

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

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

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

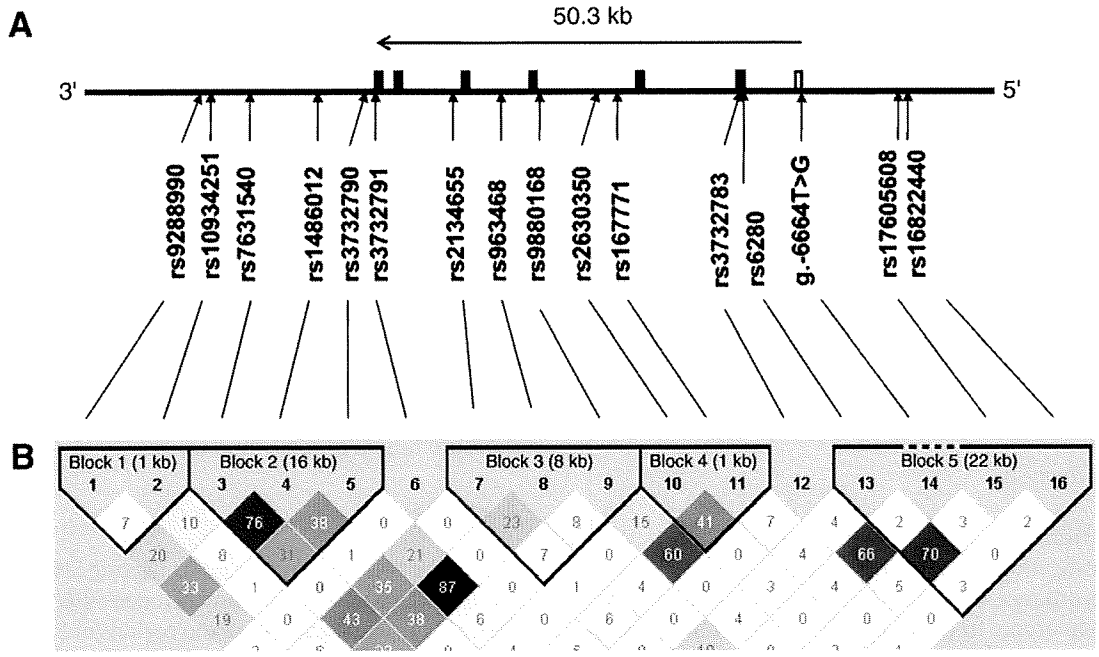

Fig. 1. Genomic structure and linkage disequilibrium (LD) of *DRD3*. (A) Genomic structure of *DRD3* and the locations of the single nucleotide polymorphisms (SNPs) analyzed in the present study. *DRD3* has seven exons (rectangles) and spans approximately 50.3 kb. Black and white rectangles represent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

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

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

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

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

Table 2
Haplotype analyses of LD blocks.

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

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

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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

Role of funding source

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

Contributors

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

Conflict of interest

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

Acknowledgements

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

Appendix A. Supplementary data

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

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