positive and negative symptoms of schizophrenia (Radonjic et al., 2008; Jentsch and Roth, 1999), and so this animal model recreates the pathophysiological conditions in the hippocampus and medial prefrontal cortex (Dissanayake et al., 2009).

The hippocampal formation has been an area of interest in schizophrenia research (Harrison, 2004) and several abnormalities of the hippocampal architecture have been reported (Heckers and Konradi, 2002). The area of CA2/3 or Dentate Gyrus in particular has received much attention in recent studies (Berretta et al., 2009; DeCarolis and Eisch, 2010).

In this study, we investigated the expression of VMAT2 in the hippocampal formation of PCP-treated mice using an immunohistochemical technique to further clarify the pathophysiology of schizophrenia.

2. Materials and methods

2.1. Animal model

All experiments were performed in accordance with the institutional guidelines of animal experimentation and with the approval of the ethics committee of the Nagoya University. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Eleven male mice (C57BL/6J, 35 weeks old) were used. Seven of them were injected with PCP (10 mg/kg/day) intraperitoneally once a day for 14 days. The other four mice were similarly injected with physiological saline as a control. All mice were bred under the same conditions.

2.2. Histology and observation

2.2.1. Tissue preparation

Animals were perfused with a tissue fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) under deep anesthesia by injecting sodium pentobarbital (40 mg/kg body weight i.p.). The brains were immediately removed, and tissue blocks were immersed in a 20% sucrose–0.01 M phosphate buffer solution for more than 3 days at 4 °C. Sections of the hippocampal regions were cut on a freezing cryostat at thickness of 30 μm and treated as free-floating sections. The sections were rinsed and stored in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for at least 3 days and up to 2 weeks prior to the subsequent immunohistochemical procedure.

2.2.2. Immunohistochemistry

The sections were rinsed in TBS (0.1 M Tris–HCl, pH 7.4, 0.9% NaCl) containing 0.3% Triton X-100 (TX) and 2% normal goat serum (NGS) for 30–60 min at room temperature. The primary antibody employed in this study was an anti-VMAT2 monoclonal antibody (Chemicon, Temecula, CA, Lot No. 3536; 1:1000). Incubation with the primary antibody was carried out for 48 h at 4 °C. The sections were then incubated in a medium containing biotinylated anti-universal (rat and/or rabbit) IgG (Vector Vectastain; 1:100) for 45 min at room temperature, followed by incubation with an avidin–biotin peroxidase complex (ABC method) for 45 min. After each incubation, the sections were rinsed in NGS-TX-TBS solution. Finally, the sections were rinsed in PBS twice for 10 min, reacted with 0.05%, 3,3′diaminobenzidine-HCl in 0.05 M Tris–HCl buffer (pH 7.6) for 2 or 3 min, and mounted onto gelatin-coated slides. Specimens were observed under a light microscope.

To clarify the specificity of the immunoreactivity to VMAT2, control experiments were conducted with sections from the animal model and wild type mice using the same immunostaining procedure except that prior to immunohistchemical labeling, the diluted anti-VMAT2 antibody was pre-absorbed with 10 μg/ml or 50 μg/ml of VMAT2 (Novus Biologicals, CO, USA) instead of the primary antibody.

2.3. Observation and analysis

We selected two random squares (30 μm × 30 μm) in the area of the stratum radiatum along the pyramidal layer in the CA2 region of the right or left hippocampus per specimen. Also, we selected two random squares (30 μm × 30 μm) in the CA4 area per specimen. These specimens were observed using a light microscope. The microscopic photographs were downloaded to a personal computer (PC) from a digital camera (HC-2000, Fuji film Co. Japan) as digital data. The digital images of immunoreactivity were processed into black (immunopositive) and white (immunonegative) using image processing software (Image J: http://rsb.info.nih.gov/ij/ Free software supplied by NIH) (Fig. 1). An appropriate threshold of distinction between black and white tones was unified consistently for every specimen. The amount of immunoreactivity was assessed by the space occupied value as the number of pixels per square observational area. Finally, we counted the number of pixels in each group. The significance of variation in differences between the PCP-treated group and the saline-treated control group was assessed by using the Mann–Whitney U test with $P < 0.05$ considered statistically significant. Since we could not suppose a normal distribution or homoscedasticity of the population structure, we used a non-parametric test in the statistical comparison.

3. Results

VMAT2-immunopositive varicose fibers were observed throughout the hippocampal formation in saline-treated mice (Fig. 2A). In contrast, less immunoreactivity was observed overall

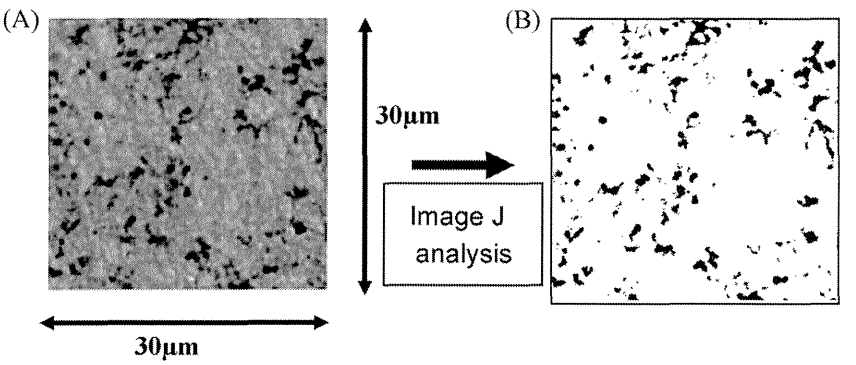

Fig. 1. (A) Microscopic photographs were uploaded to a PC as digital data in the setup area (30 μm × 30 μm). (B) The digital images were processed for dichotomization using image software (Image J: http://rsb.info.nih.gov/ij/). Only immunopositive products were extracted by computer processing. The occupancy space of immunoreactive products was automatically calculated as pixel units. The numerical value of pixels was applied to the index of immunoreactivity.

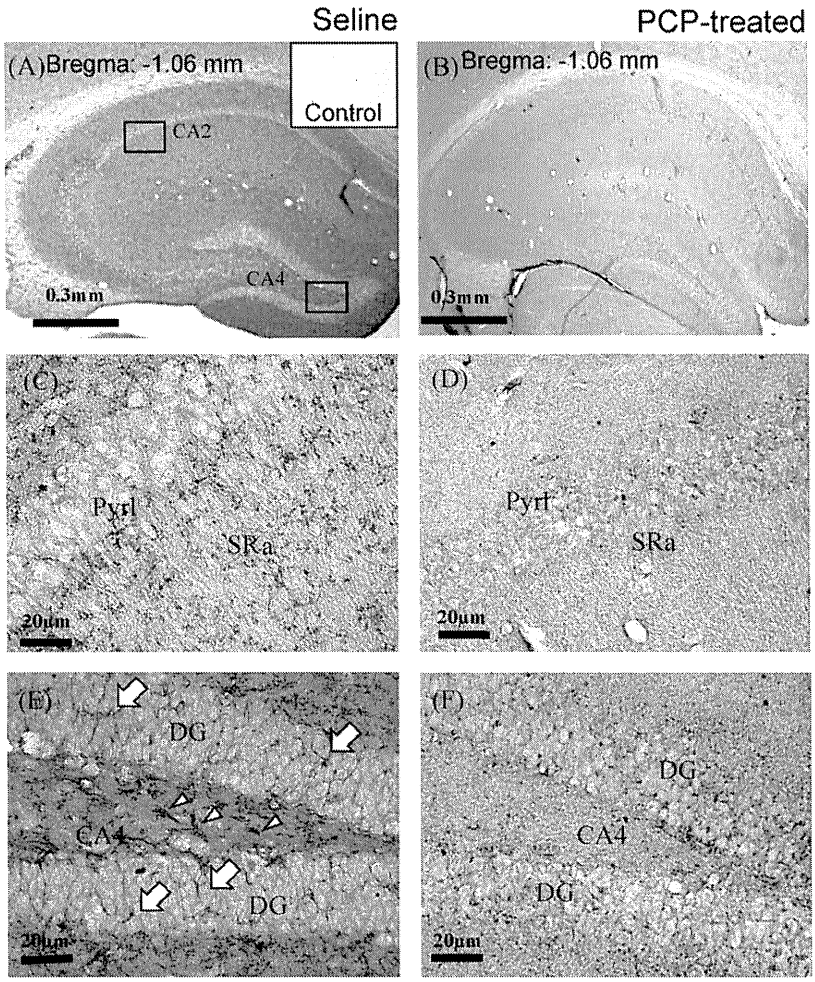

Fig. 2. (A) Immunoreactivity to VMAT2 in the saline-treated mice. Immunoreactivity was observed throughout the hippocampus. The approximate location in the brain was −1.06 mm (stereotaxic coordinate) from the bregma (http://www.mbl.org/atlas165/atlas165_start.html). Each square frame indicates the observational field in CA2 and CA4 areas. No immunoreactivity was observed in the control experiment using the pre-absorbed primary antibody with 10 μg/ml of VMAT2 protein (inset indicates control). (B) Immunoreactivity to VMAT2 in the PCP-treated mice. Overall, less immmunoreactivity was observed in the hippocampus than in the saline-treated mice (photo A). (C) Dense immunopositive varicose fibers or deposits of VMAT2 were observed in the stratum radiatum (SRa) along the pyramidal cell layer (Pyrl) in the CA2 area of saline-treated mice. (D) Little immunoreactivity was observed in the CA2 area of PCP-treated model mice compare to those of the saline-treated mice (photo C). (E) Extensive immunoreactivity was observed in the CA4 area and dense immunopositive fibers ran into the cell layer in the dentate gyrus (DG) (arrows). Immunopositive varicose deposits were detected in the CA4 area (arrowheads). (F) Immunoreactivity for VMAT2 in the CA4 area in PCP-treated mice. A significant reduction in immunoreactivity was observed compared to that in the saline-treated mice (photo E). No immunopositive dense varicose products or fibers were observed as shown in photo E. *Abbreviation*: Pyrl: pyramidal layer, SRa: stratum radiatum, DG: dentate gyrus granule cell layer.

in the PCP-treated mice (Fig. 2B). In the control experiments with the pre-absorbed primary antibody, no immunoreactivity was observed in any specimen (inset in Fig. 2A).

In the saline-treated mice, immunoreactive varicose fibers and deposits were detected in the hippocampal formation overall including the stratum radiatum (SRa) along the pyramidal layer (Pyrl) in the CA2 (Fig. 2C) and the CA4 (Fig. 2E) areas. Dense deposits (Fig. 2E, arrowheads) and some dense fibers penetrating the gyrus granule cell layer in the dentate gyrus (Fig. 2E, arrows), were also observed, especially in the CA4 region.

In the PCP-treated mice, a significant reduction in the immunoreactivity of granules to VMAT2 was observed in the hippocampal formation overall including the CA2 (Fig. 2D) and CA4 (Fig. 2F) areas. Also, the reduction of expression was observed in CA1 and CA3 area.

Additionally, we observed the other entire area of the brain precisely whether these reductions were occurred only in the hippocampus or not. The expression of VMAT immunoreactvity was observed throughout the brain, mainly in the monoaminergic neuronal tract, of the saline-treated mice (Fig. 3A). On the other hand, in the PCP-treated mice, the reduction of VMAT2 immunoreactivity were observed throughout the brain (Fig. 3B) including cerebral cortex (Fig. 3D), striatum (Fig. 3F) and substantia nigra (Fig. 3H) compared to that of the saline-treated mice respectively (Fig. 3C, E and G), same as shown in the hippocampus formation.

The significance of the reduction in VMAT2 expression of the hippocampal formation was reconfirmed by comparing immunoreactivity between the PCP-treated and saline-treated groups (Fig. 4).

4. Discussion

In this study, we investigated the expression of VMAT2 in the hippocampal formation in an animal model of schizophrenia using immunohistochemical techniques. A significant reduction in VMAT2 expression was observed in the brain of the PCP-treated mice.

4.1. VMAT2 in schizophrenia

The physiological etiology of schizophrenia has not been clarified. However, neurotransmission in the brain is likely to be disrupted because most of the neuroleptic drugs for schizophre-

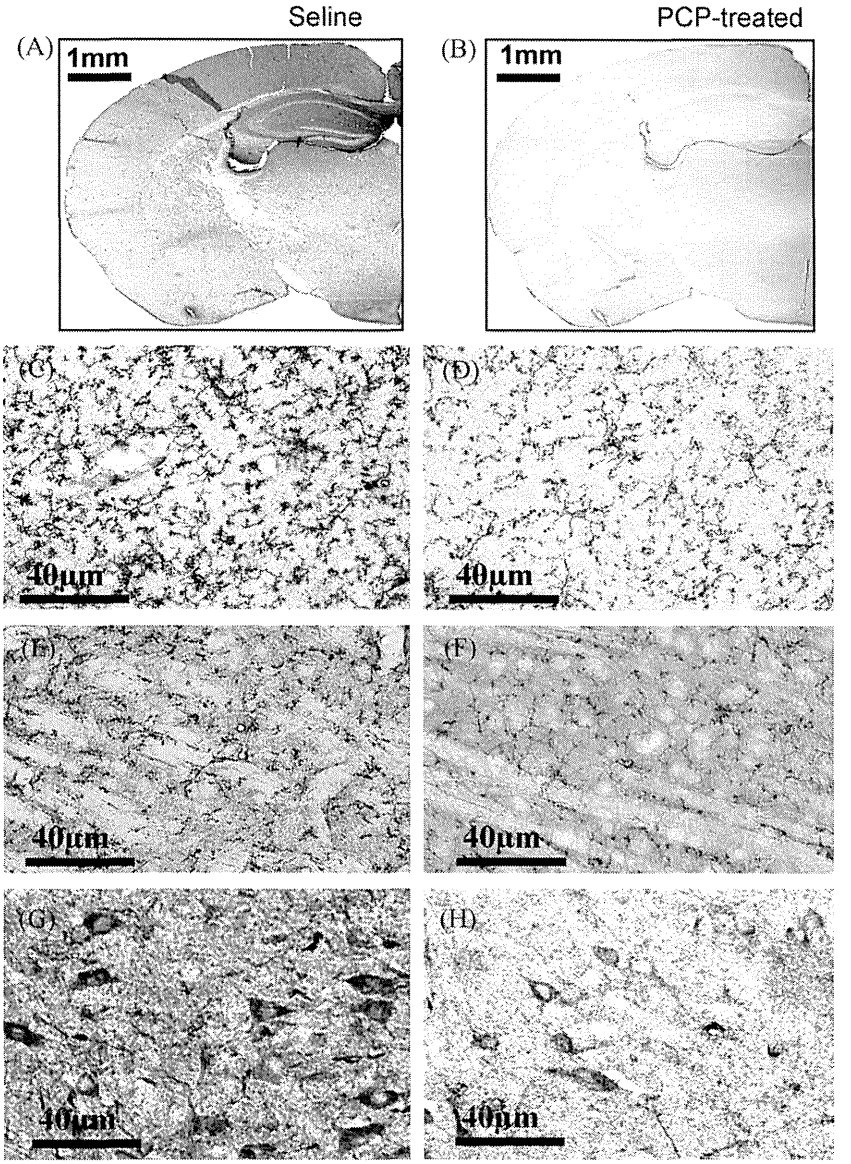

Fig. 3. (A and B) Low magnification photomicrographs of the VMAT2 immunoreactivity in the brain of the saline-treated mouse (A), and PCP-treated mouse (B). The reduction of VAMT2 immunoreactivity was observed in the PCP-treated mouse compared to that of saline-treated mouse throughout the brain. (C and D) VMAT2 immunoreactivity in the temporal cortex of the saline-treated mouse (C) and PCP-treated mice (D). The reduction of immunopositive neuropil was observed in the PCP-treated mice compared to that in the saline-treated mice. (E and F) VMAT2 immunoreactivity in the striatum of the saline-treated moue (E) and PCP-treated mice (F). The reduction of immunopositive varicose fibers was observed in the PCP-treated mice compared to that in the saline-treated mice. (G and H) VMAT2 immunoreactivity in the substantia nigra of the saline-treated mice (G) and PCP-treated mice (H). The more reduction of immunostained neurons and deposits were observed in the PCP-treated mice compared to that in the saline-treated mice.

nia block dopaminergic or serotonergic receptors (Seeman, 2002). Also, some psychostimulants such as MAP increase the concentration of dopamine in the synaptic clefts, which would induce psychosis observed in schizophrenic patients (Yui et al., 2000). Therefore, neurotransmission is clearly impaired, but how or at which stage the disruption occurs is unclear. The VMAT2 protein exists within the pre-synaptic vesicles and acts as a regulator of monoamine neurotransmitters. Changes in the concentration or density of this protein have been reported in patients with mental disorders including schizophrenia (Zubieta et al., 2001) and mood disorders (Zucker et al., 2002). This protein is thought to have a role in higher mental functions and be closely linked to mental illness.

4.2. VMAT2 in PCP-treated mice

Changes in the concentration of VMAT2 have been reported in MAP users (Boileau et al., 2008; Kitamura, 2009), in COC users (Wilson et al., 1996; Little et al., 2003), and in COC-treated animals (Brown et al., 2001). MAP and COC act as accelerators and inhibitors of the uptake dopamine during synaptic transmission and induce a psychosis similar to that observed in schizophrenic patients. PCP induces not only psychosis but also other schizophrenic-like symptoms such as cognitive deficits and negative symptoms in healthy subjects functioning as an NMDA antagonist and perhaps activating prefrontal cortical dopaminergic neurons (Umino et al., 1998). We found changes in VMAT2 expression in the brains of PCP-treated animals, similar to MAP users or COC-treated animals, though there are differences in the psychopharmacological actions of these substances.

The expression of VMAT2 was reported to be regulated mainly by the circumference of the neurotransmitter (Tong et al., 2008). Repeated administration of PCP in neonatal rats impaired prefrontal glutamatergic transmission and subcortical dopaminergic transmission (Hori et al., 2000). Acute administration of PCP

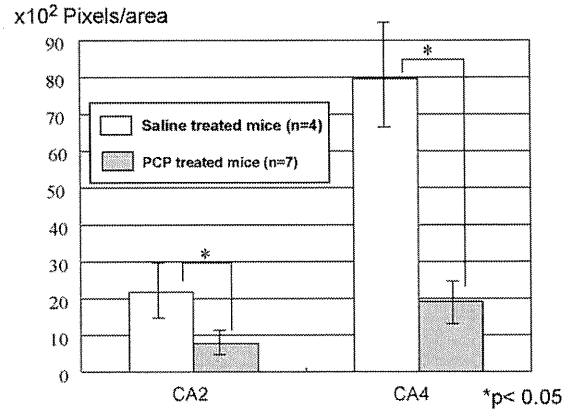

Fig. 4. Significant decreases in VMAT2 immunoreactivity were observed in the CA2 and CA4 areas of hippocampal formation statistically (Mann–Whitney *U* test with $P < 0.05$ considered statistically significant) using the data from digital image analysis in PCP-treated mice compared to saline-treated mice.

affected focal frontohippocampal activation including serotonergic transmission (Gozzi et al., 2008, 2010). PCP might therefore influence different types of neurotransmission and broad areas of the brain. Although the exact mechanism involved remains unknown, abnormal glutamatergic neurotransmission would influence monoaminergic transmission including the dopaminergic system and might to a lead the reduction in VMAT2 expression in the synaptic organization.

The principal pharmacologically treated animal models of schizophrenia are based on the concept of a deficit in dopaminergic neurotransmission of the synapse organ as stated in the dopaminergic hypothesis and/or in glutamatergic neurotransmission as stated in the glutamate hypothesis. PCP-treated animals are considered a useful for evaluating novel therapeutic candidates and confirming the pathological mechanisms of schizophrenia (Mouri et al., 2007). Recently, treatment with PCP was reported to alter the density of sigma-1 receptors in the hippocampus, which is potentially related to the cognitive dysfunction in schizophrenia (Kunitachi et al., 2009). PCP-treated mice are therefore suitable for studying the effects of antipsychotics on emotional and cognitive deficits in schizophrenia, which may confirm the actual clinical course of the disease with negative symptoms and/or the cognitive dysfunction. Anyway, investigations of the function or movement of the VMAT2 protein in PCP-treated mice may be useful for understanding the pathophysiology of schizophrenia and for behavioral pharmacology or drug discovery research.

4.3. Further study

There are several limitations to this study. First, it was unclear whether reduction in VMAT2 expression occurred after the administration of PCP, how long it lasted or if it was depended on the concentration of PCP. Second, since an immunohistochemical analysis has were weak quantitative significance, the regulation of VMAT2 expression needs to be confirmed by determining the quantity of protein directly or by in situ hybridization. Still, VMAT2 is an important mediator of neurotransmission and closely linked to the pathophysiology of schizophrenia. It is also necessary to investigate VMAT2 expression in the postmortem human brain pathologically to clarify the etiology of schizophrenia.

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References

Berretta, S., Gisabella, B., Benes, F.M., 2009. A rodent model of schizophrenia derived from postmortem studies. Behav. Brain Res. 204, 363–368.
Bickel, S., Javitt, D.C., 2009. Neurophysiological and neurochemical animal models of schizophrenia: focus on glutamate. Behav. Brain Res. 204, 352–362.
Boileau, I., Rusjan, P., Houle, S., Wilkins, D., Tong, J., Selby, P., Guttman, M., Saint-Cyr, J.A., Wilson, A.A., Kish, S.J., 2008. Increased vesicular monoamine transporter binding during early abstinence in human methamphetamine users: is VMAT2 a stable dopamine neuron biomarker? J. Neurosci. 28, 9850–9856.
Brown, J.M., Hanson, G.R., Fleckenstein, A.E., 2001. Regulation of the vesicular monoamine transporter-2: a novel mechanism for cocaine and other psychostimulants. J. Pharmacol. Exp. Ther. 296, 762–767.
DeCarolis, N.A., Eisch, A.J., 2010. Hippocampal neurogenesis as a target for the treatment of mental illness: a critical evaluation. Neuropharmacology 58, 884–893.
Desbonnet, L., Waddington, J.L., O'tuathaigh, C.M., 2009. Mutant models for genes associated with schizophrenia. Biochem. Soc. Trans. 37, 308–312.
Dissanayake, D.W., Zachariou, M., Marsden, C.A., Mason, R., 2009. Effects of phencyclidine on auditory gating in the rat hippocampus and the medial prefrontal cortex. Brain Res. 1298, 153–160.
Fleckenstein, A.E., Volz, T.J., Hanson, G.R., 2009. Psychostimulant-induced alterations in vesicular monoamine transporter-2 function: neurotoxic and therapeutic implications. Neuropharmacology 56 (Suppl. 1), 133–138.
Gozzi, A., Crestan, V., Turrini, G., Clemens, M., Bifone, A., 2010. Antagonism at serotonin 5-HT(2A) receptors modulates functional activity of frontohippocampal circuit. Psychopharmacology (Berl) 209, 37–50.
Gozzi, A., Herdon, H., Schwarz, A., Bertani, S., Crestan, V., Turrini, G., Bifone, A., 2008. Pharmacological stimulation of NMDA receptors via co-agonist site suppresses fMRI response to phencyclidine in the rat. Psychopharmacology (Berl) 201, 273–284.
Harrison, P.J., 2004. The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. Psychopharmacology (Berl) 174, 151–162.
Heckers, S., Konradi, C., 2002. Hippocampal neurons in schizophrenia. J. Neural Transm. 109, 891–905.
Hori, T., Subramaniam, S., Srivastava, L.K., Quirion, R., 2000. Behavioral and neurochemical alterations following repeated phencyclidine administration in rats with neonatal ventral hippocampal lesions. Neuropharmacology 39, 2478–2491.
Howes, O.D., Kapur, S., 2009. The dopamine hypothesis of schizophrenia: version III—the final common pathway. Schizophr. Bull. 35, 549–562.
Jentsch, J.D., Roth, R.H., 1999. The neuropsychopharmacology of phencyclidine: from NMDA receptor hypofunction to the dopamine hypothesis of schizophrenia. Neuropsychopharmacology 20, 201–225.
Kitamura, O., 2009. Detection of methamphetamine neurotoxicity in forensic autopsy cases. Leg. Med. (Tokyo) 11 (Suppl. 1), S63–S65.
Kunitachi, S., Fujita, Y., Ishima, T., Kohno, M., Horio, M., Tanibuchi, Y., Shirayama, Y., Iyo, M., Hashimoto, K., 2009. Phencyclidine-induced cognitive deficits in mice are ameliorated by subsequent subchronic administration of donepezil: role of sigma-1 receptors. Brain Res. 1279, 189–196.
Little, K.Y., Krolewski, D.M., Zhang, L., Cassin, B.J., 2003. Loss of striatal vesicular monoamine transporter protein (VMAT2) in human cocaine users. Am. J. Psychiatry 160, 47–55.
Mooslehner, K.A., Chan, P.M., Xu, W., Liu, L., Smadja, C., Humby, T., Allen, N.D., Wilkinson, L.S., Emson, P.C., 2001. Mice with very low expression of the vesicular monoamine transporter 2 gene survive into adulthood: potential mouse model for parkinsonism. Mol. Cell Biol. 21, 5321–5331.
Mouri, A., Noda, Y., Enomoto, T., Nabeshima, T., 2007. Phencyclidine animal models of schizophrenia: approaches from abnormality of glutamatergic neurotransmission and neurodevelopment. Neurochem. Int. 51, 173–184.
Nabeshima, T., Mouri, A., Murai, R., Noda, Y., 2006. Animal model of schizophrenia: dysfunction of NMDA receptor-signaling in mice following withdrawal from repeated administration of phencyclidine. Ann. N. Y. Acad. Sci. 1086, 160–168.
Pothos, E.N., 2002. Regulation of dopamine quantal size in midbrain and hippocampal neurons. Behav. Brain Res. 130, 203–207.
Pothos, E.N., Larsen, K.E., Krantz, D.E., Liu, Y., Haycock, J.W., Setlik, W., Gershon, M.D., Edwards, R.H., Sulzer, D., 2000. Synaptic vesicle transporter expression regulates vesicle phenotype and quantal size. J. Neurosci. 20, 7297–7306.
Radonjic, N.V., Petronijevic, N.D., Vuckovic, S.M., Prostran, M.S., Nesic, Z.I., Todorovic, V.R., Paunovic, V.R., 2008. Baseline temperature in an animal model of schizophrenia: long-term effects of perinatal phencyclidine administration. Physiol. Behav. 93, 437–443.
Seeman, P., 2002. Atypical antipsychotics: mechanism of action. Can. J. Psychiatry 47, 27–38.
Seeman, P., 2009. Glutamate and dopamine components in schizophrenia. J. Psychiatry Neurosci. 34, 143–149.
Taylor, S.F., Koeppe, R.A., Tandon, R., Zubieta, J.K., Frey, K.A., 2000. In vivo measurement of the vesicular monoamine transporter in schizophrenia. Neuropsychopharmacology 23, 667–675.
Tong, J., Wilson, A.A., Boileau, I., Houle, S., Kish, S.J., 2008. Dopamine modulating drugs influence striatal (+)-[11C]DTBZ binding in rats: VMAT2 binding is sensitive to changes in vesicular dopamine concentration. Synapse 62, 873–876.
Umino, A., Takahashi, K., Nishikawa, T., 1998. Characterization of the phencyclidine-induced increase in prefrontal cortical dopamine metabolism in the rat. Br. J. Pharmacol. 124, 377–385.
Volz, T.J., Farnsworth, S.J., Rowley, S.D., Hanson, G.R., Fleckenstein, A.E., 2009. Age-dependent differences in dopamine transporter and vesicular monoamine

transporter-2 function and their implications for methamphetamine neurotoxicity. Synapse 63, 147–151.
Wilson, J.M., Levey, A.I., Bergeron, C., Kalasinsky, K., Ang, L., Peretti, F., Adams, V.I., Smialek, J., Anderson, W.R., Shannak, K., Deck, J., Niznik, H.B., Kish, S.J., 1996. Striatal dopamine, dopamine transporter, and vesicular monoamine transporter in chronic cocaine users. Ann. Neurol. 40, 428–439.
Yui, K., Ikemoto, S., Ishiguro, T., Goto, K., 2000. Studies of amphetamine or methamphetamine psychosis in Japan: relation of methamphetamine psychosis to schizophrenia. Ann. N. Y. Acad. Sci. 914, 1–12.
Zubieta, J.K., Taylor, S.F., Huguelet, P., Koeppe, R.A., Kilbourn, M.R., Frey, K.A., 2001. Vesicular monoamine transporter concentrations in bipolar disorder type I, schizophrenia, and healthy subjects. Biol. Psychiatry 49, 110–116.
Zucker, M., Aviv, A., Shelef, A., Weizman, A., Rehavi, M., 2002. Elevated platelet vesicular monoamine transporter density in untreated patients diagnosed with major depression. Psychiatry Res. 112, 251–256.

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Short Communication

Immunohistochemical Study of Vesicle Monoamine Transporter 2 in the Hippocampal Region of Genetic Animal Model of Schizophrenia

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KEY WORDS VMAT2; schizophrenia; animal model; immunohistochemistry

ABSTRACT Recent research in the etiology of schizophrenia revealed that there may be some neurodevelopmental failures such as neuronal network incompetence in the brain of this disease, and neurotransmitters cannot function accurately or adequately. But, it is unknown precisely what kinds of deficit in neurotransmission may be existed histopathologically. We investigated the expression of vesicle monoamine transporter 2 (VMAT2), which has a significant role in neurotransmission, in the hippocampal formation of the animal model of schizophrenia, 14-3-3epsilon hetero knockout (KO) mouse, using an immunohistochemical staining technique to clarify the neuronal abnormalities in the model animal. As a result, the expression of VMAT2 was increased significantly in the hippocampal formation of 14-3-3epsilon hetero KO mice compared to that of the wild-type littermates. In conclusion, these findings might be related the pathophysiology of this disease includes a monoaminergic transmission abnormality, based on the investigation in a genetically-modified mouse as schizophrenic model. **Synapse 64:948–953, 2010.** ©2010 Wiley-Liss, Inc.

Schizophrenia is a major mental disease with a high prevalence of approximately 1% of the general population. Impaired regulation of monoaminergic neurotransmission, including dopaminergic, serotonergic, and catecholaminergic transmission, is thought to be one aspect of the pathophysiology of this disease (Seeman, 2009). Genomic approaches have uncovered some suspect genes as risk factors in the pathogenesis (Maier et al., 2006). It is known that most of these genes have roles in forming neuronal networks, expanding neuronal fibers, migration of neurons, etc. (Harrison, 2007; Iritani, 2007). Currently, the neurodevelopmental hypothesis of schizophrenia is generally accepted and it can explain many physiological phenomena in terms of the etiology (Rapoport et al., 2005).

Some models of this disease have been used to elucidate its pathogenesis (Bickel and Javitt, 2009; Desbonnet et al., 2009). More recently, some genetically modified animal models have been created based on the results of genomic research on schizophrenia. One such model is the 14-3-3epsilon knockout (KO) mouse. The 14-3-3epsilon protein is associated with disrupted-in schizophrenia-1 (DISC1), a promising candidate susceptibility gene for schizophrenia, which is involved in neurodevelopment, including neuronal maturation of the cerebral cortex (Mackie et al., 2007). It was reported that variation in the DISC1 gene affected hippocampus structure and function and could be a risk factor for schizophrenia (Callicott et al., 2005). The 14-3-3epsilon protein is a novel DISC1-interacting molecule, which forms a complex substance with NUDEL and LIS1, GRB2 (Taya et al., 2007). Genetically-modified 14-3-3epsilon KO mice present with a variety of symptoms and signs of schizophrenia, including working memory deficits or cognitive decline (Ikeda et al., 2008). Therefore, the

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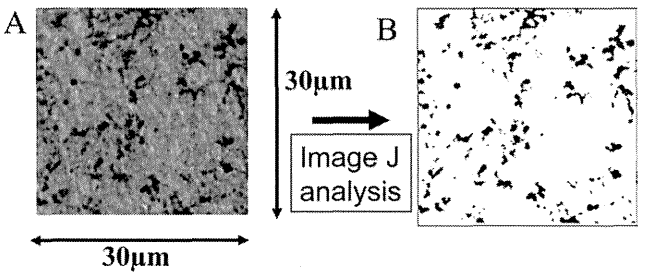


Fig. 1. (**A**) Microscopic photographs were uploaded to a Personal Computer as digital data in the setup area (30 μm × 30 μm). (**B**) The digital images were processed for dichotomization using image software. (Image J: http://rsb.info.nih.gov/ij/). Only immunopositive products were extracted by computer processing. The occupancy space of immunoreactive products was automatically calculated as pixel units. The numerical value of pixels was applied to the index of immunoreactivity.

14-3-3epsilon hetero KO mouse is thought to be a novel animal model of schizophrenia.

The VMAT2 protein package neurotransmitters such as dopamine, serotonin, norepinephrine enter synaptic vesicles for release to the synaptic cleft. Within the synaptic vesicles, VMAT2 regulates vesicle loading and consequently defines quantal size, receptor sensitivity and synapse plasticity (Pothos, 2002). Therefore, this protein has a significant relation to the pathophysiology of neuropsychiatric diseases including schizophrenia (Taylor et al., 2000), mood disorders (Zubieta et al., 2001), methamphetamine (MAP) neurotoxicity (Volz et al., 2009), and Parkinson's disease (Mooslehner et al., 2001).

In this study, we investigated the expression of VMAT2 in the representative schizophrenic animal models using an immunohistochemical technique on the hippocampal formation to clarify the pathophysiology of schizophrenia.

Totally 14 mice were used (12–15 weeks old). Eight were 14-3-3epsilon hetero KO mice (four of them were male, other were female), six were genetically untreated mice (three of them were male, other were female). Also, "the genetically untreated mice" means the 14-3-3 epsilon (+/+) littermates, and we use these wild-type littermates as control for comparison. These mice were bred under the same conditions as the KO mice. The genetic background of the mice was mixed 129/S56 × NIH Black Swiss. More precise information, including the behavior of this genetically modified animal model, was given in the previous study (Ikeda et al., 2008). All experiments were performed in accordance with the institutional guideline of animal experiment of Nagoya University for animal welfare, based on the guidelines issued by the US National Institute of Health for the humanate treatment of experimental animals. All efforts were made to minimize animals suffering and to reduce the number of animals used.

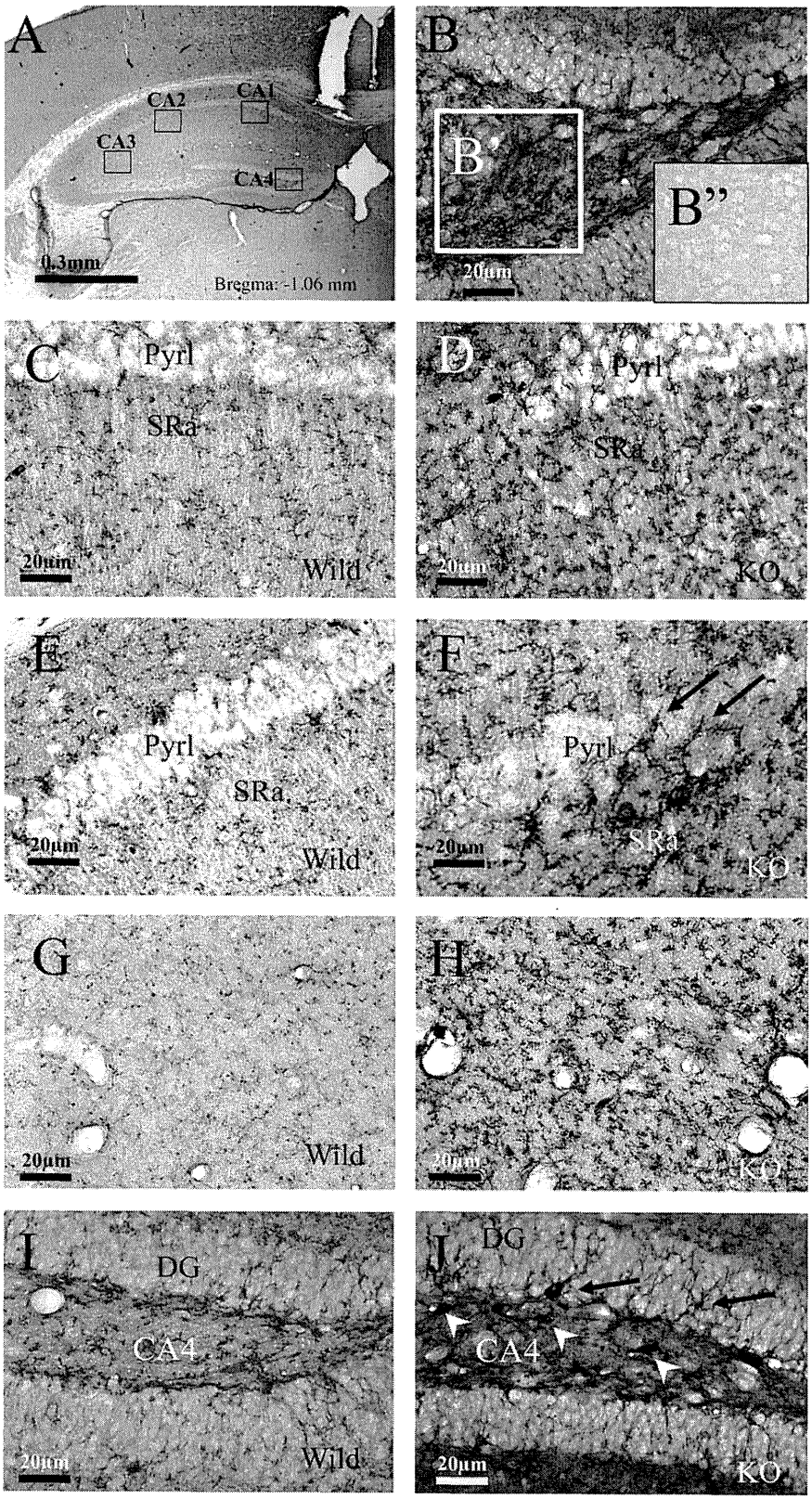
Animals were perfused with a tissue fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) under deep anesthesia by injecting sodium pentobarbital (40 mg/kg body weight i.p.). The brains were immediately removed, and tissue blocks were immersed in a 20% sucrose-0.01M phosphate buffer solution for more than 3 days at 4°C. Sections of the hippocampal regions were cut on a freezing cryostat at 30 μm thickness and treated as free-floating sections. The sections were rinsed and stored in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for at least 3 days and up to 2 weeks prior to the subsequent immunohistochemical procedure. The sections were rinsed in TBS (0.1 M Tris-HCl, pH 7.4, 0.9% NaCl) containing 0.3% Triton X-100 (TX) and 2% normal goat serum (NGS) for 30–60 min at room temperature. The primary antibody used in this study was an anti-VMAT2 polyclonal antibody (Novus Biologicals, CO; 1:1000). Incubation with the primary antibodies was carried out for 48 h at 4°C. The sections were then incubated in a medium containing biotinylated antiuniversal (rat and/or rabbit) IgG (Vectastain; 1:100) for 45 min at room temperature, followed by incubation with an avidin-biotin peroxidase complex (ABC method) for 45 min. After each incubation step, the sections were rinsed in NGS-TX-TBS solution. Finally, the sections were rinsed in PBS twice for 10 min and reacted with 0.05%, 3,3′diaminobenzidine-HCl (Toronto Research Chemicals, Canada) in 0.05 M Tris-HCl buffer (pH 7.6) for 2 or 3 min, and mounted onto gelatin-coated slides. Specimens were observed under a light microscope.

To clarify of specificity of the VMAT2 immunoreactivity, the control studies were conducted in the sections of animal model group and wild-type littermates group under the same immunostaining procedure as mentioned earlier except that prior to immunohistchemical labeling the diluted anti-VMAT2 antibody was preabsorbed with 10 μg/ml or 50 μg/ml of VMAT2 (Novus Biologicals, CO) instead of the primary antibody.

We selected three random squares (30 μm × 30 μm) in the area of the stratum radiatum along the pyramidal layer in the CA1 and CA2 area of the right or left hippocampus of three adjacent sections respectively per each mouse. Also, we selected three random squares (30 μm × 30 μm) in the CA3 and CA4 area of three adjacent sections respectively per each mouse. That is, we selected three observational areas in three adjacent sections respectively per each experimental animal for taking care of the thickness variability made by the cryostat.

These specimens were observed using a light microscope. The microscopic photographs were downloaded to personal computer from a digital camera (HC-2000, Fuji film, Japan) as digital data. The digital images of gradation expression immunoreactivity were processed into black (immunopositive) and white (immunonegative) images using imaging processing software Image J (Image J: http://rsb.info.nih.gov/ij/ Free software supplied by NIH) (Fig. 1).

An appropriate threshold of distinction between black and white tones was unified consistently throughout every specimen. The amount of immuno-


A
CA2
CA1
CA3
CA4
0.3mm
Bregma: -1.06 mm
B
B'
B"
20μm
C
Pyrl
SRa
20μm
Wild
D
Pyrl
SRa
20μm
KO
E
Pyrl
SRa
20μm
Wild
F
Pyrl
SRa
20μm
KO
G
20μm
Wild
H
20μm
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I
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CA4
20μm
Wild
J
DG
CA4
20μm
KO

Fig. 2.

reactivity was assessed by the space occupied value as the number of pixels per observational square area. The number of pixels was measured automatically by the software Image J. Finally, we weighed the number of pixels in each group indicating the space occupied value. In the quantitative analyses of the immunoreactivity, we had processed in a blind manner for removing the observer bias. The significance of variation in differences between the genetically modified group and the wild-type littermates group was assessed by Mann–Whitney U test with $P < 0.05$ considered statistically significant. Since we could not supposed the normal distribution or homoscedasticity of population structure, we used nonparametric test in statistical comparison.In the control studies of preabsorbing the primary antibodies, no immunoreactivity was observed in every specimen (Fig. 2B″).

As a result, a VMAT2-immunopositive structure of varicose fibers or deposits was observed in the stratum radiatum (SRa) along the pyramidal layer (Pyrl) of the area of CA1 (Fig. 2C) and CA2 (Fig. 2E) and in the area of CA3 (Fig. 2G) and C4 (Fig. 2I) in the wild-type littermates (Fig. 2A).

In the case of KO mice compared to the wild-type littermates, an increase in the immunoreactivity expression of VMAT2 was observed along the hippocampal region, i.e. SRa along the Pyrl of CA1 (Fig. 2D) and CA2 (Fig. 2F), and in the area CA3 (Fig. 2H) and CA4 (Fig. 2J). Some dense immunopositive varicose fibers penetrating into Pyrl of CA2 (Fig. 2F arrows) and the dentate gyrus granule cell layer (Fig. 2J arrows) were observed in the KO mice. Some cell bodies of neurons in the CA4 area were stained and stood out in bold and sharp relief (Fig. 2J arrow heads).

The significance of the increased expression was reconfirmed statistically in each observed area by a comparison of immunoreactivity expression using image analysis in the KO model group compared to the wild-type littermates group (Fig. 3).

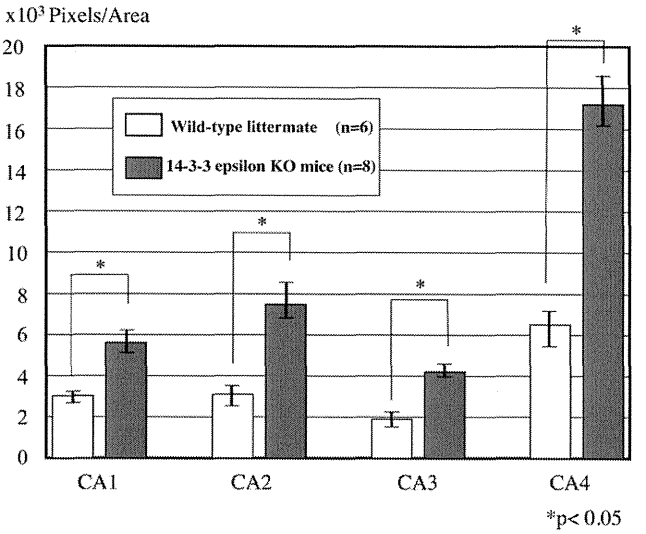

Fig. 3. Significant increases in VMAT 2 immunoreactivity were observed in the CA1, CA2, CA3, and CA4 areas statistically (Mann–Whitney U-test with $P < 0.05$ considered statistically significant) using the data from digital image analysis of 14-3-3epsilon KO mice compared to those of the wild-type littermates.

The physiological etiology of schizophrenia has not been clarified precisely. However, an abnormality in neurotransmission may likely exist in the schizophrenic brain because most available neuroleptic drugs for schizophrenia block dopaminergic or serotonergic receptors (Seeman, 2002). Also, psychostimulant drugs such as MAP induced upregulation of the dopamine concentration in the synaptic cleft, which may cause schizophrenia-like symptoms (Guilarte et al., 2003). From these facts, there is clearly a neurotransmission dysfunction in the brain in this disorder, but it is unclear how these malfunctions occur or at which stage and/or phase in neurotransmission.

Fig. 2. (**A**) General photomicrograph view of the immunoreactivity of VMAT2.The approximate location of mouse brain was −1.06 mm level in the stereotaxic coordinate from bregma (http://www.mbl.org/atlas165/atlas165_start.html). Immunoreactivity of VMAT2 was observed throughout the hippocampus region in the wild-type littermates. The each square frame indicates the observational field of CA1, CA2, CA3, and CA4. (**B**) Control studies were performed. The immmunoreactivity was observed in the normal immunostaining procedure (**B′**), but no immunoreactivity was observed in the control study of preabsorved the primary antibody with the 10 μg/ml of VMAT2 protein (**B″**). The area B′ indicated the same location of the area B″ in the field of CA4. (**C, D**) The immunopositive structure of VMAT2 was observed in the stratum radiatum (SRa) along the pyramidal cell layer (Pyrl) in the CA1 area of the wild-type littermates (C) and of the 14-3-3epsilon KO mice (D). In the CA1 area, an increase of the immunopositive structure of VMAT2 was observed in the 14-3-3epsilon KO mice compare to those of the wild-type littermates. (**E, F**) The immunopositive structure of VMAT2 was observed in the stratum radiatum (SRa) along the pyramidal cell layer (Pyrl) in the CA2 area of the wild-type littermates (E) and of the 14-3-3epsilon KO mice (F). In the CA2 area, an increase of the immunopositive structure of VMAT2 was observed in the 14-3-3epsilon KO mice compare to those of the wild-type littermates. Especially, the dense immunoreactivity was observed in the area of SRa along Pyrl and some dense immunopositive fibers were running into Pyrl (arrows). Pyrl: Pyramidal layer, SRa: Stratum radiatum(**G, H**) The immunopositive structure of VMAT2 was observed in the CA3 area of the wild-type littermates (G) and of a 14-3-3epsilon KO mice (H). In the CA3 area, an increase of granular or mossy immunoreactivity was observed in 14-3-3epsilon KO mice compare to those in the wild-type littermates. (**I, J**) The immunopositive structure of VMAT2 was observed in the field of CA4 area of the wild-type littermates (I) and of a 14-3-3epsilon KO mice (J). An increased number of VMAT2-immunopositive structures were observed in a 14-3-3epsilon KO mice compare to in the wild-type littermates. Especially, the dense immunoreactivity was observed in CA4 and some dense immunopositive fibers ran into the dentate gyrus granule cell layer (DG) (arrows). Some cell bodies of neurons in the CA4 area were stained and stood out in bold and sharp relief (arrowheads). DG: dentate gyrus granule cell layer.

The VMAT2 protein exists within the presynaptic vesicles and acts as a regulator of monoamine neurotransmitters. Concentration or density changes in this protein were reported in mental disorders including schizophrenia (Zubieta et al., 2001). This protein is thought to have a role in higher mental functions and be closely linked to mental illness. Also, gene variation in VMAT2 is thought to be a risk factor for schizophrenia (Talkowski et al., 2008). In schizophrenic patients, it was reported that the density of VMAT2 increased in an investigation of platelets (Zucker et al., 2002). In this study, we confirmed changes in the tissue expression of VMAT2 in the brains of a mouse model of schizophrenia for first time.

The 14-3-3epsilon protein works in a coordinated manner with DISC1 to help the neuronal network mature (Taya et al., 2007). Malformation of neuronal morphology in the hippocampus of impaired DISC1 function mice was reported (Faulkner et al., 2008). It is interesting that morphological abnormalities appeared in the hippocampus formation in the schizophrenic brain (Wang et al., 2008), and also in a rat model of schizophrenia (Bertrand et al., 2010) in neuroimaging studies. Also, synapse malformation in these areas of 14-3-3epsilon KO mice is suspected because of DISC1 pathway modulates the expression of synaptogenic proteins (Hennah and Porteous, 2009). The malformation and /or dysfunction of synapses may impair adequate negative- or positive-feedback and regulation of neurotransmitter concentration. In 14-3-3epsilon KO mice, a monoamine transmitter in presynaptic vesicles without negative feedback may be overexpressed due to synapse dysfunction and increased VMAT2 protein.

Some reports suggested that VMAT2 may have a neuroprotective function against neurodegeneration or exposure to neurotoxins such as MAP (Caudle et al., 2007; Guillot and Miller, 2009). The situation of lacking the role of 14-3-3epsilon protein may mimic the exposure to neurotoxins for VMAT2 expression. Moreover, these physiological conditions in this genetically modified animal may resemble the actual pathopysiological state of schizophrenia induced hyperdopaminaergic condition.

The etiology of schizophrenia has been considered multifactorial, and many aspects of the intrinsic pathophysiology in this disease have been reported (van Os and Kapur, 2009). A genetically modified animal model was developed based on recent results of a search for risk genes for schizophrenia. The philosophy behind this animal model is that a deficit of risk genes may directly indicate the pathophysiology of the disease, and this animal model is thought to be a model based on the neurodevelopmental hypothesis. This study also indicates that a deficit of the risk genes could induce changes in the expression of neurotransmission mediators.

As the further assignment, it should be necessary to investigate VMAT2 expression in the postmortem schizophrenic brain pathologically to clarify the etiology of schizophrenia.

REFERENCES

Bertrand JB, Langlois JB, Begou M, Volle J, Brun P, d'Amato T, Saoud M, Suaud-Chagny MF. 2010. Longitudinal MRI monitoring of brain damage in the neonatal ventral hippocampal lesion rat model of schizophrenia. Hippocampus 20:264–278.

Bickel S, Javitt DC. 2009. Neurophysiological and neurochemical animal models of schizophrenia: Focus on glutamate. Behav Brain Res 204:352–362.

Callicott JH, Straub RE, Pezawas L, Egan MF, Mattay VS, Hariri AR, Verchinski BA, Meyer-Lindenberg A, Balkissoon R, Kolachana B, Goldberg TE, Weinberger DR. 2005. Variation in DISC1 affects hippocampal structure and function and increases risk for schizophrenia. Proc Natl Acad Sci USA 102: 8627–8632.

Caudle WM, Richardson JR, Wang MZ, Taylor TN, Guillot TS, McCormack AL, Colebrooke RE, Di Monte DA, Emson PC, Miller GW. 2007. Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. J Neurosci 27:8138–8148.

Desbonnet L, Waddington JL, O'Tuathaigh CM. 2009. Mutant models for genes associated with schizophrenia. Biochem Soc Trans 37 (Part 1):308–312.

Faulkner RL, Jang MH, Liu XB, Duan X, Sailor KA, Kim JY, Ge S, Jones EG, Ming GL, Song H, Cheng HJ. 2008. Development of hippocampal mossy fiber synaptic outputs by new neurons in the adult brain. Proc Natl Acad Sci USA 105:14157–14162.

Guilarte TR, Nihei MK, McGlothan JL, Howard AS. 2003. Methamphetamine-induced deficits of brain monoaminergic neuronal markers: Distal axotomy or neuronal plasticity. Neuroscience 122:499–513.

Guillot TS, Miller GW. 2009. Protective actions of the vesicular monoamine transporter 2 (VMAT2) in monoaminergic neurons. Mol Neurobiol 39:149–170.

Harrison PJ. 2007. Schizophrenia susceptibility genes and their neurodevelopmental implications: focus on neuregulin 1. Novartis Found Symp 288:246–255; discussion 255–249:276–281.

Hennah W, Porteous D. 2009. The DISC1 pathway modulates expression of neurodevelopmental, synaptogenic and sensory perception genes. PLoS One 4:e4906.

Ikeda M, Hikita T, Taya S, Uraguchi-Asaki J, Toyo-oka K, Wynshaw-Boris A, Ujike H, Inada T, Takao K, Miyakawa T, Ozaki N, Kaibuchi K, Iwata N. 2008. Identification of YWHAE, a gene encoding 14–3-3epsilon, as a possible susceptibility gene for schizophrenia. Hum Mol Genet 17:3212–3222.

Iritani S. 2007. Neuropathology of schizophrenia: A mini review. Neuropathology 27:604–608.

Mackie S, Millar JK, Porteous DJ. 2007. Role of DISC1 in neural development and schizophrenia. Curr Opin Neurobiol 17:95–102.

Maier W, Zobel A, Kuhn KU. 2006. Clinical impact of recently detected susceptibility genes for schizophrenia. Dialogues Clin Neurosci 8:79–84.

Mooslehner KA, Chan PM, Xu W, Liu L, Smadja C, Humby T, Allen ND, Wilkinson LS, Emson PC. 2001. Mice with very low expression of the vesicular monoamine transporter 2 gene survive into adulthood: potential mouse model for parkinsonism. Mol Cell Biol 21:5321–5331.

Pothos EN. 2002. Regulation of dopamine quantal size in midbrain and hippocampal neurons. Behav Brain Res 130:203–207.

Rapoport JL, Addington AM, Frangou S, Psych MR. 2005. The neurodevelopmental model of schizophrenia: update 2005. Mol Psychiatry 10:434–449.

Seeman P. 2002. Atypical antipsychotics: Mechanism of action. Can J Psychiatry 47:27–38.

Seeman P. 2009. Glutamate and dopamine components in schizophrenia. J Psychiatry Neurosci 34:143–149.

Talkowski ME, Kirov G, Bamne M, Georgieva L, Torres G, Mansour H, Chowdari KV, Milanova V, Wood J, McClain L, Prasad K, Shirts B, Zhang J, O'Donovan MC, Owen MJ, Devlin B, Nimgaonkar VL. 2008. A network of dopaminergic gene variations implicated as risk factors for schizophrenia. Hum Mol Genet 17:747–758.

Taya S, Shinoda T, Tsuboi D, Asaki J, Nagai K, Hikita T, Kuroda S, Kuroda K, Shimizu M, Hirotsune S, Iwamatsu A, Kaibuchi

K. 2007. DISC1 regulates the transport of the NUDEL/LIS1/14–3-3epsilon complex through kinesin-1. J Neurosci 27:15–26.
Taylor SF, Koeppe RA, Tandon R, Zubieta JK, Frey KA. 2000. In vivo measurement of the vesicular monoamine transporter in schizophrenia. Neuropsychopharmacology 23:667–675.
van Os J, Kapur S. 2009. Schizophrenia. Lancet 374:635–645.
Volz TJ, Farnsworth SJ, Rowley SD, Hanson GR, Fleckenstein AE. 2009. Age-dependent differences in dopamine transporter and vesicular monoamine transporter-2 function and their implications for methamphetamine neurotoxicity. Synapse 63:147–151.
Wang L, Mamah D, Harms MP, Karnik M, Price JL, Gado MH, Thompson PA, Barch DM, Miller MI, Csernansky JG. 2008. Progressive deformation of deep brain nuclei and hippocampal-amygdala formation in schizophrenia. Biol Psychiatry 64:1060–1068.
Zubieta JK, Taylor SF, Huguelet P, Koeppe RA, Kilbourn MR, Frey KA. 2001. Vesicular monoamine transporter concentrations in bipolar disorder type I, schizophrenia, and healthy subjects. Biol Psychiatry 49:110–116.
Zucker M, Valevski A, Weizman A, Rehavi M. 2002. Increased platelet vesicular monoamine transporter density in adult schizophrenia patients. Eur Neuropsychopharmacol 12:343–347.

Identification of Novel Candidate Genes for Treatment Response to Risperidone and Susceptibility for Schizophrenia: Integrated Analysis Among Pharmacogenomics, Mouse Expression, and Genetic Case-Control Association Approaches

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Background: Pharmacogenomic approaches based on genomewide sets of single nucleotide polymorphisms (SNPs) are now feasible and offer the potential to uncover variants that influence drug response.

Methods: To detect potential predictor gene variants for risperidone response in schizophrenic subjects, we performed a convergent analysis based on 1) a genomewide (100K SNP) SNP pharmacogenetic study of risperidone response and 2) a global transcriptome study of genes with mRNA levels influenced by risperidone exposure in mouse prefrontal cortex.

Results: Fourteen genes were highlighted as of potential relevance to risperidone activity in both studies: *ATP2B2, HS3ST2, UNC5C, BAG3, PDE7B, PAICS, PTGFRN, NR3C2, ZBTB20, ST6GAL2, PIP5K1B, EPHA6, KCNH5,* and *AJAP1*. The SNPs related to these genes that were associated in the pharmacogenetic study were further assessed for evidence for association with schizophrenia in up to three case-control series comprising 1564 cases and 3862 controls in total (Japanese [JPN] 1st and 2nd samples and UK sample). Of 14 SNPs tested, one (rs9389370) in *PDE7B* showed significant evidence for association with schizophrenia in a discovery sample ($p_{allele} = .026$ in JPN_1st, two-tailed). This finding replicated in a joint analysis of two independent case-control samples ($p_{JPN\_2nd+UK} = .008$, one-tailed, uncorrected) and in all combined data sets ($p_{all} = .0014$, two-tailed, uncorrected and $p_{all} = .018$, two-tailed, Bonferroni correction).

Conclusions: We identified novel candidate genes for treatment response to risperidone and provide evidence that one of these additionally may confer susceptibility to schizophrenia. Specifically, *PDE7B* is an attractive candidate gene, although evidence from integrated methodology, including pharmacogenomics, pharmacotranscriptomic, and case-control association approaches.

Key Words: Expression: PDE7B, pharmacogenomics, risperidone, schizophrenia

Schizophrenia is a severe psychiatric disorder with a lifetime risk of approximately 1%. With its early onset, typically in late teens to early 20s, frequent relapse and chronic course, schizophrenia imposes a considerable burden on sufferers, their families, and society. Worldwide, it is a major source of morbidity, but it is often overlooked that it is also associated with a considerable truncation in life span, the mortality rate in individuals with schizophrenia being more than twice that of the age- and sex-matched population (1). A large number of antipsychotics have been developed as treatment agents. However, individual response to these drugs is highly variable, and identifying the optimal treatment for any patient is often a trial and error process that can span many years and even then, response is often poor. There is a pressing need both to identify new treatments and to attempt to improve the information based on which response to treatment can be predicted.

Genetic factors are generally assumed to contribute to variable treatment response (2), and on this basis, a number of pharmacogenetic studies have been performed. Here, the aim was to detect DNA sequence predictors for treatment response. Most studies have focused on genes encoding neurotransmitter receptors, such as dopamine or serotonin receptors, the logic being that antipsychotics usually have high affinities with members of these classes of receptor. Although a number of variants have been correlated with treatment response in several stud-

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ies—for example, dopamine D2 and D3 receptor variants (2)—there are no definitive predictors of response.

Pharmacogenetics has been driven by a candidate gene approach. This approach has the disadvantage that targets for study are limited by our current understanding of the mechanisms of drugs, and therefore, this method cannot identify unsuspected predictor genes. Approaches that are independent of prior functional hypotheses of gene action based on genome-wide surveys of SNPs are, however, now feasible. The genome-wide approach has its disadvantages, but one of the most important is that, with effectively random sets of SNPs, the low prior probability that any is truly associated with disease requires a stringent type I error rate to control the enormous potential for reporting false positives. One way to address this issue is to use very large (and therefore highly powered) studies in which such stringent statistical support might realistically be achieved. Another approach that is more economical in genotyping costs is to undertake multistage analysis in which candidate variants from a screening sample are validated by replication in other data sets (3). However, because the samples for pharmacogenomics require a large amount of clinical data and are preferably prospective, large samples are difficult to collect.

Another approach is to try to enhance the prior probability for a given gene by integrating pharmacogenomic data with other sources of data—for example, from studies of gene expression (4). Under the hypothesis that genes related to drug response may be regulated by exposure to that drug, genes in which expression is altered in animals exposed to that drug have a higher probability of being genuinely associated in a pharmacogenetic study than any random gene. If this is correct, genes in which expression is altered by drug exposure require less stringent statistical support.

We aimed to detect predictor genes for risperidone response in schizophrenic patients using this convergent approach (4). Specifically, we compared data from a pharmacogenetic study based on first-episode, previously drug-naive subjects with schizophrenia who were treated with risperidone with data from a pharmacotranscriptomic study based on mice exposed to the same drug. Moreover, candidate variants from genes implicated by convergent data were also tested for evidence for association to schizophrenia per se because variants that are related to drug response may also be related to disease risk (5). Evidence that this occurs can be considered an additional independent line of circumstantial support that the convergence between the pharmacogenetic and transcriptomics does not merely reflect chance.

Methods and Materials

Subjects and Collection of Clinical Data

We performed an open-labeled pharmacogenetic study involving 108 first-episode, previously antipsychotic-naive schizophrenic patients. All received risperidone monotherapy after enrollment. Details are described elsewhere (6,7). Briefly, patients were entered into the study if they 1) met DSM-IV-TR criteria for schizophrenia (and then remained in follow-up to at least 6 months), 2) were physically healthy and had all laboratory parameters within normal limits, and 3) had neither a current nor a past DSM-IV-TR diagnosis of mood disorders or substance abuse. Consensus diagnoses were made by at least two experienced psychiatrists on the basis of unstructured interviews with patients and families and review of medical records. Duration of untreated psychosis (DUP) was defined as the period from the onset of psychotic symptoms to that of first antipsychotic exposure. Sixty subjects were recruited from outpatient clinics, and 48 subjects were treated as inpatients.

Subjects received risperidone monotherapy (starting dosage: .5–4 mg/day, mean starting dosage: 2.5 mg), and dosage was adjusted in accordance with symptomatic response by trained psychiatrists (1–8 mg/day, mean dosage: 3.4 mg at 8 weeks) for 8 weeks. Patients with insomnia were prescribed brotizolam, .25 mg or .5 mg, at bedtime. No other psychotropic drugs were permitted.

Clinical symptoms were evaluated at the first visit and after 8 weeks of treatment by the use of the Positive and Negative Syndrome Scale (PANSS). Evaluations were carried out by qualified psychiatrists and psychologists (the interrater reliability was measured by intraclass correlation coefficient was .90, unpublished data).

The clinical characteristics of subjects that we used as potential covariates were selected from another report (8): sex (57 male, 51 female), age (mean 30.2 ± 9.5 years), DUP (1.5–32 months, mean 7.6 ± 7.1 months), and baseline PANSS total score (mean 83.0 ± 22.9).

Samples used in the schizophrenia case-control association analysis consisted of three sets: (1) JPN_1st: this was used for identifying genes of potential interest and comprised 540 patients with schizophrenia (275 male and 265 female; aged 43.3 ± 15.0 years) and 425 healthy controls (236 male and 189 female; aged 36.3 ± 13.9 years) from the Japanese population; 2) JPN_2nd sample (used to follow up genes of interest) comprised 545 patients with schizophrenia (282 male and 263 female; aged 50.7 ± 14.9 years) and 500 controls (279 male and 221 female; aged 40.8 ± 15.4 years) from the Japanese population; 3) Additional follow-up data for SNPs of interest were extracted from a UK genomewide association study (GWAS) of schizophrenia comprising 479 patients with schizophrenia and 2937 controls from the UK population (9).

Controls in the Japanese population were screened for past history of mental disorders. All individuals were unrelated. After explanation of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at Fujita Health University, University of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, Osaka University Graduate School of Medicine and by multiple ethics committees across the UK where sample recruitment was performed.

Microarray Experiments

See also Methods in Supplement 1.

SNP Chip. Genomewide genotyping was carried out using Illumina Sentrix human 1 Genotyping BeadChip (109,363 SNPs randomly distributed throughout the genome) according to the manufacturer's instructions (Illumina, San Diego, California). Details are given in the Supplement 1.

Mouse Expression Chip. We compared mRNA levels of the prefrontal cortex (PFC) between control ($n = 3$) and risperidone-exposed mice (2.4 mg/kg given orally, once a day for 21 days, $n = 3$). Affymetrix Mouse Gene 1.0 St. Array, which profiles the expression of 28,853 genes (Affymetrix, Santa Clara, California), was used to measure the amount of mRNA.

The procedures involving animals and their care were conducted in conformity with the international guidelines, Principles of Laboratory Animal Care (National Institutes of Health Publication 85-23, revised 1985).

Experimental Procedures and Statistical Analysis

Study 1: Pharmacogenomics. Quality control (QC) regarding population stratification (Figure S1 in the Supplement 1),

Hardy-Weinberg equilibrium (HWE), genotyping rate, and minor allele frequency was conducted by PLINK (10). Details are described in Supplement 1.

After QC, 99 samples (51 males and 48 females) and 62,935 autosomal SNPs (a mean call rate of 99.2%, indicating a high rate of successful genotyping) were analyzed to evaluate the effect of each SNP on antipsychotic response to risperidone.

To evaluate the effect of each SNP on antipsychotic response to risperidone, multiple regression analysis was carried out with a dependent variable [% PANSS change = 100 × ((PANSS at week 0) − (PANSS at Week 8))/PANSS at Week 0] and independent variables that included sex, age, duration of illness, initial PANSS score, and the genotype of each polymorphism. Each genotype was assessed using dominant, recessive, and multiplicative genetic models, respectively.

To calculate the best empiric p values based on the most significant result in each genetic model, we generated 1 million simulated data sets by randomizing the PANSS changes (the covariates stay with the genotypes) with respect to the GWAS data. This approach retains the linkage disequilibrium (LD) relationships between SNPs, and therefore allows for the appropriate degree of nonindependence in the data sets. The same multiple regression analysis model as applied to the real data were applied to each SNP in each permuted data set, and the empiric significance for a SNP was the proportion of the simulated data sets in which the test statistic was equal to, or greater than, that observed in the true data set (11–15).

SNPs were annotated to the closest genes with an up- and downstream span of 20 kb by WGAViewer (16).

Study 2: Mouse Expression Assay. In the mouse expression assay, data sets passing QC were normalized using GeneChip Operating Software (Affymetrix) and the raw intensity values exported for further analysis. Only genes called present based on Affymetrix detection p value for the presence of each gene on either chip were included. A t test was performed to assess the statistical significance of genes in which expression differed between control and risperidone-exposed mice. Power analysis was carried out using PowerAtlas (17). Our data set provides expected discovery rate (corresponding to power) of .37, an expected proportion of true positives (PTPs) of .72, and an expected proportion of true negatives of .80 at alpha set at .05. A major aim of this study was to prioritize genes showing convergent evidence in the pharmacogenomic study, thus we consider a high PTP optimal.

These data were submitted to CIBEX (http://cibex.nig.ac.jp/index.jsp, accession number: CBX77).

Study 3: Checking Overlap Results Between Pharmacogenomics (Study 1) and Mouse Expression Assay (Study 2). We checked candidate SNPs from the genes that showed convergent evidence for relevance to risperidone action from Study 1 and Study 2. Candidate genes were defined as follows: 1) genes for which there was at least one SNP with p values less than 5.0×10^{-4} in the pharmacogenomic study and in which expression significantly differed between groups at $\alpha < .05$ and 2) genes with much stronger evidence for $p < 1.0 \times 10^{-4}$ but that had weaker evidence for association in the pharmacogenomic study ($p < .05$).

Study 4: Case-Control Association Analysis of Strong Candidate Genes from Pharmacogenomics and Mouse Expression Assay. The candidate SNPs from Study 3 were further assessed for evidence for association with schizophrenia. These SNPs were genotyped by TaqMan assay (Applied Biosystems, California) in the Japanese case-control samples. Genotypes for the SNP in *PDE7B* in the UK samples were extracted from the Affymetrix GWAS data (9) after confirmation of good-quality cluster plots.

Genotype deviation from HWE was evaluated by a goodness of fit chi-square test. Marker-trait association was evaluated for allele/genotype-wise using standard contingency tables (SPSS 15.0, SPSS, Tokyo, Japan).

For SNPs analyzed in multiple samples, we conducted a meta-analysis using a random-effects model. Heterogeneity was measured using a Q statistic test in the combined studies. Odds ratios (ORs) were pooled using DerSimonian and Laird methods. The significance of the pooled OR was determined using a Z test. All data were analyzed using an R package, meta (http://www.r-project.org/index.html).

Results

Possible Predictor SNPs for Risperidone Treatment: From Pharmacogenomic Result (Study 1)

Among the 62,935 SNPs we examined in the pharmacogenomics study, 51,550 SNPs were annotated to 14,655 genes (annotation span: 5′ or 3′ ± 20 kb). For a number of genes, we had multiple SNPs with p values less than 5.0×10^{-4} because of the high LD among genotyped markers. Where this occurred, we list only the strongest associated SNP from that gene (the top 10 hits and SNPs with p value less than 5.0×10^{-4} in Table 1 and Table S1 in Supplement 1, respectively).

Table 1. Predictor Genes in the Pharmacogenomics (Top 10)

Ranking	SNP	Chr	Position[a]	Closest Gene[b]	p Value (Pharmacogenomics)
1	rs2289273	3	10,388,601	*ATP2B2*	1.60×10^{-5}
2	rs234091	1	183,186,172	*FAM129A*	2.00×10^{-5}
3	rs241202	8	28,689,604	*INTS9*	3.20×10^{-5}
4	rs4340422	19	48,604,802	*TEX101*	5.00×10^{-5}
5	rs6682786	1	23,615,883	*TCEA3*	7.30×10^{-5}
6	rs1001220	7	72,748,539	*WBSCR22*	7.70×10^{-5}
7	rs3829241	11	68,611,939	*TPCN2*	8.90×10^{-5}
8	rs460473	16	22,740,528	*HS3ST2*	1.03×10^{-4}
9	rs9792264	8	135,640,117	*ZFAT*	1.10×10^{-4}
10	rs6443999	3	186,056,249	*VPS8*	1.17×10^{-4}

Chr, chromosome; SNP, single nucleotide polymorphism.
[a]Based on Ensemble *Homo sapiens* Version 54.36p (NCBI36).
[b]SNPs are annotated to the closest genes with ± 20-kb span.

Table 2. Overlap Genes Based on the Pharmacogenomcis ($p < 5.0 \times 10^{-4}$) with Mouse Expression Assay ($p < .05$)

Ranking	SNP	Chr	Position[a]	Closest Gene[b]	*p* Value (Pharmacogenomics)	*p* Value[c] (Mouse Expression)	Fold Change
1	rs2289273	3	10,388,601	*ATP2B2*	1.60×10^{-5}	.000710	.504
8	rs460473	16	22,740,528	*HS3ST2*	1.03×10^{-4}	.00600	.259
28	rs3775003	4	96,390,234	*UNC5C*	2.20×10^{-4}	.0132	1.85
32	rs196290	10	121,398,061	*BAG3*	2.81×10^{-4}	.0283	1.33
35	rs9389370	6	136,472,958	*PDE7B*	2.88×10^{-4}	.00806	.710
53	rs1356787	4	57,012,104	*PAICS*	4.26×10^{-4}	.0368	.660
54	rs4641299	1	117,284,884	*PTGFRN*	4.27×10^{-4}	.00160	.283

Chr, chromosome; SNP, single nucleotide polymorphism.
[a]Based on Ensemble *Homo sapiens* Version 54.36p (NCBI36).
[b]SNPs are annotated to the closest genes with ± 20-kb span.
[c]Comparison between risperidone-treated mice ($n = 3$) and saline-treated mice ($n = 3$).

We also looked specifically in our data for support for genes recently suggested as associated with iloperidone based on the only other available antipsychotic GWAS data set (18) and candidate genes implicated in earlier studies (2) including *DRD2*, *DRD3*, *HTR2A*, and others (Tables S2 and S3 in Supplement 1). No strong evidence for association to any of these was found in our pharmacogenomics data set.

Genes Influenced by Risperidone Exposure in Mouse PFC (Study 2)

We examined 22,556 probes in 12,706 genes in RNA extracted from the PFC of mice treated with either risperidone or with vehicle. Of these, 754 (5.9%) and 2227 (17.5%) genes had at least one probe that showed nominally significant differences at $p <$ .01 and .05, respectively, a rate much higher than chance. The top genes with *p* value less than 5.0×10^{-4} are presented in Table S4 in Supplement 1.

Overlapping Genes Between Pharmacogenomic and Mouse Expression Assays (Study 3)

We looked to see whether the pharmacogenetic data (excluding 14 SNPs that could not be annotated to the closest gene) and expression overlapped. Seven genes containing nominally significant alteration in expression in mice also contained SNPs with *p* value less than 5×10^{-4} (Table 2). The relation between PANSS changes and physical locations of each SNP and the genotype effects to risperidone response can be seen in Figures S2 and S3 and Table S5 in Supplement 1. In addition, we found seven genes that met the more stringent threshold for expression change in the mouse and that had at least one significant SNP ($p < .05$) in the pharmacogenomic data (Table 3). It should be stressed these SNPs were not strongly associated with treatment response ($p = .0047$-$.0472$).

Consequently 14 SNPs were further assessed for case-control association analysis in Study 4.

Examining Candidate SNPs as Susceptibility Factor for Schizophrenia (Study 4)

The 14 candidate SNPs in genes showing convergent evidence from Study 3 were further tested for association with schizophrenia (Table 4). For rs242056, a proxy for rs2071999 in *AJAP1*, the genotypes significantly deviated from HWE in controls ($p = .0016$). This SNP was therefore excluded.

Of the remaining 13 SNPs, a single SNP (rs9389370) in *PDE7B* showed a nominally significant association in the JPN_1st case-control sample ($p_{allele} = .026$, two-tailed). In an attempt to extend this putative association, we used two other samples. In the second Japanese sample, we obtained significant evidence for association (second set, $P_{allele} = .02$, one-tailed) and a nonsignificant trend in the UK sample ($p_{allele} = .07$, one-tailed) (Table 4). Meta-analysis of the two replication data sets showed significant evidence for association ($p_{JPN2nd+UK} = .008$, one-tailed). As expected, in all data sets combined, the evidence was stronger than observed in the screening sample alone ($p_{all} = .0014$, two-tailed, uncorrected; $p = .018$, 13 times Bonferroni correction for number of SNPs tested in Study 4) with no evidence for heterogeneity ($p = .56$; Table 5).

Discussion

Combined Analysis as a Tool for Prioritizing Candidate Genes for Pharmacogenomics and Susceptibility

Genomewide approaches to pharmacogenomics have the capacity to provide novel insights into mechanisms and predictors of drug response. However, a major concern of this approach, which is not specific to pharmacogenomics, relates to balancing the need to set a stringent threshold for the type I error rate against the desire to achieve power to detect findings at that threshold. Unless the genetic effect sizes in pharmacogenetics are substantially greater than is typical for complex diseases (19), the sorts of sample sizes currently available for studies of

Table 3. Overlap Genes Based on the Mouse Expression Assay ($p < 1.0 \times 10^{-4}$) with the Pharmacogenomics ($p < .05$)

Ranking	Gene	Probe ID	Fold Change	*p* Value (Mouse Expression)	*p* Value (Pharmacogenomics)	SNP ID
2	*Nr3c2*	1435991-at	5.86	2.23×10^{-6}	.0297	rs2070951
3	*Zbtb20*	1439278-at	4.94	5.04×10^{-6}	.0230	rs9883949
4	*St6gal2*	1434819-at	.23	6.52×10^{-6}	.0102	rs1448110
7	*Pip5k1b*	1450389-s-at	2.46	1.02×10^{-5}	.0472	rs1414944
8	*Epha6*	1421527-at	3.18	1.46×10^{-5}	.0047	rs727229
9	*Kcnh5*	1441742-at	.44	2.72×10^{-5}	.0305	rs10141458
24	*Ajap1*	1438662-at	.66	9.21×10^{-5}	.0208	rs2071999

SNP, single nucleotide polymorphism.

Table 4. Case-Control Analysis of the Candidate SNPs from the Pharmacogenomics and Mouse Expression Data

SNP	Sample	Proxy SNPs	Phenotype	N	Genotype M/M	Genotype M/m	Genotype m/m	*p* Value Allele	*p* Value Genotype	*p* Value HWE	MAF
ATP2B2	JPN_1st		Case	536	275	208	53	.676	.184	.14	29.3
rs2289273			Control	417	209	179	29			.26	28.4
HS3ST2	JPN_1st		Case	538	163	263	112	.408	.682	.76	45.3
rs460473			Control	407	117	196	94			.50	47.2
UNC5C	JPN_1st		Case	540	336	178	26	.249	.136	.70	21.3
rs3775003			Control	406	231	159	16			.07	23.5
BAG3	JPN_1st		Case	539	234	243	62	.609	.877	.93	34.0
rs196290			Control	407	183	180	44			.98	32.9
PDE7B	JPN_1st		Case	535	259	222	54	**.0255**	.0738	.53	30.8
rs9389370			Control	422	229	165	28			.81	26.2
	JPN_2nd		Case	536	278	200	58	**.0214**[a]	.0966	.018	29.5
			Control	500	281	183	36			.41	25.5
	UK		Case	478	181	226	71	.0672[a]	.327	.97	38.5
			Control	2,932	1203	1348	381			.91	36.0
PAICS	JPN_1st		Case	540	181	274	85	.662	.808	.27	41.1
rs1356787			Control	424	134	223	67			.10	42.1
PTGFRN	JPN_1st		Case	535	319	180	36	.222	.219	.13	23.6
rs4641299			Control	413	255	141	17			.65	21.2
NR3C2	JPN_1st		Case	534	295	206	33	.284	.509	.71	25.5
rs2070951			Control	414	213	173	28			.37	27.7
ZBTB20	JPN_1st		Case	537	234	245	58	.494	.420	.61	33.6
rs9883949			Control	423	169	211	43			.05	35.1
ST6GAL2	JPN_1st	rs2241991	Case	533	224	246	63	.936	.814	.72	34.9
rs1448110		$r2 = 1$	Control	409	169	196	44			.25	34.7
PIP5K1B	JPN_1st		Case	536	173	275	88	.550	.764	.22	42.1
rs1414944			Control	420	145	208	67			.6	40.7
EPHA6	JPN_1st		Case	539	146	274	119	.877	.949	.65	47.5
rs727229			Control	419	110	217	92			.44	47.9
KCNH5	JPN_1st		Case	539	169	265	105	.224	.476	.95	44.1
rs10141458			Control	413	142	201	70			.94	41.3
AJAP1	JPN_1st	rs242056	Case	—	—	—	—	—	—	—	—
rs2071999		$r2 = .46$	Control	418	191	161	66			.0016	35.0

Bold numbers represent significant *p* value.

JPN_1st, first Japanese sample; JPN_2nd, second Japanese sample; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; M, major allele, m, minor allele; SNP, single nucleotide polymorphism; UK, United Kingdom sample.

[a]Based on one-tailed analysis.

antipsychotics have no realistic prospect of attaining the sorts of levels of significance suggested for genomewide significance (7.2×10^{-8} or 1×10^{-7}) (20,21). Although it is at least possible that the typical effects on gene expression of drugs may be much more substantial than that of SNPs on disease risk, broadly similar balances of power and type I error also apply to our genomewide expression study. Therefore, with the aim of prioritizing our findings, we attempted to cross-validate the top findings from our study using independent approaches as suggested (4). Our methods of prioritizing our findings were based on two hypotheses; one that the most highly significant sets of SNPs from our pharmacogenetic study of risperidone are likely to be enriched among genes whose expression is altered by that drug (and vice versa), the other that SNPs related to drug response may also be enriched among SNPs associated with disease. To what extent these hypotheses are correct is currently unknown.

From our data, we found 14 markers in genes that showed some degree of overlapping support in the pharmacogenomics

Table 5. Meta-Analysis of rs9389370 in PDE7B

Analysis	Sample	OR	95% CIs Lower Limit	95% CIs Upper Limit	*p* Value
	JPN_1st	1.26	1.03	1.54	**.0255**
	JPN_2nd	1.22	1.01	1.48	**.0214**[a]
	UK	1.11	.967	1.28	.0672[a]
Meta (Replication)	JPN_2nd+UK	1.15	1.03	1.29	**.0082**[a]
Meta (All)	JPN1st+JPN2nd+UK	1.17	1.06	1.30	**.0014**

Bold number represent significant *p* value.

CI, confidence interval; JPN_1st, first Japanese sample; JPN_2nd, second Japanese sample; OR, odds ratio; UK, United Kingdom Sample.

[a]Based on one-tailed analysis.

and mouse expression experiments. These top convergent candidate genes have no previous support for association with schizophrenia or risperidone response and are thus novel candidates for antipsychotic response. However, at present, they have no clinical utility in terms of predicting treatment response, and independent replication using other samples will be required. Moreover, even if replicated, the potential clinical utility for pharmacogenetics is questionable because the effect sizes in each case are small, although it is conceivable given the limited coverage of each gene that the true functional variants have much stronger effects.

Another method for prioritizing genes from genomewide data are to apply a gene ontology (GO) based approach to investigate whether sets of findings tend to converge on particular biological pathways or functions. Our previous experience of GO category analysis suggests that with respect to genetic data, these require large data sets (22). Nevertheless, in response to an anonymous reviewer's comments, for interested readers, we provide the results of our GO category analyses based on ALIGATOR (22) and David Bioinformatics Resources 2008 (http://david.abcc.ncifcrf.gov/) in Supplement 1. Although a number of categories were observed to be significant in each analysis (Tables S6 and S7 in Supplement 1), there is no overlap between the results of the two analytic approaches. Moreover, our favored approach based on ALIGATOR did not reveal any categories that were significant after correction for multiple testing, so it is likely that all of those findings represent chance positives.

Possible Predictor SNPs for Response to Risperidone

In this study, several genes were detected as possible novel predictors for treatment response to risperidone: *ATP2B2, HS3ST2, UNC5C, BAG3, PDE7B, PAICS, PTGFRN, NR3C2, ZBTB20, ST6GAL2, PIP5K1B, EPHA6, KCNH5*, and *AJAP1*. Because the multiple testing burden in SNPs is more severe, our primary analysis included selecting genes based on the more stringent thresholds in the pharmacogenomics data (Table 2) and were additionally shown to have altered expression in the mouse expression study. However, in response to review, we additionally provide data for much more weakly associated SNPs that have highly significant expression changes in the mouse brain (Table 3). Given the weak evidence for most of the latter group of SNPs, we think those are most likely to be chance positives but report the findings for others to test.

Among genes of particular interest in Table 2 is *ATP2B2*, which encodes one of four isoforms of the plasma membrane Ca^{2+} pumps of mammalian cells, showed both the strongest statistical association with treatment response ($p = 1.60 \times 10^{-5}$) and was among those genes that had the strongest association with differential expression because of exposure to risperidone ($p = .00071$). The product of this gene is thought to be involved in neurodevelopment (23) because of its influence on Ca^{2+} homeostasis and Ca^{2+} signaling. This in turn regulates multiple neuronal functions, including synaptic transmission, plasticity, and cell survival (24). Interestingly, several of the other genes with convergent evidence for a role in risperidone response might also be related to neurodevelopment via association with netrin (*UNC5C*) (25,26), interaction with heat shock proteins (*BAG3*) (27,28), cyclic adenosine monophosphate (cAMP) systems (*PDE7B*; details discussed later), glucocorticoids (*NR3C2*) (29), and ephirin (*EPHA6*) (30). Given the neurodevelopmental hypothesis of schizophrenia (31) and evidence that second-generation antipsychotics, including risperidone, have neurogenic actions in hippocampus and PFC (32), our findings suggest that genes involved in the regulation of neurodevelopment or neurogenesis are candidate genes for treatment response in schizophrenics, as well as for schizophrenia per se.

PDE7B Is Candidate Gene Either for Treatment Response and Susceptibility for Schizophrenia

We pursued the top findings from Study 3 to see whether the findings with best convergent evidence (human and mouse) for relevance to risperidone response might also influence susceptibility to schizophrenia. After correction for multiple testing, we found evidence for association between disease status and *PDE7B*, which was therefore the only gene supported across all study designs.

Phosphodiesterases (PDEs) are central in regulating degradation of cAMP and cyclic guanosine monophosphate (cGMP), which are important second messengers for many cellular functions (33). There are 21 known genes encoding PDEs in human, spread across 11 distinct PDE families (*PDE1* to *PDE11*). Among these, *PDE4B* has been reported as a candidate susceptibility gene for schizophrenia. This was on the basis of a translocation found in two affected members of a single pedigree and the observation that the protein interacts with Disrupted in Schizophrenia 1 (*DISC1*), itself another strong candidate gene for schizophrenia and affective disorders (34). Elevation of cellular cAMP leads to dissociation of *PDE4B* from DISC1 and an increase in *PDE4B* activity (34).

PDE7B degrades cAMP, but not cyclic guanosine monophosphate (cGMP), and is predominantly expressed in brain (33). To date, no direct evidence for association of *PDE7B* with schizophrenia has been reported; however, several findings provide some functional plausibility to our results. First, mRNA for *PDE7B* and dopamine D1, D2, and D3 receptors show a similar pattern of distribution, and it is thought that the dopamine D1 receptor activates *PDE7B* through the cAMP pathway (35). Second, *PDE7B* maps to 6q23-24, one of the most significant linkage regions for schizophrenia (OMIM %600511; SCZD3) (36). Lastly, association has recently been reported to the *Abelson Helper Integration Site 1* (AHI1) and *Family with sequence similarity 54 A* (*FAM54A*), which are respectively in the 5′ and 3′ regions of *PDE7B*. However, it should be noted that in those studies, SNPs in *PDE7B* were not associated with disease (37,38).

PDE inhibitors have recently emerged as being of interest as therapeutic agents for neuropsychiatric disorders, such as schizophrenia, depression, and dementia (33). Our results indicate that among these, drugs acting on *PDE7B* may be of particular value in schizophrenia, although particularly for clinical applications, our results should be treated with caution until independent replications have been reported.

Limitations and Conclusion

The major limitation in this study is that the sample sizes we used for the genomewide pharmacogenetics and gene expression studies are small. In particular, the phramacogenetics study is only highly powered to detect effects that are much larger than typical of common susceptibility alleles for diseases to date. This is less of a limitation with respect to one major goal of pharmacogenetics, namely, the identification of common markers with sufficiently large effects to be of value in guiding therapeutics. Our study suggests that in such large common effects may not exist, although being based on one of the earliest chips, the coverage of genes is incomplete, and it would be desirable to repeat this experiment with a denser set of SNPs. The extent to which clinical heterogeneity is likely to have an impact on treatment response, and therefore power to detect association to that response, is also currently

unknown. We presume it is likely to play some role, as is the possibility of imperfect adherence to treatment. More subtle effects are of potential value in informing about drug mechanisms relevant to therapeutic response, and here, power is limited. Given that limitation, we tried to minimize false negatives through the use of relaxed significance criteria but tried to control false positives by combining expression and genetic data. Nevertheless, replication of our findings are required. Our follow-up observation of association between *PDE7B*, a novel candidate gene, and schizophrenia does, however, suggest that the use of convergent data may have successfully enriched for findings of true relevance to schizophrenia and its response to treatment.

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Supplementary material cited in this article is available online.

1. Saha S, Chant D, McGrath J (2007): A systematic review of mortality in schizophrenia: Is the differential mortality gap worsening over time? *Arch Gen Psychiatry* 64:1123–1131.
2. Arranz MJ, de Leon J (2007): Pharmacogenetics and pharmacogenomics of schizophrenia: A review of last decade of research. *Mol Psychiatry* 12:707–747.
3. McCarthy MI, Abecasis GR, Cardon LR, Goldstein DB, Little J, Ioannidis JP, *et al.* (2008): Genome-wide association studies for complex traits: Consensus, uncertainty and challenges. *Nat Rev Genet* 9:356–369.
4. Le-Niculescu H, Balaraman Y, Patel S, Tan J, Sidhu K, Jerome RE, *et al.* (2007): Towards understanding the schizophrenia code: An expanded convergent functional genomics approach. *Am J Med Genet B Neuropsychiatr Genet* 144B:129–158.
5. Gorwood P, Foot E (2006): Introduction on psychopharmacogenetics. In: Gorwood P, Hamon M, editors. *Psychopharmacogenetics*. New York: Springer, 1–24.
6. Yamanouchi Y, Iwata N, Suzuki T, Kitajima T, Ikeda M, Ozaki N (2003): Effect of DRD2, 5-HT2A, and COMT genes on antipsychotic response to risperidone. *Pharmacogenomics J* 3:356–361.
7. Ikeda M, Yamanouchi Y, Kinoshita Y, Kitajima T, Yoshimura R, Hashimoto S, *et al.* (2008): Variants of dopamine and serotonin candidate genes as predictors of response to risperidone treatment in first-episode schizophrenia. *Pharmacogenomics* 9:1437–1443.
8. Lane HY, Lee CC, Liu YC, Chang WH (2005): Pharmacogenetic studies of response to risperidone and other newer atypical antipsychotics. *Pharmacogenomics* 6:139–149.
9. O'Donovan MC, Craddock N, Norton N, Williams H, Peirce T, Moskvina V, *et al.* (2008): Identification of loci associated with schizophrenia by genome-wide association and follow-up. *Nat Genet* 40:1053–1055.
10. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, *et al.* (2007): PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81:559–575.
11. Olshen AB, Jain AN (2002): Deriving quantitative conclusions from microarray expression data. *Bioinformatics* 18:961–970.
12. Mukherjee S, Tamayo P, Rogers S, Rifkin R, Engle A, Campbell C, *et al.* (2003): Estimating dataset size requirements for classifying DNA microarray data. *J Comput Biol* 10:119–142.
13. Listgarten J, Damaraju S, Poulin B, Cook L, Dufour J, Driga A, *et al.* (2004): Predictive models for breast cancer susceptibility from multiple single nucleotide polymorphisms. *Clin Cancer Res* 10:2725–2737.
14. Morley M, Molony CM, Weber TM, Devlin JL, Ewens KG, Spielman RS, *et al.* (2004): Genetic analysis of genome-wide variation in human gene expression. *Nature* 430:743–747.
15. Tomita Y, Ikeda M, Mutoh H, Inada T, Iwata N, Ozaki N, *et al.* (2007): Association study between apolipoprotein L and schizophrenia by exhaustive and rule-based combination analysis for identification of multilocus interactions. *J Biosci Bioeng* 103:303–310.
16. Ge D, Zhang K, Need AC, Martin O, Fellay J, Urban TJ, *et al.* (2008): WGAViewer: Software for genomic annotation of whole genome association studies. *Genome Res* 18:640–643.
17. Page GP, Edwards JW, Gadbury GL, Yelisetti P, Wang J, Trivedi P, *et al.* (2006): The PowerAtlas: A power and sample size atlas for microarray experimental design and research. *BMC Bioinform* 7:84.
18. Lavedan C, Licamele L, Volpi S, Hamilton J, Heaton C, Mack K, *et al.* (2009): Association of the NPAS3 gene and five other loci with response to the antipsychotic iloperidone identified in a whole genome association study. *Mol Psychiatry* 14:804–819.
19. Wellcome Trust, Case Control Consortium (2007): Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* 447:661–678.
20. Carlson CS, Eberle MA, Kruglyak L, Nickerson DA (2004): Mapping complex disease loci in whole-genome association studies. *Nature* 429:446–452.
21. Dudbridge F, Gusnanto A (2008): Estimation of significance thresholds for genomewide association scans. *Genet Epidemiol* 32:227–234.
22. Holmans P, Green EK, Pahwa JS, Ferreira MA, Purcell SM, Sklar P, *et al.* (2009): Gene ontology analysis of GWA study datasets provides insights into the biology of bipolar disorder. *Am J Hum Genet* 85:13–24.
23. Kurnellas MP, Lee AK, Li H, Deng L, Ehrlich DJ, Elkabes S (2007): Molecular alterations in the cerebellum of the plasma membrane calcium ATPase 2 (PMCA2)-null mouse indicate abnormalities in Purkinje neurons. *Mol Cell Neurosci* 34:178–188.
24. Wojda U, Salinska E, Kuznicki J (2008): Calcium ions in neuronal degeneration. *IUBMB Life* 60:575–590.
25. Kruger RP, Lee J, Li W, Guan KL (2004): Mapping netrin receptor binding reveals domains of Unc5 regulating its tyrosine phosphorylation. *J Neurosci* 24:10826–10834.
26. Bonnin A, Torii M, Wang L, Rakic P, Levitt P (2007): Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nat Neurosci* 10:588–597.
27. Calabrese V, Scapagnini G, Ravagna A, Stella G, Butterfield DA (2002): Molecular chaperones and their roles in neural cell differentiation. *Dev Neurosci* 24:1–13.
28. Frebel K, Wiese S (2006): Signalling molecules essential for neuronal survival and differentiation. *Biochem Soc Trans* 34:1287–1290.
29. Sousa N, Cerqueira JJ, Almeida OF (2008): Corticosteroid receptors and neuroplasticity. *Brain Res Rev* 57:561–570.
30. Martinez A, Soriano E (2005): Functions of ephrin/Eph interactions in the development of the nervous system: Emphasis on the hippocampal system. *Brain Res Rev* 49:211–226.
31. Weinberger DR (1995): From neuropathology to neurodevelopment. *Lancet* 346:552–557.
32. Newton SS, Duman RS (2007): Neurogenic actions of atypical antipsychotic drugs and therapeutic implications. *CNS Drugs* 21:715–725.
33. Halene TB, Siegel SJ (2007): PDE inhibitors in psychiatry—Future options for dementia, depression and schizophrenia? *Drug Discov Today* 12:870–878.
34. Millar JK, Pickard BS, Mackie S, James R, Christie S, Buchanan SR, *et al.* (2005): DISC1 and PDE4B are interacting genetic factors in schizophrenia that regulate cAMP signaling. *Science* 310:1187–1191.
35. Sasaki T, Kotera J, Omori K (2004): Transcriptional activation of phosphodiesterase 7B1 by dopamine D1 receptor stimulation through the cyclic AMP/cyclic AMP-dependent protein kinase/cyclic AMP-response element binding protein pathway in primary striatal neurons. *J Neurochem* 89:474–483.
36. Lewis CM, Levinson DF, Wise LH, DeLisi LE, Straub RE, Hovatta I, *et al.* (2003): Genome scan meta-analysis of schizophrenia and bipolar disorder. Part II: Schizophrenia. *Am J Hum Genet* 73:34–48.
37. Amann-Zalcenstein D, Avidan N, Kanyas K, Ebstein RP, Kohn Y, Hamdan A, *et al.* (2006): AHI1, a pivotal neurodevelopmental gene, and C6orf217 are associated with susceptibility to schizophrenia. *Eur J Hum Genet* 14:1111–1119.
38. Ingason A, Sigmundsson T, Steinberg S, Sigurdsson E, Haraldsson M, Magnusdottir BB, *et al.* (2007): Support for involvement of the AHI1 locus in schizophrenia. *Eur J Hum Genet* 15:988–991.

Copy Number Variation in Schizophrenia in the Japanese Population

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Background: Copy number variants (CNVs) have been shown to increase the risk to develop schizophrenia. The best supported findings are at 1q21.1, 15q11.2, 15q13.3, and 22q11.2 and deletions at the gene neurexin 1 (*NRXN1*).

Methods: In this study, we used Affymetrix 5.0 arrays to investigate the role of rare CNVs in 575 patients with schizophrenia and 564 control subjects from Japan.

Results: There was a nonsignificant trend for excess of rare CNVs in schizophrenia ($p = .087$); however, we did not confirm the previously implicated association for very large CNVs (>500 kilobase [kb]) in this population. We provide support for three previous findings in schizophrenia, as we identified one deletion in a case at 1q21.1, one deletion within *NRXN1*, and four duplications in cases and one in a control subject at 16p13.1, a locus first implicated in autism and later in schizophrenia.

Conclusions: In this population, we support some of the previous findings in schizophrenia but could not find an increased burden of very large (>500 kb) CNVs, which was proposed recently. However, we provide support for the role of CNVs at 16p13.1, 1q21.1, and *NRXN1*.

Key Words: Deletion, duplication, *NRXN1*, 16p13.1, 1q21.1, schizophrenia

Copy number variations (CNVs) are deletions and duplications of DNA ranging from a kilobase (kb) to several megabases (Mb). Recently, rare CNVs were shown to play a role in the etiology of a number of neuropsychiatric disorders, particularly schizophrenia, autism, and mental retardation (1).

Several studies have reported a greater prevalence of rare CNVs in people with schizophrenia (2-4). However, some have found no such excess (5,6) and even among the positive studies, there is marked variation in the magnitude of the observed effect. For example, in the International Schizophrenia Consortium (ISC) study (4), cases had only a 1.15-fold excess of rare CNVs, rising to 1.67-fold for deletions greater than 500 kb. An increase only among very large CNVs (>1 Mb) in cases was found by Kirov *et al.* (7). Another study showed an odds ratio of 3.37 for CNVs, rising to 4.82 for early-onset schizophrenia (2). This may, in part, reflect differences in the sensitivity of CNV assays, definitions of low-frequency CNVs, or variation in the phenotypic composition of the samples, as cases with early onset or lower IQ were particularly enriched for CNVs in one study (2).

In addition to increased CNV burden, a number of specific CNVs have been associated with schizophrenia (4,7,8). There is strong replicated evidence for deletions at 1q21.1, 15q11.2, 15q13.3, and 22q11.2 and emerging evidence for duplications at 16p13.1 (4,7). Deletions of the neurexin 1 gene (*NRXN1*) have also been reported in multiple studies on schizophrenia (2,6,7,9,10). Given the discrepancy in estimates of the effect size of CNV burden as a risk factor for schizophrenia and in particular the absence of association in the only Asian sample reported to date (5), we aimed to test for an excess burden of CNVs in a population from Japan. We also sought supportive evidence for a contribution for the specific loci listed above.

Methods and Materials

We analyzed 1139 age- and gender-matched unrelated subjects of Japanese ethnicity (575 schizophrenic patients and 564 control subjects). Control subjects were members of the general public who had no personal history of mental disorders. This was ascertained during face-to-face interviews where subjects were asked if they had suffered an episode of depression, mania, or psychotic experiences or if they had received treatment for any psychiatric disorder. Patients were entered into the study if they 1) met DSM-IV criteria for schizophrenia; 2) were physically healthy and had normal routine laboratory tests; and 3) had no mood disorders, substance abuse, neurodevelopmental disorders, epilepsy, or known mental retardation. Consensus diagnoses were made by at least two experienced psychiatrists according to DSM-IV criteria on the basis of unstructured interviews with patients and families and review of medical records. After description of the study, written informed consent was obtained from each subject. This study was approved by the ethics committees of each participating university.

We used Affymetrix 5.0 Arrays (Affymetrix, Santa Clara, California), following the manufacturer's protocols (http://www.affymetrix.com). This array includes 470K single nucleotide polymorphism (SNP) probes and 420K nonpolymorphic probes. The CNVs discussed below in more detail (at *NRXN1*, 1q21.1, and 16p13.1) were validated using the Illumina HumanHap 660W- or 610-quad bead arrays (Illumina, San Diego, California), following the manufacturer's protocols (http://www.illumina.com).

Copy number variations were called using the Birdsuite program (http://www.broadinstitute.org/science/programs/medical-and-

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Table 1. Global CNV Burden Analysis

		CNV Burden				CNVs Intersecting Genes			
CNV Type	Size	SCZ	CON	CNV Rate SCZ/CON	*p* Value	SCZ	CON	CNV Rate SCZ/CON	*p* Value
Deletions and Duplications	All	567	485	1.1/.95	.087	382	320	.74/.62	.084
	100–200 kb	285	229	.55/.45	.046	182	145	.35/.28	.074
	200–500 kb	221	192	.43/.37	.20	150	134	.29/.26	.30
	500 kb–1 Mb	48	52	.09/.10	.72	38	32	.07/.06	.31
	>1 Mb	13	12	.025/.023	.52	12	9	.023/.018	.35
Deletions Only	All	174	157	.34/.31	.30	91	87	.18/.17	.46
	100–200 kb	98	84	.19/.16	.26	52	47	.10/.09	.38
	200–500 kb	65	60	.13/.12	.42	29	35	.06/.07	.79
	500 kb–1 Mb	8	8	.015/.016	.62	8	3	.015/.006	.12
	>1 Mb	3	5	.006/.010	.86	2	2	.004/.004	.69
Duplications Only	All	393	328	.76/.64	.10	291	233	.56/.45	.075
	100–200 kb	187	145	.36/.28	.070	130	98	.25/.19	.071
	200–500 kb	156	132	.30/.26	.21	121	99	.23/.19	.18
	500 kb–1 Mb	40	44	.077/.086	.73	30	29	.058/.057	.53
	>1 Mb	10	7	.019/.014	.33	10	7	.019/.014	.33

p values are one-tailed and based on 10,000 permutations.
CNV, copy number variation; CON, control; kb, kilobase; Mb, megabase; SCZ, schizophrenia.

population-genetics/birdsuite/birdsuite-0) (11). The software first assigns copy number across regions of known copy number polymorphisms, then calls SNP genotypes (for samples and SNPs believed to have two copies of the locus), then searches for novel CNVs via a hidden Markov model, and generates an integrated sequence and copy number genotype at every locus. It takes into account genotypes within CNVs, e.g., A-null, AAB, and BBB, in addition to AA, AB, and BB calls (11).

We observed a batch effect, similar to what we reported in our previous study (7): arrays from different batches gave poor results if analyzed together. Therefore, we identified the batches and analyzed together samples within the same batch, as recommended in the Birdsuite manual (11). After initial filtering for quality control, using the standard criteria implemented in the Genotyping Console software (www.affymetrix.com), including quality control call rate (>86%), SNP call rate (>95%), and population stratification based upon principal components analysis, 1107 samples (560 cases and 547 control subjects) were retained for further analysis. They had 16,466 CNVs (eight subjects showed no CNVs). We then excluded low-confidence CNVs (logarithm of odds <10), CNVs <100 kb, and those with the lowest 1% density for probe coverage (52 segments). We removed 50 samples that had high sample-specific measures of noise (variance >2), as those had a mean of 175 CNV segments, indicating they were false-positives. We also removed 17 samples that had more than 20 apparent CNVs (the mean number of CNVs for these samples was 156), as such samples are also likely to be false-positives (4,7). The filtering left 1032 samples: 519 cases aged 43.4 ± 14.7 years (258 male and 261 female cases) and 513 control subjects aged 43.8 ± 14.5 years (252 male and 261 female control subject). They had a total of 5180 CNVs (~5 per person). Finally, following previous studies (4,7), we filtered common CNVs (found in >1% of the total sample), leaving 1052 rare and larger than 100 kb CNVs for the analysis (~1 per person). This filtering was also performed for CNVs found at >5% in the total sample, resulting in 2081 CNVs. All CNVs that passed filtering and were present in <1% of the samples are available as an University of California, Santa Cruz (UCSC)-friendly file in Supplement 1.

Copy number variations were considered to colocalize if they overlapped by at least 50% of their length, as implemented in PLINK ver1.0.4 (http://pngu.mgh.harvard.edu/~purcell/plink/) (12) as used for the analysis of CNV loci in previous datasets (4,7).

Results

The numbers of rare CNVs stratified by size in cases and control subjects are listed in Table 1. Overall, we found an excess of CNVs in subjects with schizophrenia (case-control ratio = 1.16). Although not significant ($p = .087$, one-tailed permutation test), this is similar to that reported by the largest CNV study (4) where the case-control ratio was 1.15. The effect in that study (4) was coming mostly from deletions >500 kb and duplications in the 100 kb to 200 kb range. No subcategory of CNV defined by size or nature (deletion or duplication) was significantly associated with disease in the current study. Copy number variations in the 100 kb to 200 kb range were more common in cases than in control subjects, ratio = 1.23, $p = .046$; however, this does not survive correction for the multiple testing of four size ranges and two types of CNVs. Duplications (but not deletions) within the same size range were the most significantly associated general category in the ISC study ($p = 1 \times 10^{-4}$) with virtually an identical effect (case-control ratio = 1.26). However, no specific duplications of this size overlapped between the two studies (4). We did not replicate the finding of an excess of large deletions (>500 kb) that was reported in the ISC study (4) or of deletions and duplications >1 Mb reported in the study by Kirov *et al.* (7).

Analysis of the burden of CNVs intersecting genes revealed no significant excess of genes disrupted in subjects with schizophrenia, either overall or for any size range, with similar trends to the results from the general burden analysis (Table 1).

We repeated the same analysis for CNVs <5% in the sample. This resulted in 388 and 368 deletions and 698 and 627 duplications in cases and control subjects, respectively. The trends between cases and control subjects were virtually identical to those in Table 1 (data not presented).

Although we found no enrichment of large CNVs in schizophrenia, we present the details of large CNVs (>1 Mb) in Table S1 in Supplement 2 because these have been most consistently implicated by others (4,7). Of those, one case but no control subjects had a deletion on 1q21.1, one of the most convincingly