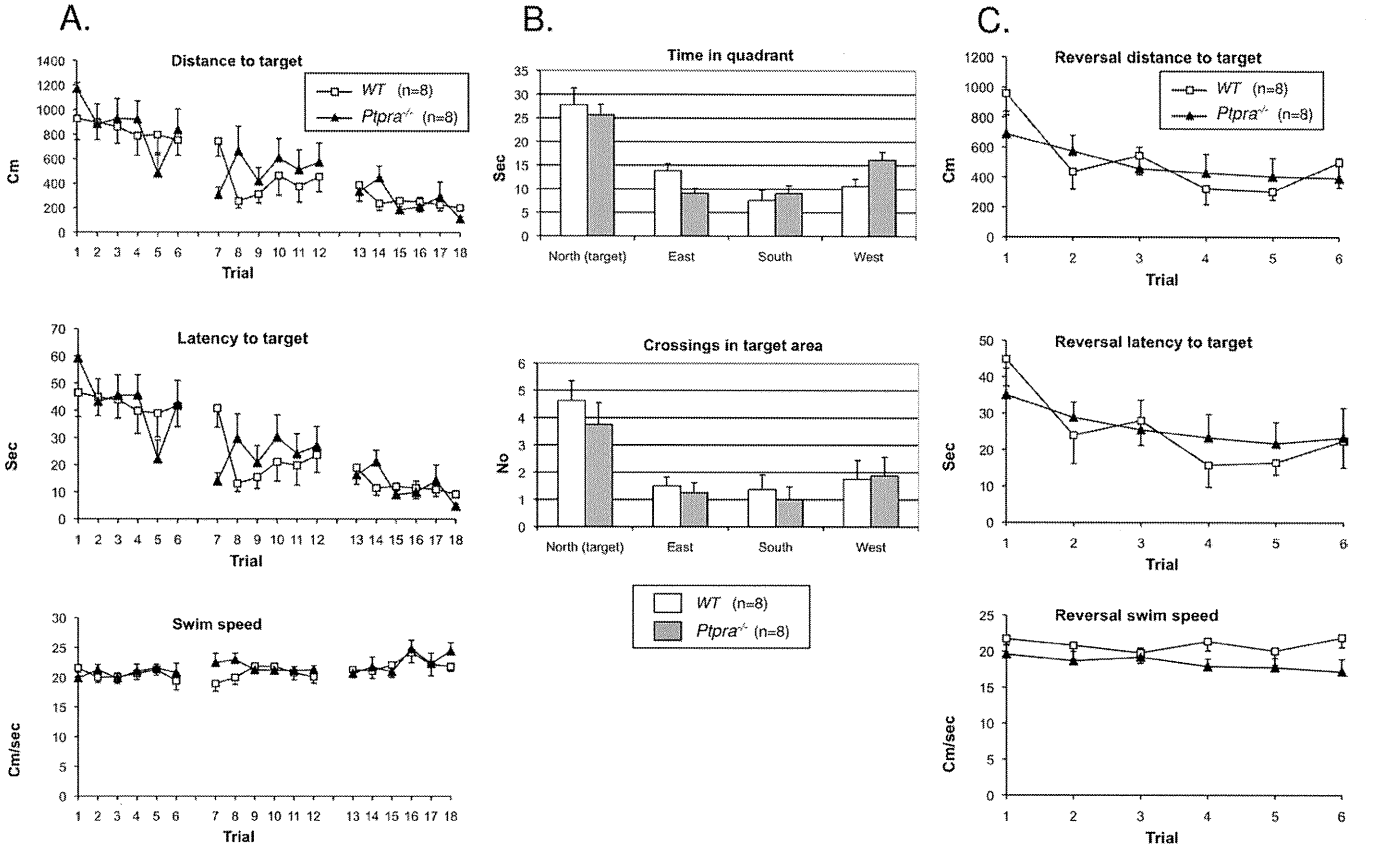

Figure 2. Morris water-maze analysis of wildtype (WT) and *Ptpra*$^{-/-}$ mice. **(A)** Training: swim distance to target, escape latency, and swim speed. Both WT ($n = 8$) and *Ptpra*$^{-/-}$ ($n = 8$) used shorter distances to locate the hidden platform over trials. No effect of genotype or genotype × trial interaction on distance to platform, escape latency, or swim speed was found. One-way repeated-measures analysis of variance (ANOVA) WT: $F(17,119) = 4{,}982$; $p < .001$ and *Ptpra*$^{-/-}$: $F(17,119) = 5.477$; $p < .001$). Two-way repeated-measures ANOVA found no effect of genotype for distance [$F(1,238) = .803$; $p = .385$] and no genotype × trial interaction [$F(17,238) = 1.231$; $p = .241$]. **(B)** Probe test: time in quadrants and crossings in platform area. Both genotypes spent significantly longer time in the northern quadrant where the platform used to be located than in other quadrants and had more crossings in the area previously occupied by the platform than in areas of identical size and position in the other quadrants. Time spent in northern quadrant compared with other quadrants ($p = .006$ or less, two-way repeated measures ANOVA, Fisher Least Significant Difference post hoc); no effect of genotype [$F(1,42) = 1.340$; $p = .266$] or genotype × quadrant interaction [$F(3,42) = 1.801$; $p = .162$]. Number of crossings in the area where the platform was during training (D = 9 cm) compared with crossings in areas of equal size and position in the other quadrants [$F(3,42) = 9.199$; $p < .001$]; no effect of genotype [$F(1,42) = 1.317$; $p = .27$] or genotype × quadrant interaction [$F(3,42) = .203$; $p = .894$]. Post hoc analysis revealed significantly more crossings in the target area for both groups ($p = .047$ or less) compared with crossings in areas equal in size and position in the other quadrants. **(C)** Reversal learning: swim distance to target, escape latency and swim speed during reversal learning. Two-way repeated measures ANOVA on distance to target showed significant effect of trial [$F(5,70) = 3.383$; $p = .009$] but no effect of genotype [$F(1,70) = .0591$; $p = .811$] or genotype × trial interaction [$F(5,70) = .757$; $p = .584$]. No effect of genotype or genotype × trial interaction was found on escape latency or swim speed.

We found that mRNA levels of 8/9 tested oligodendrocyte lineage marker genes were significantly ($p < .05$ to $< .001$) reduced (range 53%–67%) in *Ptpra*$^{-/-}$ mice (Figure 3). This phenomenon applied not only to an oligodendrocyte marker (MBP) but also to genes that are functionally involved in oligodendrocyte differentiation (e.g., *Sox10, Qk*), and oligodendrocyte lineage genes with well-documented reduced expression in human SZ brain (e.g., *Cnp1, Cldn11, Qk*) (48) or that are genetically associated with SZ, such as *Erbb4* (9) and *Qk* (54).

A Polymorphism in Human *PTPRA* Demonstrates Close Genetic Association with Schizophrenia Susceptibility

Our finding that ablation of mouse *Ptpra* mimics neuropsychological and gene expression abnormalities associated with SZ prompted us to pursue a genetic link between human *PTPRA* and SZ risk. *PTPRA* maps to 20p13, identified as a susceptibility locus by low-resolution linkage studies in two human groups (34,35). We pursued more detailed single nucleotide polymorphism (SNP) fine-mapping analysis on a third population to search for evidence for closer association between SZ and *PTPRA*.

In the first stage, 560 cases and 548 controls were genotyped using the GeneChip Human Mapping 5.0 Array (Affymetrix, Santa Clara, California). Of 21 SNPs genotyped across the *PTPRA* locus, six yielded nominally significant association with SZ (rs6132976, rs6132977, rs6132978, rs1016753, rs1178032, and rs16988201) (best uncorrected $p = .002$). To confirm this association, we performed a replication using an independent sample comprising 850 cases and 829 controls. Based on the linkage disequilibrium (LD) pattern from the first stage analysis, three SNPs (rs1016753, rs1178032, and rs16988201) were selected (rs6132976, rs6132977, and rs6132978 were represented by rs1016753; Figure 4). Analysis of imputation (Table S2 in Supplement 1) and LD pattern within the *PTPRA* locus suggested that the SNPs selected for follow-up capture all ungenotyped SNPs, which increase the risk of developing SZ. In the replication, only rs1016753 showed significant association ($p = .04$), with the same direction of association (Breslow-Day $p = .218$).

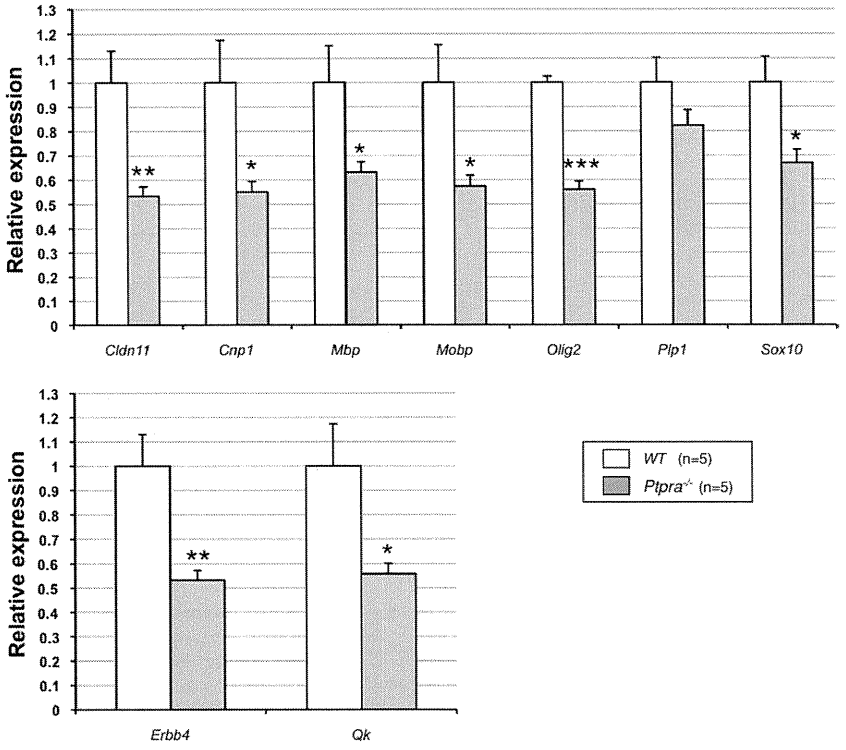

Figure 3. Reduced expression of oligodendrocyte- and myelin-related gene expression in total brain of *Ptpra*$^{-/-}$ mice. Five-month-old animals (five males/genotype) were analyzed by quantitative polymerase chain reaction. Four endogenous control genes (*Actb, B2m, Gusb,* and *Ppia*) were used for normalization. *$p < .05$; **$p < .01$; ***$p < .001$ vs. wildtype (WT).

Pooled analysis of first and second stages (1420 cases, 1377 controls) showed highly significant association of this SNP with SZ ($p = .0008$; Table S3 in Supplement 1).

Reduced *PTPRA* Expression Levels in Dorsolateral and Prefrontal Cortex from Schizophrenia Patients

As an independent approach to explore a possible involvement of *PTPRA* in schizophrenia, we examined its expression level in postmortem samples from patients, compared with healthy control subjects and to patients with bipolar disorder (35 each). The qPCR analysis (Figure 5) showed that *PTPRA* expression was significantly reduced in dorsolateral prefrontal cortex from SZ patients (13% decrease; $p = .018$), with trend-level reductions in samples from patients with bipolar disorder ($p = .078$).

Discussion

This study was prompted by implications of *Ptpra* in developmental processes linked to schizophrenia (neuronal migration, myelination); by RPTPα acting as a signaling subunit for cell adhesion molecules (NCAM and CHL1) with genes that have been related to SZ risk; and by the mapping of a SZ locus close to *PTPRA*. The avenues we explored provide independent lines of convergent evidence linking RPTPα to SZ: typical changes in neuropsychological parameters in RPTPα-deficient mice, association of the human gene with disease risk, and reduced cortical *PTPRA* expression in SZ patients.

Behavioral Characteristics of *Ptpra*$^{-/-}$ Mice Relevant to Schizophrenia

We demonstrate that *Ptpra* LOF is associated with enhanced MAMPH responsiveness (Figure 1A), defective sensorimotor gating as measured by PPI (Figure 1B), and failure to habituate to a startle response (Figure 1E). All these endpoints implicate an SZ-like profile based on current clinical knowledge. The deficits could not be accounted for by obvious sensorimotor deficits, because *Ptpra*$^{-/-}$ mice did not display differences in motility, rotarod, and beam-walk tests or in initial startle response.

The enhanced response of *Ptpra*$^{-/-}$ mice to MAMPH suggests an augmented dopaminergic system (40). In a previous study, Skelton *et al.* (55) failed to detect an altered amphetamine response in a different *Ptpra*$^{-/-}$ strain; this negative result may reflect a different dosing regime (we used 2 mg/kg whereas Skelton *et al.* used 1 mg/kg), or, more likely, a less uniform genetic background. We backcrossed our *Ptpra*$^{-/-}$ allele 10 times into inbred C57Bl/6J. In contrast, the founder animals of Skelton *et al.* were crossed into outbred Black Swiss mice (32,55); the ensuing higher genetic heterogeneity may have made the change in MAMPH responsiveness associated with loss of *Ptpra* function difficult to detect. In the absence of studies on the effect of *Ptpra* ablation on dopamine receptor expression, agonist binding, or activity, it seems premature to speculate about the mechanism of the MAMPH effect. Strikingly, haloperidol-induced catalepsy requires the *Fyn* gene, and this drug activates the FYN kinase in striatum (56). Because Fyn is a well-established RPTPα target (25,29,32,33), defective Fyn activation in absence of RPTPα may fail to inhibit striatal dopamine signaling.

Contrasting with the increased MAMPH responsiveness in *Ptpra*$^{-/-}$ mice, we observed no effect of *Ptpra* status on sensitivity to the glutamate antagonist MK-801. Although somewhat surprising given links between RPTPα and NMDA-R (30,31), our negative finding may merely be a function of the selected dose (.2 mg/kg) because no hyperactive response was seen in the control mice either. In our hands, .2 mg/kg MK-801 reproducibly induces hyperactivity in outbred NMRI mice (not shown). Therefore, pending further dose exploration in the *Ptpra*$^{-/-}$ mice, we suggest the failure to detect changes in MK-801 responsiveness is equivocal.

Ptpra$^{-/-}$ mice aged 2.5 months show a pronounced PPI deficit (Figure 1B). "Inhibitory failure" revealed by defective PPI is considered a correlate of defects in acute attention and gating associated with psychiatric diseases, including SZ. As an endophenotype, PPI is

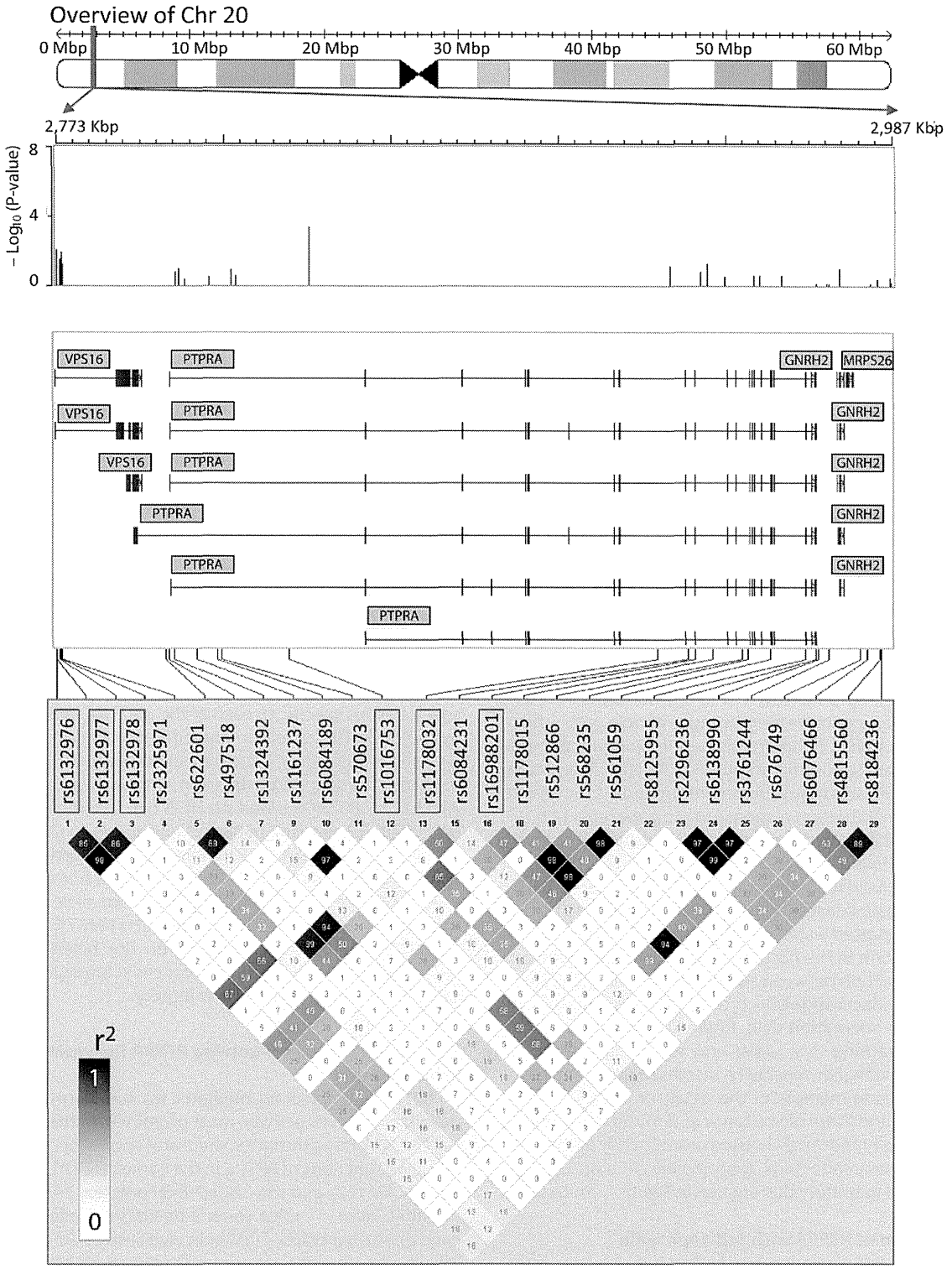

Figure 4. Genetic association of single nucleotide polymorphisms around and in the *PTPRA* gene with schizophrenia. Red boxes indicate nominally associated single nucleotide polymorphisms in the first-stage analysis (genome-wide association study screening sample). r^2 is the correlation coefficient between the two loci. The numbers are correlation coefficients calculated based on the genome-wide association study sample.

decreased in nonaffected relatives of SZ patients, suggesting it may be a proximal indicator of genetic susceptibility (39). Interestingly, the PPI deficit in *Ptpra*$^{-/-}$ mice did not persist when the same mice were retested at 5 months of age (Figure 1D). This restriction of the deficit to early adulthood suggests involvement of compensatory changes with aging. Because knockout of *Ptpra* alone cannot sustain this phenotype, *Ptpra*$^{-/-}$ mice may provide a point of entry to identify genetic or environmental parameters that will specify PPI

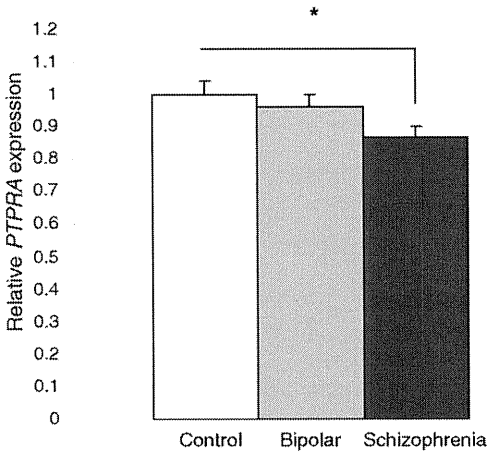

Figure 5. Reduced *PTPRA* expression levels in dorsolateral prefrontal cortex specimens from schizophrenia patients. Thirty-five patient samples for each category (healthy control subjects, patients with schizophrenia, and patients with bipolar disorder) were analyzed by quantitative polymerase chain reaction. Four endogenous control genes (*ACTB, GAPD, GUSB,* and *PPIA*) were used for normalization. *$p < .05$ versus control.

extinction or exacerbation. Such studies may provide insight into factors and processes that determine SZ prognosis.

The startle response in mice displays plasticity in terms not only of gating but also of habituation. Whereas *Ptpra*$^{-/-}$ and WT mice had an equivalent startle response at 2.5 months (Figure 1C), we observed habituation by 5 months in WT but not *Ptpra*$^{-/-}$ mice (Figure 1E). This finding is interesting because people with schizophrenia also have a deficit in habituation—for example, the eyeblink reflex in response to auditory stimuli (57). A deficit in preattentive inhibitory mechanisms to extraneous information is thought to underlie an altered habituation response in schizophrenia (42).

We found no deficit in Morris water-maze acquisition or memory in *Ptpra*$^{-/-}$ mice. Here again, our findings seem to differ from Skelton *et al.* (55) who did report such a deficit. The different genetic background may again constitute a possible explanation. In addition, Skelton *et al.* reported water-maze defects only under particular conditions (platform in the northeast, but not in the southwest quadrant); this interaction between spatial environment and genotype may primarily reflect subtle effects of *Ptpra* status on sensitivity to spatial cues or on orientation skills. Interestingly, *Ptpra*$^{-/-}$ mice display defective learning in a radial arm water-maze test (27), which may reflect the higher sensitivity, increased requirement for short-term and working memory of this assay, or both. Taken together, our findings and those of Skelton *et al.* (55) and Petrone *et al.* (27) indicate that the hippocampal system is not overly affected by *Ptpra* LOF despite the architectural abnormalities in hippocampus resulting from radial migratory dysfunction in *Ptpra*$^{-/-}$ mice (27).

Genetic Association of *PTPRA* with Schizophrenia

KO studies can be confounded by flanking markers from backcrossing or outcrossing or by inadvertent consequences of genome manipulation unrelated to changes in *Ptpra* function (e.g., altered expression of known or unknown flanking genes). A strong case can be made for a direct link between *Ptpra* and the observed phenotypes. Two independent *Ptpra*$^{-/-}$ mice both reveal effects of *Ptpra* knockdown on NMDA-R phosphorylation (27,31); an electrophysiological study shows rescue of the NMDA-R gating defect in *Ptpra*$^{-/-}$ cells by RPTPα expression and mimicking of the defect by antibodies against RPTPα (30). The two lines also show similar effects on SFK-dependent pathways, which can also be rescued by RPTPα expression (33,58,59), or mimicked by RPTPα knockdown (29,60,61). Indeed, many *Ptpra*$^{-/-}$ phenotypes can be clearly linked to deregulation of the two best-established RPTPα substrates, the tyrosine kinases c-Src and Fyn (22,62). Our finding of impaired oligodendrocyte marker expression in *Ptpra*$^{-/-}$ mice is again consistent with studies using an independent *Ptpra* LOF allele and different assays (29) and with Fyn dysfunction (63).

The collective mouse evidence thus makes *PTPRA* a valid candidate for follow-up study in humans. Accordingly, we report highly significant association of a SNP in *PTPRA* with schizophrenia in a Japanese population. The sample size (~ 2600) is enough to detect mild to moderate effects of SNPs, and the evidence of *PTPRA* association is robust because the two-stage analysis reduces the potential for type I error. On the basis of LD analysis in the first stage, we selected rs1016753 as a representative SNP for rs6132976, rs6132977, and rs6132978. Therefore, the association of rs1016753 might reflect possible association of these or other linked SNPs. Because the LD structure of *PTPRA* is relatively loose, we cannot narrow down the associated region to identify the "true" SNPs. Unbiased genome-wide association studies searching for genetic SZ risk determinants have failed to implicate the *PTPRA* locus; our focus on a particular population may have lowered the detection threshold for environmental or genetic reasons.

It remains premature to speculate on the relevance of rs1016753 or linked SNPs for *PTPRA* function. *PTPRA* expression and the choice among alternative splicing events can be surveyed. On the basis of exon array data (not shown), we performed qPCR on immortalized lymphoblastoid cell lines derived from 48 participants in the association study (43 CC carriers, 5 CG carriers), using primers directed against exons specific to each of the 3 *PTPRA* transcripts described by the National Center for Biotechnology Information (NCBI). This revealed significantly increased expression of the NM_080840.2 transcript (as defined by its "exon 1" with physical position Chr20: 2,802,142-2,802,406 based on NCBI B36 assembly), but not of the NM_002836.3 and NM_080841.2 transcripts, in CG compared with CC carriers (Table S4 in Supplement 1). Unfortunately, we were unable to perform a similar analysis on the human brain samples used for Figure 5 because of the low minor allele frequency in this cohort. At the protein level, the effect of rs1016753 allelic status and altered NM_080840.2 expression on the balance between two RPTPα isoforms with known differences in biological activity (64,65) can also be a subject for further inquiry.

Molecular Pathways Involving *PTPRA* Relevant to Schizophrenia

RPTPα is a signaling mediator for surface molecules that are devoid of catalytic activity, most prominently integrins and canonical CAMs. Among the latter, there are reports for *cis* association and signaling functions of RPTPα in the context of TAG-1 (50), contactin (66), NCAM (22), and the CHL1/NB-3 complex (24).

Adult *Ptpra*$^{-/-}$ mice show a decrease of oligodendrocyte lineage gene expression. Further in vivo studies are needed to establish whether this effect is primary or degenerative and whether it is autonomous to the OLG lineage. The Pallen group recently reported decreased MBP protein levels in the brain of P18 *Ptpra*$^{-/-}$ mice and provided strong evidence for a lineage-autonomous role for RPTPα in oligodendrocyte differentiation in vitro, with deficient Fyn activation as a plausible mechanism (29,53). Interestingly, oligodendrocyte Fyn integrates signaling in a complex between contactin-1 and integrins (67), that is, between members of two classes of cell surface molecules that rely on RPTPα for signal transduction (58,66). Abnormalities in oligodendrocyte function are a robust

biological marker of human schizophrenia (48,49), but elucidation of links between myelination and the disease remains more a matter of speculation than of hypothesis testing. A broad question is how to link the white matter abnormalities in patients to the as yet more clinically relevant pharmacologic evidence of neurotransmitter pathway dysfunction. The *Ptpra*$^{-/-}$ model may play a valuable role in exploring this issue. More specifically, manipulation of the *Ptpra* gene will be useful to explore to what extent the neurobehavioral abnormalities result from loss of *Ptpra* function in the neuronal or oligodendroglial lineage and whether or how *Ptpra* dysfunction in one lineage may impact other lineages and neurotransmitter systems.

CAMs are linked to NMDA neurotransmission, the dysfunction of which is also linked to SZ. Long-term potentiation at CA3-CA1 excitatory synapses is reduced in *Chl1*$^{-/-}$ mice (68) and in a hippocampal-specific NCAM knockout (69); NMDA-mediated behavioral alterations have also been observed in these mice (69,70). A recent study links NCAM poly-sialylation to NMDA-R signaling (71). Absence of the NCAM isoform NCAM180 leads to increased lateral ventricle size, one of the most reliable morphological features in brains of schizophrenics, and is often accompanied by cognitive impairments (70). Association studies implicate *NCAM* and *CHL1* in human SZ risk, and LOF of the corresponding genes in mice engenders intriguing phenotypic overlaps with *Ptpra* LOF in terms of cortical radial migration (72), dendrite orientation (24), impaired long-term potentiation (69), and impaired sensorimotor gating/PPI (73). Thus, phenotypes observed in *Ptpra*$^{-/-}$ mice could be mediated by the effect of RPTPα on these molecules.

The best characterized substrate and effector for RPTPα in NCAM- and CHL1/NB-3 signaling complexes is the SFK Fyn. That RPTPα is a net activator of Fyn kinase activity (32,33) would be consistent with phenotypic overlap between LOF in either gene. Like *Ptpra*$^{-/-}$ mice (27,29), *Fyn*$^{-/-}$ mice exhibit abnormal long-term potentiation, spatial learning, radial migration, myelination (62, 74), and myelin gene expression (51). Genetic association of *FYN* with SZ was reported as absent (75), although there are positive data about prefrontal function in patients (76). Interestingly, *Fyn* is required for haloperidol signaling in striatal neurons (56), and platelets from SZ patients show decreased expression and altered *FYN* splicing (77). Fyn also phosphorylates NMDA-R subunits (52). Phosphorylation of NMDA-R subunits is reduced in *Ptpra*$^{-/-}$ mice, and RPTPα associates with and controls gating of NMDA-R (27,30,31). Thus, reduced RPTPα function could contribute to a schizophrenic phenotype through impairment of Fyn activity.

Taken together, one can envision a SZ-relevant pathway as NCAM/CHL1-NB3 → RPTPα → Fyn → NMDA-R. However, this is a speculative working hypothesis. Not only are the links between Fyn and SZ relatively tenuous, there are also important phenotypic differences between *Fyn*$^{-/-}$ *and Ptpra*$^{-/-}$ mice (e.g., in hippocampal structure), RPTPα can act on other SZ-relevant SFKs (including c-Src) (14), and RPTPα directs SFKs toward only a subset of their substrates (25).

Our findings also warrant consideration of cross-talk of RPTPα with the NRG1–ERBB4 pathway. RPTPα can affect ERBB1 signaling (25, 78), and we find that *Ptpra* ablation results in reduced *Erbb4* expression (Figure 3). NRG1/ERBB4 signaling suppresses upregulation of NMDA-R by c-Src (14). *Nrg1*$^{+/-}$ mice show reduced Fyn/Pyk2-mediated phosphorylation of Y1472 in the NR2B subunit of NMDA-R, which can be rescued by the antipsychotic clozapine (13); it remains to be seen whether clozapine can reverse the reduced phosphorylation of Fyn and NR2B and the abnormal behavior in *Ptpra*$^{-/-}$ mice.

The convergent evidence reported here linking RPTPα to schizophrenia may allow for novel hypotheses and open avenues for modeling and dissection of a disease mechanism that may yield clues for therapeutic exploration.

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Supplementary material cited in this article is available online.

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Research Report

Impairment of the tyrosine hydroxylase neuronal network in the orbitofrontal cortex of a genetically modified mouse model of schizophrenia

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ABSTRACT

Important genes have been identified that are associated with susceptibility to schizophrenia. DISC1 is one of these candidate genes. The protein 14-3-3 epsilon is a DISC1-interacting molecule and is associated with axon elongation. The genetically modified 14-3-3 epsilon heterozygous knockout mice are considered to be an animal model of schizophrenia because they present endophenotypes of schizophrenia including working memory impairment. This study investigated the immunohistochemical expression of tyrosine hydroxylase (TH) to reveal the alterations in the functional structure of the axon elongation caused by the deficit of 14-3-3 epsilon. The study focused on the orbitofrontal cortex in the prefrontal cortex which is a region of interest in schizophrenia research. The investigation used eight 15-week-old knockout mice and six age-matched wild-type mice. The TH immunopositive fibers were linear and dense in the wild-type mice. These fibers were serpentine, thin and short in the knockout mice. Although it appeared that dendritic spine-like immunopositive varices were strung tightly in the fibers of wild-type mice, these were few and sparse in those of the of the knockout mice. Quantitative analysis showed a significant decrease in the total extent of the TH-immunopositive fibers in the orbital cortex of the knockout mouse. There is thought to be a dysfunction of a neurotransmitter such as dopamine and noradrenalin in the prefrontal cortex of these knockout mice.

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Abbreviation: KO, knockout; TH, tyrosine hydroxylase; VTA, ventral tegmental area; LC, locus coeruleus

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1. Introduction

Schizophrenia is a common neuropsychiatric disorder that occurs in approximately 1% of the general population. Although the etiology of schizophrenia remains unclear, it is widely thought to be a neurodevelopment disorder. Many important findings have been obtained by studies of neurochemistry, neurophysiology and neuroimaging. However, alterations in the schizophrenia brain and the etiology of schizophrenia brain and especially the morphology of neuronal fibers must be conducted by direct observation of the brain tissue itself by microscopy.

Recently, molecular biological investigations have identified several putative schizophrenic candidate genes, and most of these genes are associated with the formation of neuronal networks, expanding neuronal fibers, migration of neurons, etc. (Harrison, 2007; Iritani, 2007). One of the major candidate genes is disrupted-in-schizophrenia 1 (DISC1). This is a promising candidate susceptibility gene for schizophrenia, which was first described as a strong candidate gene in a large Scottish family in which a balanced chromosomal translocation segregates with schizophrenia and other psychiatric disorders (Blackwood et al., 2001; Brandon et al., 2009; Chubb et al., 2008). DISC1 is also involved in neurodevelopment, including axonal elongation. In this process, DISC1 interacts with a complex formed by NudE-like (NUDEL), lissencephaly-1 (LIS1) and 14-3-3 epsilon (Taya et al., 2007).

The 14-3-3 epsilon protein is a member of the 14-3-3 family and is also one of the DISC1-interacting molecules. The 14-3-3 proteins are ubiquitous proteins that are highly conserved from bacteria to humans and plants and have several molecular and cellular functions such as signal transduction, cell cycle regulation, apoptosis and stress responses (Fu et al., 2000; Muslin and Xing, 2000; Tzivion and Avruch, 2002). There are seven distinct mammalian isoforms of 14-3-3 proteins, and some of these were previously reported to have a genetic association with schizophrenia (Toyooka et al., 1999; Wong et al., 2003; Wong et al., 2005).

YWHAE, a gene encoding 14-3-3 epsilon, was reported to be a susceptibility gene for schizophrenia and genetically modified 14-3-3 epsilon heterozygous knockout (KO) mice present various endophenotypes of schizophrenia including working memory deficits or cognitive decline (Ikeda et al., 2008). Therefore, this 14-3-3 epsilon heterozygous KO mouse is considered to be a novel animal model for schizophrenia.

Tyrosine hydroxylase (TH) is one of catecholaminergic markers (Beeler et al., 2009; Nair-Roberts et al., 2008) and is the enzyme responsible for catalyzing the conversion of the amino acid L-tyrosine to catecholamines such as dopamine and noradrenalin, which are transmitters that are closely associated with the pathophysiology of schizophrenia (Howes and Kapur, 2009; Yamamoto and Hornykiewicz, 2004).

This study investigated the immunohistocheminal expression of TH in the brains of the KO mouse using a neuropathological approach to reveal the alterations in the functional structure of the axon elongation caused by the deficit of 14-3-3 epsilon.

The study focused on the orbitofrontal cortex in the prefrontal cortex which is a region of interest in schizophrenia research, because there are several reports noting a volume reduction in the orbitofrontal cortex of schizophrenia patients (Fornito et al., 2009; Nakamura et al., 2008). The ventral tegmental area (VTA) and locus coeruleus (LC) were also observed because they are the nuclei from which the TH neuronal network originates.

2. Results

2.1. *Orbital cortex*

The immunopositive fibers were linear, dense, orderly and well developed in the wild-type mice. However, these fibers were serpentine, thin and short in the KO mice. Although it appeared that dendritic spine-like immunopositive varices were strung tightly and thick in the fibers of wild-type mice, these were few and sparse in those of the KO mice. The immunopositive neuronal fibers for the two different primary antibodies showed a similar staining pattern. In addition, TH immunoreactivity was found to be present within the neurofilament-immunoreactive area (Fig. 1). In the subsequent experiments, the antibody from Affinity BioReagents Co. was used for analyses. There was a significant decrease in the total extent of the TH-immunopositive fiber in the orbital cortex of the KO mice in comparison to that of the wild-type mice (Fig. 2).

2.2. *Ventral tegmental area and locus coeruleus*

The immunopositive neurons were dense and the axonal fibers were well developed in both regions of the wild-type and KO mice. No significant differences in the neuronal density and size of the soma in the VTA and LC were observed between wild-type mice and KO mice (Fig. 3, Table 1).

3. Discussion

This study investigated the changes of TH expression in the orbitofrontal cortex located in the prefrontal cortex, and the VTA and LC of catecholamine-originating neurons in 14-3-3 epsilon heterozygous KO mice.

Although there was no significant difference in the density and size of the neurons in the VTA and LC between the wild-type and KO mice, there was a markedly significant reduction of TH-immunopositive fibers in the orbitofrontal cortex of these projective terminations in the KO mice in comparison to the wild-type mice. While the originating neurons were intact, the subsequent projective fibers showed the decrease in the immunopositive composition.

3.1. *Hypofrontality and schizophrenia*

Cognitive deficits based on prefrontal cortex dysfunction are a persistent clinical feature of schizophrenia. Hypofrontality is among the major findings of functional neuroimaging studies on schizophrenic subjects (Ingvar and Franzen, 1974). Positron

emission tomography shows that young adult neuroleptic-naive schizophrenia patients have decreased perfusion in the orbitofrontal cortex (Andreasen et al., 1997). The neuroimaging results were thought to be due to a deficit of the TH neuronal fibers in the orbitofrontal cortex.

3.2. *Tyrosine hydroxylase and schizophrenia*

Cognitive dysfunction is induced by a reduction of catecholamines in the prefrontal cortex (Brozoski et al., 1979; Mizoguchi et al., 2009). The present study observed a marked reduction in

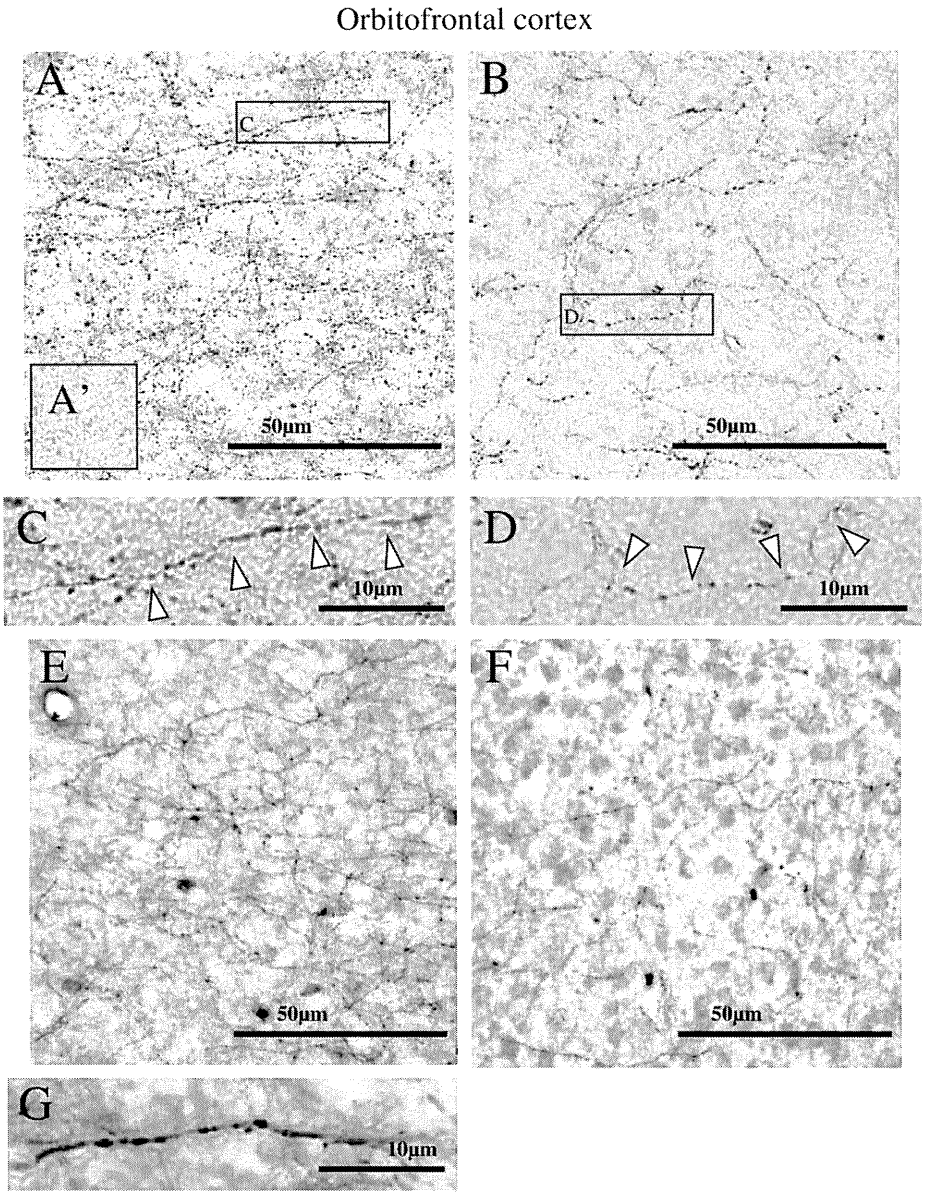

Fig. 1 – These figures show the TH-immunostained fibers in sections of orbitofrontal cortex stained using two different primary anti-TH antibodies; Bregma 1.98 mm (http://www.mbl.org/atlas165/atlas165_start.html). (A–D) Anti-tyrosine hydroxylase antibody (Affinity BioReagents Co.). (E–F) Anti-tyrosine hydroxylase antibody (Millipore Co.). (A) Wild-type mouse, low magnification. The immunopositive fibers were linear, dense, orderly and well developed. (A') No immunoreactivity was observed in the control study of the primary antibody pre-absorved with the 10 μg/ml of TH protein. (B) KO mouse, low magnification. The immunopositive fibers were serpentine, thin and short compared to wild-type mouse. (C) High-magnification figure of gray square frame in panel A. Dendritic spine-like immunopositive varices were strung tightly and thick in a fiber. (D) High-magnification figure of gray square frame in panel B; arrow head shows an immunoreactive fiber. Dendritic spine-like immunopositive varices were few and sparse in a fiber. (E) Wild-type mouse, low magnification. The immunopositive pattern of the antibody from Millipore Co. was similar to that in Figure A that used the antibody from Affinity Bioreagents. (F) KO mouse, low magnification. The immunopositive pattern of the antibody from Millipore Co. was similar to (B) which was generated using the antibody from Affinity Bioreagents. (G) A high magnification photograph of the double staining for TH (brown) and neurofilaments (red). It is noteworthy that the TH immunoreactivity (brown) was present within the neurofilament-immunoreactive area.

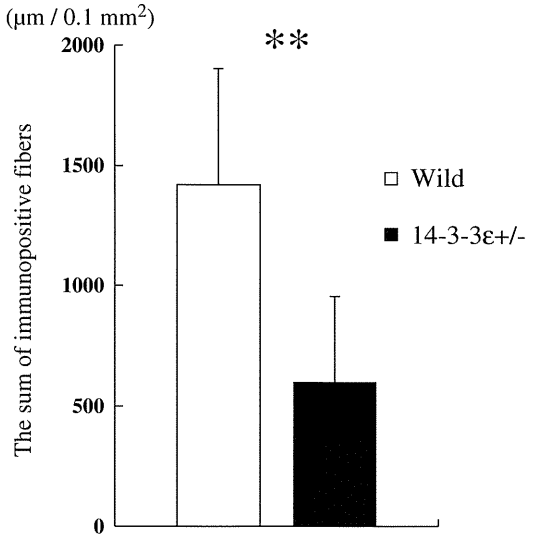

Fig. 2 – This graph shows the results of the sum of TH-immunopositive fibers (P<0.01). The error bars represent the standard deviation from the mean.**

the number of TH immunopositive fibers in the orbitofrontal cortex of the prefrontal cortex of the KO mice. This suggests that there is dysfunction of a neurotransmitter such as dopamine and noradrenalin synthesized by TH in prefrontal cortex of these KO mice. This result may be closely associated with the impaired working memory of these KO mice (Ikeda et al., 2008).

3.3. *Neuropathology of schizophrenia*

There is decreased dendritic spine density in the prefrontal cortex of a schizophrenic human brain (Glantz and Lewis, 2000; Glantz et al., 2006). Similar findings were observed in these KO mice. It was revealed that the TH neuronal network contains dopamine, which is important to the pathophysiology of schizophrenia, was disturbed by the deficiency in 14-3-3 epsilon, which was one of the DISC1-interacting molecules considered to be essential for axon elongation. This finding suggests that dysfunction of the TH neuronal network caused by the deficit of 14-3-3 epsilon may have been involved in the pathophysiology of schizophrenia and correlated with a dysfunction in the DISC1 complex.

4. Limitation

There are three limitations in this study. First, to evaluate the true length of a neuronal fiber, the three-dimensional measurement of the length from the soma to the terminal of a neuron is necessary. However, it is impossible to achieve this measurement by using two-dimensional histological experiments. Therefore, we considered that the neuronal fibers observed in an area of pre-determined thickness represented the density of the neuronal network in the limited area. Second, we observed that there was decreased expression of TH-containing neuronal fibers in the genetic model mouse in this report, but it is unknown whether these findings were specific in TH-containing fibers or not. To conclusively determine, further experiments will be necessary. Third, it will also be necessary to demonstrate the role of 14-3-3 epsilon not only in the brains of the animal model but also in the postmortem brain of patients with schizophrenic disease to conclusively determine the actual pathophysiology of this illness.

5. Experimental procedures

5.1. *Subjects (animal model)*

Eight 15-week-old 14-3-3 epsilon heterozygous KO (+/–) mice and the same number of age-matched 14-3-3 epsilon (+/+) littermate (wild-type) mice were used, and these mice were bred under the same conditions. The background of this animal model has been described in previous studies (Ikeda et al., 2008; Toyooka et al., 2003).

5.2. *Preparation*

The animals were placed under deep anesthesia by injection of sodium pentobarbital (40 mg/kg body weight i.p.) and then perfused with a tissue fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). The brains were immediately removed, and tissue blocks were immersed in a 20% sucrose–0.05 M phosphate buffer solution for more than 3 days at 4 °C. The sections of the prefrontal regions (20 μm) were cut on a freezing cryostat 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.

All animal experiments were performed according the guidelines of Nagoya University for animal experiments. All efforts were made to minimize the suffering of the animals used in this study and to reduce the number of animals used.

5.3. *Immunohistochemistry*

5.3.1. *Tyrosine hydroxylase*

The sections were rinsed in 0.1 M Tris–Cl buffered saline (TBS; pH7.4, 0.9% NaCl) containing 0.3% TritonX-100(TX) and 2% normal goat serum(NGS) two times for 15 min at room temperature. Because there may be differences in the detection of neuronal fibers by antibodies from different manufacturers, we employed two different anti-tyrosine hydroxylase primary monoclonal antibodies (Affinity Bio-Reagents, USA; catalog no. MA1-18038, lot no. 635001; 1:2000, and Millipore, USA; catalog no. AB152, lot no. NG1752018; 1:100) to ensure that our staining patterns were not related to the particular antibody that was used. The sections were incubated with the primary antibodies for 48 h at 4 °C. The sections were then incubated in medium containing biotinylated anti-universal (rat and/or rabbit) IgG (Vecstain; 1:100) for 30 min at room temperature and rinsed in NGS-TX-TBS solution, followed by incubation with an avidin-biotin peroxidase complex (ABC method) for 30 min and rinsed in TBS solution. Finally, the sections were rinsed in PBS twice for 10 min and reacted with 0.05% 3,3'diaminobenzine-HCl in

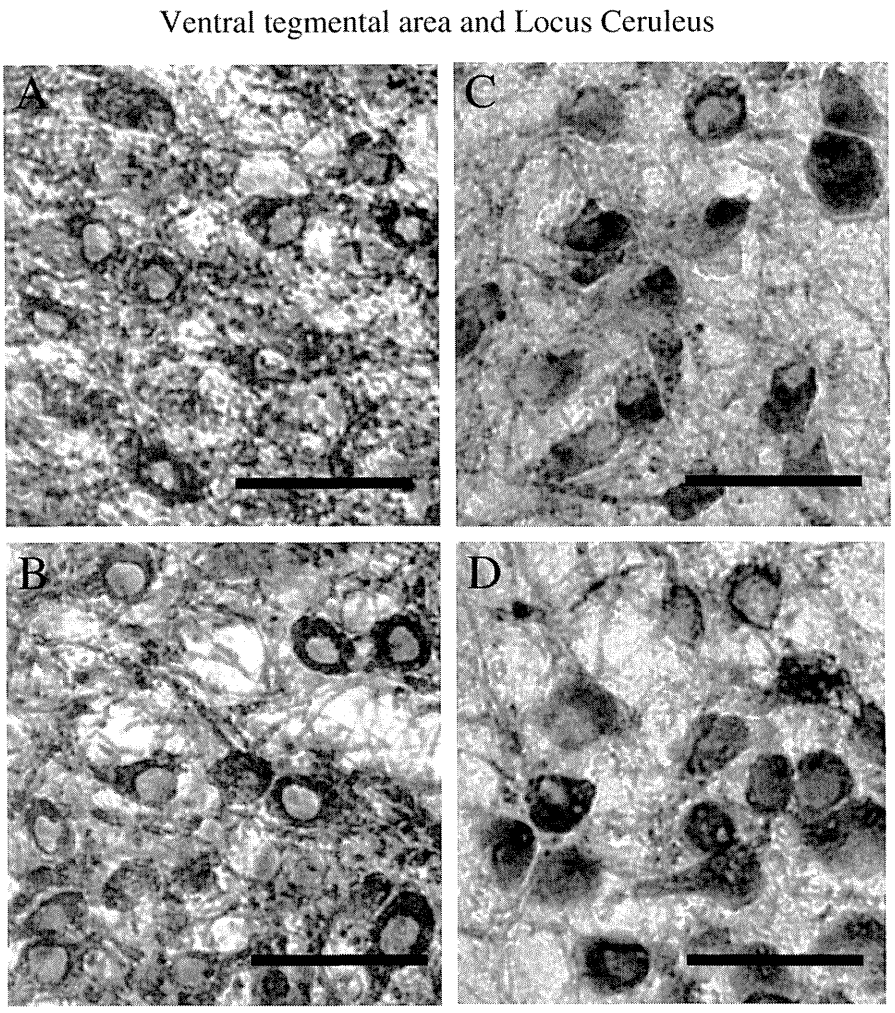

Fig. 3 – These figures show TH-immunostained neurons, including the (A) VTA of a wild-type mouse, (B) VTA of a KO mouse, (C) LC of a wild-type mouse, (D) LC of a KO mouse; scale bar=50 μm.

0.05 M Tris–HCl buffer (pH7.6) for 2 or 3 min and mounted onto gelatin-coated slides.

5.3.2. *Double staining for tyrosine hydroxylase and neurofilaments*

We also performed double immunostaining for TH and neurofilaments to confirm that the TH immunoreactivity was present within the neuronal fibers using a double labeling staining kit (Biocare Medical, LLC, USA; catalog no. MRCT525G, H, L), an anti-neurofilament antibody (Enzo Life Sciences International, Inc., USA; catalog no. NA1297, lot no. Z07518b; 1:500) and an anti-TH antibody (Affinity BioReagents, USA; catalog no. MA1-18038, lot no. 635001; 1:2000).

The sections were incubated with the primary anti-TH antibody for 48 h at 4 °C, followed by the primary anti-neurofilament antibody for 1 h at room temperature. Thereafter, the samples were rinsed in NGS-TX-TBS. The sections were then incubated in anti-mouse polymeric horseradish peroxidase and anti-rabbit polymeric alkaline phosphatase for 30 min at room temperature and rinsed in TBS solution twice

Table 1 – Difference of density and size of the neuron in the VTA and LC.

	Density (cells/mm^2)			Size (μm^2)		
	Wild-type	14-3-3ε +/–	*P* value	Wild-type	14-3-3ε +/–	*P* value
VTA	77.85±30.65	67.63±26.22	0.414	257.52±65.06	204.72±42.06	0.142
LC	317.39±59.44	324.01±55.69	0.32	168.68±30.94	175.70±48.37	0.801

This table shows the density and size of TH-immunostained neurons in VTA and LC. The data are shown as the mean±standard deviation except for the *P* value. No significant differences were observed between the wild-type mice and KO mice in the neuronal density and size of the soma in the VTA and LC.

for 10 min. Finally, the sections were reacted with 0.05% 3,3'diaminobenzine–HCl in 0.05 M Tris–HCl buffer (pH7.6) for 2 or 3 min, followed by reaction with alkaline phosphate substrate in 0.1 M Tris–HCl buffer (pH 8.3) for 20 min, then were mounted onto gelatin-coated slides.

5.4. *Observations and analysis*

Specimens were observed under a light microscope. The microscopic photographs were downloaded to a PC from a digital camera (DP21, OLYMPUS Co. Japan) as digital data. The Image J 1.41o software package (free software presented by NIH: http://www.rsb.info.nih.gov/ij/) was used to measure the following described data. All measurements were performed under the blind method.

5.4.1. *Orbitofrontal cortex*

Each TH-immunopositive fiber in the region of interest defined as 300 μm wide × 230 μm high in the coronal section (Bregma: +1.98 mm by the mouse brain atlas; http://www.mbl.org/atlas165/atlas165_start.html) of the orbitofrontal cortex was traced and the length was measured. Subsequently, the length of the immunopositive fibers was summed up.

It is impossible to measure the entire length of neuronal fibers using two-dimensional histological experiments. We therefore estimated that the length of the TH-immunopositive fibers on the section represented the density of the neuronal network and thus analyzed these data.

The significance of the variation in the differences of the total length was compared between in the wild-type mouse group and in the KO mouse group by the Mann–Whitney *U* test with $P<0.05$ considered to be statistically significant.

5.4.2. *Ventral tegmental area and locus coeruleus*

A neuron with a clearly defined nucleus was regarded as a TH-immunopositive neuron and counted in the ventral tegmental area (VTA) and locus coeruleus (LC). Neuronal density (cells/mm^2) and the size (μm^2) of the soma were measured using Image J. The average of neuronal density and size was compared between in the wild-type mouse group and in the KO mouse group by the Mann–Whitney *U* test with $P<0.05$ considered to be statistically significant.

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A Case Control Association Study and Cognitive Function Analysis of Neuropilin and Tolloid-Like 1 Gene and Schizophrenia in the Japanese Population

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Abstract

Background: Using a knock-out mouse model, it was shown that NETO1 is a critical component of the NMDAR complex, and that loss of *Neto1* leads to impaired hippocampal long term potentiation and hippocampal-dependent learning and memory. Moreover, hemizygosity of *NETO1* was shown to be associated with autistic-like behavior in humans.

Purpose of the Research: We examined the association between schizophrenia and the neuropilin and tolloid-like 1 gene (*NETO1*). First, we selected eight single nucleotide polymorphisms (SNPs) within the *NETO1* locus, based on the Japanese schizophrenia genome wide association study (JGWAS) results and previously conducted association studies. These SNPs were genotyped in the replication sample comprised of 963 schizophrenic patients and 919 healthy controls. We also examined the effect of associated SNPs on scores in the Continuous Performance Test and the Wisconsin Card Sorting Test Keio version (schizophrenic patients 107, healthy controls 104).

Results: There were no significant allele-wise and haplotype-wise associations in the replication analysis after Bonferroni correction. However, in meta-analysis (JGWAS and replication dataset) three association signals were observed (rs17795324: $p = 0.028$, rs8098760: $p = 0.017$, rs17086492: $p = 0.003$). These SNPs were followed up but we could not detect the allele-specific effect on cognitive performance measured by the Continuous performance test (CPT) and Wisconsin Card Sorting test (WCST).

Major Conclusions: We did not detect evidence for the association of *NETO1* with schizophrenia in the Japanese population. Common variants within the *NETO1* locus may not increase the genetic risk for schizophrenia in the Japanese population. Additionally, common variants investigated in the current study did not affect cognitive performance, as measured by the CPT and WCST.

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Introduction

The glutamate hypothesis of schizophrenia (GHS) emerged in the early 1980 s as an alternative to the prevailing theory of altered dopamine neurotransmission. The GHS is based on the observation that non-competitive antagonists of the N-methyl-d-asparate (NMDA) subtype of glutamate receptors, such as phencyclidine (PCP), ketamine and MK-801, induce a psychotic reaction in healthy individuals that resembles schizophrenia (both the positive and negative symptoms). When the same compounds are administered to patients with schizophrenia, exacerbation of psychotic symptoms can be the outcome [1]. Together, these observations suggest that diminished function of the NMDA receptor (NMDAR) may play a role in the pathoetiology of schizophrenia. Moreover, evidence from morphological, clinical and neuroimaging studies have provided support for the GHS by mapping cognitive impairment, alterations in blood flow and changes in neuronal morphology to particular brain areas, including the frontal and cingulate cortices, both of which are areas with extensive excitatory glutamatergic neurotransmission [2].

The N-methyl-D-aspartate receptor (NMDAR), a major excitatory ligand-gated ion channel in the central nervous system, is composed of a heterotetramer between two NR1 and two NR2 subunits. Moreover, the NMDAR is a principal mediator of synaptic plasticity [3]. It has been shown that corticolimbic NMDAR hypofunction is one of the core molecular mechanisms relevant for phenotypes observed in animal models of schizophrenia [4]. One of the genes that regulate NMDAR function is neuropilin and tolloid-like 1 gene (*NETO1*). *NETO1* maps to the 18q22-q23 and three alternative splicing variants (mRNA level) have been observed [5]. Specifically, variants 1 and 2 are detected in the retina while variant 3 is specific for fetal and adult brain. NETO1 is a transmembrane protein, which has two extracellular CUB domains, a low-density lipoprotein class A (LDLa) domain, a transmembrane domain and classical type I PDZ-domain binding motif [5] (Figure 1). Deletion of *Neto1* leads to deficits in synaptic plasticity in mice while stimulation of the AMPA receptor can partially compensate for deficits caused by *Neto1* deletion [6]. NETO1 interacts with the core NMDAR subunits, NR2A and NR2B and a scaffolding protein, postsynaptic density-95 (PSD-95), maintaining the abundance of NR2A-containing NMDARs in the postsynaptic density of the hippocampus. PSD-95 is a protein that is almost exclusively located in the postsynaptic density of neurons, and is important in anchoring synaptic proteins [7]. Increase in surface NR2A, but not NR2B, occurs in hippocampal neurons derived from dysbindin-null mutant mice (Dys-/-). Dysbindin controls hippocampal LTP by selective regulation of the surface expression of NR2A [8]. In situ hybridization studies of schizophrenia detected decreased transcript expression of the NR1 subunit, increased transcript expression of the NR2B subunit and unchanged transcript expression of the NR2A subunit in hippocampus [9]. Therefore, regulation of NR2 in hippocampus in schizophrenia may be relevant for the etiology of schizophrenia and NETO1 may play an important role in the molecular mechanism by maintaining the abundance of NR2A-containing NMDARs in the postsynaptic density of hippocampal neurons. Moreover, NETO1 interacts with kainate receptors (KAR), one of the glutamate receptors, in mouse brain. NETO1 modulates the KAR affinity for the endogenous ligand glutamate. NETO1 modulates not only kinetics, but also the amplitude of slow excitatory postsynaptic current in KAR (KAR-EPSC) [10,11]. NETO1 fundamentally alters the function and neuronal localization of GluK1-containing KAR [10,11]. Therefore, NETO1 may influence glutamate neurotransmission through modulation of KAR and NMDAR properties.

Genetic studies suggested that the *NETO1* locus could harbor genetic variants that are relevant for susceptibility to neuropsychiatric disorders. Specifically, hemizygosity of *NETO1* was shown to be associated with a autistic-like behaviors in humans [12]. Although unequivocal genome wide evidence ($p<10^{-8}$) for association at the *NETO1* locus has not been reported, it is of note that in the recent GWASs of Alzheimer's disease and schizophrenia, suggestive association signals were observed (rs1109070; $p = 0.000669$ [13] and rs9962470; $p = 0.000154$ [14] in Alzheimer's disease and schizophrenia, respectively). Moreover, several weak association signals ($P<0.05$) within the *NETO1* locus were detected in the first GWAS of schizophrenia conducted in the Japanese population (JGWAS) [15]. It is of note that in the JGWAS, genome wide evidence for association was not detected, and the non-genome wide level of statistical significance should be interpreted with caution. However due to the relatively small sample size, type II errors (false negative result) cannot be excluded – especially in the case of small odds ratios (OR) which are expected for common SNPs associated with schizophrenia [16].

Based on the aforementioned biological studies, *NETO1* can be seen as a promising candidate gene for schizophrenia. However, to the best of our knowledge, no genetic association study specifically designed to evaluate the association between *NETO1* and schizophrenia has been conducted. The goal of the present study was to evaluate the association between *NETO1* and schizophrenia based on the JGWAS. Additionally, as deficits were found in LTP and learning and memory in *Neto1*-null mice [7], we performed a cognitive function analysis that targeted the relationship between common SNPs selected based on the JGWAS and cognitive function assessed by the CPT and the WCST.

Results

In the replication sample set, we did not detect any association between eight SNPs and schizophrenia after Bonferroni correction was applied (Table 1). Using the replication sample set, we have conducted haplotype-wise analysis in order to investigate association between haplotypes within the *NETO1* locus and schizophrenia. We did not detect evidence for haplotype-wise association after Bonferroni correction was applied (Table S1). In order to maximize the power, we performed meta-analysis combining results from the JGWAS and the replication dataset. In this analysis we could detect three suggestive association signals (rs17795324: $P = 0.028$, rs8098760: $P = 0.017$, rs17086492: $P = 0.003$). In the test of heterogeneity, we detected four SNPs, which showed significant high heterogeneities (Breslow-Day test; rs17795324: $P = 0.04$, rs6566674: $P = 0.01$, rs8098624: $P = 0.003$, rs1109070: $P = 0.0001$) (Table 2). However, we could not detect any association between these three SNPs (rs17795324, rs8098760 and rs17086492) and CPT and WCST scores (Table S4). We investigated the association between the *NETO1* gene and schizophrenia stratified by gender. We could detect no association signals either for males or females in the meta-analysis after Bonferroni correction (Tables S2 and S3).

Discussion

In this study, we investigated the association between eight SNPs within *NETO1* and schizophrenia in the Japanese population. We detected associations between *NETO1* and schizophrenia in the meta-analysis, however, as the JGWAS dataset was included in the meta-analysis, evidence for association might be overestimated. In order to address this issue we tested the association between candidate SNPs from our meta-analysis and cognitive performance measured by the CPT and WCST. This analysis was of interest for us as *Neto1*-null mice show deficits in LTP and learning and memory [7], and if the three SNPs (rs17795324, rs8098760, rs17086492) were genuinely associated with schizophrenia, then carriers of risk alleles would likely have deficits in cognitive processing assessed by CPT and WCST [6]. However, we could not detect any association between these three SNPs (rs17795324, rs8098760, rs17086492) and the psychological tests we applied. We also performed meta-analysis (Method S1) of rs6566674 (the SNP that was included both in our replication sample set and another Japanese GWAS [17]), however we did not detect evidence for an association with schizophrenia. These results suggest that *NETO1* is not associated with schizophrenia in the Japanese population.

We detected four SNPs, which showed heterogeneous association (Breslow-Day test; rs17795324: $P = 0.04$, rs6566674: $P = 0.01$, rs8098624: $P = 0.003$, rs1109070: $P = 0.0001$) in the meta-analysis. These high heterogeneities may be derived from flip-flop phenomenon, that is, associations of opposite alleles at the same biallelic locus with the same disease [18]. Although the flip-

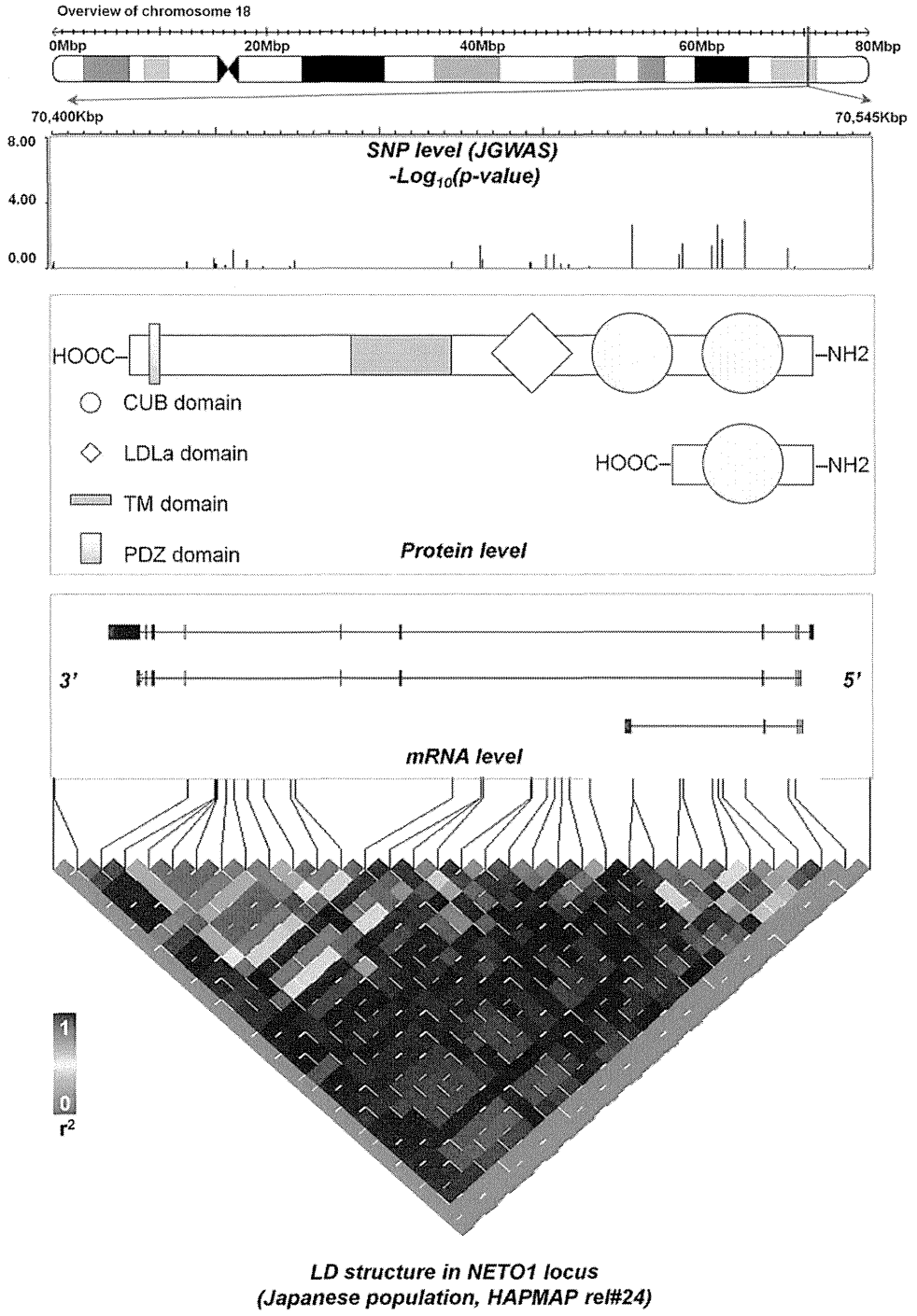

Figure 1. NETO1-gene centric data. LD: linkage disequilibrium. NETO1 maps to the 18q22-q23 and three alternative splicing variants (mRNA level) have been observed. Specifically variants 1 and 2 are detected in retina while variant 3 is specific for fetal and adult brain (mRNA level panel). NETO1 is a transmembrane protein, which has two extracellular CUB domains, a low-density lipoprotein class A (LDLa) domain, a transmembrane domain and a classical type I PDZ-domain binding motif (protein level panel).
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flop phenomenon may represent a genuine genetic association (i.e., genuinely different LD architectures across populations with different ancestral origins), it may also be an artifact due to sampling variation that leads to variability in observed LD patterns.

Several caveats should be considered when interpreting the results of our study. First, in terms of sample size, the replication dataset may not have sufficient statistical power to detect associations between SNPs with low genotype relative risk (GRR) and schizophrenia. In other words, our sample has statistical power greater than 0.8 for the detection of association signals at nominal statistical significance, of the polymorphism with a minor allele frequency of 0.1, when the GRR is 1.30. Therefore, the possibility of association between schizophrenia

Table 1. Results of JGWAS (N = 1108).

SNP	Position[a]	Minor allele	Case[b]	Control[b]	p-value[c]	OR	L95[d]	U95[d]
rs9962470	68616086	A	0.03	0.04	0.31	0.79	0.50	1.24
rs17086334	68626752	C	0.10	0.08	**0.05**	1.34	1.00	1.80
rs17795324	68654033	G	0.27	0.33	**0.003**	0.76	0.63	0.91
rs6566674	68662791	T	0.20	0.16	**0.04**	1.26	1.01	1.57
rs8098624	68669199	T	0.30	0.24	**0.002**	1.35	1.12	1.63
rs8098760	68669970	T	0.11	0.08	**0.02**	1.42	1.06	1.90
rs17086492	68674050	C	0.14	0.10	**0.001**	1.52	1.17	1.98
rs1109070	68674559	C	0.21	0.16	**0.01**	1.35	1.09	1.68

[a]based on NCBI 36.
[b]minor allele frequency.
[c]Fisher's exact test.
[d]Lower (L) and upper (U) 95% confidence intervals.
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and common SNPs with GRR<1.30 cannot be excluded. Furthermore, the JGWAS may not have sufficient power to detect associations between SNPs with low GRR and schizophrenia. Therefore, other relevant common variants in the *NETO1* region, which the JGWAS cannot identify, may exist. In addition, association between *NETO1* and schizophrenia may be specific only for the patients with early onset age. However, as in the current study, the frequency of the early onset schizophrenia patients was low (less than 5%). The power to detect such an association is inadequate. Therefore, the effect of onset age on the association between *NETO1* and schizophrenia remains to be investigated in further studies.

The second caveat is that our study design was based on the common disease, common variant hypothesis, based on which we applied a minor allele frequency threshold (>5%) and selected eight SNPs for follow-up. In the best case scenario, common variants detected in GWAS can explain only part of the heritability in cases of schizophrenia (~30%) [19] and missense or nonsense mutations on the one side and structural variations (i.e., copy number variants (CNVs)) on the other side are likely to contribute to the increased susceptibility [20]. Recently, the concept of synthetic associations has been suggested, though some there are some objections [21]. Uncommon or rare genetic variants can easily create synthetic associations that are credited to common variants. This possibility requires careful consideration in the interpretation and follow up of GWAS signals [22].

The third caveat in our association study is that cases and controls in replication samples were not matched in age. In other words, although highly unlikely, the controls may develop schizophrenia at some point in life, as they were significantly younger than cases.

The fourth caveat is related to the validity of cognitive function analysis. The premise that *NETO1* was associated with cognitive function in humans had been derived from results of a knock-out mice study [7]. *Neto1*-null mice showed impaired spatial learning measured by the Morris water maze task, the delayed matching-to-place version of the Morris water maze task and displaced-object tasks. In the current study, we investigated executive function (WCST) and vigilance/attention (CPT-IP), however, the results of these cognitive tests might not represent similar cognitive dysfunctions that were shown in the *Neto1*-null mouse study. It may be useful to examine different domains of cognitive impairment

Table 2. Allele frequencies of the eight SNPs of *NETO1*.

SNP	Position[a]	Minor allele	Replication (N = 1882)						Meta analysis (N = 2990)				
			Case[b]	Control[b]	p-value[c]	OR	L95[d]	U95[d]	p-value[c]	OR	L95[d]	U95[d]	BDp[e]
rs9962470	68616086	A	0.02	0.03	0.24	0.79	0.53	1.18	0.126	0.79	0.59	1.07	0.99
rs17086334	68626752	C	0.08	0.08	0.87	0.98	0.77	1.25	0.289	1.11	0.92	1.33	0.09
rs17795324	68654033	G	0.29	0.30	0.61	0.96	0.84	1.11	**0.028**	0.88	0.79	0.99	0.04
rs6566674	68662791	T	0.17	0.19	0.12	0.88	0.74	1.04	0.926	1.01	0.88	1.15	0.01
rs8098624	68669199	T	0.27	0.29	0.43	0.94	0.82	1.09	0.213	1.08	0.96	1.21	0.003
rs8098760	68669970	T	0.10	0.08	0.18	1.17	0.93	1.47	**0.017**	1.24	1.04	1.49	0.26
rs17086492	68674050	C	0.12	0.11	0.22	1.14	0.93	1.39	**0.003**	1.28	1.09	1.50	0.09
rs1109070	68674559	C	0.16	0.19	**0.01**	0.78	0.65	0.93	0.718	0.98	0.85	1.12	0.0001

[a]based on NCBI 36.
[b]minor allele frequency.
[c]Fisher's exact test.
[d]Lower (L) and upper (U) 95% confidence intervals.
[e]p-value of Breslow-Day test.
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associated with *NETO1* in schizophrenic patients using broader cognitive assessment tools.

In conclusion, we were not able to detect evidence for an association between *NETO1* and schizophrenia in the Japanese population. Common variants within the *NETO1* locus may not increase the genetic risk for schizophrenia in the Japanese population. Additionally, common variants investigated in the current study did not affect cognitive performance, as measured by the CPT and the WCST.

Materials and Methods

Participants

This study was approved by the Ethics Committees of the Nagoya University Graduate School of Medicine and Fujita Health University, and written informed consent was obtained from each participant. Patients were included in the study if they (1) met DSM-IV criteria for schizophrenia, (2) were physically healthy and (3) had no mood disorders, substance abuse, neurodevelopmental disorders, epilepsy or known mental retardation. A general characterization and psychiatric assessment of subjects is available elsewhere [23]. Controls were selected from the general population. Control subjects had no history of mental disorders, based on questionnaire responses from the subjects themselves during the sample inclusion step, and based on an unstructured diagnostic interview done by an experienced psychiatrist during the blood collection step. The JGWAS sample was comprised of 575 patients with schizophrenia (43.5±14.8 years (mean±s.d.), male 50%) and 564 healthy controls with no personal or family history of psychiatric illness (44.0±14.4 years (mean±s.d.), male 49.8%). All subjects were unrelated, living in the central area of the Honshu island of Japan and self-identified as members of the Japanese population. Subjects of replication samples consisted of 963 schizophrenic patients (47.7±0.5 years (mean±s.d.), male 55.2%) and 919 healthy controls (45.0±0.5 years (mean±s.d.), male 51.0%). The JGWAS and replication samples were collected independently at each university hospital.

Genotyping and data analysis

Based on the JGWAS results we initially selected SNPs with probability values, $p<0.05$ and allelic frequencies, MAF>0.05 within the *NETO1* locus. Then we identified redundant SNPs based on the linkage disequilibrium or LD pattern within the interrogated region. Specifically, if the correlation coefficient between two loci (r^2) was 0.8 or higher, only one of the two loci was selected for the association study [24]. The correlation coefficient between two loci (r^2) was calculated using Haploview v.4.1 based on the HapMap database (release no. 24, population: Japanese in Tokyo). Finally, we selected seven nonredundant SNPs within the *NETO1* locus. Moreover, one common polymorphism (rs9962470), which showed a low p-value ($p = 0.000154$) in a previous GWA study [14], was included for genotyping. All eight SNPs are intronic polymorphisms. DNA was extracted from peripheral blood according to a standard protocol [23]. Genotyping was performed using a fluorescence-based allelic discrimination assay (Taqman, Applied Biosystems, Foster City, CA, USA). Power was calculated according to the methods of Skol et al. [25].

To exclude low-quality DNA samples or genotyping probes, data sets were filtered on the basis of SNP genotype call rate (more than 90%) or checked deviation from Hardy-Weinberg equilibrium (HWE) in the control sample. Subjects whose percentage of missing genotypes was >30% or who had evidence of possible DNA contamination were excluded from subsequent analyses.

To reduce the total number of tests, eight associated markers were selected based on the JGWAS results. Next, conditional on the findings of the JGWAS, which used a less stringent nominal level, a meta-analysis was done involving the confirmation sample using the replication data and data from the JGWAS. In the replication sample, Fisher's exact test was used to compare allele frequencies between patients and control subjects. The significance level was set at $p<0.05$. In the replication sample set, log likelihood ratio tests for assessing haplotype-wise association between schizophrenia and a combination of tagging SNPs was performed using UNPHASED software v3.04. The rare haplotype frequency threshold was set at 5% [26]. In this meta-analysis, p-values were generated by a Cochran–Mantel–Haenszel stratified analysis, and the Breslow–Day test was performed for evaluations of heterogeneous associations as implemented in gPLINK v.2.050 [27].

Neurocognitive assessment

1. CPT. We used the Continuous Performance Test–Identical Pairs Version Release 4.0 (NewCPT.exe, Copyright 1982–2004 by Barbara A. Cornblatt, All Rights Reserved). The size of PC monitor used for the test was 10.4 inches as each letter was at least 2.2×1.5 cm [28]. Stimuli were flashed on the screen at a constant rate of 1 per second, with a stimulus "on" time of 50 ms. Stimuli were four-digit numbers and were presented 150 times. In each 150-trial condition, 30 of the trials (20%) were target trials and required a response. Target trials were those on which the second of a pair of two identical stimuli appeared [28]. The outcome measure was a mean, d′.

2. WCST. The WCST [29] mainly assesses executive function including cognitive flexibility in response to feedback. We used a modified and computerized version of the test: Wisconsin Card Sorting Test (Keio Version) (KWCST) [30]. The outcome measures were numbers of categories achieved (CA), total errors (TE), and perseverative errors of Milner (PEM) and Nelson types (PEN) in the first trial. We selected outcomes in the WCST, following a prior study, which used KWCST as a measure of cognitive function [31].

(1) CA: This is the number of categories for which six consecutive correct responses are achieved (eight is the maximum number of categories which can be achieved), and is the sum measure of the level of conceptual shifts in the KWCST.

(2) PEN: This is the number of incorrect responses in the same category as the immediately preceding incorrect response (maximum of 47 perseverative errors) [32].

(3) PEM: This is the number of incorrect responses in the same category as the immediately preceding correct response after the category changes.

(4) TE: This is the total number of incorrect responses [33].

3. Clinical information. Chlorpromazine (CPZ) equivalent doses were calculated based on the report by Inada [34,35]. The Positive and Negative Symptom Scale (PANSS) was used to evaluate patients [36].

4. Analysis of cognitive performance. From the sample used in the current study, we made a subset of randomly selected participants older than 18 years of age for analysis of cognitive performance. Cognitive data analysis was done for the participants who completed both WCST and CPT-IP. We checked the effect of three SNPs on cognitive performance measured by the Continuous Performance Test and the Wisconsin Card Sorting Test (107 schizophrenic patients, 104 Healthy controls). IBM SPSS statistical software, version 19 was used for all analyses. We compared age, education, CPZ equivalent doses, age at onset, duration of illness, positive scale, negative scale and General

Psychopathology Scale between schizophrenia cases and control subjects using a two-tailed t-test and Welch's t-test. We compared sex between case and control groups using Fisher's exact test. Next, we compared d′ in the CPT and CA, PEM, PEN, TE in the WCST between the case and control groups using a two-tailed t-test and Welch's test (Table S4).

Supporting Information

Method S1 Meta-analysis.
(DOC)

Table S1 Haplotype analysis of the eight SNPs of *NETO1*.
(DOC)

Table S2 Allele frequencies of the eight SNPs of *NETO1* in males.
(DOC)

Table S3 Allele frequencies of the eight SNPs of *NETO1* in females.
(DOC)

Table S4 Cognitive performance of three SNPs in *NETO1*.
(DOC)

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Author Contributions

Conceived and designed the experiments: MB BA NO. Performed the experiments: MB T. Koide T. Kikuchi KK YA NK. Analyzed the data: MB T. Koide BA KY IK MI TI TY NI NO. Contributed reagents/materials/analysis tools: T. Koide YA. Wrote the paper: MB BA NO.

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