Table 3
Impact of genetic variants in the *SIGMAR1* gene on activation of the prefrontal cortex during VFT-letter task.

Gln2Pro (rs1800866)	Schizophrenia ($N=127$)		Control ($N=216$)		p values ($F_{1,335}$ values)		
	Pro carrier ($N=70$)	Gln/Gln ($N=57$)	Pro carrier ($N=115$)	Gln/Gln ($N=101$)	Diagnosis effect	Genotype effect	Interaction
Activation of the rt. PFC	0.52 ± 1.05	0.90 ± 1.43	0.95 ± 1.09	1.24 ± 1.07	5.09×10^{-5} (16.85)	0.013 (6.24)	0.59 (0.29)
Activation of the lt. PFC	0.55 ± 1.10	0.99 ± 1.47	0.98 ± 1.22	1.05 ± 1.16	0.038 (4.34)	0.075 (3.18)	0.12 (2.39)

Means ± SD are shown. To control for confounding factors, the effect of diagnosis, the *SIGMAR1* genotype and their interaction on activation of prefrontal cortex was analyzed by two-way analysis of covariance with age, sex, years of education and performance score on the VFT-letter as covariates. Significant p-values are shown in boldface and underlined.

(Miyatake et al., 2004), suggesting that sigma-1 receptor signaling in subjects with the Gln/Gln genotype might be more active than that in Pro carriers. Because haloperidol acts as an antagonist for sigma-1 receptor (Cobos et al., 2008), haloperidol could attenuate the effect of the polymorphism on the sigma-1 receptor signaling. Patients with the Gln/Gln genotype ($N=5$, 500.0 ± 326.0 mg/day) tended to have taken more CPZeq of haloperidol than Pro carriers ($N=10$, 398.8 ± 338.2 mg/day) in patients with taking haloperidol, although it was not statistically significant ($p=0.46$). Thus, higher sigma-1 receptor signaling in patients with the Gln/Gln genotype might be reduced by haloperidol treatment.

NIRS, a neuroimaging method, is increasingly used in the investigation of frontal cortex dysfunction in schizophrenia. Compared with other neuroimaging methods such as functional magnetic resonance imaging and positron emission tomography, NIRS measurement is restraint free, easy to use, and portable, has a low running cost and is noninvasive. Using NIRS, three studies have suggested that Gln2Pro in *SIGMAR1*, Val158Met in catechol-O-methyltransferase (*COMT*) and genotypes based on a threshold of greater than 35 CAG repeats in the TATA box-binding protein (*TBP*) gene were associated with prefrontal hemodynamic response (Ohi et al., 2009; Takizawa et al., 2009a,b). The activation of the PFC during the verbal fluency task was significantly lower in carriers of the Pro allele in the *SIGMAR1* gene (when compared to individuals with the Gln/Gln genotype) and in individuals with the Val/Val genotype in the *COMT* gene (when compared to carriers of the Met allele) (Takizawa et al., 2009a,b). In addition, we have reported evidence that genotypes with greater than 35 CAG repeats in the *TBP* gene, which were more common among patients with schizophrenia, were significantly associated with PFC hypoactivation during the tower of Hanoi, a test of executive function. If combined imaging and genetics research demands a larger and wider variety of samples in the near future, NIRS has great potential as a neuroimaging modality to detect cortical function with ease and speed. Further research is needed to investigate whether these genotype effects can be replicated in different cohorts with larger sample sizes.

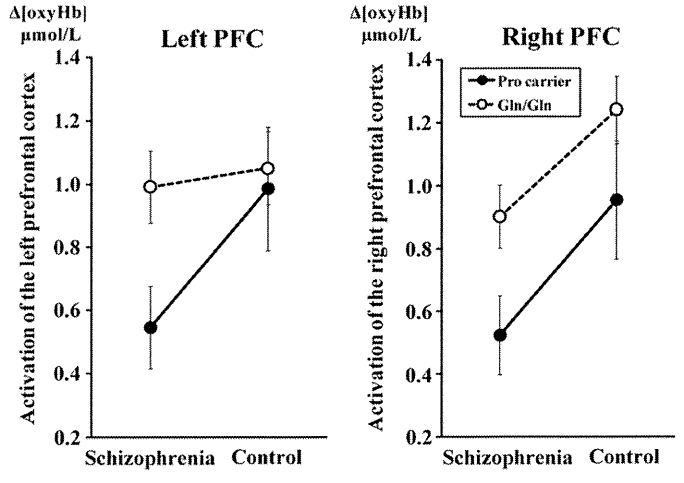

Fig. 2. Effect of the risk Gln2Pro genotype and diagnosis on activation of the bilateral PFC as measured by NIRS during the verbal fluency task. Carriers of the Pro allele in the *SIGMAR1* gene, which was more common among patients with schizophrenia, showed lower activation of the right PFC compared to individuals with the Gln/Gln genotype in both patients with schizophrenia and healthy controls (schizophrenia patients: Pro carriers $N=70$, Gln/Gln $N=57$; controls: Pro carriers $N=115$, Gln/Gln $N=101$). Closed circles represent Pro carriers. Open circles represent individuals with the Gln/Gln genotype. Bars represent the standard error. PFC: prefrontal cortex.

Several studies have suggested that sigma-1 receptor ligands are useful in the treatment of schizophrenia. In animal studies, sigma receptor antagonists such as BMY-14802 and panamesine (EMD57445) improved methamphetamine-induced behavioral sensitization in rats (Ujike et al., 1992a,b) and sigma receptor antagonists such as BMY-14802, haloperidol and NE-100 improved phencyclidine (PCP)-induced behavioral abnormalities (Ogawa et al., 1994; Okuyama et al., 1995; Takahashi et al., 2001). On the other hand, recent studies suggest that donepezil, a sigma-1 receptor agonist, plays a role in memory processing and that fluvoxamine, also a sigma-1 agonist, can improve PCP-induced cognitive deficits in mice (Hashimoto et al., 2007a; Kunitachi et al., 2009). In clinical trials, putative selective antagonists of sigma receptors such as eliprodil (SL 82.0715) and panamesine showed antipsychotic effects in schizophrenia (Frieboes et al., 1997; Huber et al., 1999; Modell et al., 1996; Muller et al., 1999), while sigma-1 receptor agonists (fluvoxamine) improved cognitive impairments and extra-pyramidal symptoms seen in schizophrenia (Furuse and Hashimoto, 2010a, b; Iyo et al., 2008). Due to the differential effects of sigma-1 agonists and antagonists, stabilization of the sigma-1 receptor may be useful in the treatment of schizophrenia. Despite the importance of prefrontal dysfunctions in schizophrenia, no drug has been approved for the treatment of this aspect of schizophrenia. We investigated the effect of rs1800866 using *in silico* analysis (Polyphen2 database; http://genetics.bwh.harvard.edu/pph2) (Adzhubei et al., 2010), which predicts whether non-synonymous SNP affects protein structure and function. However, this mutation was not predicted to affect protein structure and function with a score of 0.001 (sensitivity: 1.00; specificity: 0.06) on HumVar in this analysis. Further research will be required to clarify the effects of *SIGMAR1* functions on the pathophysiology of schizophrenia.

There were several limitations to this study. We evaluated both patients with schizophrenia and controls using SCID in the NIRS analysis, while we evaluated participants using unstructured clinical interview in the genetic association analysis. Because we did not use SCID to evaluate these subjects in the genetic association analysis, it might not be representative of the typical patients with schizophrenia and healthy subjects, despite the confirmations of the diagnosis by at least two trained psychiatrists. In the NIRS results, the main effect was significant both for diagnosis and genotype, but their interaction was not. This may indicate that both diagnosis of schizophrenia and Pro genotype reduce the PFC activity, but these findings are independent phenomena and do not mutually interact in the brain. On the other hand, a study by Takizawa et al. has found a significant interaction of diagnosis and Gln2Pro genotype (Takizawa et al., 2009a). Their results may be more straightforward than the present study because the genotype effect was seen only in the patients group. We could not exclude the possibility that many antipsychotics including haloperidol might interact with the polymorphism effect. The number of the subjects in the present study

was more than 3 timesthat of Takizawa et al. (Takizawa et al., 2009a); however, Takizawa et al. used 52-channel NIRS thatwas 26 times greater than the present study. We examined activation on PFC using two-channel NIRS because the brain activations during verbal fluency task with two 24-channel NIRS were prominent in frontal channels in schizophrenia (Suto et al., 2004). However, we might not have detected the most sensitive region affected by the Gln2Pro polymorphism due to narrow spatial coverage of the PFC.

In conclusion, the *SIGMAR1* polymorphism is associated with schizophrenia risk. Prefrontal function during verbal generation is modulated by variation in the *SIGMAR1* genotype. These findings may lead to a new target for antipsychotics.

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Conflict of interest

All authors declare that they have no conflict of interest.

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ARCHIVAL REPORTS

Loss of Function Studies in Mice and Genetic Association Link Receptor Protein Tyrosine Phosphatase α to Schizophrenia

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Background: Solid evidence links schizophrenia (SZ) susceptibility to neurodevelopmental processes involving tyrosine phosphorylation-mediated signaling. Mouse studies implicate the *Ptpra* gene, encoding protein tyrosine phosphatase RPTPα, in the control of radial neuronal migration, cortical cytoarchitecture, and oligodendrocyte differentiation. The human gene encoding RPTPα, *PTPRA,* maps to a chromosomal region (20p13) associated with susceptibility to psychotic illness.

Methods: We characterized neurobehavioral parameters, as well as gene expression in the central nervous system, of mice with a null mutation in the *Ptpra* gene. We searched for genetic association between polymorphisms in *PTPRA* and schizophrenia risk (two independent cohorts, 1420 cases and 1377 controls), and we monitored *PTPRA* expression in prefrontal dorsolateral cortex of SZ patients (35 cases, 2 control groups of 35 cases).

Results: We found that $Ptpra^{-/-}$ mice reproduce neurobehavioral endophenotypes of human SZ: sensitization to methamphetamine-induced hyperactivity, defective sensorimotor gating, and defective habituation to a startle response. *Ptpra* loss of function also leads to reduced expression of multiple myelination genes, mimicking the hypomyelination-associated changes in gene expression observed in postmortem patient brains. We further report that a polymorphism at the *PTPRA* locus is genetically associated with SZ, and that *PTPRA* mRNA levels are reduced in postmortem dorsolateral prefrontal cortex of subjects with SZ.

Conclusions: The implication of this well-studied signaling protein in SZ risk and endophenotype manifestation provides novel entry points into the etiopathology of this disease.

Key Words: Mouse model, myelination, *PTPRA*, RPTPα, schizophrenia, tyrosine phosphatase

Schizophrenia (SZ; OMIM database entry #181500) is diagnosed by the joint appearance of positive (hallucinations, delusions), negative (disturbed affective and social functioning), and cognitive symptoms. Initial hypotheses about the pathophysiologic mechanism derive from pharmacologic observations: blocking D2 receptors can alleviate positive symptoms, and *N*-methyl-D-aspartate receptor (NMDA-R) antagonists can mimic disease symptoms, which provides support for the dopaminergic and glutamatergic hypotheses. Imaging and postmortem analyses reveal that SZ is accompanied by neuropathologic abnormalities, including decreased myelin content, atypical neuronal cytoarchitecture, and altered laminar organization, suggesting abnormalities in neural development (1). Gene expression studies indicate abnormalities in myelination (2).

Schizophrenia has a significant genetic basis (3). Nonaffected kin can display quantifiable neurobehavioral abnormalities, perhaps reflecting manifestation of a subset of genetic predispositions. The identification of SZ-associated genes is starting to provide insights into disease etiology by implicating molecular signaling pathways. One of the first and most reproducible instances of genetic association with SZ is *neuregulin 1* (*NRG1*), the product of which, signaling via epidermal growth factor (EGF) receptor-like tyrosine kinases, modulates oligodendrocyte development, neuronal migration and differentiation, and glutamatergic and gamma-aminobutyric acid–ergic neurotransmission (4–8). Two other genes in the NRG1 pathway, *ERBB4* encoding a tyrosine kinase receptor for NRG1 and *PTPRZ1* encoding an ERBB4-associated protein tyrosine phosphatase in the oligodendrocyte lineage, are also genetically associated with SZ (9–11). NRG1 signaling may be functionally linked to NMDA-R modulation (11,12), perhaps via phosphorylation of the latter by the Src-family tyrosine kinases Fyn (13) and c-Src (14). Among other predisposition genes for SZ are cell adhesion molecules such as CHL1 and NCAM (15–21), the signaling activities of which also rely on Src-family kinases (SFKs).

Receptor protein tyrosine phosphatase RPTPα (encoded by human *PTPRA* and mouse *Ptpra*) is a physically associated signaling subunit of CHL1 and NCAM, through its well-documented role in regulating Fyn (22–24). RPTPα can also modulate SFKs downstream of EGF-receptor (*ERBB1*) activation (25). *Ptpra* is abundantly ex-

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pressed in the developing central nervous system and remains highly expressed in the adult (26,27). In mice, loss of *Ptpra* function is associated with neurodevelopmental defects in peripheral myelination (28), oligodendrocyte differentiation and myelin basic protein (MBP) expression (29), radial cortical migration (27), misorientation of apical dendrites of deep layer pyramidal neurons (24), reduced NMDA-R phosphorylation, and impairments in synaptic plasticity and short-term memory (27,30,31). Many of these effects reflect a function of RPTPα in regulating SFKs (24,25,29,31–33). Interestingly, *PTPRA* maps to a chromosomal region (20p13) that has been linked to SZ (34,35).

Given the multifold involvement of RPTPα in neurodevelopmental and signaling pathways associated with SZ, we set out to explore whether loss of *Ptpra* function in mice engendered neurobehavioral abnormalities or gene expression signatures relevant to SZ. Positive findings led us to pursue association between polymorphisms at the *PTPRA* locus and disease risk and changes in *PTPRA* expression in dorsolateral prefrontal cortex of patients.

Methods and Materials

Full details of all procedures can be found in Supplement 1.

Mice

Generation of RPTPα-deficient ($Ptpra^{-/-}$) mice has been described previously (33). The allele was backcrossed 10 times with C57Bl/6J mice. Control wildtype (WT) mice were generated by intercross of $Ptpra^{+/-}$ heterozygotes.

Mouse Motor and Neurobehavioral Testing

Spontaneous exploratory locomotor activity and drug-induced hyperactivity were generally assessed as in Butini *et al.* (36), and prepulse inhibition and acute startle responses as in Andreasen *et al.* (37).

Gene Expression Analysis

RNA was extracted from mouse whole brain and human dorsolateral prefrontal cortex and subject to quantitative polymerase chain reaction (qPCR) analysis.

Genetic Association

This was performed essentially as in Ikeda *et al.* (38), followed by inclusion of a second independent cohort.

Results

$Ptpra^{-/-}$ Mice Display Enhanced Psychostimulant-Induced Hyperactivity, Deficient Sensorimotor Gating, and Failure to Habituate to a Startle Response

Dissection of multifactorial diseases is helped by the identification of genetically based quantitative nonapparent "endophenotypes" that are proximal consequences of genetic predisposition but precede or are not necessarily accompanied by manifestation of the disease itself. This reductionist approach is particularly relevant to the dissection of psychiatric disease and to its animal modeling (39).

RPTPα participates in several processes implicated in pharmacologic and neurodevelopmental descriptions of SZ, and $Ptpra^{-/-}$ mice manifest neuropathologic abnormalities reminiscent of those reported in patients. To determine whether loss of function (LOF) of mouse *Ptpra* results in behavioral and neuropsychological abnormalities associated with SZ, we focused on three models: locomotor response to psychostimulants, prepulse inhibition (PPI) as a measure of sensorimotor gating, and the water-maze test for spatial memory.

The studies were performed on a previously described *Ptpra* null allele (27). We first assessed sensorimotor capabilities to exclude the possibility of compounding effects (Table S1 in Supplement 1). Latency to fall off a beam or from an accelerating rotarod revealed no obvious abnormality in general sensorimotor capability of $Ptpra^{-/-}$ mice [beam walk: $F(1,33) = 0$, $p = 1$ and $F(1,33) = .298$, $p = .589$ respectively]. Spontaneous exploratory locomotor activity was also unaffected by *Ptpra* allelic status [$F(1,33) = 1.983$, $p = .169$). We concluded that *Ptpra* LOF does not engender sensorimotor abnormalities that would affect the subsequent analyses.

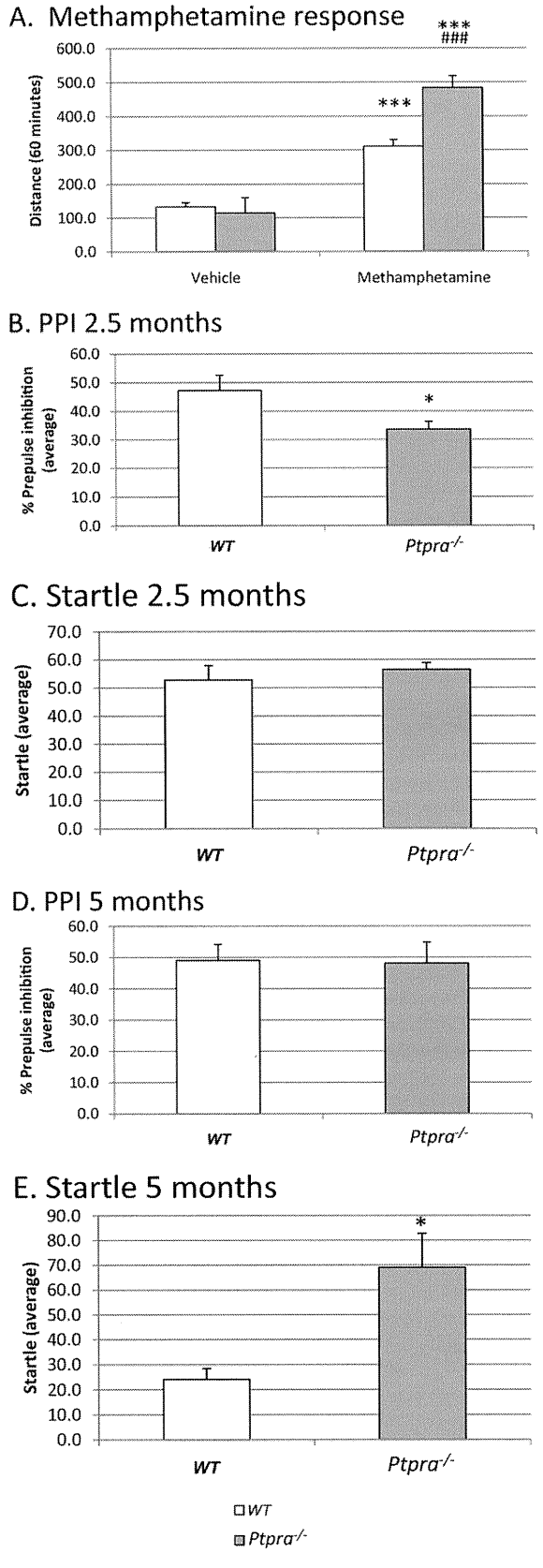
We subsequently asked whether *Ptpra* LOF altered the locomotor response to the psychostimulant methamphetamine (MAMPH), a pharmacologic model inspired by the dopaminergic hypothesis of SZ (40). We found locomotor activity after acute MAMPH administration (2 mg/kg) to be significantly higher in $Ptpra^{-/-}$ mice than in controls [main effect of genotype $F(1,31) = 5.753$, $p = .023$; drug treatment: $F(1,31) = 72.386$, $p < .001$; genotype × drug treatment: $F(1,31) = 8.797$, $p = .006$; post hoc multiple comparisons: MAMPH (WT) vs. MAMPH ($Ptpra^{-/-}$): $p < .001$; Figure 1A].

Given the functional association of RPTPα with NMDA-R (27,30,31), we also investigated the effect of administration of the noncompetitive NMDA-R antagonist MK-801, known for its ability to induce psychotic symptoms in healthy humans. However, at the dose used (.2 mg/kg), MK-801 did not significantly increase locomotor activity in WT mice (not shown).

We next assessed PPI of the startle response, a suggested SZ endophenotype. PPI denotes attenuation of a startle motor response to a sensory (acoustic) stimulus when the latter is immediately (< 500 msec) preceded by a milder stimulus. Used as an operational measure of sensorimotor gating, PPI is impaired in SZ individuals and their unaffected relatives, and antipsychotics can reverse impairment of PPI in experimental models (41). It constitutes a preattentive process akin to a reflex response. We tested the mice on two occasions (2.5 months apart) to determine 1) whether any PPI abnormality persisted and 2) whether normal habituation to the startle response occurred, because impaired habituation is a hallmark of SZ (42). Such a longitudinal design is rarely applied to knockout mice, partly because of difficulties in the use of test batteries in rodents (43,44).

At the initial age of analysis (2.5 months), we observed no difference in startle response between $Ptpra^{-/-}$ and WT mice [Figure 1C; $F(1,14) = .332$, $p = .576$]. In contrast, $Ptpra^{-/-}$ mice manifested a significant reduction in PPI [Figure 1B; $F(1,14) = 6.006$, $p = .032$). This genotype-dependent difference in PPI disappeared at a more advanced age [5 months; $F(1,13) = .034$, $p = .857$; Figure 1D], suggesting a critical time period for manifestation of this abnormal phenotype. Strikingly, however, at the more advanced age, the typical habituation (reduced response) to the acoustic startle stimulus alone observed in WT animals [startle at 2.5 months compared with 5 months: $F(1,14) = 11.797$, $p = .014$] did not occur in $Ptpra^{-/-}$ mice [startle at 2.5 months compared with 5 months: $F(1,13) = .013$, $p = .914$), leading to a significant difference in acoustic startle response during this retesting at 5 months (Figure 1E).

Finally, we subjected $Ptpra^{-/-}$ mice to a water-maze test, a hippocampal-dependent model of spatial memory. Impaired hippocampal-based function in SZ is well documented (45). Detailed analysis revealed no genotype differences in place finding (swim distance and latency to target; Figure 2A) nor in the probe test (recall of spatial position of the platform; Figure 2B) or reversal learning (Figure 2C).



Loss of *Ptpra* Function Leads to Reduced Central Nervous System Levels of Myelin Markers and SZ-Associated genes

Increased MAMPH sensitivity, impaired PPI, and failure to habituate to a startle response are commonly accepted indicators for modeling SZ-associated states in mice. To assess whether the relevance of *Ptpra*$^{-/-}$ mice as a model for SZ-associated abnormalities extends beyond neuropsychological parameters, we began assessing SZ-associated gene expression markers. Imaging analysis, postmortem brain studies, genetic association studies, and gene expression studies reveal that abnormal oligodendroglial function and myelination are commonly associated with SZ (2,46–49). One of the major targets of RPTPα, the Src family kinase Fyn, plays important roles in myelination (50–53). A transient defect in peripheral myelination has been documented in the strain of *Ptpra*$^{-/-}$ mice studied here (28); an independently generated *Ptpra*$^{-/-}$ strain was recently reported to display impaired oligodendrocyte differentiation in vitro and reduced MBP immunostaining in vivo (29). We therefore investigated the expression of myelin related genes in the brains of our strain of *Ptpra*$^{-/-}$ mice.

Figure 1. Methamphetamine (MAMPH) sensitivity, prepulse inhibition (PPI), and startle response in wildtype (WT) and *Ptpra*$^{-/-}$ mice. **(A)** Influence of genotype on locomotor response to MAMPH challenge. Methamphetamine resulted in pronounced hyperactivity in both genotypes ($p < .001$ vs. vehicle [WT] and vehicle [*Ptpra*$^{-/-}$], respectively). However, the locomotor response to MAMPH was exaggerated in *Ptpra*$^{-/-}$ compared with WT mice ($p < .001$, MAMPH [WT] vs. MAMPH [*Ptpra*$^{-/-}$]). The study was run as a within-subject design in which each individual mouse served as its own control by injecting them with vehicle, MAMPH (2 mg/kg), or MK801 (.2 mg/kg, data not shown, see text) in a semirandomized order ensuring representation of all treatment groups on each test day over 3 days. Compounds were dosed intraperitoneally immediately before test start, $n = 7$ or 8 (*Ptpra*$^{-/-}$) and 8 or 9 (WT). The animals were 3.5 months old at testing. Data are represented as mean distance traveled (± SEM) over 60 min. Statistical evaluation was performed by applying two-way analysis of variance (ANOVA) with genotype and drug as factors followed by Fishers Least Significant Difference test for multiple comparisons. ***$p < .001$ vs. vehicle (*Ptpra*$^{-/-}$) and vehicle (WT), respectively. ###$p < .001$ vs. WT-methamphetamine. **(B)** Effect of genotype on PPI of the acoustic startle response at age 2.5 months. *Ptpra* gene disruption leads to reduced prepulse inhibition of the acoustic startle response compared with WT mice at age 2.5 months ($p < .05$). Data from four prepulse intensities (pp 4, pp 8, pp 16, and pp 24) are collapsed and expressed as mean ± SEM, $n = 7$ (*Ptpra*$^{-/-}$) and 8 (WT). Statistical evaluation was performed by applying two-way ANOVA with genotype and sex as factors. *$p < .05$ vs. WT. **(C)** Effect of genotype on acoustic startle response at age 2.5 months. No effect of *Ptpra* gene disruption is seen on the startle response to a 120-dB noise burst at age 2.5 months compared with WT mice ($p > .05$). Data are expressed as mean ± SEM, $n = 7$ (*Ptpra*$^{-/-}$) and 8 (WT). Statistical evaluation was performed by applying two-way ANOVA with genotype and sex as factors. **(D)** Effect of genotype on prepulse inhibition of the acoustic startle response at age 5 months. At 5 months, the reduction of PPI noted at 2.5 months was no longer evident in *Ptpra*$^{-/-}$ mice ($p > .05$ vs. WT), indicating a critical time period for manifestation of this phenotype. Data from four prepulse intensities (pp 4, pp 8, pp 16, and pp 24) are collapsed and expressed as mean ± SEM, $n = 7$ for both genotypes. Statistical evaluation was performed by applying two-way ANOVA with genotype and sex as factors. **(E)** Effect of genotype on acoustic startle response at age 5 months. At 5 months, *Ptpra*$^{-/-}$ mice displayed an increased startle response to a 120-dB noise burst compared with WT mice ($p = .013$). This difference is due to a significantly reduced startle response in WT mice at age 5 months compared with 2.5 months ($p = .014$). This habituated response to a startle inducing stimulus is not evident in *Ptpra*$^{-/-}$ mice, because the startle response at 5 months of age is similar to that at 2.5 months ($p = .914$). Data are expressed as mean ± SEM, $n = 7$ (*Ptpra*$^{-/-}$ and WT). Intergroup comparisons were performed by applying two-way ANOVA with genotype and sex as factors. Intragroup comparisons were performed by applying one-way repeated-measure ANOVA with age and genotype as factors. *$p < .05$ versus WT.

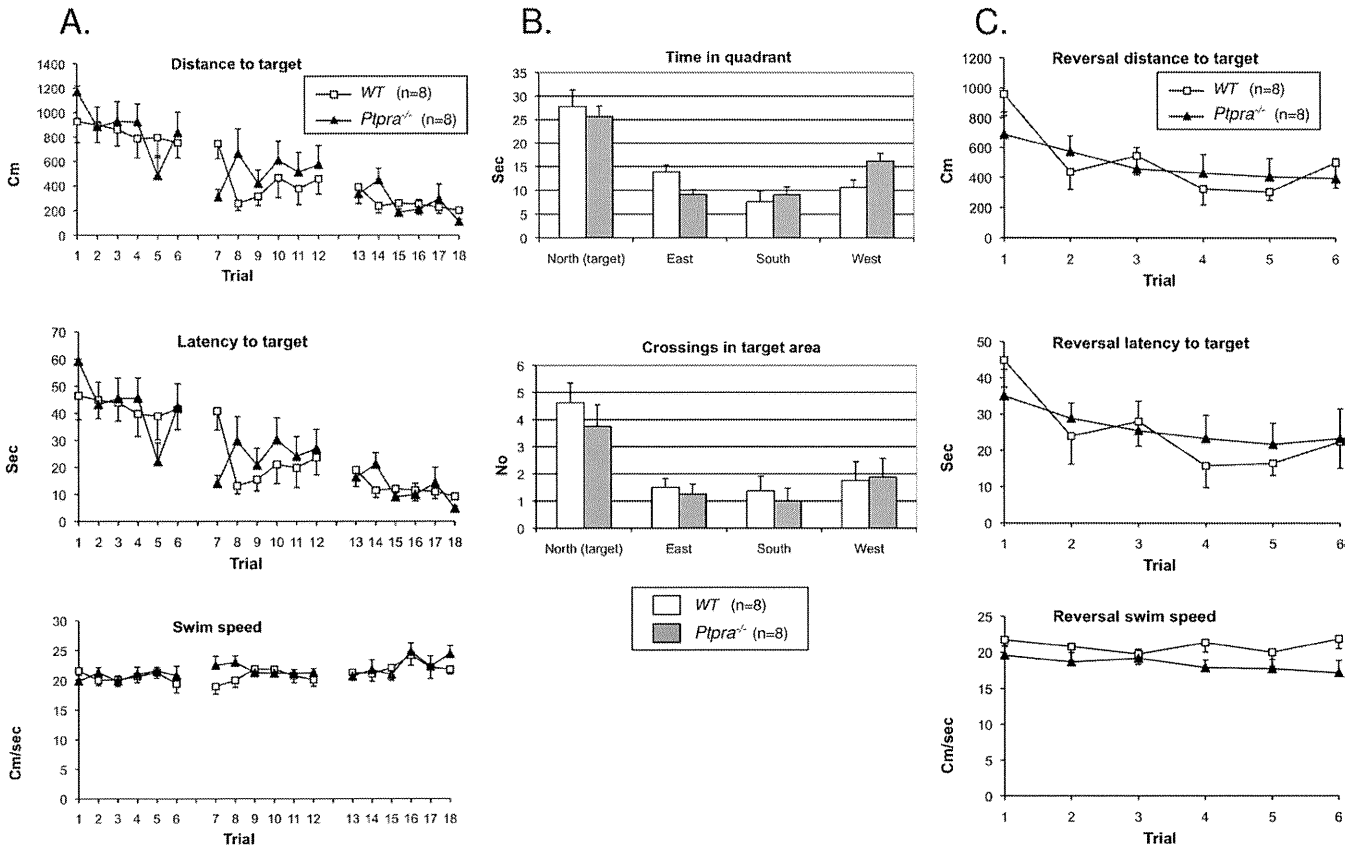


Figure 2. Morris water-maze analysis of wildtype (WT) and *Ptpra*$^{-/-}$ mice. **(A)** Training: swim distance to target, escape latency, and swim speed. Both WT ($n = 8$) and *Ptpra*$^{-/-}$ ($n = 8$) used shorter distances to locate the hidden platform over trials. No effect of genotype or genotype × 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 $p = .218$).

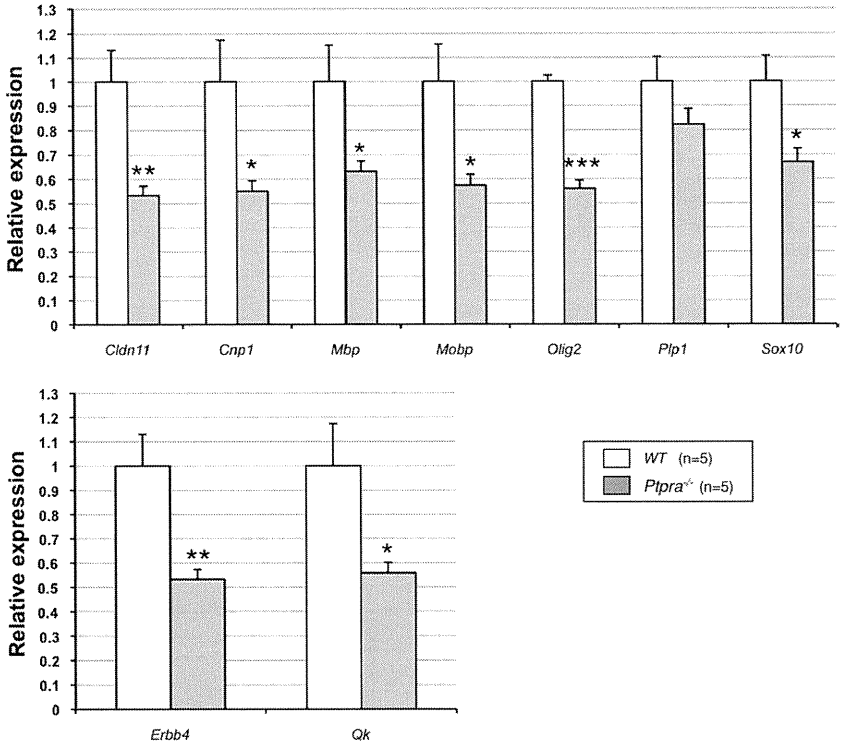


Figure 3. Reduced expression of oligodendrocyte- and myelin-related gene expression in total brain of *Ptpra*$^{-/-}$ mice. Five-month-old animals (five males/genotype) were analyzed by quantitative polymerase chain reaction. Four endogenous control genes (*Actb, B2m, Gusb,* and *Ppia*) were used for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

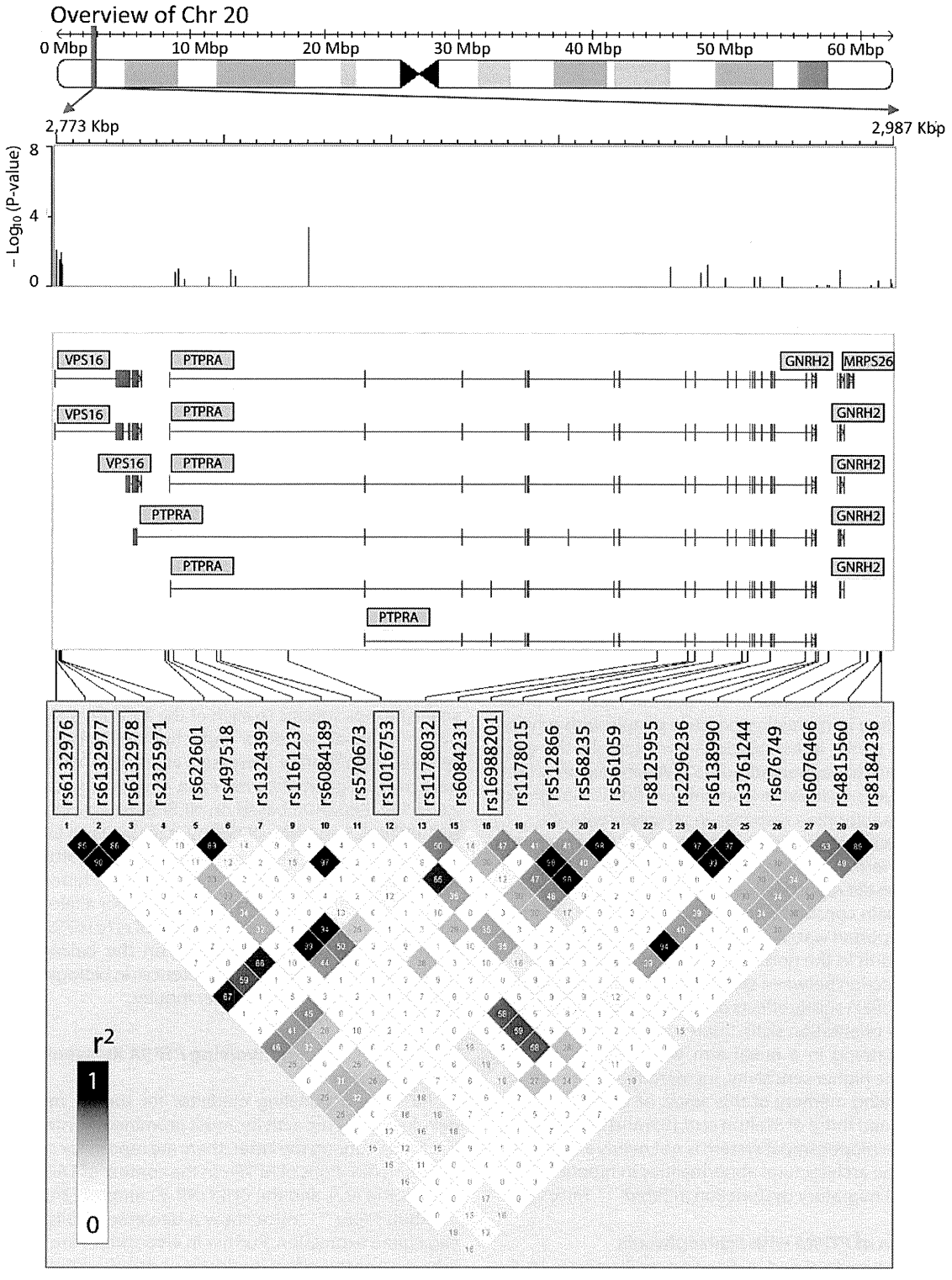


Figure 4. Genetic association of single nucleotide polymorphisms around and in the *PTPRA* gene with schizophrenia. Red boxes indicate nominally associated single nucleotide polymorphisms in the first-stage analysis (genome-wide association study screening sample). r^2 is the correlation coefficient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

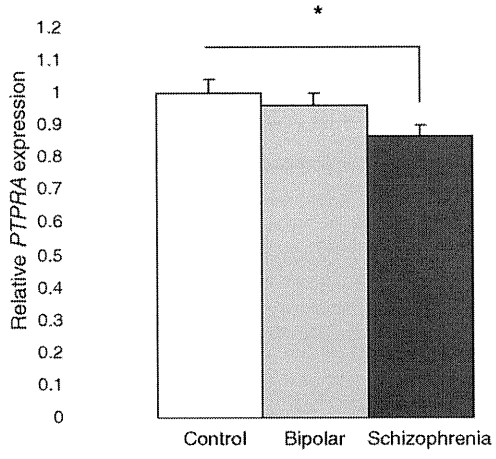


Figure 5. Reduced *PTPRA* expression levels in dorsolateral prefrontal cortex specimens from schizophrenia patients. Thirty-five patient samples for each category (healthy control subjects, patients with schizophrenia, and patients with bipolar disorder) were analyzed by quantitative polymerase chain 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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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

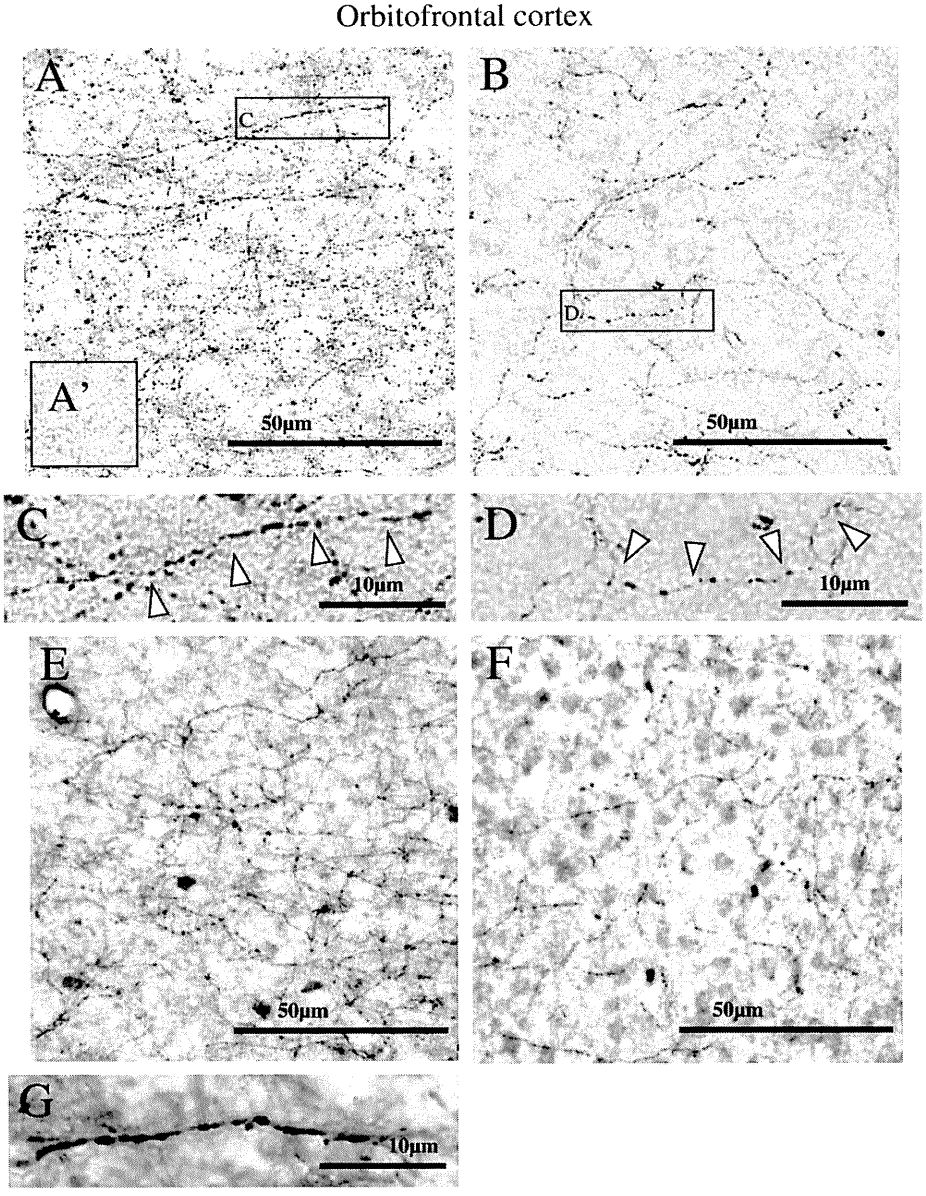

Fig. 1 – These figures show the TH-immunostained fibers in sections of orbitofrontal cortex stained using two different primary anti-TH antibodies; Bregma 1.98 mm (http://www.mbl.org/atlas165/atlas165_start.html). (A–D) Anti-tyrosine hydroxylase antibody (Affinity BioReagents Co.). (E–F) Anti-tyrosine 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.

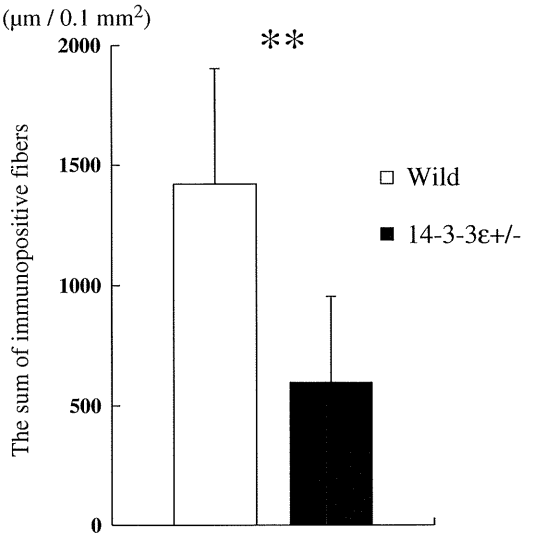

Fig. 2 – This graph shows the results of the sum of TH-immunopositive fibers ($P<0.01$). The error bars represent the standard deviation from the mean.**

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

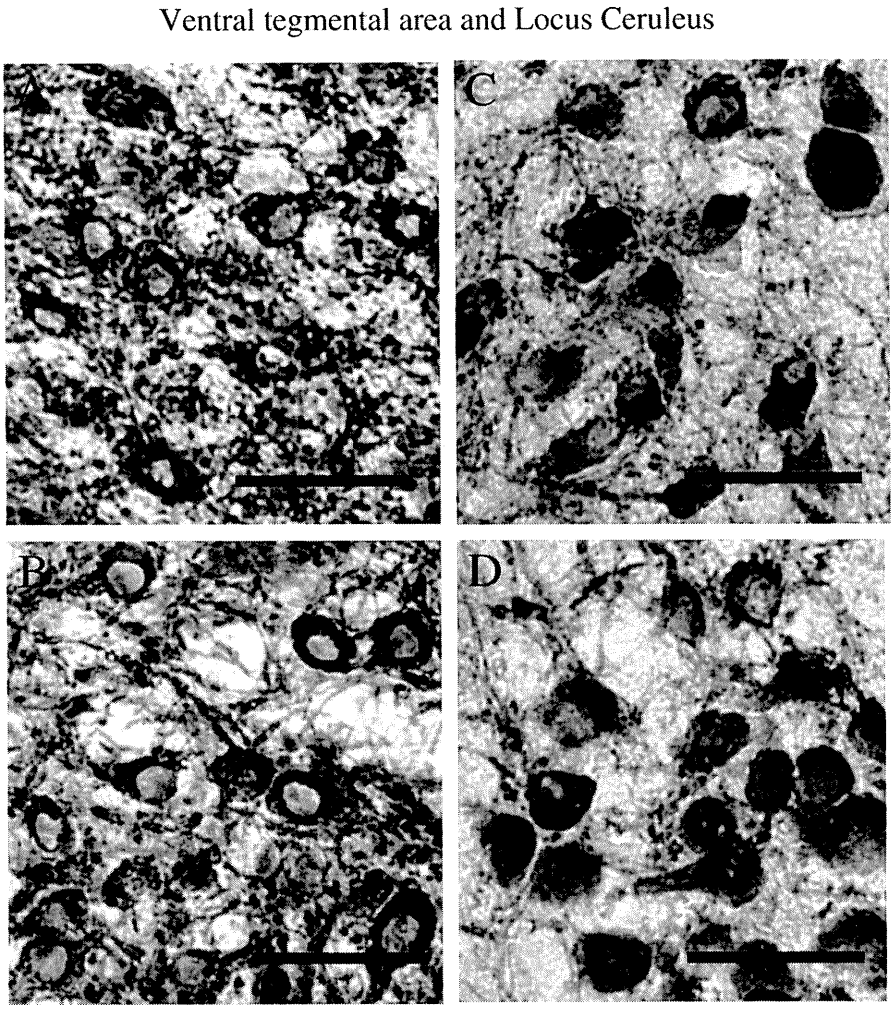

Fig. 3 – These figures show TH-immunostained neurons, including the (A) VTA of a wild-type mouse, (B) VTA of a KO mouse, (C) LC of a wild-type mouse, (D) LC of a KO mouse; scale bar=50 μm.

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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