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Matrix Metalloprotease-9 Inhibition Improves Amyloid β-Mediated Cognitive Impairment and Neurotoxicity in Mice[S]

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ABSTRACT

In Alzheimer's disease (AD), the expression of matrix metalloproteases (MMPs), which are capable of degrading extracellular matrix proteins, is increased in the brain. Previous studies with cultured glial cells have demonstrated that amyloid β (Aβ) protein can induce the expression of MMPs, which could be involved in the degradation of Aβ. In the present study, we investigated the role of MMP-2 and MMP-9 in cognitive impairment induced by the injection of Aβ in mice. The intracerebroventricular injection of Aβ25-35, Aβ1-40, and Aβ1-42, but not Aβ40-1, transiently increased MMP-9, but not MMP-2, activity and protein expression in the hippocampus. Immunohistochemistry revealed the expression of MMP-9 to be increased in both neurons and glial cells in the hippocampus after Aβ treatment. The Aβ-induced cognitive impairment in vivo as well as neurotoxicity in vitro was significantly alleviated in MMP-9 homozygous knockout mice and by treatment with MMP inhibitors. These results suggest the increase in MMP-9 expression in the hippocampus to be involved in the development of cognitive impairment induced by Aβ1-40. Thus, specific inhibitors of MMP-9 may have therapeutic potential for the treatment of AD. Our findings suggest that, as opposed to expectations based on previous findings, MMP-9 plays a causal role in Aβ-induced cognitive impairment and neurotoxicity.

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Alzheimer's disease (AD), the most common neurodegenerative disorder in humans, is characterized by deterioration of cognitive and mental functions, including learning and memory. The formation of extracellular deposits of amyloid β (Aβ) peptide, leading to the formation of neuritic plaques and neurofibrillary tangles in the cortex and hippocampus, is a prominent pathological feature of AD (Yamada and Nabeshima, 2000; Selkoe and Schenk, 2003). Aβ, a spontaneously aggregating peptide of 39 to 43 amino acids, is the primary protein component of senile plaques, a pathological hallmark of AD (Hardy and Selkoe, 2002; Takuma et al., 2005a). Neurotoxicity mediated by Aβ has been well demonstrated both in vivo and in vitro and has been shown to involve oxidative stress, the perturbation of intracellular cal-

ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid β; AAV/Aβ, a viral vector carrying Aβ cDNA; MMP, matrix metalloprotease; LTP, long-term potentiation; MK-801, 5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); GM6001, *N*-[(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide; NORT, novel-object recognition test; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; NeuN, neuron-specific nuclear antigen; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; ANOVA, analysis of variance; NMDA, *N*-methyl-D-aspartate.

cium homeostasis, and the activation of an apoptotic pathway (Takuma et al., 2005a).

In animal experiments, we have demonstrated that the intrahippocampal injection of Aβ, including Aβ1-40, Aβ1-42, and Aβ25-35, induces hippocampal damage, learning, and memory deficits (Yamada et al., 2005; Alkam et al., 2007; Wang et al., 2007), and impairment of the cholinergic system, which play important roles in cognitive deficits associated with aging and neurodegenerative diseases (Yamada et al., 1999; Tran et al., 2001). A recent study has shown that intraventricular infusion of Aβ1-42 induces learning deficits in 9-month-old but not 2.5-month-old mice, and these learning deficits are shown 12, but not 6, weeks after infusion of Aβ1-42 in 9-month-old mice, suggesting that Aβ infusion results in age-dependent and delayed learning deficits without role of Aβ deposition and inflammation (Malm et al., 2006). In addition, we have shown that the oral administration of a viral vector carrying Aβ cDNA (AAV/Aβ) reduced the amount of Aβ accumulated and attenuated cognitive impairment in Tg2576 mice, suggesting AAV/Aβ to be safe and effective for the treatment of AD and that the accumulation of Aβ is the event initiating the decades-long pathological cascade leading to the disease (Mouri et al., 2007). Whereas plaques and amyloid fibrils have been viewed by some as resistant to proteolytic degradation, it is possible that certain proteases contribute to endogenous mechanisms leading to the clearance of plaques.

Matrix metalloproteinases (MMPs) function to remodel the pericellular environment, primarily through the cleavage of extracellular matrix proteins and cell surface components (Yong et al., 2001). Gelatinases (MMP-2 and MMP-9) are capable of cleaving collagen IV and V, laminin, and chondroitin sulfate proteoglycan, which are associated with cell adhesion (Yong et al., 2001). Furthermore, MMP-9 degrades Aβ and amyloid plaques (Yan et al., 2006) and has been implicated specifically in cerebral ischemia (Lo et al., 2002), kainate-induced neuronal injury (Szklarczyk et al., 2002), hippocampal long-term potentiation (LTP) and memory (Nagy et al., 2006), and methamphetamine dependence (Mizoguchi et al., 2007a,b). Thus, gelatinases are involved in neuronal activity-dependent synaptic plasticity and cell death in the brain.

It is interesting that MMP-9 is increased in the brains of AD patients (Backstrom et al., 1996). Moreover, MMP-9 expression in astrocytes is induced in the presence of Aβ peptide (Deb et al., 2003). MMP-9 is expressed in the cytoplasm of neurons, neurofibrillary tangles, senile plaques, and vascular walls in brain tissue from AD patients (Asahina et al., 2001). Although MMP-9 has been found to cleave the Aβ peptide at several sites (Backstrom et al., 1996; Yan et al., 2006), its potential role in Aβ-induced cognitive dysfunction and neurotoxicity has not yet been elucidated.

In the present study, we investigated the role of MMP-2 and MMP-9 in cognitive impairment induced by the intracerebroventricular injection of Aβ in mice, as well as Aβ-induced neurotoxicity in primary cultured neurons. Our findings suggest that, as opposed to expectations based on previous findings, MMP-9 plays a causal role in Aβ-induced cognitive impairment and neurotoxicity.

Materials and Methods

Animals. Male ICR mice (6 weeks old; Charles River Japan, Yokohama, Japan), weighing 20 ± 5 g at the beginning of the experiments, were used. We also used MMP-9 homozygous knockout [MMP-9(−/−)] mice and wild-type (FVB/N) mice (10–12 weeks old) obtained from The Jackson Laboratory (Bar Harbor, ME).

All experiments were performed in accordance with the Guidelines for Animal Experiments of the Kanazawa University (Kanazawa, Japan) and Nagoya University Graduate School of Medicine (Nagoya, Japan), the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (Tokyo, Japan), and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD).

Drugs. The doses of all drugs are expressed as those of the salt. Aβ25-35, Aβ1-40, and Aβ40-1 (Bachem California, Torrance, CA) were dissolved in distilled water at a concentration of 1 mg/ml and stored at −30°C before use and incubated for aggregation at 37°C for 4 days before the injection. Aβ peptides were injected intracerebroventricular at a volume of 3 μl. Vehicle and Aβ40-1 were injected as the control. All peptides were injected as described previously (Alkam et al., 2007; Wang et al., 2007). In brief, a microsyringe with a 28-gauge stainless steel needle 3.0 mm long was used for all experiments. Mice were anesthetized lightly with ether, and the needle was inserted unilaterally 1 mm to the right of the midline point equidistant from each eye, at an equal distance between the eyes and the ears and perpendicular to the plane of the skull. A single shot of the same volume (3 μl) of peptide or vehicle was delivered gradually within 3 min. Mice exhibited normal behavior within 1 min after the injection. MK-801 (Sigma-Aldrich, St. Louis, MO) at a dose of 0.1 to 0.3 mg/kg was given 30 min before Aβ25-35. GM6001 (Calbiochem, San Diego, CA) at a dose of 5 μg was intracerebroventricularly injected with Aβ in a total volume of 5 μl.

Novel-Object Recognition Test. The NORT was carried out as described previously (Mizoguchi et al., 2008). The experimental apparatus consisted of a Plexiglas open-field box (30 × 30 × 35 cm high), with a sawdust-covered floor. The apparatus was located in a sound-attenuated room and was illuminated with a 20-W bulb.

In a standard procedure, the NORT consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects for two consecutive days (habituation session, days 1–2). During the training session, two novel objects were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 min (day 3). The objects were constructed from a golf ball, wooden column, and wall socket, which were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention sessions, the animals were placed back into the same box 24 h after the training session (day 4), but one of the familiar objects used during training had been replaced with a novel object. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index in the retention session, a ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as a ratio of the time spent exploring the object that was replaced by the novel object in the retention session, over the total exploring time.

Repeated Training NORT. The procedure of repeated training NORT is the same with the standard NORT, except that the number of training sessions was increased from one to four, and then the

mice were subjected to the retention session. During the four training sessions (days 3–4, twice a day), mice were repeatedly exposed to the same two objects in the test box. During the retention session (day 5), one of the two familiar objects used during four training sessions was replaced with a novel object.

Gel Zymography. Samples were prepared as described previously (Mizoguchi et al., 2007a,b). In brief, brain tissues were homogenized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.05% Brij 35, and 0.02% NaN_3, pH 7.6) with 1% Triton X-100 and centrifuged at 12,000*g* for 10 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid kit (Bio-Rad, Osaka, Japan). The supernatant was incubated with gelatin-Sepharose 4B (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) that had been washed three times with the lysis buffer, with constant shaking, for 24 h at 4°C. After centrifugation at 500*g* for 2 min, the pellet was resuspended in 500 μl of the lysis buffer and washed three times. The pellet was resuspended in 150 μl of lysis buffer containing 10% dimethyl sulfoxide and shaken for 2 h, and then it was used for assaying gelatinase activity of MMP-2 and MMP-9.

The samples were subjected to electrophoresis in a 10% SDS-polyacrylamide gel electrophoresis containing 0.1% gelatin under nonreducing conditions. Gels were washed twice for 30 min in 2.5% Triton X-100 to remove SDS, washed for 30 min in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 2 μM $ZnCl_2$, 200 mM NaCl, and 0.02% Briji 35, pH 7.4) at room temperature, and further incubated for 24 h in the same buffer at 37°C. Gels were then stained for 3 h in Coomassie Blue (1% Coomassie Brilliant Blue G-250, 30% methanol, and 10% acetic acid) and destained in 40% methanol/7% acetic acid until clear bands of gelatinolysis occurred on a dark background. Total activity including pro-MMP activity was analyzed with the ATTO Densitograph Software Library Lane Analyzer (Atto Instruments, Tokyo, Japan).

Western Blotting. Brain tissues were homogenized in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, and 0.005% Brij 35, pH 7.4) and microwaved for 15 s according to the protocol for immunoblotting with monoclonal antibodies. The homogenate was centrifuged at 13,000*g* for 30 min to pellet insoluble material. The protein concentration in the supernatant was determined using a Protein Assay Rapid kit. The sample was boiled in 2× sample buffer (0.25% bromphenol blue, 12% 2-mercaptothanol, 20% glycerol, 4% SDS, and 0.1 M Tris-HCl, pH 6.8) and subjected to SDS-polyacrylamide gel electrophoresis on a 4% stacking gel and 8% separating gel, and then transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The same concentration (20–40 μg) of protein per lane was loaded for all Western blotting. The band intensities of the film were analyzed by densitometry. The amount of MMP-9 was calculated versus the amount of β-actin protein. The primary polyclonal rabbit or goat antibodies used in the present study were anti-MMP-9 (1:1000; Abcam plc, Cambridge, UK) and β-actin (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The secondary antibodies, horseradish peroxidase-linked anti-rabbit and anti-goat IgG, were used at 1:2000 and 1:5000 dilutions, respectively (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Immunoreactive materials on the membrane were detected using enhanced chemiluminescence Western blotting detection reagents (GE Healthcare) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry.

In Situ Zymography. Mice were intracardially perfused with ice-cold saline before being frozen at −80°C using optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). The brains were sectioned at 20 μm in a cryostat. We adapted an in situ zymography method to localize net gelatinolytic activity in brain sections as described previously (Szklarczyk et al., 2002; Mizoguchi et al., 2007b). Nonfixed sections were incubated for 24 h at 37°C in a humid dark chamber with a reaction buffer containing 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, and 2 mM sodium azide, pH 7.6, and 100 μg/ml FITC-labeled DQ-gelatin (Invitrogen, Carlsbad, CA) intramolecularly quenched. After the incubation, sections were washed in PBS, fixed in 4% paraformaldehyde, and mounted on slides. Some sections were incubated with the broad-spectrum MMP inhibitor 1,10-*O*-phenanthroline (1 mM; Invitrogen). Samples were observed with an FITC filter, and the images were analyzed using an AxioVision 3.0 system (Carl Zeiss, Jena, Germany). The cleavage of gelatin-FITCs by tissue gelatinases releases quenched fluorescence representative of net proteolytic activity. Sections incubated without FITC-labeled DQ-gelatin were not fluorescent.

Double Immunostaining. Polyclonal rabbit anti-MMP-9 antibody (1:250; Abcam plc), monoclonal mouse anti-neuron-specific nuclear antigen (NeuN) antibody (1:200; Millipore Bioscience Research Reagents, Temecula, CA), anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Millipore Bioscience Research Reagents), and anti-F8/40 antibody (1:100; Sigma-Aldrich) served as primary antibodies. Affinity-purified FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG served as secondary antibodies. Samples were observed with an AxioVision 3.0 system.

Neuronal Cultures. Cortical neurons were prepared from mouse embryos at 17 days of gestation as described previously (Takuma et al., 2005b). In brief, slices of cerebral cortex were digested with trypsin and triturated in Neurobasal medium containing 2% B27 supplement, 0.5 mM glutamine, and 4.4 mM sodium bicarbonate at 4°C. Cells were separated from debris and diluted in 1 ml of the medium. After centrifugation for 2 min, the cell pellet was resuspended in Neurobasal medium containing 2% B27 supplement, 0.5 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin, and plated at 2×10^6 cells/ml into 24-well plates coated with 50 μg/ml poly(D-lysine).

Aβ1-40 (Peptide Institute Inc., Osaka, Japan) at a concentration of 10 μM was added to cultured cortical neurons from ICR mice for 24 h. Cells were fixed with 3% paraformaldehyde in PBS(−) at 4°C and washed three times. Polyclonal rabbit anti-MMP-9 antibody and monoclonal mouse anti-NeuN antibody served as primary antibodies in 1% bovine serum albumin and 0.1% Triton X-100 in PBS(−). Affinity-purified FITC-conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG served as secondary antibodies. Samples were observed with an AxioVision 3.0 system and LSM 510 confocal microscope (Carl Zeiss).

Cell Death Assay. Cell death was evaluated by measuring lactate dehydrogenase (LDH) activity released into the medium using a colorimetric assay as described previously (Takuma et al., 2005b). In brief, cells in 96-well plates were exposed to experimental treatments, and then 50 μl of culture supernatant was collected from each well. Supernatants were reacted with a tetrazolium salt at room temperature for 30 min, and stop solution was added. Absorbance at 405 nm was measured on a Benchmark microplate reader (Bio-Rad). Total cellular LDH activity was determined by lysing the cells. The amount of LDH activity released from cells was expressed as a percentage of total LDH activity.

An MMP-2/-9 inhibitor II (Calbiochem) at doses of 10 and 30 μM was added to the culture medium 30 min before treatment with Aβ1-40 at a dose of 10 μM in cultured cortical neurons from ICR mice. Three or 5 days after treatment, the amount of LDH released from the cultured neurons was measured. Total cellular LDH activity was determined by lysing the cells. The amount of LDH activity released from the cells was expressed as a percentage of total LDH activity.

Statistical Analysis. All data are expressed as the mean ± S.E. Statistical significance was determined using a one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test for multigroup comparisons. p values less than 0.05 were taken to indicate statistically significant differences. Student's t test was used for two-group comparisons.

Results

Dose- and Time-Dependent Changes in MMP-9 Activity in the Hippocampus after Intracerebroventricular Injection of Aβ25-35 or Aβ1-40 in Mice. We investigated whether MMP-2 and MMP-9 activities were induced by the intracerebroventricular injection of Aβ25-35 and Aβ1-40 using gel zymography method. In Fig. 1A, lane 8 represents a zymographic control marker, murine recombinant MMP-9, whose molecular mass is approximately 105 kDa. The injection of Aβ25-35 transiently and dose-dependently increased MMP-9, but not MMP-2, activity in the hippocampus compared with the activity in the vehicle-treated control group [$F_{(2,22)} = 0.84, p > 0.05$ (Fig. 1B); $F_{(2,22)} = 7.58, p < 0.05$ (Fig. 1C) by one-way ANOVA]. MMP-9 activity was markedly increased to 500 to 1300% of control levels on day 1, but it returned to the basal levels on days 3 and 7 after injection. Likewise, the intracerebroventricular injection of Aβ1-40 dose-dependently increased MMP-9, but not MMP-2, activity in the hippocampus [$F_{(4,33)} = 0.25$, $p > 0.05$ (Fig. 2B); $F_{(4,33)} = 3.22, p < 0.05$ (Fig. 2C) by one-way ANOVA] on day 1. Again, the MMP-9 activity returned to the basal levels of vehicle or Aβ40-1-injected mice on days 3 and 7 ($p < 0.05$ by t test; Fig. 2F). It should be noted that the injection of Aβ25-35 and Aβ1-40 produced a lower molecular weight band that may be an active form of MMP-9 (Figs. 1A and 2, A and D). Moreover, the intracerebroventricular injection of Aβ1-40 tended to increase MMP-9, but not MMP-2, activity in the frontal cortex on day 1, but the change was not statistically significant (Supplemental Fig. 1). It was confirmed that the intracerebroventricular injection of the more fibrinogenic Aβ1-42 at 900 pmol significantly increased MMP-9 activity in the hippocampus on day 1 after injection [vehicle-injected control mice (n = 6), 100 ± 27; Aβ1-42-injected mice (n = 8), 460 ± 57]. The injection of Aβ1-42 also induced a minimal increase in MMP-2 activity [vehicle-injected control mice (n = 6), 100 ± 5; Aβ1-42-injected mice (n = 8), 120 ± 4].

Spatial Changes in Net Proteolytic Activity in the Hippocampus after Intracerebroventricular Injection of Aβ1-40. We analyzed the spatial changes in gelatinase

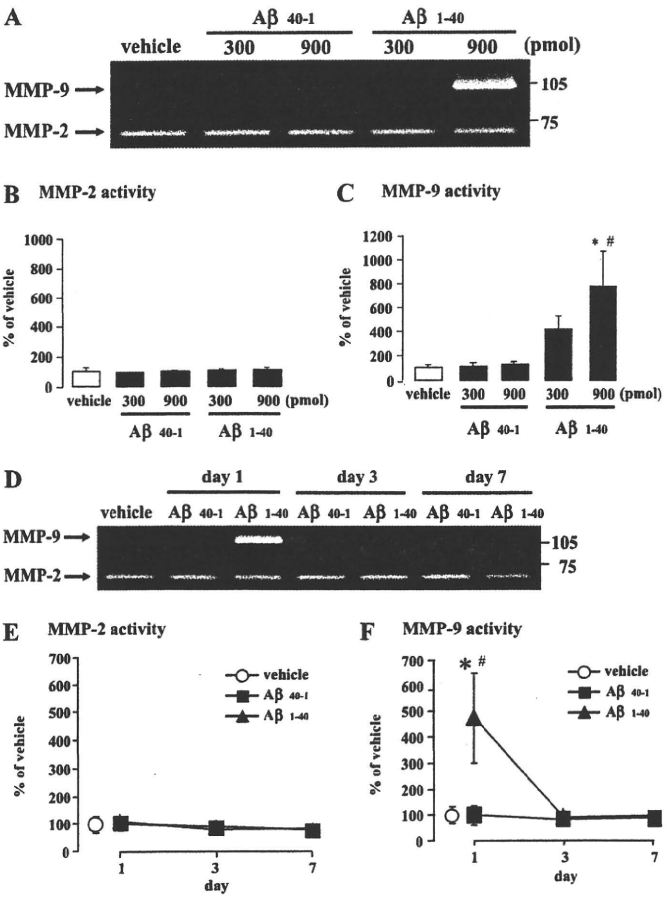

Fig. 2. Effect of intracerebroventricular injection of Aβ1-40 on MMP-2 (B and E) and MMP-9 (C and F) activities in the hippocampus. A and D, gel zymography. Mice were injected with either vehicle or Aβ1-40 at a dose of 300 or 900 pmol and then killed on day 1, 3, and 7 after the injection. Values are the mean ± S.E. [B and C; vehicle, n = 8; Aβ40-1 (300 pmol), n = 4; Aβ40-1 (900 pmol), n = 8; Aβ1-40 (300 pmol), n = 9; Aβ1-40 (900 pmol), n = 9; E and F, vehicle, n = 11; Aβ40-1 (900 pmol; day 1), n = 12; Aβ40-1 (900 pmol; day 3) n = 4; Aβ40-1 (900 pmol; day 7), n = 4; Aβ1-40 (900 pmol; day 1), n = 13; Aβ1-40 (900 pmol; day 3), n = 4; Aβ1-40 (900 pmol; day 7), n = 4]. *, $p < 0.05$ versus vehicle-injected mice. #, $p < 0.05$ versus Aβ40-1-injected mice (day 1).

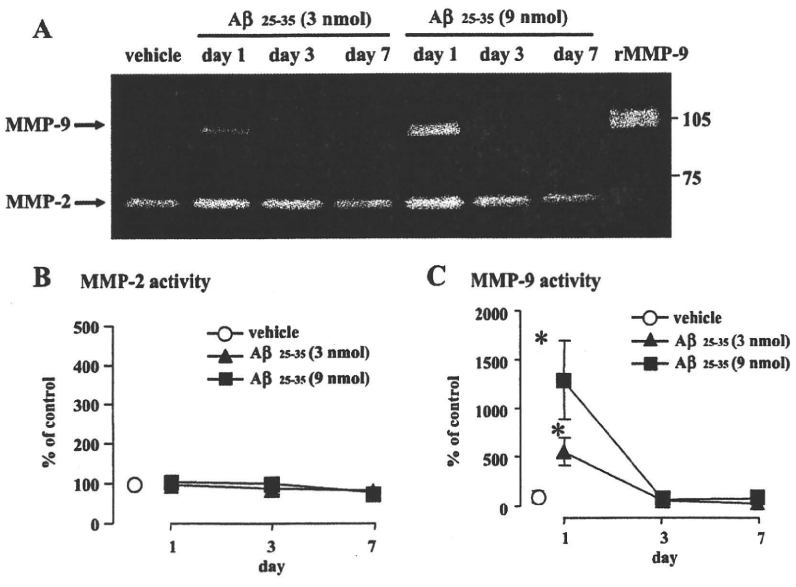

Fig. 1. Effect of intracerebroventricular injection of Aβ25-35 on MMP-2 (B) and MMP-9 (C) activities in the hippocampus. A, gel zymography. Mice were injected with either vehicle or Aβ25-35 at a dose of 3 or 9 nmol and then killed on day 1, 3, and 7 after the injection. Values are the mean ± S.E [vehicle; n = 9; Aβ25-35 (3 nmol; day 1), n = 8; Aβ25-35 (3 nmol; day 3), n = 5; Aβ25-35 (3 nmol; day 7), n = 3; Aβ25-35 (9 nmol; day 1), n = 8; Aβ25-35 (9 nmol; day 3), n = 4; Aβ25-35 (9 nmol; day 7), n = 3]. *, $p < 0.05$ versus vehicle-injected mice.

activity in the hippocampus after the intracerebroventricular injection of Aβ1-40 by in situ zymography. Brain sections were incubated with gelatin conjugated to a quenched fluorescence dye, and the cleavage of gelatin by gelatinase results in an increase in fluorescence. The signal was completely inhibited by the zinc chelator phenantroline, a broad-spectrum MMP inhibitor, indicating that the fluorescence is associated with MMP activity (Supplemental Fig. 2, compare B with A). In vehicle- and Aβ40-1-injected groups, gelatinase activity was observed in the CA1–CA4 layers and dentate gyrus of the hippocampus, indicating that constitutive gelatinolytic activity was localized to the main neuronal layers of the hippocampus. Twenty-four hours after the intracerebroventricular injection of Aβ1-40, an intense signal was visualized in the hippocampus compared with the Aβ40-1-injected group (Supplemental Fig. 2, compare D with C). In addition, the gelatinase activity was markedly increased in the molecular layer of the hippocampus in the Aβ1-40-treated group compared with the Aβ40-1-injected group (Supplemental Fig. 2, E and F).

The Intracerebroventricular Injection of Aβ1-40 Increases MMP-9 Protein Expression in the Brain. Next, we examined whether MMP-9 protein levels were also increased in the hippocampus by the intracerebroventricular injection of Aβ1-40 on day 1. A Western blot analysis revealed the hippocampal protein level to be increased 24 h after Aβ1-40 was injected at 900 pmol compared with levels in the vehicle- and Aβ40-1-treated groups [$F_{(2,15)} = 5.55, p < 0.05$ (Fig. 3A) by one-way ANOVA].

To determine the cell types in which the expression of MMP-9 is induced by the injection of Aβ1-40, double immunostaining for MMP-9 with NeuN, a neuronal marker (Fig. 3D); F4/80, a microglial marker (Fig. 3E); or GFAP, an astroglial marker (Fig. 3F), was performed. In the Aβ1-40-treated group, strong immunolabeling of MMP-9 was visualized in the CA3 layers of the hippocampus compared with the Aβ40-1-injected group (Fig. 3, compare B with C), in which the majority of the immunoreactivity was colocalized to NeuN-positive cells, suggesting the expression of MMP-9 in neurons. However, some MMP-9 immunoreactivity was observed in F4/80- or GFAP-positive cells in the hippocampus (Fig. 3, D–F).

Role of the Aβ-Induced Increase in MMP-9 Expression in Aβ-Induced Impairment of Recognition Memory. To examine the role of MMP-9 in Aβ1-40-induced cognitive dysfunction, we investigated the effect of GM6001, a broad-spectrum MMP inhibitor (Galardy et al., 1994; Wang and Tsirka, 2005), on Aβ1-40-induced impairment of recognition memory in the NORT. Cotreatment with GM6001 dose-dependently suppressed the Aβ1-40-induced increase in hippocampal MMP-9 activity compared with 2.5% dimethyl sulfoxide, and the effect of GM6001 (5 μg) was statistically significant [$F_{(2,16)} = 3.80, p < 0.05$ (Supplemental Fig. 3A) by one-way ANOVA]. However, GM6001 had little effect on MMP-2 activity, and there was no significant difference in activity between the 2.5% dimethyl sulfoxide-injected and GM6001-injected mice (Supplemental Fig. 3A). As shown in Supplemental Fig. 3B, the intracerebroventricular injection of Aβ1-40 significantly reduced exploratory preference for the novel object in the retention session [$F_{(3,18)} = 5.68, p < 0.05$ (Supplemental Fig. 3B) by one-way ANOVA], without affecting total exploration time in the training and retention sessions [data not shown; training: $F_{(3,18)} = 0.29, p > 0.05$ by one-way ANOVA; retention: $F_{(3,18)} = 2.05, p > 0.05$ by one-way ANOVA], indicating the impairment of recognition memory in Aβ1-40-injected mice. Simultaneous treatment with GM6001 (5 μg) in Aβ1-40-injected mice caused a significant improvement in exploratory preference in the retention session [$F_{(3,18)} = 5.68, p < 0.05$ (Supplemental Fig. 3B) by one-way ANOVA], without affecting the exploratory preference in the training session [$F_{(3,18)} = 0.34, p > 0.05$ (Supplemental Fig. 3B) by one-way ANOVA] or total exploration time in the training or retention session [data not shown; training: $F_{(3,18)} = 0.29, p > 0.05$ by one-way ANOVA; retention: $F_{(3,18)} = 2.05, p > 0.05$ by one-way ANOVA].

In addition to the pharmacological studies, we investigated the role of MMP-9 in Aβ1-40-induced impairment of recognition memory using MMP-9(−/−) mice. In the standard (one-training) procedure of NORT, the exploratory preference to the novel object in the retention session was markedly reduced in MMP-9(−/−) mice (49.3 ± 2.9; $n = 10$) compared with wild-type mice (69.0 ± 2.3; $n = 10$) without a change in total exploration time in the training and retention sessions. Thus, it was difficult to assess the Aβ-induced memory impairment in MMP-9(−/−) mice in a standard one-training NORT. Accordingly, the number of training sessions was increased from one to four, and the mice were subjected to the retention session (repeated training NORT). There was no difference in total exploration time in the first and fourth training sessions between Aβ40-1-treated wild-type and MMP-9(−/−) mice, respectively [first training: $F_{(3,21)} = 2.28$, $p > 0.05$ by one-way ANOVA; fourth training: $F_{(3,21)} = 1.75$, $p > 0.05$ by one-way ANOVA] (Fig. 4A). As shown in Fig. 4B, there was no difference in exploratory preference in the retention session between Aβ40-1-treated wild-type and MMP-9(−/−) mice, indicating that the Aβ40-1-treated MMP-9(−/−) mice could recognize the novel object 24 h after four training sessions [$F_{(3,21)} = 17.9, p < 0.05$ by one-way ANOVA for retention] (Fig. 4B). Under these conditions, Aβ1-40-injected wild-type mice showed a marked impairment of exploratory preference in the retention session [$F_{(3,21)} = 17.9, p < 0.05$ by one-way ANOVA] (Fig. 4B), without exhibiting a change in exploratory preference in the training sessions [first training: $F_{(3,21)} = 2.00, p > 0.05$ by one-way ANOVA; fourth training: $F_{(3,21)} = 1.99, p > 0.05$ by one-way ANOVA] (Fig. 4B) or in total exploration time in the training sessions [first training: $F_{(3,21)} = 2.28, p > 0.05$ by one-way ANOVA;

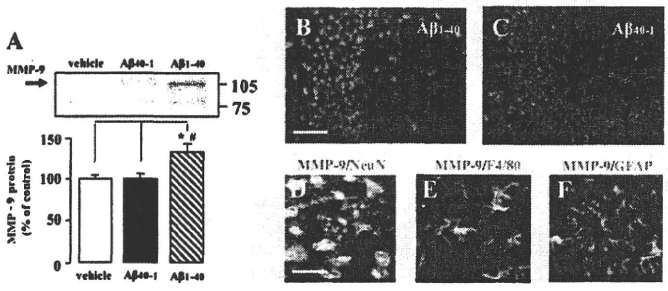

Fig. 3. Effect of intracerebroventricular injection of Aβ1-40 on the MMP-9 protein level in the hippocampus. A, Western blot analysis: Mice were injected with either vehicle, Aβ40-1, or Aβ1-40 at a dose of 900 pmol and then killed 1 day later. Values are the mean ± S.E. (A; $n = 6$). *, $p < 0.05$ versus vehicle-injected mice. #, $p < 0.05$ versus Aβ40-1-injected mice. B to F, double immunostaining for MMP-9 (B–F; green) and NeuN (D; red), F4/80 (E; red), or GFAP (F; red) in the CA3 layer of the hippocampus. Scale bar, 100 μm (B) and 10 μm (D).

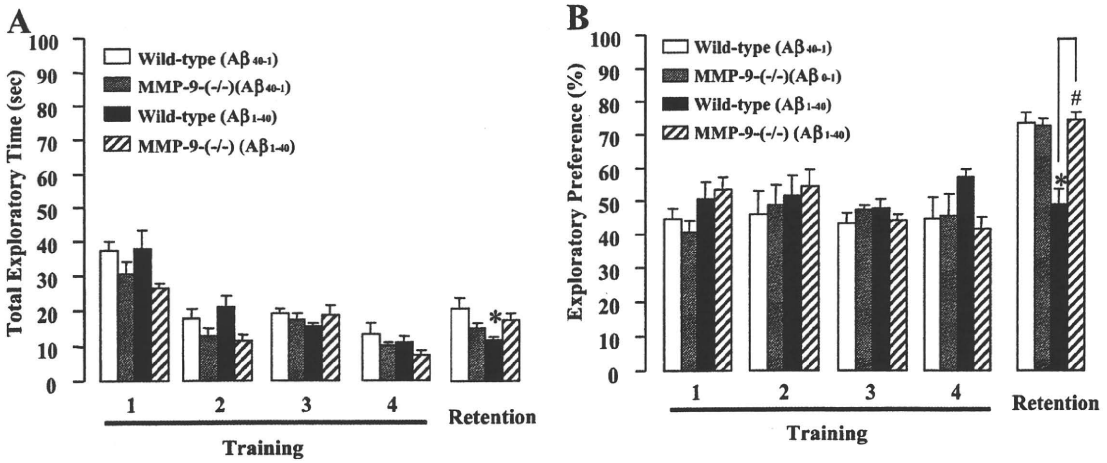

Fig. 4. Effect of Aβ1-40 on total exploratory time (A) and exploratory preference (B) in repeated training NORT in wild-type and MMP-9(−/−) mice. Mice were intracerebroventricularly injected with Aβ1-40 at a dose of 900 pmol. Values are the mean ± S.E. (n = 5–7). *, $p < 0.05$ versus wild type (Aβ40-1). #, $p < 0.05$ versus wild type (Aβ1-40).

fourth training: $F_{(3,21)} = 1.75$, $p > 0.05$ by one-way ANOVA] (Fig. 4A). There was a slight but significant difference in total exploration time in the retention session between Aβ40-1- and Aβ1-40-injected wild-type mice [$F_{(3,21)} = 4.86$, $p < 0.05$ by one-way ANOVA] (Fig. 6A).

Pretreatment with MK-801 Inhibits Aβ25-35-Induced Increase in MMP-9 Expression and Impairment of Recognition Memory. To examine the mechanism by which Aβ injection induces the MMP expression, we investigated the effect of MK-801, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, on the Aβ25-35-induced increase in MMP-9 expression and impairment of recognition memory in the NORT. Pretreatment with MK-801 had no effect on the hippocampal MMP-9 activity in vehicle-treated mice. However, the NMDA receptor antagonist completely inhibited the Aβ25-35-induced increase in MMP-9 expression in the hippocampus [$F_{(3,15)} = 6.17$, $p < 0.05$ by one-way ANOVA] (Fig. 5A). Moreover, pretreatment with MK-801 (0.1–0.3 mg/kg) in Aβ25-35-injected mice caused a dose-dependent and significant improvement of exploratory preference in the retention session [$F_{(5,51)} = 2.74$, $p < 0.05$ by one-way ANOVA] (Fig. 5B), without affecting exploratory preference in the training session [$F_{(5,51)} = 0.65$, $p > 0.05$ by one-way ANOVA] (Fig. 5B) or total exploration time in the training [$F_{(5,51)} = 0.93$, $p > 0.05$ by one-way ANOVA] or retention session [$F_{(5,51)} = 0.87$, $p > 0.05$ by one-way ANOVA]. Post hoc analysis indicated that MK-801 at 0.3 mg/kg, but not 0.1 mg/kg, significantly improved exploratory preference in Aβ25-35-treated mice ($p < 0.05$). MK-801 at 0.1 to 0.3 mg/kg in the vehicle-treated control group had no effect on exploratory preference or total exploration time in the training and retention sessions (Fig. 5B).

Effect of Aβ1-40 on MMP-9 Expression in Primary Cultured Cortical Neurons. Treatment with Aβ1-40 (Supplemental Fig. 4E), but not Aβ40-1 (Supplemental Fig. 4C), at a dose of 10 μM for 24 h induced MMP-9 expression in primary cultured cortical neurons compared with vehicle treatment (Supplemental Fig. 4A). Immunoreactivity was observed in NeuN-positive cells, indicating that Aβ1-40 treatment can induce MMP-9 expression in neurons (Supplemental Fig. 4G). NeuN-positive cells differed between the Aβ1-40- (Supplemental Fig. 4F) and Aβ40-1-treated (Supplemental Fig. 4D) groups, suggesting that cell death was not induced drastically 24 h after Aβ1-40 treatment (Supplemental Fig. 4, compare F with D).

A Specific Inhibitor of MMP-2/-9 Attenuated Aβ1-40-Induced Neurotoxicity in Primary Cultured Cortical Neurons. Finally, we investigated the role of MMP-9 in Aβ1-40-induced neurotoxicity by measuring LDH activity released into the culture medium. Aβ1-40 treatment for 3 or 5 days markedly induced the release of LDH from cultured cortical neurons [3 days (Fig. 6A): $F_{(5,18)} = 17.6$, $p < 0.05$ by one-way ANOVA; 5 days (Fig. 6B): $F_{(5,18)} = 40.4$, $p < 0.05$ by one-way ANOVA]. However, cotreatment with rather specific MMP-2/-9 inhibitor reduced the amount of LDH released from cultured cortical neurons [3 days (Fig. 6A): $F_{(5,18)} = 17.6$, $p < 0.05$ by one-way ANOVA; 5 days (Fig. 6B): $F_{(5,18)} =$

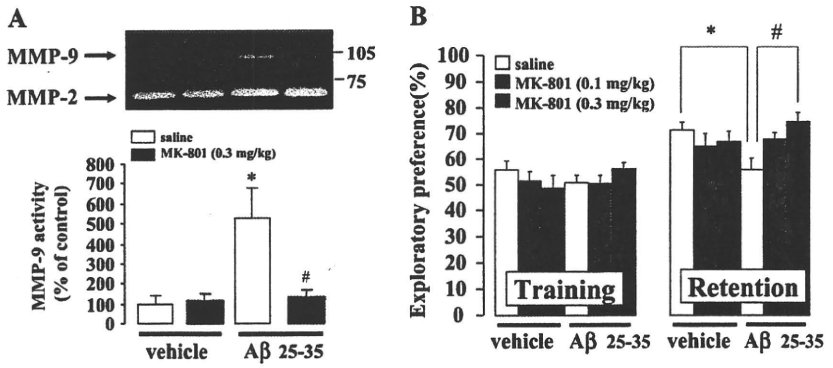

Fig. 5. Effect of MK-801 on Aβ25-35-induced increase in hippocampal MMP-9 activity (A) and memory impairment (B). Mice were given MK-801 at 0.1 to 0.3 mg/kg 30 min before receiving an intracerebroventricular injection of Aβ25-35 at 3 nmol. Values are the mean ± S.E. (n = 4–5 for A; n = 9–10 for B). *, $p < 0.05$ versus saline + vehicle. #, $p < 0.05$ versus saline + Aβ25-35.

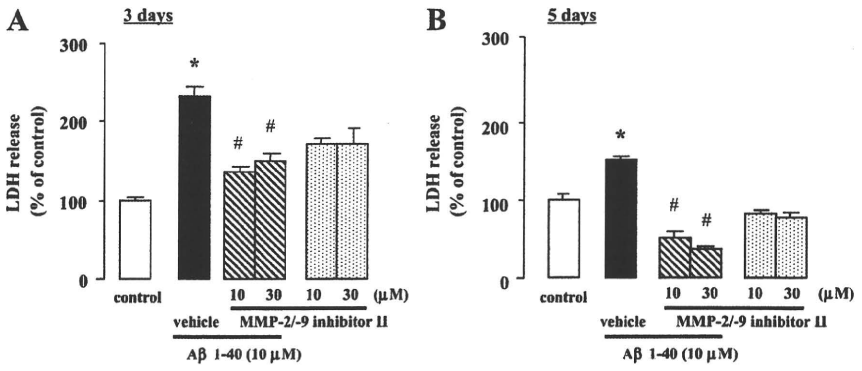

Fig. 6. Effect of a specific MMP-2/-9 inhibitor on Aβ-induced neurotoxicity in primary cultured cortical neurons. A, MMP-2/-9 inhibitor II at a dose of 10 or 30 μM was added simultaneously with Aβ1-40 at a dose of 10 μM to primary cultured cortical neurons from ICR mice. Three or 5 days later, the amount of LDH released from cultured neurons was measured. Total cellular LDH activity was determined by lysing the cells. The amount of LDH activity released from the cells was expressed as a percentage of total LDH activity. Values are the mean ± S.E. for four determinations per experimental condition. *, $p < 0.05$ versus control. #, $p < 0.05$ versus Aβ1-40 + vehicle.

40.4 $p < 0.05$ by one-way ANOVA], although the inhibitor itself had no effect on the release.

In addition to the pharmacological studies, we investigated the role of MMP-9 in Aβ1-40-induced neurotoxicity in primary cultured neurons from MMP-9(−/−) mice. Treatment with Aβ1-40 at 10 μM for 5 days markedly increased the amount of LDH released from cultured cortical neurons of wild-type mice [$F_{(5,20)} = 3.47$, $p < 0.05$ by one-way ANOVA] (Fig. 7). The Aβ1-40-induced release was almost completely suppressed in the neurons from MMP-9(−/−) mice compared with those from wild-type mice (Fig. 7).

Discussion

In the Aβ25-35-, Aβ1-40-, and Aβ1-42-injected mice, MMP-9 activity and protein expression were transiently increased in the hippocampus. We demonstrated using inhibitors of MMPs and MMP-9(−/−) mice that the increase in MMP-9 expression in the hippocampus is associated with the development of cognitive impairment and neurotoxicity induced by Aβ. Thus, specific inhibitors of MMP-9 may have a therapeutic potential for the treatment of AD.

Members of the MMP subfamily, the gelatinases MMP-2 and MMP-9, are initially expressed as inactive proenzymes and cleaved into active forms after cellular release (Van den Steen et al., 2002); this property places these proteases in a unique position to regulate levels of substrates in the extracellular space. Our present study apparently indicated that the activity of MMP-2 was constantly expressed, whereas that of MMP-9 was very weak in the hippocampus of vehicle-injected mice. The intracerebroventricular injection of Aβ led to a transient induction of MMP-9 expression in the hippocampus. In addition, we demonstrated that the Aβ-induced expression of MMP-9 was localized to neuronal and glial cells in the hippocampus. Previous study has revealed that both MMP-2 and MMP-9 are expressed in the presence of Aβ (Deb and Gottschall, 1996) and highly expressed and secreted by astrocytes (Muir et al., 2002; Deb et al., 2003). In contrast, MMP-9 is synthesized in neurons of the human hippocampus (Backstrom et al., 1996) and is expressed in the cytoplasm of neurons, neurofibrillary tangles, vascular walls, and senile plaques in the brain tissues of AD patients (Asahina et al., 2001). Our findings are consistent with these previous reports.

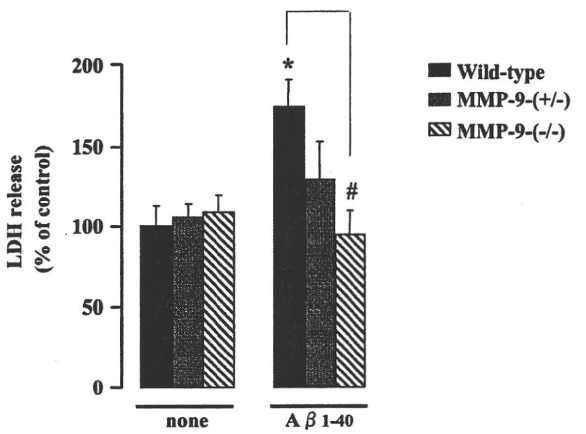

Fig. 7. Aβ-induced neurotoxicity in primary cultured cortical neurons from MMP(−/−) mice. Primary cultured cortical neurons from wild-type, MMP-9(+/−), or MMP-9(−/−) mice were treated with Aβ (10 μM). Five days later, the amount of LDH released from cultured neurons was measured. Total cellular LDH activity was determined by lysing the cells. The amount of LDH activity released from the cells was expressed as a percentage of total LDH activity. Values are the mean ± S.E. for three to five determinations per experimental condition. *, $p < 0.05$ versus control. #, $p < 0.05$ versus Aβ1-40 + vehicle.

To clarify the mechanism underlying Aβ-induced expression of MMP-9, we focused on the role of NMDA receptors, because it has been reported that Aβ activates NMDA receptors (Snyder et al., 2005), and MMP-9 expression is induced via NMDA receptors (Meighan et al., 2006; Nagy et al., 2006; Tian et al., 2007). Pretreatment with MK-801 inhibited the Aβ-induced increase in MMP-9 expression and cognitive impairment, indicating that MMP-9's induction and cognitive dysfunction are induced by Aβ treatment associated at least in part with the activation of NMDA receptors in this model. In fact, an excessive amount of glutamate in the synaptic microenvironment and the persistent influx of Ca^{2+} through NMDA receptors are considered major causes of neurodegeneration in AD (Wenk, 2006). For example, in the rat magnocellular nucleus basalis, Aβ-induced toxicity was effectively reduced by MK-801, and Aβ promoted an excitotoxic pathway that includes astroglial depolarization, extracellular glutamate accumulation, NMDA receptor activation culminating in intracellular Ca^{2+} overload, and cell death (Harkany et al., 2000). Direct injection of Aβ1-40 in the hippocampus caused neuronal loss in the CA1 area and treatment with an NMDA antagonist, memantine, reduced the Aβ-induced neuronal degeneration (Miguel-Hidalgo et al., 2002) as well as working memory deficits (Yamada et al., 2005). These findings support the hypothesis that NMDA receptors play a central role in Aβ-induced neurotoxicity. In addition, the expression and activity of MMP-9 depend on the activation of NMDA receptors and are associated with the development of LTP (Meighan et al., 2006; Nagy et al., 2006). The activation of NMDA receptors promotes the development of dendritic

spines through MMP-mediated cell adhesion molecules (Tian et al., 2007). Together with our findings that Aβ treatment increased MMP-9 expression in primary cultured neurons, the activation and expression of MMP-9 are directly and/or indirectly regulated by Aβ through the activation of NMDA receptors in neuronal cells.

MMP-9 might be induced as a protection to destroy the plaques and amyloid fibrils. The growing list of proteases can degrade soluble Aβ in vitro, namely, neprilysin (Howell et al., 1995), insulin-degrading enzyme (Kurochkin and Goto, 1994), and MMP-9 (Yan et al., 2006), suggesting a role for these proteases in regulating endogenous basal levels of Aβ in vivo. Notably, MMP-9 was reported to cleave insoluble Aβ in vitro (Yan et al., 2006). The view of the function of MMPs in the long-lasting synaptic plasticity is expanding, and evidence suggests that MMP-9 is up-regulated and becomes proteolytically active selectively during the maintenance phase of LTP at CA3-CA1 synapses in the hippocampus (Nagy et al., 2006), and similar findings have been recently made in rat prefrontal cortex. These reports suggest that MMPs function in cellular processes that contribute to learning and memory. Therefore, although we assumed that MMP inhibitors potentiate the Aβ-induced cognitive dysfunction and neurotoxicity, our findings do not support such an assumption. In Supplemental Fig. 3, we showed that MMP inhibitor treatment ameliorated Aβ-induced impairment of recognition memory, suggesting that the transient increase in hippocampal MMP-9 activity is functionally associated with the development of Aβ-induced cognitive deficits. The findings made with a pharmacological inhibitor were further supported by the result that the intracerebroventricular injection of Aβ1-40 impaired recognition memory in wild-type but not MMP-9(−/−) mice. Accordingly, even if MMP-9 can degrade Aβ/plaques, it may randomly and nonselectively destroy the extracellular matrix and neural membranes, leading to neuronal dysfunction and cognitive impairment.

Recent evidence has linked MMPs to various pathological conditions in the central nervous system, including ischemia, multiple sclerosis, Parkinson's disease, and malignant glioma. This implies that, in addition to its known function to degrade extracellular macromolecules, MMP may serve as a mediator that leads to apoptotic and/or necrotic cell death. In fact, recent studies indicate that MMP-9 has direct neurotoxic effects. Jourquin et al. (2003) demonstrated the increased release and activity of MMP-9 after stimulation with neurotoxic kainate in organotropic cultures and reduced neuronal cell death by the inhibition of MMP-9. Conversely, incubation with recombinant MMP-9 induced neuron death in the organotropic cultures. Alternatively, MMP-3 is reported to play a major role in degenerative human brain disorders such as Parkinson' disease (Kim et al., 2005). In the present study, MMP inhibitor II, reported to be highly selective for MMP-2 and MMP-9 (Tamura et al., 1998), blocked the Aβ-induced release of LDH, indicating that MMP-9 is crucial in Aβ-induced neuronal cell death. Genetic evidence also showed that Aβ-induced neurotoxicity was markedly reduced in primary cultured cortical neurons from MMP-9(−/−) mice compared with those from wild-type mice (Fig. 7). However, there is a report that GM6001, a broad-spectrum metalloproteinase inhibitor, acts synergistically with Aβ to enhance neurotoxicity in cultured neurons (Ethell et al., 2002). The discrepancy may reflect differences in the cell death assay and the specificity of inhibitors used because GM6001 can inhibit all MMPs and a-disintegrin-and-a-metalloproteinases.

In conclusion, we have demonstrated for the first time that MMP-9 activity and protein expression are transiently increased in the hippocampus by the intracerebroventricular injection of Aβ25-35, Aβ1−40, and Aβ1−42. We hypothesize that Aβ-induced secondary dysfunction such as MMP activation, could result in learning deficits by impairing synaptic function in the hippocampus. The present findings highlight the contribution of neural/glial MMP-9 to Aβ-induced neurotoxicity and cognitive impairment and support the case for highly selective MMP-9 inhibitors that could reduce deleterious proteolytic activity and neuronal death. Thus, specific inhibitors of MMP-9 may have therapeutic potential for the treatment of AD.

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Neonatal polyI:C treatment in mice results in schizophrenia-like behavioral and neurochemical abnormalities in adulthood

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ABSTRACT

It has been reported that viral infection in the first and second trimesters of pregnancy in humans increases the risk of subsequently developing schizophrenia. To develop a mouse model of immune activation during the early postnatal period, neonatal ICR mice were repeatedly injected with polyriboinosinic–polyribocytidilic acid (polyI:C; an inducer of strong innate immune responses) for 5 days (postnatal day 2–6) which may correspond, in terms of brain development, to the early second trimester in human. Cognitive and emotional behavior as well as the extracellular level of glutamate in the hippocampus were analyzed at the age of 10–12 weeks old. PolyI:C-treated mice showed anxiety-like behavior, impairment of object recognition memory and social behavior, and sensorimotor gating deficits, as compared to the saline-treated control group. Depolarization-evoked glutamate release in the hippocampus was impaired in polyI:C-treated mice compared to saline-treated control mice. Furthermore, to investigate the effect of neonatal immune activation on the expression levels of schizophrenia-related genes, we analyzed mRNA levels in the hippocampus 2 and 24 h after polyI:C treatment. No significant differences or only transient and marginal changes were observed between polyI:C-treated and saline-treated control mice in the expression levels of schizophrenia-related genes in the hippocampus.

1. Introduction

Schizophrenia is a chronic mental disorder characterized by psychosis (e.g., hallucinations and delusions), flattened emotions and impaired cognitive function, which affects about 1% of the general population. Although the disease etiology remains poorly understood, various hypotheses, including dopamine hyperfunction, glutamatergic hypofunction, GABAergic deficits, stress vulnerability and impaired neurodevelopment have been proposed as the etiology/pathophysiology of schizophrenia (Nawa and Takei, 2006; Tan et al., 2008). Among them, the neurodevelopmental hypothesis of schizophrenia supported by clinical, neuroimaging, neuropathologic, and genetic studies, is a more fundamental theory that does not conflict with other hypotheses (Sawa and Snyder, 2002; Ross et al., 2006).

The estimated heritability of schizophrenia is approximately 80% (Burmeister et al., 2008), and recent studies have identified possible candidate susceptibility genes for schizophrenia, such as *dysbindin*, *neuregulin1* and *disrupted-in-schizophrenia 1* (*DISC1*) (Harrison and Weinberger, 2005; Gogos and Gerber, 2006; Ross et al., 2006). Not only genetic factors, but also environmental factors are important in the etiology of mental disorders, and are believed to interact in most cases of schizophrenia (Caspi and

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Moffitt, 2006; Burmeister et al., 2008). One of the most significant environmental risk factors for schizophrenia is maternal viral infection in the first (Brown et al., 2000, 2004) and second (Mednick et al., 1988; Clarke et al., 2006) trimester of pregnancy, which could affect brain development by releasing stress hormones, producing hypoxia, hyperthermia, or malnutrition, or by triggering proinflammatory cytokine responses in the mother, the placenta, or the fetus (Patterson, 2007).

Polyriboinosinic–polyribocytidilic acid (polyI:C) is a synthetic analogue of double-stranded RNA that leads to the pronounced but time-limited production of pro-inflammatory cytokines after administration to mammalian organisms through the activation of toll-like receptor 3 (Wang et al., 2004). Maternal immune activation by polyI:C exposure in rodents is known to precipitate a wide spectrum of behavioral, cognitive and pharmacological abnormalities in adult offspring (Shi et al., 2003; Zuckerman et al., 2003; Meyer et al., 2005, 2006; Ozawa et al., 2006; Cameron et al., 2007; Smith et al., 2007).

There are some concerns about the prenatal polyI:C treatment model of schizophrenia. First, prenatal treatment with polyI:C in pregnant dams is reported to increase the rate of abortion. Some of the dams administrated polyI:C could not bear live pups due to abortion (Ozawa et al., 2006; our unpublished observation). Second, it is controversial to match pregnancy stages between rodents and humans. Glial proliferation and migration, as well as establishment of the blood–brain barrier, peak during the early postnatal period in rodents (Clancy et al., 2001; Nawa and Takei, 2006; Mouri et al., 2007), but such a critical developmental stage occurs *in utero*, more specifically in the second trimester of pregnancy in humans (Adinolfi et al., 1976; Nawa and Takei, 2006). The correspondence of fetal development progression in different species can be compared using database-driven websites. The programs are based on statistical algorithms that integrate hundreds of empirically derived developing neuronal events in ten mammalian species, including rats, mice and humans (http://translatingtime.net/; see also Clancy et al., 2007).

In the present study, we sought to develop a novel mouse model in which immune activation is induced during the brain development matching the stage of fetus in the second trimester of pregnancy in humans. Accordingly, neonatal ICR mice were repeatedly injected with polyI:C for 5 days from postnatal day 2–6, which correspond to post-conception day 128–158 for cortical events and 93–115 for limbic events of brain development in humans (http://translatingtime.net). Cognitive and emotional behaviors as well as glutamatergic neurotransmission in the hippocampus were analyzed in the adult mice. Furthermore, we analyzed changes in the gene expression in the hippocampus of polyI:C-treated mice using DNA microarray and real-time reverse transcription (RT)-PCR.

2. Experimental procedures

2.1. Animals

ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and maintained under standard specific pathogen-free environmental conditions. Pregnant females were monitored for the parturition date, which was taken as postnatal day (PD) 0. They were housed under a standard 12-h light/dark cycle (lights on at 9:00) at a constant temperature of 23 ± 1 °C, with free access to food and water throughout the experiments. We used male mice exclusively to minimize any potential variability due to sex-specific effects in behavioral performance. The animals were handled in accordance with the guidelines established by the Institutional Animal Care and Use Committee of Nagoya University, the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Treatment

All litters were randomly divided into saline and polyI:C-treated groups. From PD 2 to 6, mice were injected s.c. daily with either pyrogen-free saline or polyI:C (Sigma–Aldrich, St. Louis, MO) at a dose of 5 mg/kg. The time needed for polyI:C injection in each mouse was less than 1 min, which minimized the influence of separation of neonates from the mothers on maternal behaviors directed towards the pups. Animals were weaned at PD 21, and divided by gender at PD 28. Both groups were derived from at least 3 different litters to preclude possible differences in individual maternal behavior as a mitigating factor in any subsequent long-lasting changes induced in the offspring.

2.3. Nissl staining

Mice were deeply anesthetized with pentobarbital and perfused intracardially with 4% paraformaldehyde in phosphate-buffered saline (PBS). The brains were removed, post-fixed with the same fixative and cryoprotected with 30% sucrose containing PBS. Twenty micrometers thick coronal brain sections were cut on a cryostat and mounted on slides. Nissl staining was done according to standard procedures (Takuma et al., 2007). Nissl stained sections were analyzed using a light microscope (Axio Imager; Zeiss, Jene, Germany).

2.4. Behavioral analysis

Behavioral analysis of polyI:C-treated mice was carried out at the age of 10–12 weeks old.

2.5. Locomotor activity under a novel environment

Each mouse was placed in a standard transparent rectangular rodent cage (25 cm × 30 cm × 18 cm) under moderately light conditions (15 lx). Locomotor activity was then measured for 120 min using an infrared sensor (NS-AS01; Neuroscience, Tokyo, Japan) placed over the cage (Kamei et al., 2006).

To investigate the effect of neonatal treatment with polyI:C on the sensitivity to MK-801 (Sigma–Aldrich, St. Louis, MO) or methamphetamine hydrochloride (METH; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), each mouse was allowed a 120-min habituation period before MK-801 (0.1–0.3 mg/kg, i.p.) or METH (2 mg/kg, i.p.) treatment. Locomotor activity was then measured for 180 min immediately after MK-801 or METH treatment.

2.6. Open field test

Mice were placed in the center of the arena and were allowed to explore the open field (diameter: 60 cm, height: 35 cm) for the following 5 min under moderately light conditions (80 lx), while their activity was measured automatically using the Ethovision automated tracking program (Brainscience Idea Co., Ltd., Osaka, Japan) (Lee et al., 2005; Wang et al., 2007). The open field was divided into an inner circle (diameter: 40 cm), and an outer area surrounding the inner circle. The movement of mice was measured via a camera mounted above the open field. Measurements included distance and time spent in the inner and outer sections.

2.7. Novel object recognition test

A novel object recognition test was carried out as described previously (Kamei et al., 2006; Nagai et al., 2007). Mice were individually habituated to an open-box (30 × 30 × 35 high cm) for 3 days. During the training session, two novel objects were placed in the open field and the animals were allowed to explore for 10 min under moderately light conditions (10 lx). The time spent exploring each object was recorded. During retention sessions, the animals were placed back into the same box 24 h after the training session, one of the familiar objects used during training was replaced by a novel object, and the mice were allowed to explore freely for 5 min. The preference index in the retention session, the ratio of the amount of time spent exploring the novel object over the total time spent exploring both objects, was used to measure cognitive function. In the training session, the preference index was calculated as the ratio of time spent exploring the object that was replaced by a novel object in the retention session, to the total exploration time.

2.8. Social interaction test

We used the experimental paradigm described by Tremolizzo et al. (2005) to measure social behavior (e.g., social interaction, aggression and escape behavior). PolyI:C-treated or saline-treated control mice were individually housed in a home cage (29 cm × 18 cm × 12 cm) for 2 days before the trial. We used 10–15-week-old male ICR mice as intruders which had not shown aggressive behavior. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage under bright light conditions (75 lx). The duration of social interaction (close following, inspection, anogenital sniffing, and other social body contacts except aggressive behavior), aggression (attacking/biting and tail rattling) and escape behavior were analyzed. Four trials, with an inter-trial interval of 30 min, were used to analyze social behavior using the same intruder mouse.

2.9. Prepulse inhibition test

The prepulse inhibition (PPI) test was carried out as described previously (Takahashi et al., 2007; Arai et al., 2008). After the animals were placed in the chamber under moderately bright light conditions (180 lx) (San Diego Instruments, San Diego, CA), they were allowed to habituate for 10 min, during which 65 dB

background white noise was present. The animals then received 10 startle trials, 10 no-stimulus trials and 40 PPI trials. The inter-trial interval was between 10 and 20 s and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. PPI trials consisted of a prepulse (20 ms burst of white noise at 69, 73, 77 or 81 dB intensity) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the four prepulse trials (69, 73, 77 or 81 dB) was presented 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the formula: $100 \times [1 - (PPx/P120)]\%$, in which PPx was the mean of the 10 PPI trials (PP69, PP73, PP75 or PP80) and P120 was the basal startle amplitude.

2.10. In vivo microdialysis

The *in vivo* microdialysis study was carried out in mice that had previously not been used for behavioral experiments. To measure extracellular glutamate release in the hippocampus, *in vivo* microdialysis was carried out as described previously (Murai et al., 2007). Mice at the age of 10–12 weeks old were anesthetized with pentobarbital Na (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA). A guide cannula (AG-4 EICOM Corp., Kyoto, Japan) was implanted into the hippocampus [AP: −3.3, ML: +2.8 from the bregma, DV: −2.5 mm from the skull] according to the atlas (Paxinos and Franklin, 2004). A dialysis probe (A-I-4-01; membrane length 1 mm, EICOM Corp.) was implanted into the hippocampus and Ringer solution (147 mM NaCl, 4 mM KCl and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1.0 μl/min, 2 days after implantation of the guide cannula. The dialysate was collected every 10 min and the amount of glutamate in the dialysate was determined using HPLC (HTEC-500, EICOM Corp.) with electrochemical detection. Three samples were taken to establish baseline levels of extracellular glutamate. For depolarization stimulation, 60 mM KCl-containing Ringer solution was delivered through the dialysis probe for 20 min in order to induce the K^+-evoked release of glutamate.

In some experiments, Ca^{2+}-free Ringer solution (149.3 mM NaCl and 4 mM KCl) with tetrodotoxin [TTX: (1 μM), Nacalai Tesque Co., Ltd., Kyoto, Japan)] was perfused for 60 min, following the measurement of basal glutamate levels.

2.11. DNA microarray

Neonatal mice were decapitated 2 or 24 h after the final treatment with polyI:C and their brains were removed. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany). DNA microarray and expression profiling were carried out as described previously (Ibi et al., 2008). Purified total RNA was used for expression profiling with GeneChip mouse genome 430 2.0 arrays for DNA (Affymetrix, Santa Clara, CA), containing 45,101 probe sets, according to the manufacturer's protocol.

2.12. Quantitative analyses of schizophrenia susceptibility gene expression by real-time RT-PCR

Total RNA isolated from the hippocampus was converted into complementary DNA (cDNA) using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Levels of mRNA expression were quantified using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed in a volume of 25 μl with 500 ng cDNA and 500 nM primers in the Power SYBR Green Master Mix (Applied Biosystems). The primers are described in Table 1.

2.13. Statistical analysis

Data are expressed as the mean ± SE. Differences between two groups were analyzed by two-tailed Student's *t*-test. Differences in body weight gain, locomotor activity, social interaction test, PPI test and K^+-induced (60 mM) glutamate release in microdialysis were analyzed by repeated analysis of variance (ANOVA). Multiple group comparisons were made by one-way ANOVA, followed by Bonferroni's test when *F* ratios were significant ($p < 0.05$).

3. Results

3.1. General appearance of polyI:C-treated mice

No mice died following repeated neonatal treatment with polyI:C at a dose of 5 mg/kg for 5 days. There was no apparent difference in maternal behaviors directed towards the saline- and polyI:C-treated pups. Repeated treatment with polyI:C in neonatal mice slightly decreased the body weight gain (less than 10%) in adolescence, but the effect was not statistically significant (Fig. 1a). There was no difference in exploratory locomotor activity between saline- and polyI:C-treated mice in adult (Fig. 1b). Furthermore, Nissl staining showed no apparent abnormalities of anatomy in the frontal cortex and hippocampus of polyI:C-treated mice in adult (Fig. 1c and d).

Table 1
Primers for schizophrenia-related and other genes.

Target gene	Forward	Reverse
GAD67	GCGGGAGCGGATCCTAATA	TGGTGCATCCATGGGCTAC
Reelin	CCCAGCCCAGACAGACAGTT	CCAGGTGATGCCATTGTTGA
BDNF	TAAATGAAGTTTATACAGTACAGTGGTTCTACA	AGTTGTGCGCAAATGACTGTTT
COMT	GGGCACCCCAGGACCTTAT	GTCTGGAAGGTAGCGGTCTTTC
14-3-3 ε	GCCGAGCGATACGACGAA	CCACGTCCATCCCTGCTACT
PRODH	TCCTGAGCGCGTGTATCG	AGCAAGAAGACGCGCTTGA
mGlu3	ACCACCGCGGTCAGCTT	AGAGCCCGTCACTGAATTCCT
PPP3CC	CGACCGAGCGCGTCAT	GTTAGCCGTCGGGTTGGA
RGS4	CCTGCGAACACAGTTCTTCACA	TGGCTTACCCTCTGGCAAGT
DISC1	AGCTTCTCGGAGCCATGTACA	TCCCCCTGGAGAGACTGAAA
ATF4	CATGGCGCTCTTCACGAAAT	GAGGAATGTGCTTAACTCGAAGGT
FEZ1	TGGTCCTATGAAGGGCTGAGA	CGGTCCAGCAGCTCTGTCA
Npas3	GAACTCCAAGTCCGACGAGAAG	CGGTCAGGCTCCGGATCT
NUDEL	AAGCCAGAGATTTAAGGCAAGAAC	ACTTGCGGGTCACTTCCTGTT
LIS1	CACATTGATTTCATATGTACGTTGCA	ATCTTCGGTGAACTCTTAACAATCTG
KIF5A	AGAACAACCTGGAACAGCTTACAA	CGAAGTCGTTTTTCCAATTTAGGA
KIF5B	ACATTCTGCCCAGATTGCAAA	TGTGCCCGGGTGAGTTG
IFITM3	GCCTATGCCTACTCCGTGAAGT	GCCTGGGCTCCAGTCACAT
NRG1	GGGACCAGCCATCTCATAAAGT	CGCCTCCATTCACACAGAAA
DTNBP1	AGAAGGCCCTGGAAATGGA	AACTTCTGCCGCTCCTTCAG
PDE4B	GACTTGTCACCAAAAGCGATGTC	CAGGTCATCGCCGTGTTG
TNF-α	GCCAGCCGATGGGTTGT	GCAGCCTTGTCCCTTGAAGA
β-Actin	CGATGCCCTGAGGCTCTTT	TGGATGCCACAGGATTCCA

GAD67 (glutamic acid decarboxylase 67), BDNF (brain-derived neurotrophic factor), COMT (catechol-O-methyltransferase), PRODH (proline dehydrogenase), PPP3CC (protein phosphatase 3, catalytic subunit, gamma isoform), RGS4 (regulator of G-protein signaling 4), DISC1 (disrupted-in-schizophrenia 1), ATF4 (activating transcription factor 4), FEZ1 (fasciculation and elongation protein zeta 1), NPAS3 (neuronal PAS domain protein 3), NUDEL (nuclear distribution gene E-like homolog 1), LIS1 (platelet-activating factor acetylhydrolase, isoform 1b, beta1 subunit), KIF5A (kinesin family member 5A), KIF5B (kinesin family member 5B), IFITM3 (interferon-induced transmembrane 3), NRG1 (neuregulin 1), DTNBP1 (dystrobrevin binding protein 1), and PDE4B (phosphodiesterase 4B, cAMP specific).

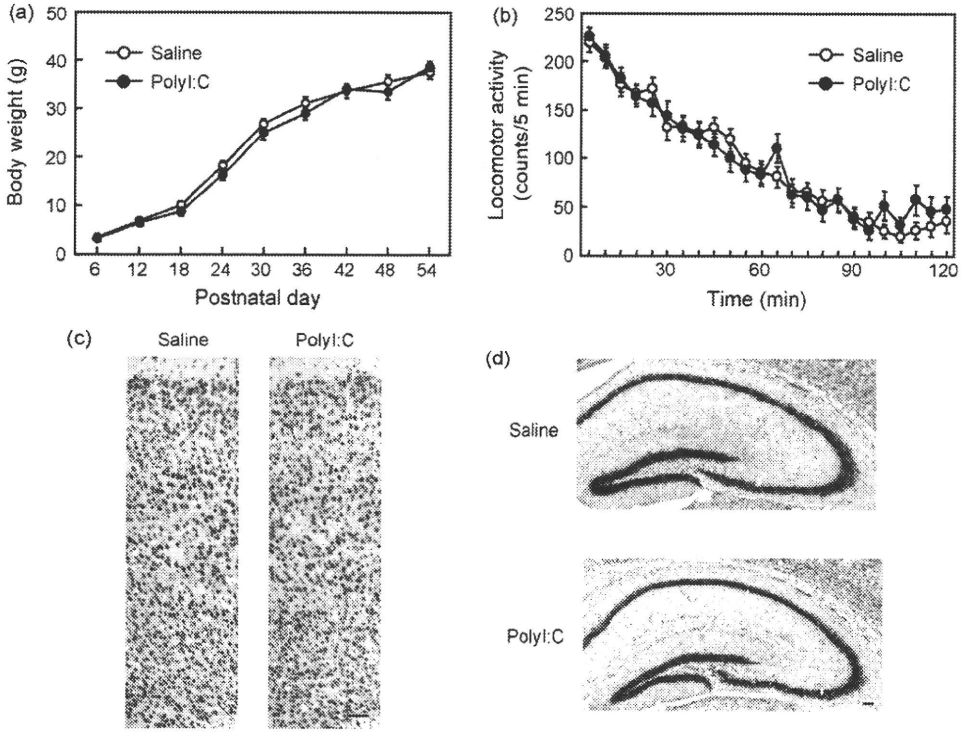

Fig. 1. General appearance and brain anatomy in polyI:C-treated mice. (a) Body weight gain after polyI:C treatment. (b) Exploratory locomotor activity under a novel environment. Gross brain anatomy in the (c) frontal cortex and (d) hippocampus. Scale bar: 200 μm. Values indicated the mean ± SE (a: $n = 11$–12, b: $n = 21$).

3.2. Effect of neonatal treatment with polyI:C on performance in the open field test

To investigate the effect of neonatal polyI:C treatment on general behavior in adult, an open field test was carried out, in which the conflict between the drive to explore a new environment and a natural aversion to illuminated open areas was used to examine both anxiety and motor activity (Wang et al., 2007). The time spent in the inner sector of the open field by the polyI:C-treated group was significantly less than by the saline-treated control group (Fig. 2a). In contrast, the time spent in the outer sector by the polyI:C-treated group was significantly more than by the control group (Fig. 2b). The distance traveled in the outer sector by the polyI:C-treated group was also significantly greater than by the control group ($p < 0.05$), although there was no difference in the inner sector between the groups (data not shown; $p > 0.05$). There was no difference between saline-treated and polyI:C-treated groups in the total distance traveled in the open field test, including inner and outer sectors (data not shown), suggesting that neonatal treatment with polyI:C does not affect motor function in adult. These results indicate that mice with neonatal polyI:C treatment avoid the inner sector and spend more time in the outer sector of the open field, suggesting increased anxiety of polyI:C-treated mice in adult.

3.3. Effect of neonatal treatment with polyI:C on performance in the novel object recognition test

During the training session, both polyI:C-treated and saline-treated control mice spent equal amounts of time exploring either of the two objects (Fig. 3b), and there was no biased exploratory preference in either group (Fig. 3a), suggesting no differences in motivation and curiosity about novel objects, and motor function between polyI:C-treated and saline-treated control mice. In the retention session, which was carried out 24 hr after the training session, the level of exploratory preference to the novel object in polyI:C-treated mice was significantly decreased compared to saline-treated control mice (Fig. 3b). Total exploration time in the retention

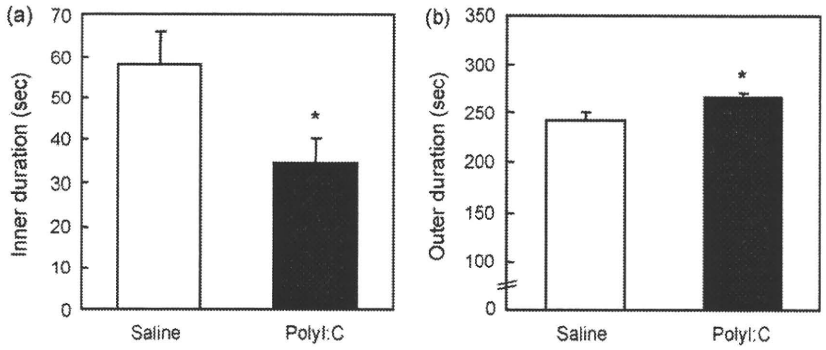

Fig. 2. Effect of neonatal polyI:C treatment on anxiety-like behavior in mice. Individual mice were allowed to explore the open field freely for 5 min. Time spent in (a) inner and (b) outer sectors. Values indicated the mean ± SE ($n = 7$–8). *$p < 0.05$ vs. saline-treated control group (two-tailed t-test).

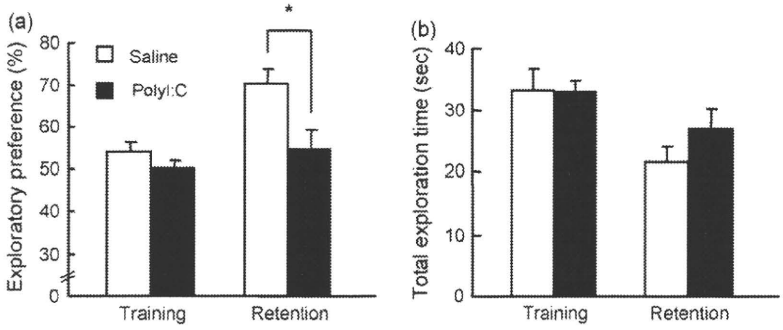

Fig. 3. Effect of neonatal polyI:C treatment on recognition memory in mice. (a) Exploratory preference. (b) Total exploration time. The retention session was carried out 24 h after the training session. Values indicated the mean ± SE ($n = 8$). *$p < 0.05$ vs. saline-treated control group (two-tailed t-test).

session did not differ between groups. These results suggest that polyI:C-treated mice have impaired recognition memory in adult.

3.4. Effect of neonatal treatment with polyI:C on performance in the PPI test

Repeated measures ANOVA of the data (73–77 dB) revealed significant effects of treatment [$F_{(1,14)} = 4.658$, $p < 0.05$] and prepulse intensity [$F_{(1,14)} = 14.644$, $p < 0.01$], but no interaction of treatment and prepulse intensity [$F_{(1,14)} = 0.061$, $p > 0.05$] (Fig. 4a). There was no difference in startle amplitude between control and polyI:C-treated groups (Fig. 4b). These results suggest that neonatal treatment with polyI:C induces PPI deficits of acoustic startle response in adulthood, suggesting an impairment of sensorimotor gating function.

3.5. Effect of neonatal treatment with polyI:C on performance in the social interaction test

In saline-treated control mice, repeated exposure to the same unfamiliar intruder mouse caused a gradual decrease in the social interaction time. In contrast, polyI:C-treated mice exhibited marked reduction of the social interaction time in trials 2–4, compared with the control group, although there was no difference in trial 1 between groups. Therefore, repeated measures ANOVA of the data (trials 2–4) revealed significant effects of treatment on social interaction [$F_{(1,10)} = 5.446$, $p < 0.05$] (Fig. 5a), but not escape [$F_{(1,10)} = 2.520$, $p = 0.146$] or aggressive behavior [$F_{(1,10)} = 0.590$, $p = 0.460$] (Fig. 5b and c), suggesting that mice receiving neonatal polyI:C treatment may possess the same level of curiosity as control mice (trial 1), but curiosity may be easily lost following repeated exposure (trials 2–4). Impaired social interaction in polyI:C-treated mice appears not to be due to the change in escape or aggressive behavior.

3.6. Effect of neonatal treatment with polyI:C on sensitivity to MK-801 or METH

Sensitivity to METH, an indirect dopaminergic agonist, or MK-801, a non-competitive N-methyl D-aspartate (NMDA) receptor blocker, is increased in individuals with schizophrenia (Lieberman et al., 1987; Duncan et al., 1999) as well as in several animal models of schizophrenia (Sakae et al., 2008). To investigate the sensitivity to METH or MK-801 in polyI:C-treated mice, locomotor activity was measured following the drug treatment. There were no differences between polyI:C-treated and saline-treated control mice in METH- or MK-801-induced hyperactivity. Repeated measures ANOVA of the data revealed no significant effects of treatment on METH-induced hyperactivity at 2 mg/kg [$F_{(1,16)} = 0.553$, $p = 0.468$], or MK-801-induced hyperactivity at 0.1 mg/kg [$F_{(1,15)} = 1.001$, $p = 0.333$] and 0.3 mg/kg [$F_{(1,16)} = 3.145$, $p = 0.0952$].

3.7. Effect of neonatal treatment with polyI:C on glutamate release in the hippocampus

Hypofunction of the glutamatergic system, such as impaired glutamate neurotransmission, has been proposed in the pathophysiology of schizophrenia (Javitt, 2004; McGuire et al., 2008). We focused on glutamate neurotransmission in the hippocampus

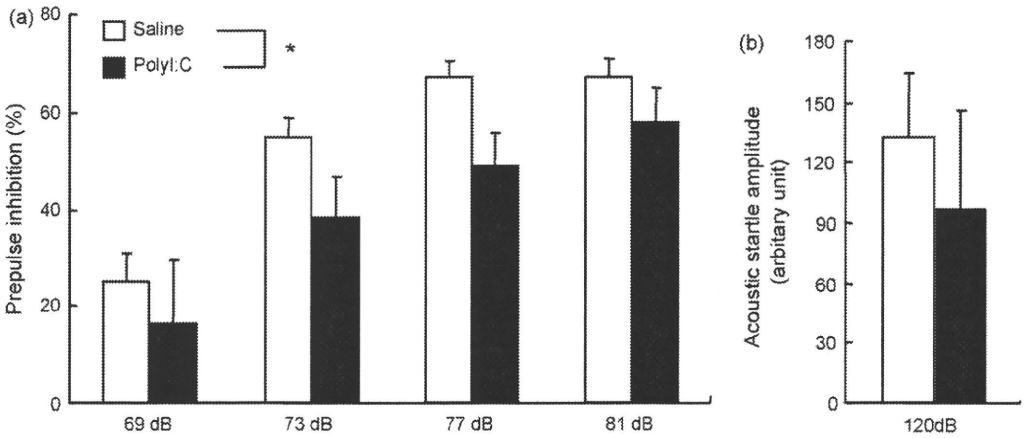

Fig. 4. Effect of neonatal polyI:C treatment on PPI in mice. (a) PPI (%) at four different prepulse intensities (69, 73, 77 and 81 dB). (b) Acoustic startle amplitude as measured in trials without prepulse. Values indicated the mean ± SE ($n = 8$). *$p < 0.05$ vs. saline-treated control group.

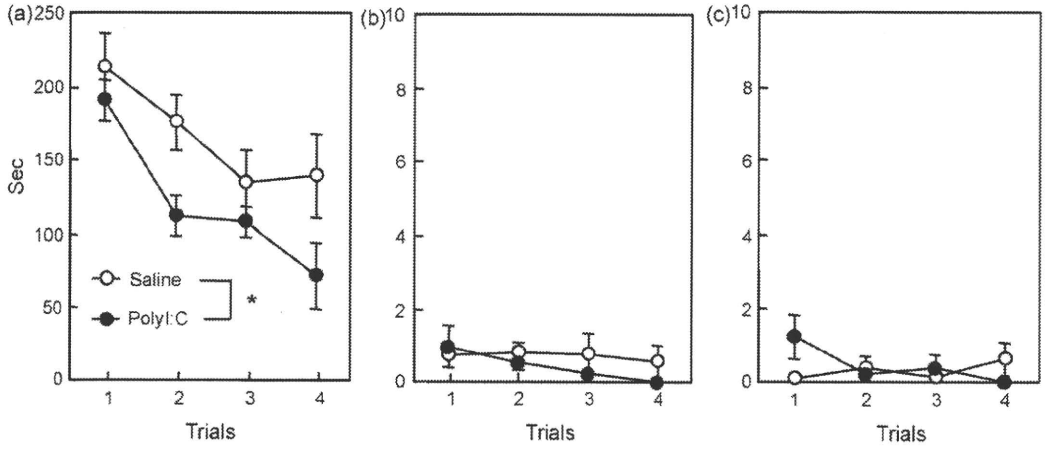

Fig. 5. Effect of neonatal polyI:C treatment on social behavior in mice. (a) Social interaction, (b) escape behavior and (c) aggressive behavior for 5 min in each trial. Each trial for 5 min was separated by a 30-min interval. Values indicated the mean ± SE ($n = 8$). $^{*}p < 0.05$ vs. saline-treated control group.

because hippocampal function is impaired in schizophrenia patients (Tamminga and Holcomb, 2005) and the hippocampus is developed and maturated during early neonatal period (Clancy et al., 2001).

PolyI:C-treated mice showed a significant increase in the basal level of glutamate in the hippocampus compared with the level in the saline-treated control group [saline-treated control ($n = 7$): 3.39 ± 0.68 pmol/10 μl, polyI:C-treated group ($n = 7$): 6.45 ± 1.01 pmol/10 μl]. Importantly, the elevated basal level of extracellular glutamate in the hippocampus of polyI:C-treated mice fell to the level in the control group by administering Ca^{2+}-free Ringer with TTX through the dialysis probe (Fig. 6a: $F_{(3,34)} = 7.134$, $p < 0.01$). The results suggest that increased basal glutamate levels in polyI:C-treated mice originally derived from neurons.

Following the measurement of basal glutamate levels, depolarization-evoked glutamate release in the hippocampus was evoked by delivering 60 mM KCl-containing Ringer through the dialysis probe for 20 min. The magnitude of high K^{+}-induced glutamate release in the hippocampus of polyI:C-treated mice was significantly decreased compared with the response in saline-treated control mice (Fig. 6b). Repeated measures ANOVA of the data revealed significant effects of group [$F_{(1,12)} = 9.697$, $p < 0.01$] and time [$F_{(3,36)} = 19.368$, $p < 0.01$], but no interaction (e.g., group × time) [$F_{(3,36)} = 2.109$, $p = 0.116$]. These results suggest that neonatal treatment with polyI:C impairs glutamatergic neurotransmission in the hippocampus in adult.

3.8. Changes in gene expression in the hippocampus 24 h after final polyI:C treatment

Scatter plots and hierarchical clustering analysis showed no obvious changes in global expression profiles between saline-treated control and polyI:C-treated mice; therefore, we analyzed changes in individual gene expression levels between polyI:C-treated and saline-treated control groups. Table 2 shows the genes whose expression ratio in the polyI:C-treated group was altered more than twofold from the level in the saline-treated control group. Student's *t*-test indicated that expression levels of some genes (*Srd5a2l*, *Creg2* and *Ptgds*) were significantly altered among the genes described in Table 2.

3.9. Effect of neonatal treatment with polyI:C on the expression of schizophrenia-related genes in the hippocampus

We analyzed the mRNA levels of schizophrenia-related genes in the hippocampus 2 and 24 h after the final treatment of polyI:C, using real-time RT-PCR. No marked differences or only transient and marginal changes were observed between polyI:C-treated and saline-treated control mice (Table 3). Among the genes examined, mRNA levels of *phosphatase 3, catalytic subunit, gamma isoform* (*PPP3CC*), *activating transcription factor 4* (*ATF4*), *kinesin family member 5B* (*KIF5B*) and *interferon-induced transmembrane protein 3* (*IFITM3*, also known as *interferon-inducible 1-8U*), were signifi-

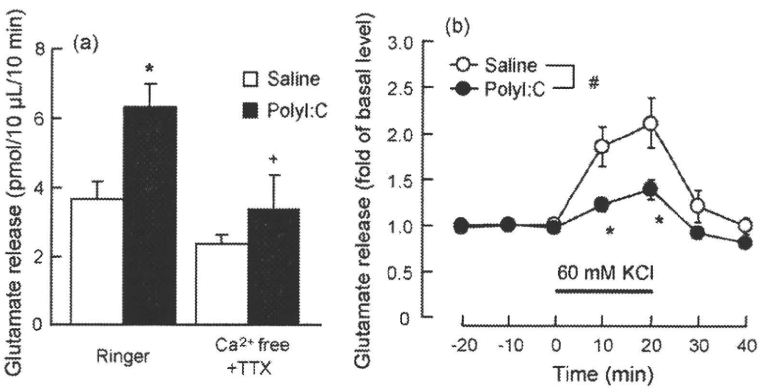

Fig. 6. Effect of neonatal polyI:C treatment on depolarization-evoked glutamate release in the hippocampus of mice. (a) Basal extracellular levels of glutamate and (b) K^{+}-induced (60 mM) glutamate release in the hippocampus of saline- or polyI:C-treated mice were determined by a microdialysis method. Each fraction was collected for 10 min. Values indicated the mean ± SE (a: $n = 6$–13, b: $n = 7$–8). (a) $^{*}p < 0.05$ vs. Ringer-perfused saline-treated control group. $^{+}p < 0.05$ vs. Ringer-perfused polyI:C-treated group. (b) $^{*}p < 0.05$ vs. saline-treated control group (two-tailed *t*-test). $^{\#}p < 0.05$ vs. saline-treated control group.

Table 2
Genes whose expression ratio in polyI:C-treated group was altered more than 2-fold from the level in saline-treated control group.

Gene title	Public ID	Gene symbol	Probe	Expression ratio	*p*-value
Histocompatibility 2, K1, K region	S70184	*H2-K1*	1427746_x_at	3.87	0.21
C-src tyrosine kinase	BB339034	*Csk*	1439744_at	3.47	0.07
Histocompatibility 2, K1, K region	L23495	*H2-K1*	1424948_x_at	3.11	0.16
Histocompatibility 2, K1, K region	BC011306	*H2-K1*	1425336_x_at	2.69	0.17
Beta-2 microglobulin	BF715219	*B2m*	1449289_a_at	2.65	0.10
P lysozyme structural	AV066625	*Lzp-s*	1436996_x_at	2.65	0.25
Beta-2 microglobulin	AI099111	*B2m*	1452428_a_at	2.6	0.05
Similar to H-2 class I histocompatibility antigen, L-D alpha chain precursor	M34962	*LOC547343*	1451683_x_at	2.49	0.16
Lymphocyte antigen 6 complex, locus A	BC002070	*Ly6a*	1417185_at	2.42	0.20
Histocompatibility 2, D region	M69068	*H2-L*	1451931_x_at	2.4	0.12
Histocompatibility 2, D region locus 1	M86502	*H2-D1*	1425545_x_at	2.35	0.12
Histocompatibility 2, D region locus 1	L36068	*H2-D1*	1451784_x_at	2.27	0.12
Eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	NM_012011	*Eif2s3y*	1417210_at	2.25	0.32
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1452077_at	2.17	0.33
Interferon induced transmembrane protein 3	BC010291	*Ifitm3*	1423754_at	2.16	0.12
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1426438_at	2.1	0.37
DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	AA210261	*Ddx3y*	1426439_at	2.03	0.44
RIKEN cDNA 1500015O10 gene	BB392676	*1500015O10Rik*	1460049_s_at	0.49	0.35
Steroid 5 alpha-reductase 2-like	BB825787	*Srd5a2l*	1439241_x_at	0.49	<0.05
Cellular repressor of E1A-stimulated genes 2	AV338277	*Creg2*	1436850_at	0.47	<0.05
Prostaglandin D2 synthase (brain)	AB006361	*Ptgds*	1423859_a_at	0.45	<0.05
Chloride intracellular channel 6	BQ176424	*Clic6*	1454866_s_at	0.44	0.41
Transthyretin	BC024702	*Ttr*	1451580_a_at	0.43	0.41
Folate receptor 1 (adult)	BG245669	*Folr1*	1450995_at	0.41	0.33
Inactive × specific transcripts	L04961	*Xist*	1427263_at	0.38	0.32
EST	BF537798	–	1438403_s_at	0.38	<0.05
Inactive × specific transcripts	L04961	*Xist*	1427262_at	0.38	0.31
Prostaglandin D2 synthase (brain)	AB006361	*Ptgds*	1423860_at	0.37	<0.05
Potassium voltage-gated channel, Isk-related subfamily, gene 2	NM_134110	*Kcne2*	1449421_a_at	0.37	0.38

Neonatal mice were sacrificed 24 h after final treatment with polyI:C for 5 days. Values indicated the mean of 3 mice.

cantly increased 2 h after the final treatment of polyI:C, compared to the saline-treated control group. At 24 h after polyI:C treatment, only the *IFITM3* mRNA level was significantly increased in the polyI:C-treated group compared to the saline-treated control group. In addition, the gene expression of tumor necrosis factor-α (TNF-α) was markedly increased in the polyI:C-treated group, suggesting a proinflammatory response in the hippocampus following polyI:C treatment.

Table 3
Expression levels of schizophrenia-related genes in the hippocampus after the final polyI:C treatment in neonatal mice.

	2 h ΔCt				24 h ΔCt			
	Saline	Poly I:C		*p*-value	Saline	Poly I:C		*p*-value
GAD67	4.198	4.108		0.613	4.096	3.975		0.466
Reelin	4.864	4.834		0.792	5.415	5.465		0.787
BDNF	5.024	5.130		0.709	5.308	5.454		0.366
COMT	5.675	5.628		0.597	5.086	5.143		0.588
14-3-3 ε	1.851	1.790		0.491	2.483	2.345		0.427
PRODH	12.674	12.348		0.181	12.849	12.930		0.627
mGlu3	0.090	0.158		0.952	6.893	6.890		0.983
PPP3CC	7.522	7.311	↑	< 0.05	7.982	8.053		0.611
RGS4	6.557	6.282		0.314	5.519	5.679		0.288
DISC1	13.511	13.164		0.073	12.124	12.135		0.937
ATF4	4.708	4.473	↑	< 0.05	5.071	5.059		0.942
FEZ1	3.820	3.624		0.117	3.023	3.076		0.696
Npas3	7.168	6.984		0.213	6.845	6.845		1.000
NUDEL	5.501	5.399		0.262	6.023	6.035		0.938
LIS1	2.048	1.965		0.316	1.313	1.454		0.342
KIF5A	3.644	3.572		0.688	4.338	4.510		0.353
KIF5B	5.506	5.306	↑	< 0.05	6.635	6.691		0.774
IFITM3	5.703	5.100	↑	< 0.01	6.784	5.893	↑	<0.001
NRG1	8.058	8.010		0.816	8.260	8.274		0.878
DTNBP1	5.691	5.588		0.211	4.571	4.612		0.747
PDE4B	7.070	6.948		0.306	6.834	7.003		0.157
TNF-α	13.507	13.048	↑	< 0.05	14.282	14.291		0.951

Neonatal mice were sacrificed 2 and 24 h after final treatment with polyI:C. Values indicate the mean of 8 mice. ↑: gene expression was significantly increased. ΔCt: the *Ct* (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. ΔCt value is calculated by subtracting the number of cycles of the target gene from that of β-actin.

4. Discussion

We demonstrated in the present study that neonatal polyI:C treatment at 5 mg/kg for 5 days (PD 2–6) in ICR mice resulted in emotional and cognitive impairment, which was associated with the dysfunction of hippocampal glutamatergic neurotransmission in adulthood. Table 4 summarizes the differences of behavioral changes between prenatal and postnatal polyI:C injection models. Impairments of social interaction, memory and prepulse inhibition were evident in both prenatal and postnatal polyI:C injection models. PolyI:C treatment in pregnant dams caused an increase in sensitivity to METH and an impairment of latent inhibition in offspring, while neonatal treatment of polyI:C had no effect on these behaviors. The results suggest that there may be some commonality but also difference between the effects of neonatal and prenatal polyI:C treatment on phenotypic changes in adulthood (Meyer et al., 2005, 2008; Ozawa et al., 2006; Smith et al., 2007; Zuckerman et al., 2003; Zuckerman and Weiner, 2005).

Previous studies demonstrated that prenatal polyI:C treatment-induced cytokines, such as TNF-α and interleukin 1β, 6 and 10, in the serum of both mother and fetus, and polyI:C induced an increase in BBB permeability (Meyer et al., 2006; Chen et al., 2007). In the present study, we found that neonatal polyI:C treatment in mice significantly increased *TNF-α* mRNA levels in the hippocampus 2 h after final polyI:C treatment, compared with the saline-treated control (Table 3). Furthermore, *IFITM3* mRNA levels induced by type I interferon (Liu et al., 2002) were significantly increased, at least up to 24 h after polyI:C treatment. These findings suggested that repeated polyI:C treatment in neonatal

Table 4
Comparison of behavioral changes between prenatal and postnatal polyI:C injection models.

	Present study	Ozawa et al. (2006)	Meyer et al. (2005, 2008)	Smith et al. (2007)	Zuckerman et al. (2003) and Zuckerman and Weiner (2005)
Animal	ICR mouse	BALB/c mouse	C57BL6/J mouse	C57BL6/J mouse	Wistar rat
Time of polyI:C treatment	PD 2–6	GD12–GD17	GD9	GD12.5	GD15
Dose of polyI:C	5 mg/kg	5 mg/kg	5 mg/kg	20 mg/kg	4 mg/kg
Locomotor activity	=	=	=	↓	=
Methamphetamine-induced hyperactivity	=	↑	↑	n/a	↑
MK-801-induced hyperactivity	=	n/a	=	n/a	↑
Social interaction	↓	n/a	n/a	↓	n/a
Anxiety-like behavior in open-field	↑	↓	↑	↑	n/a
Object recognition memory	↓	↓	n/a	n/a	n/a
Prepulse inhibition	↓	↓	↓	↓	n/a
Latent inhibition	=	n/a	↓	↓	↓

PD: postnatal day, GD: gestation day, ↑: higher than vehicle-treated control, ↓: lower than vehicle-treated control, =: no difference, and n/a: not reported.

mice causes an inflammatory response in the hippocampus, while there are some differences in cytokine responses between gestational and neonatal immunochallenge induced by polyI:C.

It is proposed that hypofunction of the glutamatergic system plays a crucial role in the disease (Coyle and Tsai, 2004; Li et al., 2007). In the present study the animal model of early postnatal immune activation with polyI:C treatment showed an increased basal extracellular glutamate level in the hippocampus with an impairment of high K^+-induced glutamate release in the hippocampus in adulthood. These results suggest that activity-dependent changes in extracellular glutamate levels in the hippocampus are suppressed, leading to a lower signal/noise ratio in glutamatergic neurotransmission in polyI:C-treated mice. Such dysfunction of glutamatergic neurotransmission in the hippocampus may be associated with cognitive deficits in polyI:C-treated mice.

Real-time RT-PCR 2 h after the final treatment indicated that *PPP3CC*, *ATF4*, *KIF5B* and *IFITM3* mRNA levels in the hippocampus were significantly increased, but the changes were transient and disappeared 24 h later (Table 3). The only exception was the gene encoding *IFITM3* gene. Levels of *IFITM3* mRNA in the hippocampus were markedly increased at least from 2 to 24 h after the final polyI:C injection. Interestingly, it has been reported that mRNA levels of *IFITM3* are markedly increased in the brains of patients with schizophrenia (Arion et al., 2007), bipolar disorder (Iwamoto et al., 2004), and autism (Garbett et al., 2008), although the pathophysiological role of IFITM3 in mental disorders remains to be determined. Because the present study was conducted in ICR mice, an outbred stock, there is a considerable genetic heterogeneity (Chia et al., 2005). Accordingly, we repeated the experiment in C57BL/6 mice, and confirmed that neonatal polyI:C treatment significantly increased *IFITM3* mRNA levels in the hippocampus (data not shown). Thus, further studies to clarify the effect of *IFITM3* expression on brain development are warranted.

The neonatal immune activation model with polyI:C in mice exhibited emotional and cognitive impairments, but some behavioral changes, such as METH-induced hyperactivity and latent inhibition, were not evident in the neonatal polyI:C treatment model. Because schizophrenia is considered to develop as a result of the interactions of several genetic and environmental factors (Tsuang, 2000; Sawa et al., 2004), partial and subtle manifestation of schizophrenia-related behavioral changes in mice caused by a single environmental factor (e.g., viral infection during neurodevelopment) supports this hypothesis. Additional genetic factors and/or other environmental insults to prenatal viral infection may be required for full manifestation of the clinical symptoms and pathophysiology of schizophrenia in rodent models. Thus, the present model of neonatal immune activation has advantages for testing gene–environmental interactions for schizophrenia. Testing the effect of neonatal polyI:C treatment in mutant mice with susceptibility genes for schizophrenia would be intriguing for the development of an animal model of schizophrenia with gene–environmental interactions.

There is a disadvantage in the neonatal polyI:C treatment model: neonatal mice are directly exposed to immune activation with polyI:C, while the neuroimmunological factors involved in the human epidemiological association in schizophrenia may be due to complex mother-to-fetus immunological insults taking place in the maternal host, placenta and/or amnion following maternal virus infection. Accordingly, much attention should be paid when interpreting the behavioral and neurochemical abnormalities manifested in neonatal polyI:C treatment model, in relation to the pathogenesis/pathophysiology in schizophrenia.

Disclosure/conflict of interest

The authors declare that there are no conflicts of interest in the publication of the present work.

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