noncoding region of the *BDNF* gene reported by Shintani et al. [1992]. It has been reported that the 132C > T in exon V of the *BDNF* gene was significantly associated with late-onset Alzheimer's disease [Kunugi et al., 2001], or schizophrenia [Szekeres et al., 2003]. In addition, it has been reported that the BDNF 196G > A (val66met) is associated with personality traits in healthy subjects [Sen et al., 2003; Itoh et al., 2004], suggesting the role of *BDNF* gene in personality traits and temperament. Considering the role of personality traits in substance use disorders [Howard et al., 1997], it is likely that the *BDNF* gene may be implicated in the vulnerability of drug abuse.

MAP is the most popular abused drug in Japan. Use of MAP induces a strong psychological dependence, and repeated usage frequently results in psychotic states, which symptoms are similar to those of paranoid-type schizophrenia [Sato et al., 1992; Ujike, 2002]. It has been demonstrated that BDNF plays a role in the survival and differentiation of midbrain dopaminergic neurons in vivo [Hyman et al., 1991] and in vitro [Spina et al., 1992], and that chronic BDNF treatment enhances locomotor activity and conditioned reward to cocaine [Hoger et al., 1999]. In addition, it is likely that BDNF could modulate the release of dopamine through the activation of TrkB receptors [Blochl and Sirrenberg, 1996]. Furthermore, it has been reported that locomotor behaviors by amphetamine was increased to a greater degree in the BDNF heterozygous (+/−) knock-out mice, and that striatal dopamine concentrations were significantly higher in the BDNF heterozygous (+/−) knock-out mice [Dluzen et al., 2001]. Moreover, it has been reported recently that pretreatment with intra-nucleus accumbens injection of BDNF antibody or TrkB antibody suppressed significantly the release of dopamine and dopamine-related behaviors induced by administration of MAP, suggesting the implication of BDNF in MAP-induced dopamine release and MAP-induced abnormal behaviors [Narita et al., 2003]. Taken together, it is of interest to study the influences of the *BDNF* gene SNPs in MAP abuse vulnerability. In this study, we analyzed the frequency of two known SNPs (196G > A (val66met) in exon XIIIA and 132C > T in exon V) of *BDNF* gene between MAP abusers and healthy subjects in Japan.

## MATERIALS AND METHODS

### Subjects and Samples

This study was performed after obtaining the approval of the ethics committees of each affiliated institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA). All subjects provided written informed consent for the use of their DNA samples for this study. The subjects were 189 patients (150 males and 39 female; age, 36.6 ± 11.9 (mean ± SD)) with MAP dependence and a psychotic disorder meeting the ICD-10-DCR criteria (F15.2 and F15.5) who were outpatients or inpatients of psychiatric hospitals of the JGIDA. Two hundred and two volunteers were recruited as healthy controls. All controls have no significant lifetime history of use of any addictive substance (158 males and 44 females; 37.2 ± 10.6 (mean ± SD)), the majority of whom were medical staff with no past history and no family history of drug dependence or psychotic disorders. Diagnoses were made by two trained psychiatrists by interview and available information including hospital records. Detailed characteristics of patients were shown in the Supplement 1 (see the online Supplement 1 at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html).

### Genotyping

The genomic DNA was extracted from peripheral leukocytes by standard procedures. Polymerase chain reaction (PCR) and the PCR-based restriction fragment length polymorphism (RFLP) assay were performed to genotype the DNA sequence variants of the *BDNF* gene. Detailed methods for genotyping were shown in the Supplement 2 (see the online Supplement 2 at http://www.interscience.wiley.com/jpages/0148-7299:1/suppmat/index.html).

### Statistical Analysis

Fisher's exact test was used for categorical comparisons, and Student's *t*-test was employed for age difference. Significance for the results was set at $P < 0.05$.

## RESULTS

Both the genotype and allele frequencies for the patients and controls are shown in Tables I and II. The genotype distribution for patients groups and control groups did not deviate significantly from the Hardy–Weinberg equilibrium. No significant differences were found in the frequency of the genotype or allele in these two SNPs between patients and controls (132C > T in exon V: genotype, $P = 0.586$, allele, $P = 0.594$; 196G > A (val66met) in exon XIIIA: genotype, $P = 0.889$, allele, $P = 0.713$). As for the 132C > T substitution, there was no individual who was homozygous for the 132T allele in exon V. Within patients, we analyzed the effects of prognosis psychosis (transient or prolonged), spontaneous relapse (positive or negative), and poly-substance abuse (yes or no) on the *BDNF* gene SNPs (132C > T in exon V and 196G > A in exon XIIIA). The genotypic and allelic distribution of two SNPs was not

TABLE I. Genotype and Allele Frequencies of the Brain-Derived Neurotrophic Factor (BDNF) 132C > T (in Exon V) Gene Polymorphism of in Controls and Methamphetamine (MAP) Abusers

| | | Genotype | | | | Allele | | |
|---|---|---|---|---|---|---|---|---|
| 132C > T | n | CC | CT | TT | *P* | C | T | *P* |
| Control | 202 | 183 (90.6%) | 19 (9.4%) | 0 (0%) | | 385 (95.3%) | 19 (4.7%) | |
| Abuser | 189 | 175 (92.6%) | 14 (7.4%) | 0 (0%) | 0.586 | 364 (96.3%) | 14 (3.7%) | 0.594 |
| Prognosis of psychosis | | | | | | | | |
| Transient | 94 | 87 (92.6%) | 7 (7.4%) | 0 (0%) | 0.664 | 181 (96.3%) | 7 (3.7%) | 0.671 |
| Prolonged | 66 | 62 (93.9%) | 4 (6.1%) | 0 (0%) | 0.612 | 128 (97.0%) | 4 (3.0%) | 0.620 |
| Spontaneous relapse | | | | | | | | |
| Positive | 64 | 60 (93.8%) | 4 (6.3%) | 0 (0%) | 0.611 | 124 (96.9%) | 4 (3.1%) | 0.619 |
| Negative | 116 | 107 (92.2%) | 9 (7.8%) | 0 (0%) | 0.685 | 223 (96.1%) | 9 (3.9%) | 0.692 |
| Poly-substance abuse | | | | | | | | |
| No | 56 | 51 (91.1%) | 5 (8.9%) | 0 (0%) | 1 | 107 (95.5%) | 5 (4.5%) | 1 |
| Yes | 122 | 114 (93.4%) | 8 (6.6%) | 0 (0%) | 0.414 | 236 (96.7%) | 8 (3.3%) | 0.424 |

Statistical analysis was performed by a Fisher's exact test (vs. control).

TABLE II. Genotype and Allele Frequencies of the BDNF 196G > A (val66met) (in Exon XIIIA) Gene Polymorphism in Controls and MAP Abusers

| | | Genotype | | | | Allele | | |
|---|---|---|---|---|---|---|---|---|
| 196G > A (val66met) | n | GG | GA | AA | *P* | G | A | *P* |
| Control | 202 | 70 (34.7%) | 107 (53.0%) | 25 (12.4%) | | 247 (61.1%) | 157 (38.9%) | |
| Abuser | 189 | 70 (37.0%) | 96 (50.8%) | 23 (12.2%) | 0.889 | 236 (62.4%) | 142 (37.6%) | 0.713 |
| Prognosis of psychosis | | | | | | | | |
| Transient | 94 | 32 (34.0%) | 53 (56.4%) | 9 (9.6%) | 0.778 | 117 (62.2%) | 71 (37.8%) | 0.856 |
| Prolonged | 66 | 25 (37.9%) | 30 (45.5%) | 11 (16.7%) | 0.472 | 80 (60.6%) | 52 (39.4%) | 0.918 |
| Spontaneous relapse | | | | | | | | |
| Positive | 64 | 27 (42.2%) | 30 (46.9%) | 7 (10.9%) | 0.571 | 84 (65.6%) | 44 (34.4%) | 0.403 |
| Negative | 116 | 39 (33.6%) | 62 (53.4%) | 15 (12.9%) | 0.972 | 140 (60.3%) | 92 (39.7%) | 0.866 |
| Poly-substance abuse | | | | | | | | |
| No | 56 | 21 (37.5%) | 27 (48.2%) | 8 (14.3%) | 0.791 | 69 (61.6%) | 43 (38.4%) | 1 |
| Yes | 122 | 47 (38.5%) | 60 (49.2%) | 15 (12.3%) | 0.762 | 154 (63.1%) | 90 (36.9%) | 0.676 |

Statistical analysis was performed by a Fisher's exact test (vs. control).

significantly different between transient type of psychosis and prolonged type of psychosis (Tables I and II). Furthermore, the genotypic and allelic distribution of two SNPs was not significantly different between positive spontaneous relapse and negative spontaneous relapse (Tables I and II). Moreover, the genotypic and allelic distribution of two SNPs was not significantly different between poly-substance abuse and non-poly-substance abuse (Tables I and II). In addition, we found that two SNPs were not in linkage disequilibrium with each other.

## DISCUSSION

The present study suggests that two SNPs (132C > T in exon V and 196G > A (val66met) in exon XIIIA) of the *BDNF* gene may not be susceptible to MAP abuse in Japanese samples. Using a European American sample and an African American sample, it has been reported that the *BDNF* gene could contribute to vulnerabilities to poly-substance abuse [Uhl et al., 2001]. It is possible that difference in ethnicity might contribute to discrepancy between our study and other study. Frequency of A allele of 196G > A (val66met) in Japanese population [Momose et al., 2002; Nakata et al., 2003; Itoh et al., 2004; this study] is higher than that of Caucasian population [Egan et al., 2003; Hakansson et al., 2003; Sen et al., 2003], suggesting the ethnic difference in this SNP (val66met) [Shimizu et al., 2004]. First, it has been reported that the 196G > A (val66met) of the *BDNF* gene is associated with Parkinson's disease in Japanese subjects [Momose et al., 2002]. However, lack of association between the BDNF 196G > A (val66met) and Parkinson's disease in a Swedish population was reported [Hakansson et al., 2003]. Second, it has been reported that the 196G > A (val66met) of the *BDNF* gene is associated with bipolar disorder in Caucasian [Neves-Pereira et al., 2002; Sklar et al., 2002]. However, no association between 196G > A (val66met) of the *BDNF* gene and bipolar disorder in Japanese population was detected [Nakata et al., 2003], suggesting that the *BDNF* gene may confer a susceptibility to bipolar disorder in Caucasian, but not in Japanese population. Thus, it is likely that ethnic differences may contribute to inconsistent findings between Caucasian sample and Japanese sample.

In this study, we investigated two SNPs; one (132C > T in exon V) in the noncoding region and the other (196G > A (val66met) in exon XIIIA) in the coding region. Whereas BDNF 196G > A (val66met) SNP does not affect the function of a mature BDNF protein, it has been shown to dramatically alter the intracellular trafficking and packaging of pro-BDNF, and, thus, the regulated secretion of the mature BDNF protein [Egan et al., 2003]. At cellular levels, marked deficits were observed in the intracellular distribution, processing, and secretion of met-BDNF, suggesting that pro-BDNF may play a critical role in synaptic targeting and activity-dependent secretion at synapses [Egan et al., 2003]. Remarkably, healthy human subjects with the met allele exhibit impaired hippocampal activity and memory function [Egan et al., 2003]. However, it is currently unknown whether the BDNF 132C > T SNP could affect on the function, synthesis, or secretion of BDNF. There are still other known SNPs in the *BDNF* gene sequences, and it is possible that there are more unknown SNPs. Further studies of other SNPs and unknown SNPs should be done to clarify the involvement of the *BDNF* gene in substance abuse vulnerability.

In conclusion, we failed to detect evidence for a role of two SNPs (196G > A (val66met) in exon XIIIA and 132C > T in exon V) of the *BDNF* gene in the pathogenesis of MAP abusers in our Japanese sample. Therefore, it is unlikely that the two SNPs (196G > A (val66met) in exon XIIIA and 132C > T in exon V) of *BDNF* gene are associated with Japanese MAP abusers.

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# Fyn Is Required for Haloperidol-induced Catalepsy in Mice*[S]

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**Fyn-mediated tyrosine phosphorylation of *N*-methyl-D-aspartate (NMDA) receptor subunits has been implicated in various brain functions, including ethanol tolerance, learning, and seizure susceptibility. In this study, we explored the role of Fyn in haloperidol-induced catalepsy, an animal model of the extrapyramidal side effects of antipsychotics. Haloperidol induced catalepsy and muscle rigidity in the control mice, but these responses were significantly reduced in Fyn-deficient mice. Expression of the striatal dopamine $D_2$ receptor, the main site of haloperidol action, did not differ between the two genotypes. Fyn activation and enhanced tyrosine phosphorylation of the NMDA receptor NR2B subunit, as measured by Western blotting, were induced after haloperidol injection of the control mice, but both responses were significantly reduced in Fyn-deficient mice. Dopamine $D_2$ receptor blockade was shown to increase both NR2B phosphorylation and the NMDA-induced calcium responses in control cultured striatal neurons but not in Fyn-deficient neurons. Based on these findings, we proposed a new molecular mechanism underlying haloperidol-induced catalepsy, in which the dopamine $D_2$ receptor antagonist induces striatal Fyn activation and the subsequent tyrosine phosphorylation of NR2B alters striatal neuronal activity, thereby inducing the behavioral changes that are manifested as a cataleptic response.**

Typical antipsychotic agents, such as haloperidol and chlorpromazine, have extrapyramidal side effects (EPS)[2] that resemble Parkinson disease. Drug-induced catalepsy, the impairment of movement initiation, in rodents is an animal model of EPS and is mainly caused by blockade of the dopamine $D_2$ receptor ($D_2$-R) (1, 2).

Haloperidol-induced responses are also dependent on *N*-methyl-D-aspartate receptor (NMDA-R) activity, because prior administration of the NMDA-R antagonist MK-801 attenuates haloperidol-induced catalepsy (3, 4). $D_2$-R and NMDA-R are co-expressed in close proximity along the dendrites of medium spiny neurons in the striatum, and they are functionally coupled in terms of controlling extrapyramidal functions (5).

The NMDA-Rs are hetero-oligomeric ligand-gated ion channels composed of a single NR1 subunit and one type of NR2 (A–D) subunit (6). The most abundant receptor subunits in the striatum are NR1, NR2A, and NR2B (7, 8). These three subunits are involved in extrapyramidal functions (5), and we have found that an NR2B-selective antagonist attenuates haloperidol-induced catalepsy (9).

Phosphorylation of tyrosine residues on the NMDA-R has been reported to modulate its channel characteristics (10, 11). Depriving the striatum of dopaminergic input increases the tyrosine phosphorylation of the striatal NMDA-R and the motor response (12, 13), but infusing the striatum with a tyrosine kinase inhibitor, genistein, attenuates both the tyrosine phosphorylation and the motor response induced by dopaminergic deprivation (13).

Fyn is a member of the Src family kinases (SFKs) and is associated with the NMDA-R at postsynaptic densities. Fyn phosphorylates NMDA-R subunits and modifies their channel activity (14). One of the NMDA-R subunits, NR2B, is preferentially phosphorylated by Fyn, and its phosphorylation has been implicated in several brain functions, including ethanol tolerance, long term potentiation, and seizure susceptibility (15–18). The Tyr-1472 of NR2B is a particularly key site for Fyn-mediated phosphorylation (17, 19).

To investigate the role of Fyn in the cataleptic behavior induced by haloperidol, we studied these haloperidol effects in Fyn-deficient mice and biochemically analyzed the Fyn-mediated signal transduction initiated by haloperidol. Based on our results, we discuss the significance of Fyn activation in haloperidol-induced catalepsy within the scope of signal transduction from $D_2$-R inhibition to modulation of the extrapyramidal system.

## MATERIALS AND METHODS

*Animals*—Fyn tyrosine kinase-deficient mice were generated by inserting the β-galactosidase gene (*lacZ*) into the reading frame of the *fyn* gene as described previously (20). Because the *lacZ* introduced is expressed in both heterozygous ($+/fyn^Z$) and homozygous ($fyn^Z/fyn^Z$) mice, heterozygous mice were mainly used as the controls instead of wild-type mice to compensate for the possible effect of *lacZ* expression as a foreign gene. The background of this mutant's strain is C57BL/6J. Genotypes were analyzed by the PCR. All animals were maintained under standard laboratory conditions as described previously (15). All experimental procedures were in accordance with the 1996 National Institutes of Health guidelines and were approved by the Animal Care Committee of the Chiba University Graduate School of Medicine,

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[2] The abbreviations used are: EPS, extrapyramidal side effects; BSS, balanced salt solution; $D_2$-R, dopamine $D_2$-receptor; HAL, Haloperidol; NMDA, *N*-methyl-d-aspartate; NMDA-R, *N*-methyl-d-aspartate receptor; PKA, protein kinase A; PKC, protein kinase C; SFKs, Src family kinases; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

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*Pharmacological Agents*—Haloperidol, the dopamine $D_2$-R-selective antagonist L-741,626 (21), and the $D_2$-R-selective agonist (−)-quinpirole were purchased from Sigma. The drugs were administered by intraperitoneal injection in a volume of 10 μl/g body weight. All solutions were prepared immediately prior to the experiments. To exclude the effect of drug tolerance, no animals were used more than once in the pharmacological experiments.

*Antibodies*—Goat polyclonal anti-$D_2$-R antibody (N19) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-tyrosine hydroxylase (TH) antibody was obtained from Chemicon (Temecula, CA). A mouse monoclonal anti-phosphotyrosine antibody (Tyr(P)-100) was purchased from Cell Signaling Technology (Beverly, MA). Phosphorylation site-specific rabbit polyclonal antibody against p-Src (Y418) and p-Src (Y529) was obtained from BIOSOURCE (Camarillo, CA) and against p-NR2B (Y1472) was from Sigma. Rabbit polyclonal anti-NR2B antibody was a gift from Dr. Masahiko Watanabe (22). Anti-Src mouse monoclonal antibody (GD11) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-Fyn rat monoclonal antibody (γC3) was raised by Dr. Masahiro Yasuda (23). The anti-βIII tubulin mouse monoclonal antibody was purchased from Promega (Madison, WI).

*Assessment of Catalepsy*—Catalepsy was measured by a bar test (24). The test was carried out 1 h after intraperitoneal injection of haloperidol (0–1.0 mg/kg) or L-741,626 (0–10 mg/kg). A 3-mm-diameter wooden bar was fixed horizontally 4 cm above the floor of a Plexiglas cage. The animals were placed inside the test cage and allowed to acclimatize for 5 min prior to performing the bar test. Both forepaws were then gently placed on the bar, and the length of time during which each mouse maintained the initial position was measured (maximum cut-off time, 180 s).

*Analysis of Rigidity*—Muscle rigidity after haloperidol administration was assessed by a mechanographic technique using a modified device designed for rat experiments (25). The mouse was placed in a narrow, well ventilated plastic tube to restrict body movement, and one hind leg was bound to a force sensor (AD4937-5N, A & D Co. Ltd., Tokyo, Japan) that records linear reciprocating motion (15-mm distance, 15 cycles/min) via a crank and motor. The raw data from the force sensor were analyzed on a Macintosh computer connected to an A/D converter and software (PowerLab 4s, chart version 3.6 ADInstruments, Mountain View, CA), and the resistance of the flexor and extensor muscles to forced extension and flexion of the knee and ankle joint was measured. The mice were attached to the above device, and the difference in muscle resistance before and after the administration of vehicle or haloperidol (1.0 mg/kg) was recorded. The mean amplitude of 10 consecutive waves at each time point was calculated. Spikes that indicated spontaneous movements of the mice were excluded from the count.

*In Situ Hybridization Histochemistry*—The distribution of $D_2$-R gene expression in the striatum of the control and Fyn-deficient mice was compared. The probe was prepared as follows. A cDNA fragment encoding the sequence of mouse $D_2$-R (1.3 kbp, a gift from Dr. T. Kaneko, Kyoto University) was cloned into the pBluescript II/KS− vector, and the clone was digested and used as a DNA template to synthesize an antisense or sense digoxigenin-labeled cRNA probe. The probe was prepared with T7 or T3 RNA polymerase and a digoxigenin RNA labeling kit (Roche Applied Science). Staining was performed as reported previously (26). The sense cRNA probe was employed as the control, and no signals in the brain were detected with it.

*Immunoblot Analysis*—One hour after administration of the vehicle, haloperidol (1.0 mg/kg), or L-741,626 (5 mg/kg), the striatum was immediately dissected and frozen in liquid nitrogen. The striatum was placed in buffer containing 10% sucrose, 3% SDS, 10 mM Tris-HCl, pH 6.8, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride and homogenized with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). Samples were spun down (20,000 × *g*, 15 min) to remove insoluble material, and the protein concentration was determined with the BCA protein assay reagent (Pierce). After the addition of 40 mM dithiothreitol, the samples were boiled for 5 min, and an equal amount of protein (40 μg per lane) from each sample was separated by electrophoresis on 10% polyacrylamide gels. The gels were transferred onto Immobilon membranes (Millipore, Bedford, MA). The membranes were blocked with 1% bovine serum albumin in Tris-buffered saline (TBS; pH 7.5) containing 0.1% Tween 20 (TBS-T) or 10% skim milk in TBS-T for 1 h and probed with primary antibodies (1:750 dilution for TH, 1:4000 for anti-βIII tubulin, and 1:1000 for other antibodies). After washing three times with TBS-T, the membranes were incubated with horseradish peroxidase-labeled secondary antibodies (anti-goat, anti-rabbit, anti-rat, or anti-mouse IgG, 1:20,000 dilution, all purchased from The Jackson Laboratories, West Grove, PA). After washing three times, the signals were detected with ECL Plus (Amersham Biosciences) and ATTO Cool Saver (ATTO Corp., Tokyo, Japan). The membranes were then incubated with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50 °C for 30 min, washed, blocked, and reprobed with other antibodies.

*Immunoprecipitation and Western Blotting*—The procedures for immunoprecipitation were as described previously (15). Striatum obtained 1 h after vehicle or haloperidol (1.0 mg/kg) administration was placed in lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, and 1 mM sodium orthovanadate) and homogenized with a Polytron homogenizer. Samples were spun down to remove insoluble material, and the protein concentration was determined. Equal amounts of protein (500 μg) were then used for immunoprecipitation. Samples were precleared with protein G-Sepharose (Amersham Biosciences), incubated for 1 h at 4 °C with 1 μg of the anti-Fyn or anti-Src antibody, and then incubated for 1 h at 4 °C with 10 μl of protein G-Sepharose. After three washes with lysis buffer, the pelleted protein G-Sepharose was boiled for 5 min in 30 μl of SDS sample buffer, and 15 μl of the supernatant was subjected to SDS-PAGE. The separated proteins were subsequently blotted onto Immobilon, probed with each antibody, and visualized as described above.

*Primary Cultures of Striatal Neurons*—Primary cultures of striatal neurons were prepared from the fetal striata of wild-type and Fyn-deficient mice at embryonic day 17. Striata from 6 to 8 fetal brains were dissected and placed in Hanks' balanced salt solution (Invitrogen) and then were transferred into a dissociation medium containing Hanks' balanced salt solution, 0.05% DNase I, and 1% trypsin/EDTA and incubated at 37 °C for 7 min. After sedimentation, the supernatant was removed, and the pellet was washed three times with Hanks' balanced salt solution containing 1% penicillin/streptomycin. The tissue was gently placed in Hanks' balanced salt solution containing 0.05% DNase I and triturated with a plastic pipette until a homogeneous suspension was obtained. After centrifugation at 130 × *g* for 8 min, the cell pellet was resuspended in Neurobasal/B27 medium (Invitrogen) containing 0.5 mM L-glutamine and penicillin/streptomycin (100 units/ml). The cell cultures were seeded at a density of $3 \times 10^5$ cells/cm$^2$ on 0.1% polyethyleneimine-coated cover glasses in 1.9 cm$^2$/well dishes (Nunclon, Nunc). Cells were maintained at 37 °C under a humidified 5% $CO_2$

atmosphere. The cultured striatal neurons were identified immunocytochemically with anti-GAD65 antibody (Chemicon) and anti-MAP2 antibody (Sigma). More than 95% of both the wild-type and Fyn-deficient neurons were double-labeled by anti-GAD65 and anti-MAP2 (supplemental Fig. S1).

*Calcium Imaging*—Calcium imaging was carried out as described previously (27). Briefly, striatal primary cells were incubated with 10 μM fura-2/AM (Dojindo) for 1 h at 30 °C in balanced salt solution (BSS) consisting of (in mM) NaCl 130, KCl 5.4, glucose 5.5, HEPES 10, and $CaCl_2$ 2, and adjusted to pH 7.4 with NaOH. After washing, the cover glasses that contained cultured neurons were mounted on the stage of an inverted fluorescence microscope (IX50; Olympus) and perfused with BSS at a flow rate of 1.8 ml/min. The perfusion medium was prewarmed and maintained at 32.6 ± 1.1 °C in the measurement dish. Fluorescence images obtained by alternate excitation with 340 and 380 nm light through the ×20 objective lens and CCD camera (C2400-8; Hamamatsu Photonics, Hamamatsu, Japan) were fed into an image processor (Argus 50, Hamamatsu) for ratiometric analysis. The effect of the $D_2$-R antagonist L-741,626 on the channel activity of NMDA receptors was investigated in the presence of the selective $D_2$-R agonist quinpirole in the perfusion medium. As shown in supplemental Fig. S2, quinpirole alone had a dose-dependent inhibitory effect on the channel activity of the NMDA receptors of the striatal primary neurons of the control mice consistent with its inhibitory effect reported in the striatal slice culture (28). Quinpirole was observed to have almost the same degree of the inhibitory effect on Fyn-deficient neurons (49% decrease at 50 μM).

*Preparation of Protein Samples from the Cultured Neurons*—Striatal neurons were cultured in 1.9 $cm^2$/well dishes (Nunclon, Nunc) as described above. Each solution used in the following experiments was pre-warmed to 37 °C in a water bath. Culture dishes were warmed on a heat block to 37 °C. The culture medium was removed, and the cultured cells were incubated with BSS for at least 5 min. The cells were then incubated with the following: 1) BSS for 7 min followed by incubation in quinpirole (50 μM) in BSS for 7 min, or 2) in quinpirole (50 μM) in BSS for 7 min followed by a mixture of quinpirole (50 μM) and L-741,626 (10 μM) in BSS for 7 min. The solution was removed, and the cells were immediately lysed in 150 μl of SDS sample buffer.

*Statistical Analyses*—The results of the catalepsy assessment and calcium imaging were evaluated by the Kruskal-Wallis test followed by the Mann-Whitney *U* test. The results of the muscle rigidity analysis were evaluated by a two-way repeated measure ANOVA. The results of Western blotting were evaluated by one-way ANOVA followed by Bartlett's test. All data are expressed as the mean ± S.E.

## RESULTS

Haloperidol induced catalepsy in the $+/fyn^Z$ mice, and the duration of the catalepsy increased in a dose-dependent manner (Fig. 1). By contrast, the duration of the catalepsy in the $fyn^Z/fyn^Z$ mice was significantly shorter (Fig. 1). At the 1.0 mg/kg dose, there was no difference in the cataleptic response between the $+/fynZ$ mice and the wild-type mice (154.5 ± 47.1 s). The $D_2$-R-selective antagonist L-741,626 was confirmed to induce catalepsy in the control mice (supplemental Fig. S3), as reported previously in rats (29), but the duration of the catalepsy was significantly reduced in $fyn^Z/fyn^Z$ mice (supplemental Fig. S3). Because there was no significant difference in the temporal patterns of the locomotor activity between $+/fyn^Z$ mice and $fyn^Z/fyn^Z$ mice (30), the altered cataleptic response in the $fyn^Z/fyn^Z$ mice was concluded not to be due to a locomotion defect.

Because Fyn-deficient mice are more fearful than control mice (31),

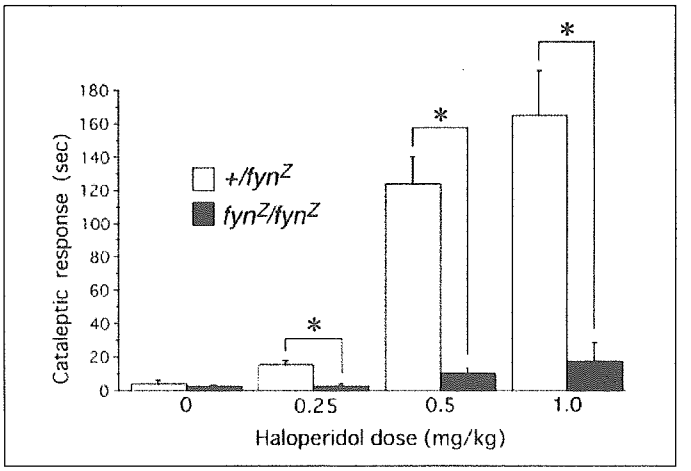


FIGURE 1. **Assessment of catalepsy following haloperidol administration.** The cataleptic response to haloperidol administration increased dose-dependently in $+/fyn^Z$ mice but was significantly reduced in $fyn^Z/fyn^Z$ mice. Eight to twelve animals were used in each group. The *columns* represent the means, and the *bars* represent the S.E. Statistically significant differences were identified by the Mann-Whitney *U* test; *, $p < 0.001$.

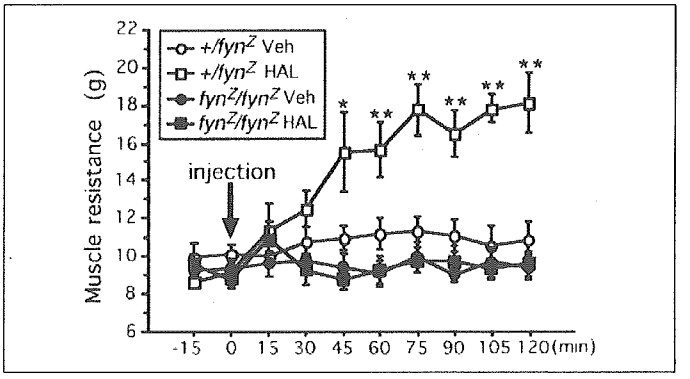


FIGURE 2. **Mean amplitude of muscle resistance before and after haloperidol (*HAL*) injection.** In $+/fyn^Z$ mice, muscular rigidity increased as early as 45 min after haloperidol administration (1 mg/kg), and the increase persisted for more than 2 h. By contrast, no increase in muscle rigidity was detected in the $fyn^Z/fyn^Z$ mice after haloperidol administration. Administration of vehicle (*Veh*) alone did not affect rigidity in either genotype. Six animals were used in each group. Data are expressed as means ± S.E. Statistically significant differences were identified by two-way repeated measure ANOVA; *, $p < 0.05$; **, $p < 0.005$.

we suspected that they might avoid a procedure like the "bar test" and that the duration of catalepsy would be misleadingly short as a result. We therefore also measured muscular rigidity to minimize any such emotional influence on the response to haloperidol. Haloperidol induced a marked increase in hind limb muscle rigidity in the $+/fyn^Z$ mice that was detectable as early as 45 min after administration (1.0 mg/kg) and persisted for more than 2 h, but no increase in muscle rigidity was detected in the $fyn^Z/fyn^Z$ mice (Fig. 2 and supplemental Fig. S4).

To exclude the possibility that the failure to respond to haloperidol was because of a difference in $D_2$-R expression, *in situ* hybridization of $D_2$-R mRNA and Western blotting of $D_2$-R protein were performed on the striatum of $+/fyn^Z$ and $fyn^Z/fyn^Z$ mice. As shown in Fig. 3, no clear difference was observed in either striatal $D_2$-R gene expression (Fig. 3*A*) or the protein level (Fig. 3*B*). Western blotting analysis of striatal TH was also performed to determine whether there was any difference between the two genotypes in the abundance of the rate-limiting enzyme in dopamine biosynthesis, but little difference in the amount of TH protein was found (Fig. 3*C*).

The effect of haloperidol on protein tyrosine phosphorylation in the

striatum of the $+/fyn^Z$ and $fyn^Z/fyn^Z$ mice was compared by Western blotting. One hour after haloperidol administration (1.0 mg/kg), a marked increase in tyrosine phosphorylation of several proteins, including 60-, 110-, and 180-kDa proteins, was observed in the striatum of the $+/fyn^Z$ mice but not of the $fyn^Z/fyn^Z$ mice (Fig. 4*A*).

Because the 60-kDa protein corresponds in size to SFKs, we measured tyrosine phosphorylation of the activation-related Tyr-418 residue on SFKs. As shown in Fig. 4*B*, phospho-Tyr-418 increased after haloperidol injection of the $+/fyn^Z$ mice, but no such effect was observed in the $fyn^Z/fyn^Z$ mice. Basal Tyr(P)-418 immunoreactivity was much lower in the $fyn^Z/fyn^Z$ mice. Because the anti-pY418 antibody recognizes both Fyn and Src, we immunoprecipitated Fyn and Src, and we examined the phosphorylation of the activation-related residue, Tyr-418, and of the inhibition-related residue, Tyr(P)-529, by Western blotting in the $+/fyn^Z$ mice. As shown in Fig. 4*C*, Fyn but not Src was activated at Tyr-418 by haloperidol, and no change was observed in the phosphorylation at Tyr-529. Because the 180-kDa protein corresponds in size to the NR2B subunit, we also measured the phosphorylation of the Tyr-1472 of NR2B, the key phosphorylation site, by Fyn. The results showed that phospho-Tyr-1472 increased in the $+/fyn^Z$ mice but not in the $fyn^Z/fyn^Z$ mice (Fig. 4*D*). The basal level of Tyr(P)-1472 in the $fyn^Z/fyn^Z$ mice was not significantly different from the basal level in the $+/fyn^Z$ mice. Marked increases in tyrosine phosphorylation of the 60-, 110-, and 180-kDa proteins and up-regulation of Tyr(P)-418 and Tyr(P)-1472 were also observed following L-741,626 administration to the control mice, but no such effects were observed in the $fyn^Z/fyn^Z$ mice (supplemental Fig. S5).

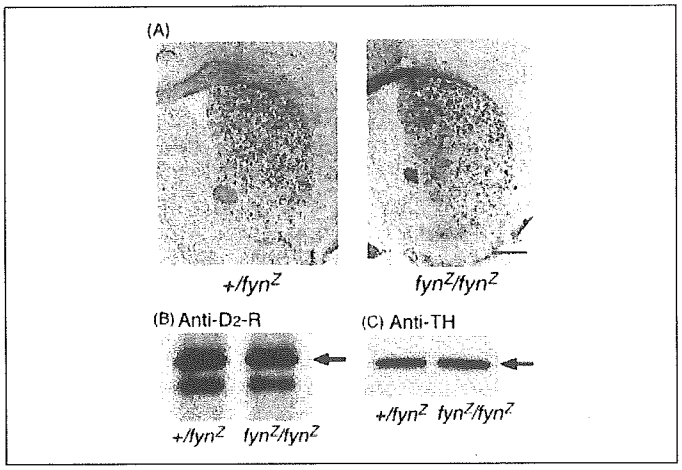


FIGURE 3. **Expression of dopaminergic markers in the striatum.** *A, in situ* hybridization histochemistry for dopamine $D_2$ receptor mRNA. Strong $D_2$ receptor expression in the striatum was seen in both genotypes, and there was little difference in its distribution. *Scale bar*, 500 μm. *B*, Western blotting analysis of the dopamine $D_2$ receptor in the striatum. The intensity of the upper band (*arrow*) around 85 kDa, corresponding to the $D_2$-receptor, was essentially the same in both genotypes. *C*, Western blotting analysis of striatal tyrosine hydroxylase (*TH*). The intensity of the band at around 70 kDa differed little between the two genotypes.

There were no sex differences in the results of either the behavioral or biochemical studies (data not shown). The same findings in regard to the haloperidol-induced enhancement of tyrosine phosphorylation were also observed in the wild-type mice, and no clear difference was detected between $+/fyn^Z$ mice and wild-type mice (data not shown). To investigate whether the Fyn-mediated increase in NMDA receptor phosphorylation by $D_2$-R blockade affects NMDA receptor activity, we prepared striatal primary cultures and assessed the channel activity of NMDA receptors by the calcium imaging method.

After 4–7 days of culture, we loaded 10 μM fura-2/AM into the primary cells and measured the increase in intracellular calcium concentration ($[Ca^{2+}]_i$) by calcium fluorimetry. Exposure to 3 μM NMDA/10 μM glycine for 30 s induced a robust response in more than 95% of the cells analyzed, and repeated applications of NMDA/glycine at 5-min intervals evoked reproducible responses (data not shown), indicating little desensitization of the NMDA receptors under our experimental conditions.

We first examined the effect of a $D_2$-R-selective antagonist,

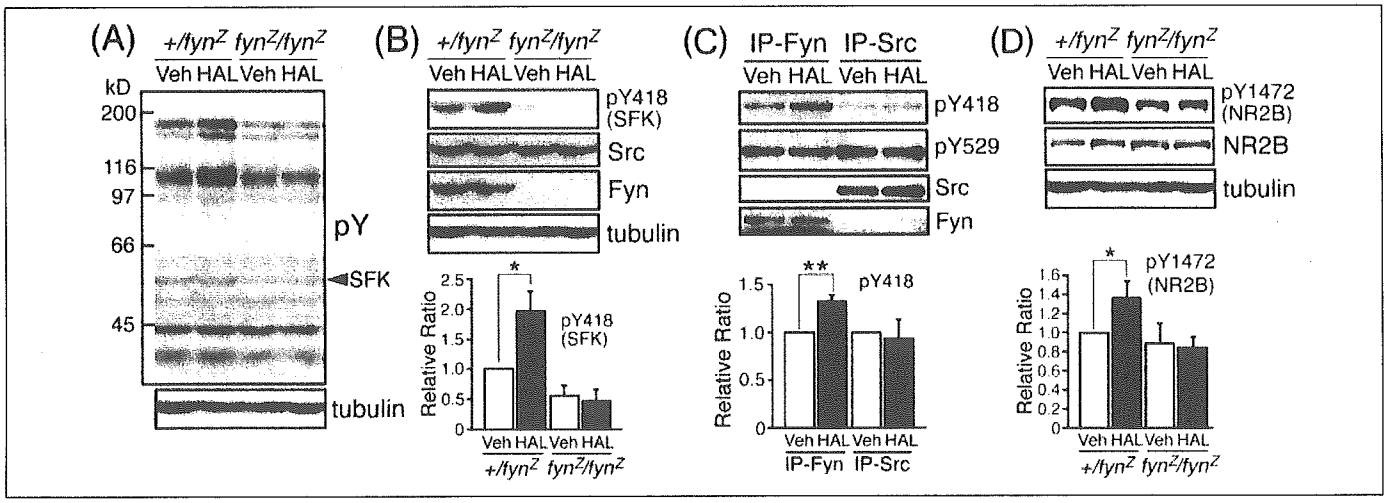


FIGURE 4. **Tyrosine phosphorylation in the striatum after haloperidol administration.** *A*, haloperidol (*HAL*) administration (1.0 mg/kg) increased the tyrosine phosphorylation of several proteins, including 60-, 110-, and 180-kDa proteins, in $+/fyn^Z$ mice compared with vehicle (*Veh*) administration, but no such difference was found in the $fyn^Z/fyn^Z$ mice. *B*, the same tendency toward a haloperidol-induced increase in tyrosine phosphorylation was observed in regard to Tyr-418 of Src family kinases (pY418) in the $+/fyn^Z$ mice, but no such increase was observed in the $fyn^Z/fyn^Z$ mice. The amounts of Src and Fyn were unchanged. Densitometric analysis revealed a significant increase in the band density of the Tyr(P)-418 of Src family kinases after injection in $+/fyn^Z$ mice with haloperidol. No such increase was detected in the $fyn^Z/fyn^Z$ mice, and the basal band density was also much lower in $fyn^Z/fyn^Z$ mice. *C*, haloperidol-induced increase in the phosphorylation of the Src family kinases Src and Fyn in the control mice demonstrated by immunoprecipitation (*IP*) and Western blotting. Haloperidol (1.0 mg/kg) induced an increase in Tyr(P)-418 following anti-Fyn immunoprecipitation but not following anti-Src immunoprecipitation. No clear change was observed in Tyr(P)-529. The amounts of Src and Fyn were unchanged. Densitometric analysis revealed an increase after haloperidol injection in the band of Tyr(P)-418 on Fyn but not Src. *D*, there was also a significant increase in the band density of Tyr(P)-1472 on NR2B following haloperidol injection in $+/fyn^Z$ mice. No such difference was detected in the $fyn^Z/fyn^Z$ mice. The basal level of Tyr(P)-1472 in the $fyn^Z/fyn^Z$ mice tended to decrease in comparison with the $+/fyn^Z$ mice, but the difference was not significant. The amount of NR2B was unchanged. *HAL*, haloperidol (1.0 mg/kg); *Veh*, vehicle. Four to six animals were used in each group. The *columns* represent means, and the *bars* represent means ± S.E. One-way ANOVA; *, $p < 0.01$; **, $p < 0.05$.

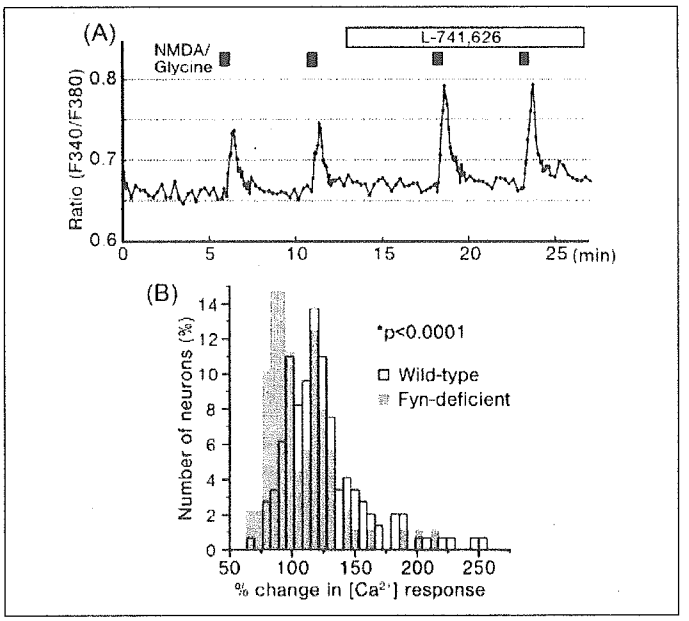


FIGURE 5. **Effect of a $D_2$-R selective antagonist, L-741,626, on the channel activity of NMDA receptors in primary striatal neurons assessed by $Ca^{2+}$ fluorimetry.** *A*, $[Ca^{2+}]_i$ responses of wild-type neurons in the presence of quinpirole (50 $\mu$M). A representative trace of wild-type neurons exposed to two applications of 3 $\mu$M NMDA, 10 $\mu$M glycine (control response) and two applications of 3 $\mu$M NMDA, 10 $\mu$M glycine 5 min after starting L-741,626 (10 $\mu$M) addition to the perfusion buffer. The addition of L-741,626 significantly enhanced NMDA/glycine-induced $[Ca^{2+}]_i$ responses compared with the control responses. *B*, histogram of $[Ca^{2+}]_i$ responses induced by 3 $\mu$M NMDA, 10 $\mu$M glycine in the presence of L-741,626 (10 $\mu$M) relative to those in the absence of L-741,626 (control response). Large numbers (74.7%) of the wild-type neurons (*open bars*) exhibited larger NMDA/glycine-induced $[Ca^{2+}]_i$ responses after the addition of L-741,626 (mean change, 125.7 ± 34.1% of the control responses), whereas the number of Fyn-deficient neurons (*filled bars*) showing larger responses was significantly decreased (46.6%, mean change, 105.8 ± 26.9% of the control responses). There was also a group of Fyn-deficient neurons that exhibited larger responses after L-741,626 addition that peaked at around 120% of the control responses. Eight wild-type and six Fyn-deficient striatal neuronal cultures were used. Mann-Whitney *U* test.

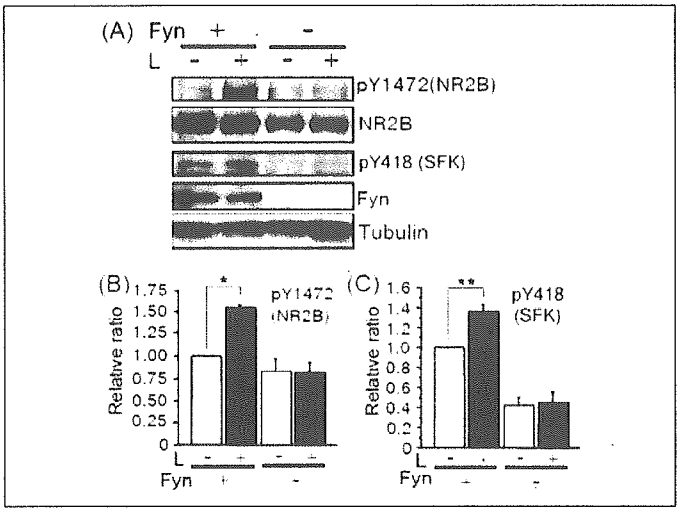


FIGURE 6. **Western blot analysis of striatal primary neurons.** *A*, neurons were exposed to or not exposed to L-741,626 (10 $\mu$M) in the presence of quinpirole. L-741,626 (10 $\mu$M) markedly increased the tyrosine phosphorylation at Tyr-1472 of NR2B (*pY1472*) and at Tyr-418 of Src family kinases (*pY418*) in the wild type, but no such difference was found in Fyn deficiency. *B*, densitometric analysis revealed a significant increase in the band density of the Tyr(P)-1472 of NR2B in the wild type. No such increase was detected in Fyn deficiency. *C*, there was also a significant increase in the band density of the Tyr(P)-418 of the Src family kinases when L-741,626 was added to the wild type. No such difference was detected in Fyn deficiency. Six wild-type and six Fyn-deficient striatal neuronal cultures were used. The *columns* represent means, and the *bars* represent S.E. One-way ANOVA; *, $p < 0.0001$; **, $p < 0.005$.

L-741,626, on the channel activity of NMDA receptors of wild-type neurons in the presence of a $D_2$-R-selective agonist, quinpirole (50 $\mu$M). After confirming that the responses evoked were reproducible by two successive applications of 3 $\mu$M NMDA, 10 $\mu$M glycine (control responses), we added 10 $\mu$M L-741,626 to the perfusion buffer BSS. After 5 min, we measured the 3 $\mu$M NMDA, 10 $\mu$M glycine-induced increases in $[Ca^{2+}]_i$ and compared them with the control responses. As shown in Fig. 5*A*, larger responses than the control responses were detected in the presence of L-741,626, but in some neurons, almost identical responses were observed both in the presence and absence (control) of L-741,626. The distribution of changes in $[Ca^{2+}]_i$ responses after the addition of L-741,626 is shown in Fig. 5*B* (*open bars*). After the addition of L-741,626, a large number of neurons (74.7%) showed larger responses than the control responses (mean change, 125.7 ± 34.1%).

To determine whether the larger NMDA/glycine-induced responses induced by L-741,626 were mediated by Fyn, we performed the same experiments on primary cultures prepared from Fyn-deficient mice. After the addition of L-741,626, only 46.6% of the Fyn-deficient neurons showed larger responses than the control responses, and L-741,626 addition had little enhancing effect on the responses (mean change, 106.8 ± 26.9% of the control responses; see Fig. 5*B*, *filled bars*). A subset of Fyn-deficient neurons exhibited larger responses after L-741,626 addition, and their responses peaked at around 120% of the control responses. In addition, the numbers of neurons exhibiting larger responses after L-741,626 addition was lower in the presence of the Src family inhibitor PP2 (10 $\mu$M) (mean change, 99.9 ± 15.7% of the control responses).

We then examined the effect of L-741,626 on Fyn activation and NMDA receptor phosphorylation in the presence of quinpirole (50 $\mu$M) by Western blot analysis, as shown in Fig. 6. Primary cells were exposed or not exposed (control) to 10 $\mu$M L-741,626 for 7 min prior to sample preparation. In the wild-type cells, immunoreactivity for anti-pY1472 antibody and anti-pY418 antibody in the L-741,626-treated cell extracts was stronger than in the control cell extracts. By contrast, when Fyn-deficient cells were used, there were no significant differences in immunoreactivity for anti-pY1472 antibody and anti-pY418 antibody between the L-741,626-exposed cell extracts and the control cell extracts.

## DISCUSSION

The results of this study show that Fyn is required for haloperidol-induced catalepsy. We also found that haloperidol induces Fyn activation and a Fyn-dependent increase in NR2B phosphorylation in mouse striatum. We used striatal primary neurons to verify that $D_2$-R blockade induced Fyn activation, enhancement of NR2B phosphorylation, and potentiation of the channel activity of NMDA receptor at the cellular level, and the latter two effects were significantly reduced in Fyn-deficient neurons. On the basis of these findings, we propose a new molecular mechanism that underlies haloperidol-induced catalepsy in which the $D_2$-R antagonist induces Fyn activation in the striatum, and the subsequent phosphorylation of the NR2B subunit by the activated Fyn increases the channel activity of NMDA receptors, which leads to changes in neural transmission and results in the cataleptic response.

Because haloperidol-induced catalepsy and muscular rigidity are mainly caused by blockade of dopamine $D_2$-Rs in the striatum (1, 2), sensitivity to haloperidol should be altered by changes in dopaminergic transmission. However, there were no clear differences between Fyn-deficient mice and control mice in the expression pattern of the $D_2$-R gene or the amounts of $D_2$-R protein and tyrosine hydroxylase, and measurements by microdialysis showed no significant difference in striatal basal dopamine levels (32). Thus, it is rather unlikely that the

reduced sensitivity to haloperidol in the Fyn-deficient mice is because of defective dopaminergic transmission.

We found that haloperidol increased phosphorylation of the Tyr-418 residues of Fyn. The catalytic activity of SFKs is controlled through autocatalytic phosphorylation and dephosphorylation, particularly at amino acid residues Tyr-418 and Tyr-529 (14), and the phosphorylated Tyr-529 intramolecularly interacts with an Src homology 2 domain to form a loop, thereby suppressing kinase function. Intermolecular autophosphorylation at Tyr-418, on the other hand, activates SFKs by displacing Tyr-418 from the substrate-binding site, thus allowing the kinase to gain access to substrates (33). We found that the haloperidol-induced increase in Tyr-418 phosphorylation occurred specifically in Fyn and did not occur in Src, whereas phosphorylation of Tyr-529 was the same in both Src and Fyn. Thus, Fyn is specifically activated by haloperidol *in vivo*.

Haloperidol also increased the phosphorylation of Tyr-1472 in the NR2B subunit, and because no increase was observed in Fyn-deficient mice, the haloperidol-induced phosphorylation of NR2B subunit must be dependent on Fyn. Moreover, we confirmed this $D_2$-R antagonist induced Fyn-mediated enhancement of NR2B phosphorylation at the cellular level in primary cultures of striatal neurons.

Fyn-mediated phosphorylation of NR2B and potentiation of NMDA-R channel activity are involved in several brain functions, including ethanol tolerance (15), seizure susceptibility (23), and long term potentiation (17). Activation of EphB receptors has also been reported to result in increased phosphorylation of NR2B and an increase in NMDA-R channel activity measured by $Ca^{2+}$ imaging in hippocampal primary cultures (34). The use of HEK293T cells transfected with a mutant NR2B construct in this study also showed that Fyn-mediated tyrosine phosphorylation of NR2B is required for the increase in NMDA-R channel activity. In our study, NMDA-R channel activity in most wild-type striatal neurons was increased by the blockade of $D_2$-R, and the proportion of such neurons was significantly reduced in Fyn deficiency. Thus, the increased NMDA-R activity after $D_2$-R blockade in most of the striatal neurons was Fyn-dependent. However, NMDA-R may also be activated by a Fyn-independent pathway, because a certain proportion of the neurons in the Fyn-deficient striatal culture exhibited increased NMDA-R activity.

It has been repeatedly observed that the NMDA-R antagonist MK-801 attenuates haloperidol-induced catalepsy (9, 35–37), and we recently reported that prior exposure to the NR2B-selective antagonist CP-101,606 significantly reduces haloperidol-induced catalepsy (9). Thus, haloperidol-induced catalepsy is specifically dependent on NR2B function, and activation of NR2B function by Fyn-mediated phosphorylation is likely to be required for catalepsy to occur.

NMDA-R dysfunction is hypothesized to be the pathogenetic mechanism responsible for schizophrenia, because NMDA-R antagonists cause psychotic states resembling schizophrenia (38–40), and mice with reduced NMDA-R expression have been reported to display schizophrenia-related behaviors (41). Because unmedicated schizophrenic patients exhibit attenuated EPS shortly after haloperidol administration compared with healthy controls (42), the lower responsiveness to haloperidol in Fyn-deficient mice may mimic a feature of schizophrenia.

Several mutant mice, including mice deficient in the $D_2$-R (24), $A_{2A}$-adenosine receptor (43), retinoid X receptor γ1 (44), and protein kinase A (PKA) (45), show reduced cataleptic responses to haloperidol. The reduced cataleptic response in one of them, the PKA-deficient mutant, is likely to be caused by a molecular mechanism similar to that in Fyn deficiency, because an increase in PKA-mediated serine phosphorylation of striatal NR1 subunits increases following haloperidol administration (46). The scaffolding protein RACK1 binds to both Fyn and NR2B, and the three molecules form a complex in rat hippocampus (16, 47). Dissociation of RACK1 from this RACK1-Fyn-NR2B complex facilitates Fyn-mediated phosphorylation of NR2B (47). Because PKA activation has been demonstrated to dissociate RACK1 from this complex (16), the above PKA-RACK1-Fyn pathway may also exist downstream of $D_2$-R in the striatum.

Another molecule that may act between $D_2$-R and Fyn is PKC. Activation of G-protein-coupled receptors, such as muscarinic and metabotropic glutamate receptors, in the hippocampus increases NMDA-evoked currents via protein kinase C (PKC) (48). The increase in NMDA-R function is mediated by the activation of SFKs, because the PKC-induced NMDA-R up-regulation is blocked by an inhibitor of Src and Fyn and does not occur in Src-deficient cells (48). $D_2$-R is another G-protein-coupled receptor, and because haloperidol administration acutely increases PKC activity in the rat striatum (49), Fyn activation after $D_2$-R blockade may be mediated by the PKC pathway.

Other molecules, including receptor tyrosine kinases (34, 50) and a cytokine receptor (51), have also been reported to be involved in SFK-mediated phosphorylation and activation of NMDA-R, and they may be involved in the striatal activation of Fyn after $D_2$-R inhibition.

In this study we found that blockade of $D_2$-R causes Fyn activation, Fyn-mediated NMDA-R phosphorylation, and potentiation of its channel activity in the striatal neurons that may be responsible for haloperidol-induced catalepsy. Further investigation should focus on the above-postulated Fyn-activation mechanisms initiated by $D_2$-R blockade, and these transduction steps should be drug targets for controlling not only motor function but higher cognitive brain function.

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# Immune Activation During Pregnancy in Mice Leads to Dopaminergic Hyperfunction and Cognitive Impairment in the Offspring: A Neurodevelopmental Animal Model of Schizophrenia

Kimiyoshi Ozawa, Kenji Hashimoto, Takashi Kishimoto, Eiji Shimizu, Hiroshi Ishikura, and Masaomi Iyo

***Background:*** *Maternal viral infection is associated with increased risk for schizophrenia. It is hypothesized that the maternal immune response to viruses may influence fetal brain development and lead to schizophrenia.*

***Methods:*** *To mimic a viral infection, the synthetic double strand RNA polyriboinosinic-polyribocytidilic acid (poly I:C) was administered into pregnant mice. Behavioral evaluations (thigmotaxis, methamphetamine [MAP]-induced hyperactivity, novel-object recognition test [NORT]), sensorimotor gating (prepulse inhibition [PPI]), and biochemical evaluation of the dopaminergic function of the offspring of phosphate-buffered saline (PBS)-treated dams (PBS-mice) and that of poly I:C-treated dams (poly I:C-mice) were examined.*

***Results:*** *In juveniles, no difference was found between the poly I:C-mice and PBS-mice. However, in adults, the poly I:C-mice exhibited attenuated thigmotaxis, greater response in MAP-induced (2 mg/kg) hyperlocomotion, deficits in PPI, and cognitive impairment in NORT compared with the PBS-mice. Cognitive impairment in the adult poly I:C-mice could be improved by subchronic administration of clozapine (5.0 mg/kg) but not haloperidol (.1 mg/kg). Increased dopamine (DA) turnover and decreased receptor binding of $D_2$-like receptors, but not $D_1$-like receptors, in the striatum were found in adult poly I:C-mice.*

***Conclusions:*** *Prenatal poly I:C administration causes maturation-dependent increased subcortical DA function and cognitive impairment in the offspring, indicating a neurodevelopmental animal model of schizophrenia.*

**Key Words:** Poly I:C, prenatal immune activation, neurodevelopment, dopamine, cognition, clozapine

Schizophrenia is a severe brain disorder characterized by disturbances of thoughts, perceptions, volition, and cognition, which affects about 1% of the world population today. Despite strong evidence of a major genetic component, specific genes have not been identified (Harrison and Owen 2003; Harrison and Weinberger 2005). However, twin studies have demonstrated the role of environmental factors, because the rate of schizophrenia among identical twins is only about 50% (Tsuang and Faraone 1995). Taking into account both genetic and environmental factors, multiple converging evidence supports the hypothesis that a disruption of neurodevelopment may play a key role in the pathogenesis of schizophrenia (Murray et al 1992; Pearce 2001; Waddington et al 1998; Weinberger 1987; Weinberger 1995; Wyatt 1996). Although this neurodevelopmental hypothesis is very attractive, as it can explain much of what is known about schizophrenia, the hypothesis is still unproven.

According to a number of epidemiological studies, one of the most plausible candidate risk factors is maternal viral infection during pregnancy. An association between exposure to influenza and increased risk for schizophrenia in the offspring was demonstrated (Barr et al 1990; Brown et al 2004a; Kendell and Kemp 1989; Mednick et al 1988; Westergaard et al 1999). It has also been reported that several viruses such as poliovirus (Suvisaari et al 1999), rubella, measles, varicella-zoster (Brown and Susser 2002), and retrovirus (Yolken et al 2000) might be associated with schizophrenia, suggesting that a specific virus itself does not cause the disorder. Instead, a maternal immune response common to various viruses could influence fetal brain development and, in consequence, lead to schizophrenia. Interestingly, maternal serum levels of tumor necrosis factor-α (TNF-α) (Buka et al 2001) or interleukin-8 (IL-8) (Brown et al 2004b) are elevated in mothers of patients with schizophrenia. In addition, it is reported that prenatal injection of the inflammatory cytokines (interleukin-1 beta [IL-1β], interleukin-6 [IL-6], and TNF-α) could inhibit the development of dendrites in embryonic cortical neurons, consistent with the neuropathology of schizophrenia (Gilmore et al 2004). Taken together, it is likely that the maternal immune response, in particular the proinflammatory cytokines, could interfere with normal fetal brain development and that maternal infections during pregnancy could be potential risk factors for schizophrenia (Gilmore et al 2004; Nawa et al 2000; Kronfol and Remick 2000).

Some studies in rodents have shown that prenatal immune challenges can lead to morphological brain abnormalities and behavioral alteration in the adult offspring. Administration of the bacterial endotoxin lipopolysaccharide (LPS) in rats caused sensorimotor gating deficits, increased tyrosine hydroxylase (TH) immunoreactivity in the nucleus accumbens, and increased serum cytokines in the offspring of pregnant dams (Borrell et al 2002). To mimic a viral infection, the synthetic double-strand RNA polyriboinosinic-polyribocytidilic acid (poly I:C) is more appropriate than LPS because double-strand RNA made as a byproduct of viral replication is thought to represent the viral equivalent of bacterial endotoxins and to play a significant role in interferon (IFN) induction by viruses (Majde 2000; Traynor et al 2004). The prenatal administration of poly I:C seems to have advantages compared with viral infection. Namely, it elicits a nonspecific immune response common to various viruses and does not cause virus-specific organ diseases like pneumonia by influenza, which may have an influence on embryonal brain

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developments by hypoxia (Majde 2000; Traynor et al 2004). Recently, it has been reported that the maternal injection of poly I:C caused sensorimotor gating deficits in the adult offspring in mice (Shi et al 2003) and caused a loss of latent inhibition, increased sensitivity to amphetamine, and an abnormal neuronal morphology in the hippocampus of adult offspring in rats (Zuckerman et al 2003).

The present study was undertaken to examine whether or not prenatal poly I:C administration leads to behavioral and biochemical abnormalities considered relevant to schizophrenia in the offspring. We focused on the following three points. First, we examined whether or not the abnormalities in the offspring of poly I:C treated dams (poly I:C-mice) emerge according to maturational delay. Since the onset of schizophrenia is usually early adulthood, an animal model of this disorder should be validated by checking to see whether it can reproduce the delay between the early neurodevelopmental insult and the emergence of abnormalities relevant to the insult. Second, we examined whether or not poly I:C-mice are impaired in their dopaminergic systems, which have been implicated in the positive symptoms of schizophrenia (Davis et al 1991; Abi-Dargham et al 2000). Third, we examined whether or not poly I:C-mice show cognitive impairment, which is recognized as the core characteristic of schizophrenia (Mohamed et al 1999; Elvevag and Goldberg 2000; Freedman 2003; Mueser and McGurk 2004). Furthermore, we examined the effects of the atypical antipsychotic drug clozapine and typical antipsychotic drug haloperidol on cognitive impairment in the adult poly I:C-mice.

## Methods and Materials

### Animals

Balb/c mice (Japan SLC, Hamamatsu, Shizuoka, Japan) were mated at about 10 weeks of age, and the first day after copulation was defined as embryonal day 0 (E0) of the pregnancy. Male and female Balb/c juvenile mice (5 weeks old, weight 14–22 g) or adult mice (9–10 weeks old, weight 20–29 g) bred in our laboratory were used for the experiments. They were housed under controlled temperature and 12-hour light/12-hour dark cycles (7:00 AM to 7:00 PM light on) with ad libitum food and water. All experiments were performed between 9:00 AM and 7:00 PM, and each animal was used only as a juvenile or at adulthood. Animals administered antipsychotic drugs or methamphetamine (MAP) were not used for neurochemical evaluation of the dopaminergic function. All experiments were performed in accordance with the Guide for Animal Experimentation of the Chiba University Graduate School of Medicine.

### Prenatal Administration of Poly I:C

Every 6 consecutive days from E12 to E17, the pregnant mice were injected intraperitoneally (IP) with poly I:C (5.0 mg/kg) (CALBIOCHEM, San Diego, California) dissolved in .2 mL phosphate buffered saline (PBS) per 20 g body weight or an equivalent volume of PBS. The offspring were separated from their mothers after 3 weeks, and male and female mice were caged separately in groups of two to four.

### Thigmotaxis Test (in the Juvenile and Adult Offspring)

Thigmotaxis is tendency to remain close to the walls. To assess the behavioral pattern under moderately stressful conditions, PBS-mice and poly I:C-mice were examined by a thigmotaxis test. The apparatus consisted of an open-topped acrylic box (50 × 50 × 30 cm high) with the floor area marked into 25 squares of 10 cm each. The mice were placed individually near the center of the box and their movements were recorded by videotaping for 10-minute periods. We quantified the number of entries into the nine center squares and the time spent in the same center area. Both the juvenile and adult offspring were examined to confirm whether or not a maturational delay was found in the alteration of the behavioral pattern.

### Prepulse Inhibition (in the Juvenile and Adult Offspring)

The mice were tested for their acoustic startle responses in a startle chamber (SR-LAB, San Diego Instruments, San Diego, California) using standard methods described previously (Swerdlow and Geyer 1998). After an initial 30-minute acclimation period, the test sessions began. They consisted of six trial types: 1) pulse alone, 40-millisecond broadband burst; pulse preceded 100 milliseconds by a 20-millisecond prepulse (PP) that was 2) 3 dB (PP68); 3) 6 dB (PP71); 4) 10 dB (PP75); or 5) 15 dB (PP80) over background (65 dB); and 6) background only (no stimulus). The amount of prepulse inhibition (PPI) is expressed as the percentage decrease in the amplitude of the startle response caused by presentation of the prepulse (%PPI). Both the juvenile and adult offspring were examined.

### Novel-Object Recognition Test (in the Juvenile and Adult Offspring)

To assess the cognitive function, the mice were examined by novel-object recognition test (NORT). The mice were individually habituated in the same box used in the thigmotaxis test for 5 days before the training session. During the training session, two novel-objects (LEGO blocks [LEGO Group, New York, New York] which were different from each other in shape and color) were placed into the open field, and each mouse was allowed to explore freely for 10 minutes. The time spent exploring each object was recorded. During retention tests, the mice were placed back into the same box 1 hour or 1 day after the training, and one of the familiar objects used during training was replaced by a novel object, which the mice were allowed to explore freely for 5 minutes. A preference index, a ratio of the amount of time spent exploring any one of the objects (training session) or the novel one (retention session) over the total time spent exploring both objects, was used to measure recognition memory. Both the juvenile and adult offspring were examined.

### Effect of Antipsychotic Drugs on the Cognitive Deficits Induced by Maternal Immune Activation (in the Adult Offspring)

In this experiment, mice that had not been examined by any test previously were used. Vehicle (.1 mL/10 g, .1 % acetic acid in PBS), clozapine (5 mg/kg) (Novartis Pharmaceuticals Ltd, Basel, Switzerland), or haloperidol (.1 mg/kg) (Dainippon Pharmaceuticals Ltd, Osaka, Japan) was administered IP for 2 weeks. The doses of clozapine and haloperidol were used as reported previously (Zuckerman et al 2003). Then, after a 1-day buffer, to avoid the sedative effects of the drugs, the mice were examined by NORT following the training session described above. The habituation was done concurrently with the last 4 days of treatment and 1-day buffer before the training.

### Spontaneous and Methamphetamine-Induced Locomotor Activity (in the Juvenile and Adult Offspring)

The locomotor activity of the mice was monitored under an infrared ray passive sensor system (SCANET-SV10, Melquest Ltd., Toyama, Japan) and the activity was integrated every 10 minutes. An apparatus with the infrared beam sensor was set on the top of

a conventional polypropylene cage, and the number of movements were counted and relayed to a computer. Mice were individually placed in the activity chambers and allowed 2 hours of free exploration as a spontaneous activity, at the end of which they were injected IP with 2 mg/kg methamphetamine (Dainippon Pharmaceuticals Ltd, Osaka, Japan) and placed once again into the chambers for an additional 2 hours to measure MAP-induced activity. Both the juvenile and adult offspring were examined.

### Measurement of Dopamine and Its Metabolites (in the Adult Offspring)

The mice were killed by decapitation. Their striatum, hippocampus, and frontal cortex were quickly dissected from the brain on ice. After being weighed, dissected tissues were stored at -80°C until assay. Tissue concentrations of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) using methods described previously (Koike et al 2005).

### Dopamine Receptor Binding Assays (in the Adult Offspring)

The striatum of the mice was homogenized for 30 seconds in 1 mL of ice-cold 50 mmol/L Tris-HCl buffer (pH 7.4; Wako Pure Chemical Industries Ltd., Osaka, Japan), including 120 mmol/L sodium chloride (NaCl) and 5 mmol/L potassium chloride (KCl), and centrifuged (39,000g, 15 minutes, 4°C). The resulting pellets were suspended in the same buffer and recentrifuged. Final pellets were suspended in the same buffer for the assay of DA $D_2$-like receptors or in the same buffer containing 2 mmol/L calcium dichloride ($CaCl_2$) plus 1 mmol/L magnesium dichloride ($MgCl_2$) for the assay of DA $D_1$-like receptors. Homogenates were added to tubes containing the [$^3$H]SCH 23390 (Amersham Biosciences, Buckinghamshire, England) (for $D_1$-like receptors; .5 nmol/L) or [$^3$H]raclopride (PerkinElmer Life and Analytical Sciences Inc., Boston, Massachusetts) (for $D_2$ -like receptors; .8 nmol/L), and the samples were incubated for 90 minutes at room temperature. Nonspecific binding was estimated in the presence of 10 μm SCH 23390 (Sigma-Aldrich, St Louis, Missouri) ($D_1$-like receptors) and 10 μm haloperidol ($D_2$-like receptors). After the addition of 4 mL of ice-cold buffer to the assay tubes, the samples were rapidly filtered using a Brandell 24-channel cell harvester (Biochemical Research Laboratory, Gaithersburg, Maryland), through Whatman GF/B glass filters (Whatman International Ltd., Florham Park, New Jersey) pretreated with .5% polyethyleneimine for at least 2 hours. The filters were washed three times with 4 mL aliquots of ice-cold buffer. The radioactivity trapped by the filters was determined by a liquid scintillation counter.

### Statistical Analysis

All data are shown as mean ± SEM. Behavioral results were analyzed by two-way analysis of covariance (ANCOVA) with treatment (PBS or poly I:C) and age (juvenile or adult) as independent factors, followed by *t* test with the Bonferroni method as the test of the simple main effect. The results of NORT were analyzed by two-way repeated measures analysis of variance (ANOVA) with treatment and age as independent factors and the retention session (1 hour and 1 day) as a within-subject factor followed by one-way ANOVA with treatment as an independent factor and the retention session as a within-subject factor in each age. The results of the drug effects on NORT at adulthood were analyzed by one-way repeated measures ANOVA with the drug group as an independent factor and the retention session as a within-subject factor, followed by the *t* test with the Bonferroni method as a post hoc test. These tests were made with SPSS 12.0J (SPSS Inc., Chicago, Illinois). Other data were statistically analyzed by an unpaired *t* test with the Microsoft EXCEL program (Microsoft Corp., Redmond, Washington). The value $p < .05$ was regarded as statistically significant. In the *t* test with the Bonferroni method, each *p*-value was adjusted so as to make the total type I error be .05.

**Table 1.** Effects of Maternal Immune Activation on the Pregnancy

| Treatment | Number of Dam | Body Weight Change from E12 to E17 | Litter Sizes |
|---|---|---|---|
| PBS | 12 | 8.3 ± .7 | 6.3 ± .5 |
| Poly I:C | 22 | 3.6 ± .4[a] | 2.9 ± .2[a] |
| Poly I:C (abortion) | 31 | −1.3 ± .5 | 0 |

PBS, phosphate buffered saline; Poly I:C, polyriboinosinic-polyribocytidilic acid.
[a]Reduction is significant compared to PBS treated group.

## Results

### Poly I:C Administration During Pregnancy

Polyriboinosinic-polyribocytidilic acid administration to the pregnant mice significantly decreased the body weight gain from E12 to E17 ($p < .001$, two-tailed *t* test) and the litter size ($p < .001$, two-tailed *t* test) compared with the PBS treated mice (Table 1). In addition, more than half of the 53 dams that were administered poly I:C (5.0 mg/kg) could not bear live pups due to abortion. However, no obvious malformations were found in the pups of poly I:C treated dams. No differences were found in body weight between the poly I:C-mice and PBS-mice when they were juveniles and at adulthood.

### Thigmotaxis

The prepubertal and postpubertal effects of poly I:C administration during pregnancy on thigmotaxis are illustrated in Figure 1. Two-way ANOVA revealed that the number of center entries was significantly affected by the treatment [$F(1,92) = 5.51$, $p = .021$], age [$F(1,92) = 15.5$, $p < .001$], and treatment × age interaction [$F(1,92) = 4.77$, $p = .031$] and that the time spent in the center area was significantly affected by the treatment [$F(1,92) = 11.7$, $p = .001$], age [$F(1,92) = 7.59$, $p = .007$], and treatment × age interaction [$F(1,92) = 4.64$, $p = .034$]. The tests of simple main effect by the *t* test with the Bonferroni method revealed that the poly I:C-mice showed significantly more number of entries into the center area ($p = .001$) and more time spent in the center area ($p < .001$) than the PBS-mice at adulthood. In contrast, in juveniles, no differences were found in either the number of entries to the center area ($p = .916$) or the time spent in the center area ($p = .411$) between the poly I:C-mice and PBS-mice (Figure 1A and B).

### Prepulse Inhibition

The prepubertal and postpubertal effects of poly I:C administration during pregnancy on prepulse inhibition are illustrated in Figure 2. Two-way repeated measures ANOVA with treatment and age as independent factors and prepulse as a within-subject factor revealed that the %PPI was significantly affected by age [$F(1,42) = 12.8$, $p < .001$], prepulse [$F(3,126) = 97.4$, $p < .001$], prepulse × age interaction [$F(3,126) = 5.09$, $p = .002$], and prepulse × treatment × age interaction [$F(3,126) = 2.83$, $p = .041$] without a significant difference between treatment [$F(1,42) = .052$,

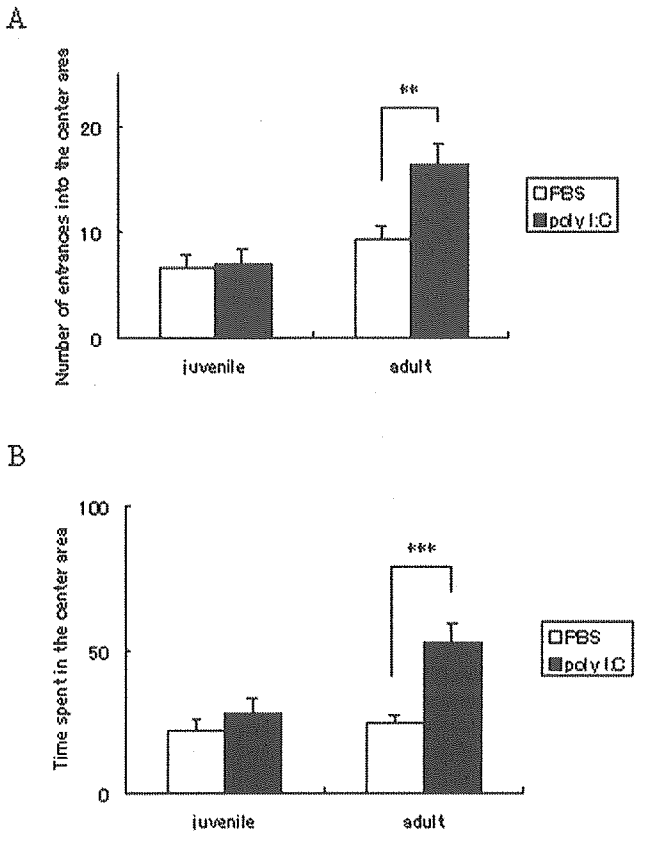


**Figure 1.** Attenuated thigmotaxis in adult offspring of poly I:C-treated dams. **(A)** Number of entries into the center area by the juvenile and adult offspring of poly I:C-treated and PBS-treated dams. **(B)** Time spent in the center area by the juvenile and adult mice of the two groups. Data are given as mean ± SEM. Juvenile mice (PBS-mice: $n$ = 20, 10 male mice; poly I:C-mice: $n$ = 20, 10 male mice) and adult mice (PBS-mice; $n$ = 30, 15 male mice; poly I:C-mice: $n$ = 26, 13 male mice) were examined. Juvenile mice displayed no differences between the two groups. However, at adulthood, the poly I:C group showed significantly more entries into the center area and more time spent in the center than the PBS group. $^{**}p < .01$, $^{***}p < .001$. poly I:C, polyriboinosinic-polyribocytidilic acid; PBS, phosphate-buffered saline.

$p$ = .822], treatment × age interaction [$F(1,42)$ = .008, $p$ = .929], and prepulse × treatment interaction [$F(3,126)$ = .684, $p$ = .564]. At adulthood, one-way repeated measures ANOVA with treatment as an independent factor and prepulse as a within-subject factor revealed that the %PPI was significantly affected by prepulse [$F(3,84)$ = 101, $p < .001$] and prepulse × treatment interaction [$F(3,84)$ = 4.71, $p$ = .004] without significant difference between treatments [$F(1,28)$ = .68, $p$ = .797]. The test of the simple main effect by the $t$ test with the Bonferroni method revealed that the poly I:C-mice showed significantly decreased %PPI in PP80 compared with the PBS-mice ($p$ = .005) (Figure 2B). In contrast, in juveniles, the %PPI was significantly affected by prepulse [$F(3,42)$ = 24.6, $p < .001$] without a significant difference between treatment [$F(1,14)$ = .008, $p$ = .930] and prepulse × treatment interaction [$F(3,42)$ = .323, $p$ = .808] (Figure 2A).<aqr;19> Then, two-way ANOVA with treatment and age as independent factors revealed that the %PPI at a prepulse of 80 dB was significantly affected by age [$F(1,42)$ = 22.7, $p < .001$] and treatment × age interaction [$F(1,42)$ = 5.75, $p$ = .021] without significant difference between treatment. The test of the simple main effect by $t$ test with the Bonferroni method revealed

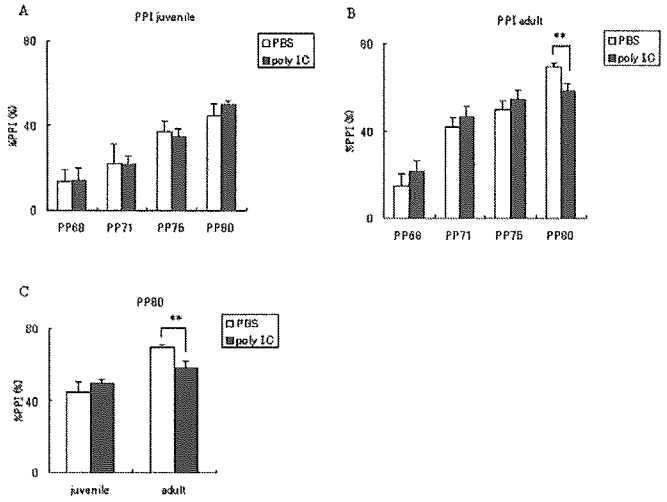


**Figure 2.** Deficits in prepulse inhibition in adult offspring of poly I:C-treated dams. **(A)** %PPI of the juvenile mice of the two groups. **(B)** %PPI of the adult mice of the two groups. **(C)** Comparison of %PPI at prepulse 80 dB from figures **(A)** and **(B)**. Data are given as mean ± SEM. Juvenile mice (PBS-mice: $n$ = 9, 1 male mouse; poly I:C-mice: $n$ = 7, 2 male mice) and adult mice (PBS-mice: $n$ = 18, 5 male mice; poly I:C-mice: $n$ = 12, 5 male mice) were examined. In the juveniles, no difference was found between the two groups in %PPI. However, at adulthood, the poly I:C group showed decreased %PPI at a prepulse of 80 dB (15 dB higher than background). Data are given as mean ± SEM. $^{**}p < .01$. poly I:C, polyriboinosinic-polyribocytidilic acid; PPI, prepulse inhibition; PBS, phosphate-buffered saline.

that the poly I:C-mice showed significantly decreased %PPI in PP80 compared with the PBS-mice ($p$ = .005) (Figure 2C). These analyses support the fact that the poly I:C-mice showed deficits in PPI at a prepulse of 80 dB at adulthood but not as juveniles.

## Cognitive Function

The prepubertal and postpubertal effects of poly I:C administration during pregnancy on cognitive function in NORT are illustrated in Figure 3. Two-way repeated measures ANOVA with treatment and age as independent factors and the retention session as a within-subject factor revealed that the preference indexes in the retention sessions was significantly affected by session [$F(1,53)$ = 50.4, $p < .001$] and treatment × age interac-

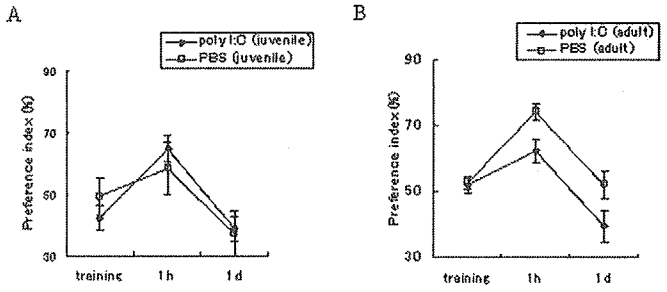


**Figure 3.** Impaired novel-object recognition memory in adult offspring of poly I:C-treated dams. **(A)** Exploratory preference of the juvenile mice of the two groups. **(B)** Exploratory preference of the adult mice of the two groups. Data are given as mean ± SEM. Juvenile mice (PBS-mice: $n$ = 11, 6 male mice; poly I:C-mice: $n$ = 10, 6 male mice) and adult mice (PBS-mice: $n$ = 19, 12 male mice; poly I:C-mice: $n$ = 17, 11 male mice) were examined. As juveniles, no difference was found between the two groups in every session. However, at adulthood, the poly I:C group showed a lower preference index in the retention sessions. poly I:C, polyriboinosinic-polyribocytidilic acid; PBS, phosphate-buffered saline.

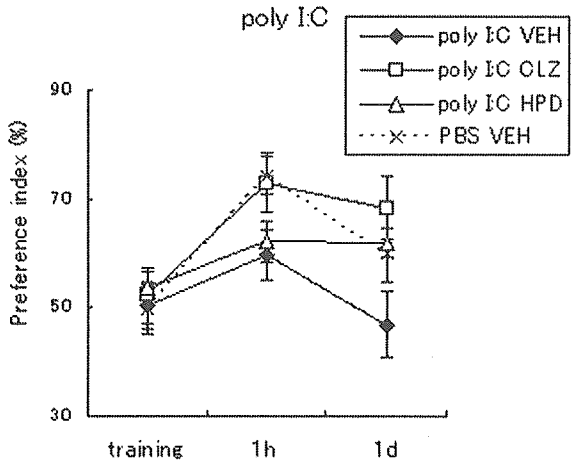


**Figure 4.** Effects of antipsychotic drugs on the cognitive deficits induced by maternal immune activation. Exploratory preference of the three drug groups of poly I:C-mice and the vehicle group of PBS-mice that were subchronically administered with clozapine (CLZ) or haloperidol (HPD) or vehicle (VEH). From the adult offspring of poly I:C-treated dams, 12 mice ($n$ = 12, 8 male mice) in the clozapine group, 12 mice ($n$ = 12, 8 male mice) in the haloperidol group, and 12 mice ($n$ = 12, 8 male mice) in the vehicle group were examined. From the adult offspring of PBS-treated dams, 15 mice ($n$ = 15, 5 male mice) in the vehicle group were examined. The clozapine group of poly I:C-mice showed higher preference indexes in the retention sessions than the vehicle group of poly I:C-mice ($p$ = .011, post hoc analysis following significant two-way repeated measures ANOVA). The preference indexes of the clozapine group of poly I:C-mice seemed to recover to the levels of those of the vehicle group of PBS-mice. Data are given as mean ± SEM. poly I:C, polyriboinosinic-polyribocytidilic acid; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

tion [$F(1,53)$ = 4.62, $p$ = .036] without significant difference between treatment [$F(1,53)$ = 1.19, $p$ = .28], age [$F(1,53)$ = 3.83, $p$ < .056], and session × treatment × age interaction [$F(1,53)$ = .080, $p$ = .778]. At adulthood, one-way repeated measures ANOVA with treatment as an independent factor and the retention session as a within-subject factor revealed that the preference indexes were significantly affected by the session [$F(1,34)$ = 40.9, $p$ < .001] and by treatment [$F(1,34)$ = 8.48, $p$ = .006] without significant difference between treatment × session interaction [$F(1,34)$ = .29, $p$ = .865] (Figure 3B). In contrast, when the mice were juveniles, the preference indexes in the retention sessions were significantly affected by the session [$F(1,19)$ = 15.4, $p$ = .001] without significant difference between the treatment [$F(1,19)$ = .345, $p$ = .564] and treatment × session interaction [$F(1,19)$ = .164, $p$ = .690] (Figure 3A). These analyses support the fact that poly I:C-mice showed impaired memory in the retention sessions compared with PBS-mice at adulthood but not as juveniles. As can be seen, this impairment was preserved in the 1-day retention session equally to the 1-hour retention session. Furthermore, no sex deference was found in the NORT.

### Effects of Antipsychotic Drugs on the Cognitive Impairment Induced by Maternal Immune Activation

The effects of subchronic (2 weeks) administration of the typical antipsychotic haloperidol (.1 mg/kg, IP) and the atypical antipsychotic clozapine (5.0 mg/kg, IP) on the cognitive impairment induced by prenatal poly I:C (5.0 mg/kg, IP) treatment in adult offspring are illustrated in Figure 4. One-way repeated measures ANOVA revealed that the preference index in the retention sessions was significantly affected by the treatment [$F(3,47)$ = 4.19, $p$ = .010] and session [$F(1,47)$ = 6.77, $p$ = .012)] without significant difference between treatment × session interaction [$F(1,47)$ = 1.23, $p$ = .309]. Post hoc analysis following the significant ANOVA revealed that the clozapine treated poly I:C-group showed higher preference indexes than the vehicle treated poly I:C-group in the retention sessions ($p$ = .011) (Figure 4). No sex deference was found in any of the experiments.

### Spontaneous and Methamphetamine-Induced Locomotor Activity

The prepubertal and postpubertal effects of poly I:C administration during pregnancy on spontaneous and MAP-induced (2 mg/kg) locomotion activity are illustrated in Figure 5. As can be seen, at adulthood, the poly I:C-mice were more active than the PBS-mice in MAP-induced locomotion activity (Figure 5B). However, no difference in MAP-induced hyperactivity was seen in the juveniles (Figure 5A). Spontaneous locomotion activity before MAP administration did not differ between the poly I:C-mice and PBS-mice both as juveniles and adults (Figure 5A and B). We integrated the activities for 60 minutes preceding MAP administration as a pre-MAP activity and for 60 minutes following MAP administration as a post-MAP activity. Two-way ANCOVA with treatment and age as independent factors and pre-MAP activity as a covariate factor revealed that post-MAP activity was significantly affected by treatment × age interaction [$F(1,59)$ = 4.71, $p$ = .034] without significant difference between treatment [$F(1,59)$ = 2.64, $p$ = .110] and age [$F(1,59)$ = 3.47, $p$ = .068]. The test of the simple main effect by $t$ test with the Bonferroni method revealed that the poly I:C-mice showed significantly increased post-MAP activity compared with the PBS-mice ($p$ = .003) at adulthood but not as juveniles ($p$ = .734) (Figure 5C). These data suggest that poly I:C-mice show hypersensitivity to MAP with maturational delay. No sex deference was found in any of the experiments.

### Monoamine Concentration in the Brain

High-performance liquid chromatography (HPLC) analysis revealed that the contents of DOPAC ($p$ < .001, two-tailed $t$ test) and HVA ($p$ = .0173, two-tailed $t$ test) in the striatum were significantly increased in the adult poly I:C-mice. In contrast, no difference in the DA levels in the striatum of the adult offspring of the two groups was shown ($p$ = .3417, two-tailed $t$ test) (Figure 6A). The value (1.52 ± .106 [mean ± SEM]) of (DOPAC + HVA)/DA in the striatum of the adult poly I:C-mice was significantly ($p$ < .001, two-tailed $t$ test) higher than that (.80 ± .068 [mean ± SEM]) in the adult PBS-mice (Figure 6B). Significant sex differences in the values of (DOPAC+HVA)/DA in the adult PBS-mice ($p$ < .001, two-tailed $t$ test) and poly I:C-mice ($p$ = .0047, two-tailed $t$ test) were shown.

In the frontal cortex, no difference between the two groups was found for DA ($p$ = .787, two-tailed $t$ test), DOPAC ($p$ = .595, two-tailed $t$ test), and HVA ($p$ = .724, two-tailed $t$ test). Furthermore, the value (1.70 ± .25 [mean ± SEM]) of (DOPAC + HVA)/DA in the adult poly I:C-mice was not different from that (1.66 ± .18 [mean ± SEM]) in the adult PBS-mice ($p$ = .881, two-tailed $t$ test) (Table 2).

### Dopamine Receptor Binding Assay

Receptor binding assays revealed that the level of $D_2$-like receptors was decreased significantly in the adult poly I:C-mice ($p$ = .031, two-tailed $t$ test) (Figure 7B). However, no difference was found in the level of $D_1$-like receptors between the two groups ($p$ = .26, two-tailed $t$ test) (Figure 7A). Furthermore, no

sex difference was found in the level of $D_1$-like or $D_2$-like receptors of the two groups.

## Discussion

The major findings of the present study are that maternal administration of poly I:C into pregnant mice causes abnormality of the dopaminergic system, deficits in sensory-motor gating, and cognitive impairment in adult offspring but not juvenile offspring and that cognitive impairment in the adult offspring of poly I:C treated dams could be improved by the subchronic administration of clozapine but not haloperidol. Therefore, we propose that the adult offspring of poly I:C treated pregnant mice may be an animal model of schizophrenia.

First, poly I:C-mice exhibited a greater response to MAP administration compared with PBS-mice at adulthood but not as juveniles. These findings are consistent with previous data reporting that amphetamine-induced (1.0 mg/kg) hyperactivity in the adult offspring of poly I:C treated dams is greater than that of PBS treated dams in rats (Zuckerman et al 2003). Furthermore, we found that DA turnover in the striatum of adult poly I:C-mice was significantly higher than in PBS-mice and that the levels of $D_2$-like receptors, but not $D_1$-like receptors, in the striatum of

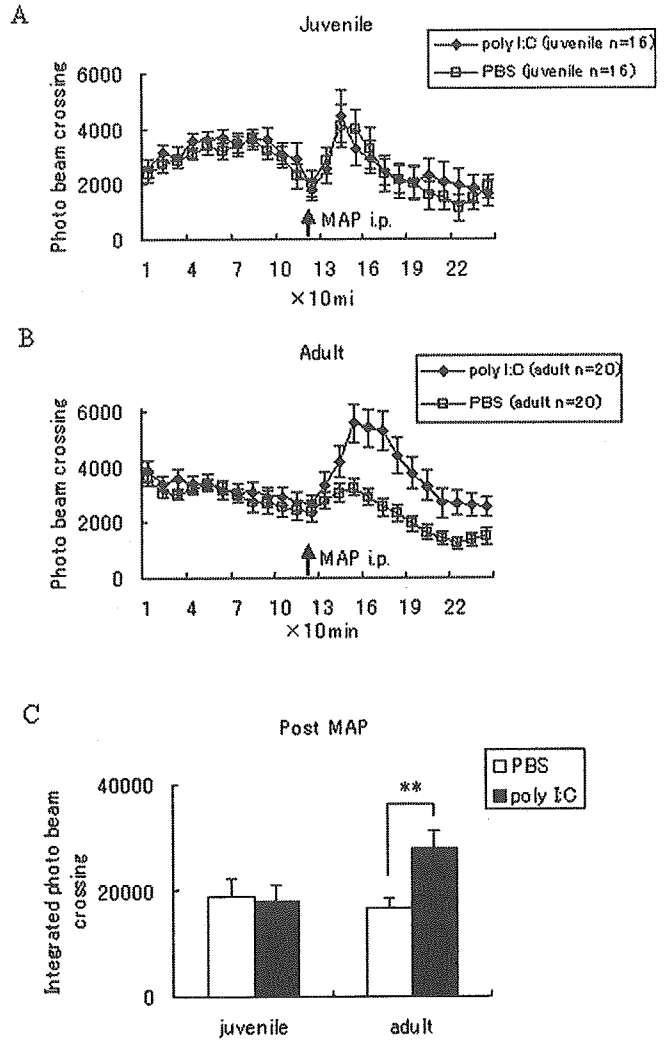


**Figure 5.** Enhanced locomotor activity induced by methamphetamine (MAP) in adult poly I:C-mice. **(A)** Number of movements, in 10-minute blocks, of the juvenile offspring of poly I:C-treated and PBS-treated dams. Twenty-four blocks were divided into the former 12 blocks before the injection of MAP and the latter 12 blocks after injection. The arrow indicates the point of injection of MAP. Sixteen juvenile poly I:C-mice ($n$ = 16, 8 male mice) and 16 PBS-mice ($n$ = 16, 8 male mice) were examined. The juvenile mice displayed no differences between the two groups. **(B)** Number of movements, in 10-minute blocks, of the adult poly I:C-mice and PBS-mice. Twenty adult poly I:C-mice ($n$ = 20, 8 male mice) and 20 adult PBS-mice ($n$ = 20, 8 male mice) were examined. At adulthood, the poly I:C group showed higher activities in the latter 12 blocks than the PBS group, without difference in the spontaneous activities in the former 12 blocks. Data are given as mean ± SEM. **(C)** Number of movements integrated for six blocks after injection of MAP (post-MAP) of the juvenile and adult poly I:C-mice and PBS-mice. As juveniles, the poly I:C-mice showed activity equal to the PBS-mice. However, at adulthood, the poly I:C-mice showed higher activity compared with the PBS-mice. Data are given as mean ± SEM. $^{**}p < .01$. MAP, methamphetamine; poly I:C, polyriboinosinic-polyribocytidilic acid; PBS, phosphate-buffered saline.

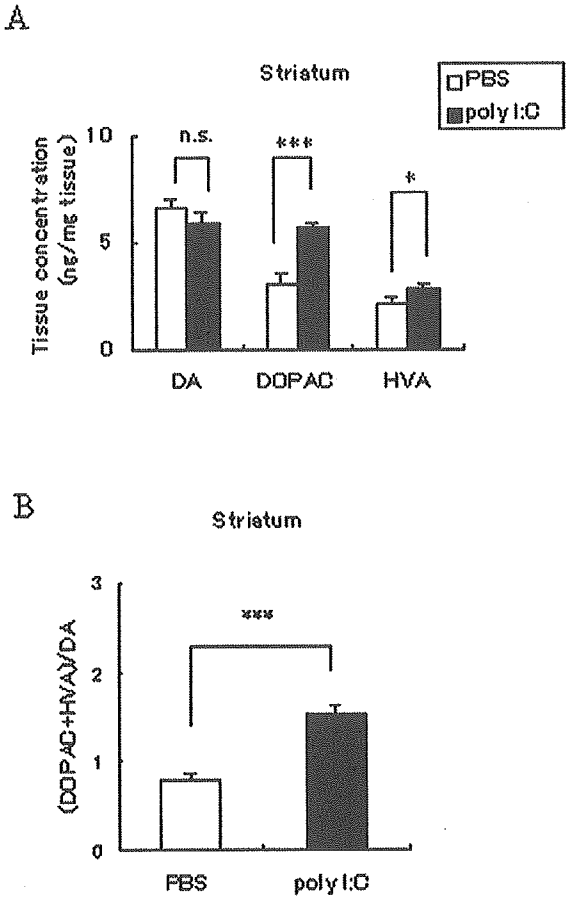


**Figure 6.** Effect of maternal immune activation on the dopamine metabolism in the striatum of the adult offspring. **(A)** Tissue concentration of DA, DOPAC, and HVA (ng/mg wet weight of tissue) in adult striatal tissue samples of the two groups. The DA levels did not significantly differ between the two groups ($p$ = .342). However, the metabolite DOPAC ($p < .001$) and HVA ($p$ = .0173) levels significantly increased in the poly I:C-mice. **(B)** Ratio of dopamine metabolites to dopamine ([DOPAC+HVA]/DA) in adult striatal tissue samples of the control and poly I:C-mice. The poly I:C-mice showed significantly higher dopamine turnover in this region ($p < .001$). Twelve experimental adult mice ($n$ = 12, 6 male mice) and 17 PBS-mice ($n$ = 17, 10 male mice) were examined. Data are given as mean ± SEM. A two-tailed $t$ test was used. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; poly I:C, polyriboinosinic-polyribocytidilic acid; PBS, phosphate-buffered saline.

**Table 2.** Effects of Maternal Immune Activation on the Dopamine Metabolism in the Frontal Cortex and of the Adult Offspring

| | DA (pg/mg Tissue) | DOPAC (pg/mg Tissue) | HVA (pg/mg Tissue) | Ratio (DOPAC + HVA)/DA |
|---|---|---|---|---|
| Frontal Cortex | | | | |
| PBS ($n$ = 17) | 227 ± 53 | 65.9 ± 9 | 183 ± 13 | 1.66 ± .18 |
| Poly I:C ($n$ = 12) | 253 ± 84 | 77.2 ± 19 | 190 ± 18 | 1.70 ± .25 |

DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; PBS, phosphate buffered saline; Poly I:C, polyriboinosinic-polyribocytidilic acid.

adult poly I:C-mice were significantly lower than those of PBS-mice. There are several reports demonstrating the increases in [$^3$H]spiperone binding to $D_2$-like receptors in the caudate putamen of subjects with schizophrenia treated with antipsychotic drugs up until death (Lee et al 1978; Mackay et al. 1982; Owen et al 1978; Seeman et al 1984). In contrast, there are several reports demonstrating that [$^3$H]spiperone binding to $D_2$-like receptors was decreased (64% to 88% of control subjects) in the caudate putamen of schizophrenic patients who were free of antipsychotic drugs at death (Reynolds et al 1980; Mackay et al 1982). Furthermore, a recent study demonstrated that there is a decrease in the density of [$^{125}$I]iodosulpride (or [$^3$H]spiperone) binding to $D_2$-like receptors in the caudate putamen from subjects with schizophrenia who were drug-free at death, suggesting that decreased $D_2$-like receptors might be reflective of these receptors being subjected to an increased dopaminergic drive in the caudate putamen (Dean et al 2004). Taken together, it is likely that the discrepancy of the $D_2$-like receptor binding in the caudate putamen in subjects with schizophrenia may be due to medication by antipsychotic drugs at death, since the chronic administration of antipsychotic drugs increases the density of $D_2$-like receptors in animals (Clow et al 1979; Wan et al 1996). Interestingly, adult monkeys with neonatal temporal limbic damage, an analogue of rats with neonatal lesions in the ventral hippocampus, showed a stimulus-dependent disinhibition of subcortical DA release and reduction of striatal $D_2$-like receptor binding, which might be due to downregulation by stimulus-dependent DA activity (Heinz et al 1999). Thus, it is likely that reduction of the striatal $D_2$-like receptors in adult poly I:C-mice might be due to a physiological response to enhanced DA turnover by striatal presynaptic neurons. It is important to examine whether a change of $D_2$-like receptor binding is due to the $D_2$ receptor or $D_3$ receptor. However we could not discriminate the subtypes in this study, since we used [$^3$H]raclopride, which binds to the both subtypes. Therefore, further studies to assess binding to the $D_3$ receptor are needed. Anyway, our findings suggest that prenatal poly I:C injection causes a maturation-dependent increased subcortical DA function in adult offspring.

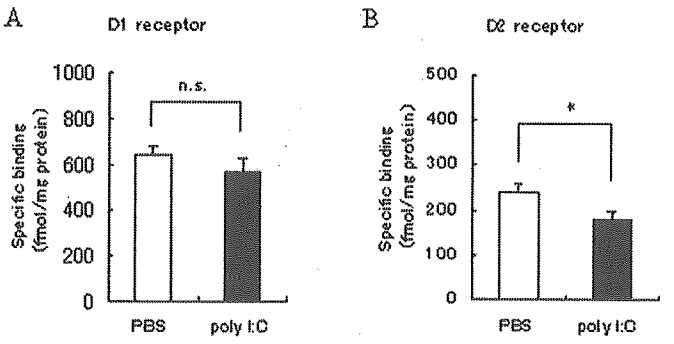


**Figure 7.** Effect of maternal immune activation on dopamine receptors in the striatum of the adult offspring. **(A)** Specific binding of the dopamine $D_1$ receptor (fmol/mg protein) in the striatal tissue samples of the adult male offspring of PBS or poly I:C treated dams. No significant difference was found in the level of $D_1$ receptor between the control mice and the poly I:C-mice ($p$ = .26). **(B)** Specific binding of the dopamine $D_2$ receptor (fmol/mg protein) in the striatal tissue samples of the control (PBS) and poly I:C adult male mice. The level of $D_2$ receptor decreased significantly in the poly I:C-mice compared with the PBS-mice ($p$ = .031). Adult mice (PBS group: $n$ = 14, 8 male mice; poly I:C group: $n$ = 10, 6 male mice) were examined. Data are given as mean ± SEM. A two-tailed $t$ test was used. *$p < .05$. PBS, phosphate-buffered saline; poly I:C, polyriboinosinic-polyribocytidilic acid.

Second, we found that prenatal poly I:C administration causes maturation-dependent deficits in sensory-motor gating, PPI. Deficits in PPI are observed in several mental illnesses, including schizophrenia (Braff et al 2001). In addition, deficits in PPI are generally recognized as an indicator of an animal model of schizophrenia (Lipska et al 2000). Polyriboinosinic-polyribocytidilic acid mice display deficits in PPI at a prepulse of 80 dB, 15 dB above background noise. These findings are partially consistent with previous data reporting that maternal poly I:C administration during pregnancy caused deficits in PPI in the adult offspring of treated mice (Shi et al 2003). However, to our knowledge, this is the first report to show that the deficits in PPI emerge with maturational delay.

Third, we found that prenatal poly I:C administration causes maturation-dependent cognitive impairment and that cognitive impairment in adult poly I:C-mice could be improved after subchronic administration of clozapine but not haloperidol. Memory function, one of the most important elements of cognitive function, has been known to be impaired in schizophrenia. A number of reports demonstrated impairment in the recognition memory of patients with schizophrenia (Conklin et al 2002; Weiss et al 2004; Aleman et al 1999; Achim and Lepage 2003). Moreover, the N-methyl-D-asparate (NMDA) receptor blocker MK-801, which is used to make a pharmacological animal model of schizophrenia, impairs memory in the NORT in rats (Maria at al 2005). It is well known that clozapine has more efficacy than haloperidol for cognitive impairment in schizophrenia (Sharma et al 2003; Potkin et al 2001; Freedman 2003). Interestingly, we found recently that, in the NORT, cognitive impairment in mice after repeated administration of the NMDA receptor antagonist phencyclidine could be improved by subsequent subchronic administration of clozapine but not haloperidol. Recently, it was demonstrated that a bath application of clozapine, but not haloperidol, to rat brain slices induced a robust potentiation of NMDA-evoked current and glutamatergic excitatory postsynaptic potentials (EPSPs) (Wittmann et al 2005). We speculate that this action of clozapine on the NMDA receptor might be related to the effect of the drug on memory in the NORT in the poly I:C-mice. Thus, it is likely that the deficits in the NORT in adult poly

I:C-mice would be a useful model for cognitive impairment in schizophrenia.

In this study, maternal administration of poly I:C was done during the period from E12 to E17, generally equivalent to the human second trimester of pregnancy. During this period, newly generated neurons migrate toward the region where the neocortex and the hippocampus are to be, initiating the construction of these regions (Soriano et al 1994; Caviness and Takahashi 1995). We used 6 consecutive days, since the precise critical point was unknown. In the previous two reports, poly I:C (4.0 mg/kg, intravenous [IV]) was administered into pregnant rats at E15 (Zuckerman et al 2003), and poly I:C (20.0 mg/kg, IP) was administered into pregnant mice at E9.5 (Shi et al 2003). Several reports demonstrated that the second trimester is the period of highest risk for schizophrenia (Mednick et al 1988). In contrast, a recent study using the serological method to diagnose maternal influenza infection has suggested that the period of high risk is in the first half of pregnancy rather than the second trimester (Brown et al 2004a). Therefore, further studies on the mechanisms underlying how maternal poly I:C injection causes abnormal behaviors in adult offspring are needed to pinpoint when poly I:C administration to pregnant dams should take place. Moreover, because the frequency of miscarriage was high in our model, we had to better regulate the degree of maternal immunity activation. A limitation of this study might be that a too high immune activation had been induced during pregnancy.

Polyriboinosinic-polyribocytidilic acid mice showed alteration in the thigmotaxis test according to maturational delay. We found that poly I:C-mice exhibited attenuated thigmotaxis in adulthood, which is often interpreted as low anxiety levels (Simon et al 1994). However, in the light/dark box test, the adult poly I:C-mice were not different from the PBS-mice (data not shown), suggesting that adult poly I:C-mice may not necessarily be anxious. As neurodevelopmental animal models of schizophrenia, it has been demonstrated that rats with neonatal lesions in the ventral hippocampus (Sams-Dodd et al 1997) and mice of dams infected with the influenza virus (Shi et al 2003) showed potentiated thigmotaxis. On the other hand, rats with reversible inactivation of the neonatal ventral hippocampus showed attenuated thigmotaxis (Lipska et al 2002). Furthermore, Simon et al (1994) reported that the DA $D_2$ receptor agonist RU24926 decreased thigmotaxis in mice, whereas the DA $D_1$ agonist SKF38393 increased thigmotaxis in mice, indicating the relation between dopaminergic function and thigmotaxis. Therefore, it seems that increases in thigmotaxis observed in the adult poly I:C-mice may be due to enhanced dopaminergic function regulated by reduced DA $D_2$-like receptors, although further studies are needed.

In conclusion, the present results suggest that prenatal poly I:C injection causes a maturation-dependent increased subcortical DA function, deficits in sensory-motor gating, and cognitive impairment in adult offspring. Therefore, it is likely that adult poly I:C-mice could be a neurodevelopmental animal model of schizophrenia. Additionally, it is suggested that cognitive impairment in the NORT in adult poly I:C-mice would be a useful animal model for evaluating new drugs for the treatment of cognitive impairments in schizophrenia (Braff et al., 2001; Lipska and Weinberger, 2000).

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# Posterior cingulate gyrus metabolic changes in chronic schizophrenia with generalized cognitive deficits

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**Abstract**

*N*-Methyl-D-aspartate (NMDA) receptor antagonists are known to induce schizophrenia-like psychotic symptoms and cognitive deficits in humans, and have been shown to cause neuronal damage in the posterior cingulate gyrus (PCG) of rodents. Patients with chronic schizophrenia exhibit generalized cognitive deficits, but it remains unclear whether or not the PCG is related to their cognitive dysfunction. To determine what biochemical changes may occur in the PCG of patients with chronic schizophrenia, and to ascertain whether or not such abnormalities may be related to the incidence of cognitive deficits, we obtained cognitive scores and proton magnetic resonance spectra (MRS) from the PCG and the left and right medial temporal lobes (MTL) of 19 patients with schizophrenia and 18 age- and sex-matched normal healthy controls. Compared to the normal controls, the patients with chronic schizophrenia showed significantly worse cognitive performance on verbal and visual memory tests, verbal fluency tests, and the Trail Making Test. The ratio of *N*-acetylaspartate to creatine and phosphocreatine (NAA/Cr) in the PCG of the patients was significantly lower than that of the controls. Moreover, the NAA/Cr in the PCG of the healthy controls exhibited age-related decline, whereas in the patients with schizophrenia, the corresponding values were consistently low, regardless of age. These findings are thus in accord with current speculation about neuronal dysfunction in the PCG based on the NMDA hypofunction hypothesis regarding the pathophysiology of chronic schizophrenia.
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*Keywords:* Cingulate gyrus; Proton magnetic resonance spectroscopy ($^1$H-MRS); Retrosplenial cortex; Schizophrenia; *N*-Acetylaspartate (NAA); Wechsler Memory Scale-Revised (WMS-R)

## 1. Introduction

Cognitive deficits are a key feature of schizophrenia (Elvevag and Goldberg, 2000; Gold and Weinberger, 1995; Sharma and Antonova, 2003), not only at the time of the first episode (Bilder et al., 1992; Heydebrand et al., 2004; Mohamed et al., 1999), but also in patients with chronic schizophrenia (Braff et al., 1991; Pantelis et al., 1997). Impairments of cognitive functions such as memory, executive function, and attention are characteristic of most patients with schizophrenia (Egan et al., 2001; Weickert et al., 2000). It remains controversial whether or not the cognitive deficits observed in patients with schizophrenia are better characterized as generalized features, or as reflective of relatively independent

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