Title: Pharmacological and proteomic analyses of neonatal polyI:C-treated adult mice

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Running title: Antipsychotics and neonatal polyI:C mice

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Abstract

Perinatal virus infection is an environmental risk factor for neurodevelopmental disorders such as schizophrenia. We previously demonstrated that neonatal treatment with a viral mimetic, polyriboinosinic-polyribocytidilic acid (polyI:C), in mice leads to emotional and cognitive deficits in adolescence. Here, we investigated the effects of antipsychotics on polyI:C-induced behavioral abnormalities. We also performed a proteomic analysis in the hippocampus of polyI:C-treated adult mice using two-dimensional electrophoresis to understand the changes in protein expression following neonatal immune activation. Neonatal mice were subcutaneously injected with polyI:C for 5 days (postnatal day 2-6). At 10 weeks, sensorimotor gating, emotional and cognitive function were analyzed in behavioral tests. Clozapine improved PPI deficit and emotional and cognitive dysfunction in polyI:C-treated mice. However, haloperidol improved only PPI deficit. Proteomic analysis revealed that two candidate proteins were obtained in the hippocampus of polyI:C-treated mice, including aldehyde dehydrogenase family 1 member L1 (ALDH1L1) and collapsin response mediator protein 5 (CRMP5). These data suggest that the neonatal polyI:C-treated mouse model may be useful for evaluating antipsychotic activity of compounds. Moreover, changes in the protein expression of ALDH1L1 and CRMP5 support our previous findings that astrocyte-neuron interaction plays a role in the pathophysiology of neurodevelopmental disorders induced by neonatal immune activation.

Keywords: animal model, antipsychotic drugs, behavior, viral infection, polyI:C, proteomics, schizophrenia

Highlights

- Neonatal polyI:C treatment caused behavioral abnormalities in adulthood.
- Antipsychotics ameliorated behavioral abnormalities in neonatal polyI:C-treated mice.
- Proteomic analysis revealed the changes in ALDH1L1 and CRMP5.

Introduction

Brain abnormalities in the brain developmental period cause various neuropsychiatric disorders, including schizophrenia (Schmidt-Kastner et al., 2012) and autism spectrum disorders (ASD) (Grabrucker, 2012), and the pathoetiology of these neuropsychiatric disorders involves both genetic and environmental factors (Caspi and Moffitt, 2006; Lang et al., 2007).

Virus infection is an environmental factor during pregnancy that increases the risk of psychiatric disorders (Benros et al., 2016). Epidemiological studies suggest that brain developmental abnormalities induced by viral-mediated excessive immune reactions during the perinatal period may be involved in the development of neuropsychiatric disorders in adolescence (Brown and Derkits, 2010). These findings have encouraged the development of animal models to investigate the influence of environmental factors (Mouri et al., 2013).

A double-stranded RNA analog polyriboinosinic-polyribocytidilic acid (polyI:C) activates toll-like receptor 3 (TLR3) and induces an immune reaction similar to virus infection in a robust manner (Alexopoulou et al., 2001). In rodents, offspring born from polyI:C-treated pregnant mice exhibit schizophrenia-like behavioral and neuropathological abnormalities in the adult brain (Meyer et al., 2006). Neonatal polyI:C-treated mice have behavioral abnormalities including increased anxiety-like behavior, impairment of object cognitive memory, social behavior, and sensorimotor gating in adulthood (Ibi et al., 2009). In addition, TLR3 was activated in polyI:C-treated cultured neurons and neonatal polyI:C-treated mice, which impairs neural development and is observed in schizophrenia

64 (Chen et al., 2017). Postnatal lipopolysaccharide (LPS)-treated adult male mice have depressive,
65 anxiety-like behavior deficits, and postnatal LPS-treated adult female mice exhibited PPI deficits that
66 resemble ASD (Custodio et al., 2018).

67 The behavioral abnormalities in neonatal polyI:C-treated mice are partly improved by
68 administration of nicotine or D-serine (Nagai et al., 2012; Yu et al., 2010). However, it is unclear
69 whether antipsychotics ameliorate behavioral abnormalities in neonatal polyI:C-treated adult mice.

70 Transcriptome and proteome analyses reveal biomarkers and novel mechanisms of mental
71 disorders. We previously identified interferon-induced transmembrane protein 3 (IFITM3) by
72 microarray analysis as a candidate gene that increases after neonatal polyI:C treatment (Ibi et al., 2009).
73 The induction of IFITM3 expression in astrocytes from neonatal polyI:C treatment impairs endocytosis
74 and has non-cell autonomous effects that affect subsequent neurodevelopment (Ibi et al., 2013). We
75 also identified astrocyte-derived humoral factors that affect neuronal development by two-dimensional
76 fluorescence difference gel electrophoresis (2D-DIGE). PolyI:C-treated cultured astrocytes have an
77 increased extracellular level of metalloproteinase-3 and follistatin-related protein 1 (Yamada et al.,
78 2014; 2018). Although neonatal polyI:C treatment changes gene and protein expression in the neonatal
79 brain, the molecular dynamics in adulthood remain unclear.

80 Here, we examined the effect of antipsychotics on behavioral abnormalities in neonatal
81 polyI:C-treated adult mice. We also analyzed changes in protein expression in the hippocampus of
82 neonatal polyI:C-treated adult mice.

83

Materials and methods

Animals

Pregnant ICR mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) and were 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. 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 (NIH), 8th edition, 2011.

PolyI:C and antipsychotics treatment

PolyI:C and antipsychotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). PolyI:C was dissolved in pyrogen-free saline. Antipsychotics were suspended in saline containing 1% carboxymethylcellulose sodium salt (CMC). PolyI:C treatment was performed as previously described (Ibi et al., 2009). Briefly, all litters were randomly divided into saline- and polyI:C-treated groups. From PD 2 to 6, mice were injected subcutaneously (s.c.) daily with either pyrogen-free saline (control group) or polyI:C at a dose of 5 mg/kg (polyI:C group). Animals were weaned at PD 21, divided by

gender at PD 28, and group-housed post-weaning until use for behavioral and neurochemical analyses at 10 weeks. Haloperidol (0.3 mg/kg) or clozapine (5 mg/kg) was orally (p.o.) administered to mice through an oral gavage needle 60 min before the behavioral test. The dose of each drug was selected according to previous pharmacological reports with minor modifications (Maeda et al., 2007; Sun and Lau, 2000; Yang et al., 2011).

Behavioral analyses

Behavioral analyses were started at 10-12 weeks of age in the following order: PPI (Day 1), open field (Day 2), novel object recognition (Day 3-7), and social interaction test (Day 8-10). Two or three independent experiments were performed for each behavioral experiment, and each mouse was used sequentially for the 4 behavioral tests as described above.

Prepulse inhibition (PPI) test

The PPI test was performed as described previously (Takahashi et al., 2007). After the animals were placed in the chamber (San Diego Instruments, San Diego, California), they were allowed to habituate for 10 min during which they were subjected to 65 dB background white noise. Animals then received 10 startle trials, 10 no-stimulus trials, and 40 PPI trials. The intertrial interval was between 10 and 20 sec, and the total session lasted 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 msec. PPI trials consisted of a prepulse (20 msec burst of white noise at 69, 73,

77, or 81 dB intensity) followed, 100 msec later, by the startle stimulus (120 dB, 40 msec white noise). Each of the four prepulse trials (69, 73, 77, or 81 dB) was performed 10 times. Sixty different trials were presented pseudo-randomly, ensuring that each trial was performed 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured for 100 msec after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer, which calculated the maximal response over the 100 msec. Basal startle amplitude was determined as the mean amplitude of the 10 startle trials. PPI was calculated according to the following formula: $100 \times [1-(PPx/P120)]$ %, in which PPx is the mean amplitude of the 10 PPI trials (PP69, PP73, PP75, or PP80), and P120 is the basal startle amplitude.

Open field test

Mice were placed at the center of an open field (diameter, 60 cm; height, 35 cm) and allowed to explore it for 5 min 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 as well as the travel distance ratio of the inner distance vs. total travel distance in the open field.

Novel object recognition test

A novel object recognition test was performed as described previously (Nagai et al., 2007). Mice were individually habituated to an open box (30 × 30 × 35 (height) cm) for 3 days. During the training session, two novel objects were placed in the open field and animals were allowed to explore for 10 min. The objects were a golf ball, wooden cylinder, and square pyramid, which were different in shape and color but similar in size. An animal was considered to be exploring the object when its head was touching, facing or sniffing the object. The time spent exploring each object was recorded by video camera and analyzed in a double-blind manner. During retention sessions, animals were placed back into the same box 24 h after training, one of the familiar objects used during training was replaced by a novel object, and mice were allowed to explore the two objects freely for 5 min. The preference index in the retention session, which was the ratio of the amount of time spent exploring the novel object to 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.

Social interaction test

We used an experimental paradigm previously described (Ibi et al., 2009; Tremolizzo et al., 2005) to measure social behavior (e.g., social interaction, aggression, and escape behavior). PolyI:C-treated or control mice were individually housed in cages (29 × 18 × 12 cm) for 2 days before the trial.

We used 10-12-week-old male ICR mice had not shown aggressive behavior as intruders. In the first trial (5 min duration), an intruder mouse was introduced into the resident's home cage. 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.

Sample preparation

Brains were removed rapidly, and the hippocampus was dissected on an ice-cold plate. Each tissue was frozen quickly and stored in a freezer at -80°C until assayed. Protein was extracted by ProteoExtract subcellular proteome extraction kit (Merck, Darmstadt, Germany). The homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C. After centrifugation, protein concentration in the supernatant was determined using the Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was used in the following experiment. For two-dimensional electrophoresis, samples were desalted by the 2D clean-up kit (GE Healthcare, Little Chalfont, Buckinghamshire, England) before measurement of protein concentration.

Fluorescence two-dimensional difference gel electrophoresis (2D-DIGE)

Proteins (25 μg) were labeled, as specified by the manufacturer, with fluorescent dyes

(CyDyes Cy3 and Cy5) specifically developed for the 2D-DIGE system (GE Healthcare). Labeled protein samples were diluted with an equal volume of a solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM dithiothreitol (DTT), and 1% pharmalyte (pH 3-10) before loading on the gel. First dimension isoelectric focusing was performed with immobilized pH gradient (IPG) gels (18-cm Immobiline dry strips pH 3-10 NL, GE Healthcare). IPG strips were rehydrated for 12 h in 7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT, 1% pharmalyte pH 3-10, and 0.001% bromophenol blue. Labeled samples were loaded at the rehydration of IPG strips, and isoelectric focusing was performed for a total of 80,536 volt-hours using an Ettan IPGphor Isoelectric Focusing System (GE Healthcare). After focusing, the gel strips were equilibrated for a 25-min equilibration step in 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, and 0.001% bromophenol blue, and 65 mM DTT was added for the first step. A second 10-min equilibration step was performed in the same solution, but containing 4% iodoacetoamide instead of DTT. SDS-PAGE was performed with 12% polyacrylamide gels (20 × 20 × 0.1 cm slab) at 25 mA/gel. The gel images were acquired using a Typhoon 8610 scanner (GE Healthcare) and analyzed with PDQuest software (Bio-Rad laboratories). For subsequent mass spectrometry, the proteins were stained with silver (Blum et al., 1987), and spots were excised manually.

Protein identification

In-gel digestion was performed using modified protocols (Shevchenko et al., 1996). Excised

198 spots were digested with trypsin (Promega, Madison, WI, USA), and the resulting peptides were
199 analyzed using liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis. Each peptide
200 was dried after in-gel digestion, reconstituted in reverse phase buffer, and transferred to an HTC PAL
201 (CTC Analytics AG, Industriestrasse, Zwingen, Switzerland). MAGIC 2002 (Michrom Bioresources,
202 Auburn, CA, USA: Magic C18 (0.1×50 mm, Michrom Bioresources) system was used for the high-
203 performance liquid chromatography. Chromatographic separation was accomplished by loading the
204 peptide onto a Nano-Column (AMR Inc. Tokyo, Japan). MS analysis was performed using Pico View
205 nanospray ion source (New Objective, Inc. Woburn, MA, USA) mounted on an LCQ (Thermo Electron,
206 San Jose, CA, USA) ion trap mass spectrometer (triple play scanning sequence data dependent mode;
207 mass range 300 to 20000; high voltage 2.50 kV). The raw data were converted to data format, and data
208 analysis to identify proteins was performed automatically using the Mascot sequence database-
209 searching software (MatrixScience, London, UK).
210
211 **Immunoblotting**
212 Equivalent amounts of protein (20 μg) were separated by SDS-PAGE and transferred to a
213 PVDF membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 3% BSA in
214 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl. After blocking, a rat anti-collapsin response mediator
215 protein 5 (CRMP5, 1:2000, Abcam, Cambridge, United Kingdom) or a mouse anti-aldehyde
216 dehydrogenase family 1 member L1 (ALDH1L1, 1:2000, Abnova, Taipei, Taiwan) was added and

incubated overnight at 4°C. The membranes were then washed with washing buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween 20]. After incubation with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated IgG for 60 min, the membranes were washed with washing buffer. The immune complex was detected using an enhanced chemiluminescence kit (GE Healthcare), and protein images were captured using Lumivision Pro HS II (AISIN, Kariya, Japan).

Statistical analysis

Statistical significance was determined using a Student's t-test or one-way or two-way analysis of variance (ANOVA) with or without repeated measures followed by Newman-Keuls multiple comparison test when F ratios were significant ($p<0.05$). Two-way ANOVA with repeated measures was used for PPI experiments (Fig. 1A). A Student's t-test was used to analyze the effect of polyI:C (Fig. 2-4 and 6). One-way ANOVA was used to analyze the effects of antipsychotics in saline or polyI:C-treated groups (Fig. 1B, 2-4).

Results

Effects of antipsychotics on PPI deficits of startle response in polyI:C-treated mice

The PPI test was performed at the age of 10-12 weeks to assess the sensorimotor gating function in polyI:C-treated mice. Two-way ANOVA with repeated measures revealed significant effects of prepulse intensity and polyI:C treatment (prepulse, $F(3,42)=19.70$, $p<0.01$; polyI:C, $F(1,14)=40.72$, $p<0.01$; prepulse × polyI:C, $F(3,42)=13.78$, Fig. 1A). PolyI:C-treated mice showed a marked impairment of PPI compared with that of the control group at all prepulse intensities (69, 73, 77, and 81 dB) ($p<0.01$, Fig. 1A). Two-way ANOVA with repeated measures revealed significant effects of antipsychotics in polyI:C-treated groups (antipsychotics, $F(2,21)=4.57$, $p<0.05$; prepulse, $F(3,63)=8.42$, $p<0.01$; antipsychotics × prepulse, $F(6,63)=1.85$, $p=0.10$, Fig. 1A) and the control group (antipsychotics, $F(2,21)=5.37$, $p<0.05$; prepulse, $F(3,63)=109.41$, $p<0.01$; antipsychotics × prepulse, $F(6,63)=1.64$, $p=0.15$, Fig. 1A). Compared with that in vehicle-treated mice, antipsychotics significantly improved PPI deficits in polyI:C-treated mice ($p<0.01$ and $p<0.05$, respectively, Fig. 1A), whereas these drugs reduced PPI in the control group ($p<0.05$, Fig. 1A). Single treatment with antipsychotics had no effects on the acoustic startle amplitude in the polyI:C- or saline-treated groups (Fig. 1B).

Effects of antipsychotics on emotional deficits in polyI:C-treated mice in an open field test

To investigate the effects of antipsychotics on emotional deficits in polyI:C-treated adult mice,

an open field test was performed at the age of 10-12 weeks 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. In control mice, the time spent in the inner sector (41.9±2.5 sec) was significantly less than that in the outer sector (258.1±2.5 sec) ($p<0.01$ by Student's t-test, Fig. 2), indicating a natural aversion to illuminated open areas under our experimental conditions. The time spent in the inner sector was significantly decreased while the time spent in the outer sector was significantly increased in polyI:C-treated mice compared with that of control mice ($p<0.01$ by Student's t-test, Fig. 2A and B). One-way ANOVA analysis revealed significant effects of antipsychotics on the time spent in inner sectors ($F(2,21)=4.10$, $p<0.05$, Fig. 2A), outer sectors ($F(2,21)=4.12$, $p<0.05$, Fig. 2B), and total distance traveled ($F(2,21)=5.12$, $p<0.05$, Fig. 2C) in the polyI:C-treated group. Post-hoc analysis revealed that clozapine, but not haloperidol, significantly increased the time spent in the inner sector ($p<0.01$, Fig. 2A), and decreased the time in the outer sector in polyI:C-treated mice ($p<0.01$, Fig. 2B) without alteration of total distance traveled (Fig. 2C). Haloperidol had no effect on the time spent in the inner and outer sector (Fig. 2A and B), but resulted in a slight but significant reduction of the total distance traveled in polyI:C-treated mice ($p<0.05$, Fig. 2C). Antipsychotics themselves had no effect on performance in the control group (Fig. 2).

Effects of antipsychotics on deficits of object recognition memory in polyI:C-treated mice

To examine the effect of antipsychotics on neonatal polyI:C treatment-induced memory

impairment, a novel object recognition test was performed at the age of 10−12 weeks. During the training session, there was no biased exploratory index in either group ($p=0.85$ by Student's t-test, Fig. 3A), and both groups of mice spent equal amounts of time exploring one of two objects ($p=0.34$ by Student's t-test, Fig. 3B), which suggested no differences in motivation and curiosity about novel objects and in motor function between the two groups. A retention session was performed 24 h after the training session. The level of exploratory index to the novel object was significantly decreased in polyI:C-treated mice compared with that of control mice ($p<0.01$ by Student's t-test, Fig. 3C). Total exploration time in the retention session did not differ between the two groups ($p=0.25$ by Student's t-test, Fig. 3D), suggesting that polyI:C-treated mice have impaired recognition memory in adulthood. In polyI:C-treated mice, antipsychotics had no effect on the exploratory index in the training session when the drugs were administered 60 min before the training session ($F(2,19)=0.17$, $p=0.85$, Fig. 3A). Haloperidol, but not clozapine, produced a slight but significant reduction of total exploration time in the training session ($p<0.05$, Fig. 3B). One-way ANOVA analysis revealed significant effects of antipsychotics on the level of exploratory index in the retention session in polyI:C-treated mice ($F(2,19)=11.00$, $p<0.01$, Fig. 3C). Treatment with clozapine significantly improved cognitive impairment in polyI:C-treated mice during the retention session ($p<0.01$, Fig. 3C). In contrast to clozapine, haloperidol had no effect on the level of exploratory index to the novel object in the retention session in polyI:C-treated mice ($p>0.05$, Fig. 3C). The total exploration time in polyI:C-treated mice was not affected by antipsychotics in the retention session (antipsychotics, $F(2,19)=2.72$, $p=0.10$ by

one-way ANOVA analysis, Fig. 3D). In control mice, antipsychotics had no effect on the level of exploratory index (training session, $F(2,19)=0.04$, $p=0.95$ by one-way ANOVA analysis, Fig. 3A; retention session, $F(2,19)=2.33$, $p=0.12$ by one-way ANOVA analysis, Fig. 3C), or total exploration time (training session, $F(2,19)=1.04$, $p=0.37$ by one-way ANOVA analysis, Fig. 3B; retention session, $F(2,19)=0.80$, $p=0.46$ by one-way ANOVA analysis, Fig. 3D).

Effects of antipsychotics on deficits of social behavior in polyI:C-treated mice

Social interactions in polyI:C-treated mice were investigated at the age of 10−12 weeks. In control mice, repeated exposure to an unfamiliar intruder mouse (4 trials) caused a gradual decrease in social interaction time. The polyI:C-treated mice exhibited a marked reduction in the social interaction time in all 4 trials compared with that of control mice (polyI:C, $F(1,14)=15.37$, $p<0.01$; trial, $F(3,42)=104.48$, $p<0.01$; polyI:C × trial, $F(3,42)=0.23$, $p=0.87$ by repeated two-way ANOVA, data not shown). Therefore, the total social interaction time was evaluated in the following analysis: One-way ANOVA revealed a significant effect of antipsychotics on social interaction ($F(2,21)=4.30$, $p<0.05$, Fig. 4A) in the polyI:C-treated group. Treatment with clozapine significantly increased the total social interaction time in polyI:C-treated mice ($p<0.05$, Fig. 4A). The behavioral components of social interaction improved by clozapine included inspection, anogenital sniffing, and other social body contacts except aggressive behavior in polyI:C-treated mice. Treatment with haloperidol failed to improve social interaction deficits in polyI:C-treated mice ($p>0.05$, Fig. 4A). In control mice,

antipsychotics had no effect on social interaction ($F(2,21)=1.95$, $p=0.17$ by one-way ANOVA, Fig. 4A). Antipsychotics had no significant effect on escape (saline-treated groups, $F(2,21)=0.58$, $p=0.57$; polyI:C-treated groups, $F(2,21)=1.25$, $p=0.31$ by one-way ANOVA, Fig. 4B) or aggressive behavior (saline-treated groups, $F(2,21)=1.94$, $p=0.07$; polyI:C-treated groups, $F(2,21)=0.83$, $p=0.45$ by one-way ANOVA, Fig. 4C) in polyI:C-treated and control mice.

2D-DIGE analysis in the hippocampus of polyI:C-treated mice

To explore candidate molecules responsible for the abnormal behaviors in polyI:C-treated adult mice, we applied a proteomic approach using 2D-DIGE analysis. Fig. 5A shows the expression levels of proteins extracted from the hippocampus of polyI:C- and saline-treated mice. We used automatic image matching and spot identification using PDQuest software to identify 1065.3±0.2 and 1065.8±0.2 unique spots in control and polyI:C-treated mice, respectively. No differences were observed between the two groups in the total number of protein spots, and more than 65% of all spots were matched to the reference gel, which allowed excellent comparison between the two groups. Significant changes in the relative abundance of two protein spots (SSP3707 and SSP7512) were identified in polyI:C-treated mice (Fig. 5B, Table 1). One spot (SSP3707) exhibited a 1.6-fold increase in polyI:C-treated mice ($p<0.05$, Fig. 5B, Table 1). Another protein spot (SSP7512) decreased more than 0.6-fold in polyI:C-treated mice ($p<0.01$, Fig. 5B, Table 1). These two proteins were identified using LC-MS/MS (Table 1) as ALDH1L1 and CRMP5, and there were no gross variations in the

326 theoretical MW/pI of the proteins.

327 Immunoblot analysis also revealed changes in the expression levels of ALDH1L1 and
328 CRMP5 in the hippocampus of polyI:C-treated mice (Fig. 6). The expression level of ALDH1L1 was
329 significantly increased in polyI:C-treated mice ($p<0.05$, Fig. 6A), whereas that of CRMP5 was
330 decreased in polyI:C-treated mice ($p<0.01$, Fig. 6B).

331

Discussion

In this study, neonatal polyI:C-treated mice showed deficits of sensorimotor gating, anxiety and motor activity, objective cognitive memory, and social behavior. These behavioral abnormalities are consistent with our previous reports (Ibi et al., 2009; Yu et al., 2010) and sensitive to antipsychotics, especially clozapine. Thus, this animal model has some predictive validity and enables to evaluate antipsychotic activity of compounds.

Haloperidol improves positive symptoms. It acts mainly by blocking the dopamine D_2 receptor. However, it is ineffective for negative symptoms and causes side effects such as extrapyramidal symptoms (Ananth et al., 2001). Clozapine is used for the treatment of refractory schizophrenia (De Oliveira and Juruena, 2006). Although a detailed mechanism of action for clozapine is still unknown, selective suppression of the mesolimbic dopamine nervous system independent of the dopamine D_2 receptor blocking effect is conceivable (Beloate et al., 2016). Clozapine improves both positive and negative symptoms, possibly by binding receptors other than the dopamine D_2 receptor such as the serotonin 5-HT_{2A} and dopamine D_4 receptor (Arnt and Skarsfeldt, 1998).

The effects of antipsychotics have been investigated using various model mice. We have also evaluated the effects of antipsychotics in neonatal polyI:C-administered transgenic mice with a dominant-negative form of the disrupted-in-schizophrenia 1 gene (DN-DISC1), which is a gene-environment interaction model (Ibi et al., 2010; Nagai et al., 2011). Cognitive dysfunction of neonatal polyI:C-administered DN-DISC1 mice is restored by treatment with clozapine, but not haloperidol. In

this study, impairment of PPI in polyI:C-treated mice was improved by haloperidol and clozapine. It has been reported that clozapine reverses PPI deficit induced by serotonin 5-HT_{2A} receptor agonist 2,5-dimethoxy-4-iodoamphetamine (de Oliveira et al., 2017). Amphetamine fails to induce PPI deficit in dopamine D_2 receptor knockout mice whereas D_3 and D_4 receptor knockout mice show PPI deficit by amphetamine treatment (Ralph et al., 1999). These findings suggest that ameliorating effects of clozapine and haloperidol on PPI deficits in polyI:C-treated mice may be mediated through the blockade of serotonin 5-HT_{2A} and dopamine D_2 receptors, respectively. Haloperidol and clozapine at the dose used in the present study reduced PPI by itself in control mice. Consistent with this result, clozapine is reported to impair PPI performance (Le Pen and Moreau, 2002).

Clozapine, but not haloperidol, improved deficits of anxiety and recognition memory. These results are consistent with clinical findings that clozapine is superior to haloperidol in its effect on cognitive impairment in schizophrenia patients (De Oliveira and Juruena, 2006). It has been reported that dopamine D_4 receptor antagonist blocks the ability of clozapine to reverse the novel object recognition deficit in phencyclidine-induced animal model of schizophrenia (Miyauchi et al., 2017). Furthermore, serotonin 5-HT_{2A} antagonist treatment improves phencyclidine-induced memory deficit (Idris et al., 2010). Both serotonin 5-HT_{2A} and dopamine D_4 receptors may play an important role in ameliorating effect of clozapine on cognitive impairment in neonatal polyI:C-treated mice.

Neonatal polyI:C administration increases IFITM3 mRNA in the hippocampus of neonatal mice 24 h after the last treatment (Ibi et al., 2009). IFITM3 protein in the hippocampus of neonatal

mice are increased, at least up to 72 h, after the final polyI:C treatment, but there is no significant difference in adulthood (Ibi et al., 2013). However, neonatal polyI:C treatment decreases spine density of cortical pyramidal neurons, impairs glutamatergic neurotransmission in the hippocampus, and induces behavioral abnormalities in adulthood (Ibi et al., 2009), which suggests that neonatal polyI:C administration leads to dynamic changes in molecules in the adult brain. Accordingly, we measured the protein expression levels in the hippocampus of neonatal polyI:C-treated mice by 2D-DIGE, and identified ALDH1L1 and CRMP5, whose protein expression levels were significantly altered between control and polyI:C-treated mice.

ALDH1L1 is an enzyme that converts 10-formyltetrahydrofolate to tetrahydrofolate and CO_2 in a $NADP^+$-dependent reaction (Krupenko, 2009). The expression of ALDH1L1 is elevated in schizophrenia patients compared with that in healthy subjects (Barley et al., 2009). ALDH1L1 is expressed in radial glia cells (Anthony and Heintz, 2007) and astrocytes in the brain (Cahoy et al., 2008). Taken together, the increase in ALDH1L1 protein induced by neonatal polyI:C treatment may suggest a role for astrocytes in brain dysfunction in adulthood.

The expression of CRMP5 is prominent in the neocortex and hippocampus and is a cytoplasmic protein expressed in postnatal neurons (Ricard et al., 2001). CRMP5 is located in the filopodia of growth cones and controls the dynamics of filopodia and growth cone morphology, which affects neuronal axon formation (Hotta et al., 2005). TLR3 is present in growth cones, and polyI:C inhibits axon formation (Cameron et al., 2007). CRMP5-deficient mice have abnormal axon-Schwann cell

389 interactions, and impaired long-term depression (Camdessanche et al., 2012). Thus, it is possible that
390 the decrease of CRMP5 protein levels in the hippocampus may be involved in the behavioral and
391 neuropathological abnormalities of neonatal polyI:C-treated adult mice.

392 The relationship between ALDH1L1 and CRMP5 as well as their role in behavioral
393 abnormalities in neonatal polyI:C-treated adult mice remains unclear. ALDH1L1 is a marker of glial
394 cells, while CRMP5 is expressed in neurons. We have proposed that astrocyte-neuron interaction has
395 a crucial role in neuropathological and behavioral abnormalities of neonatal polyI:C-treated mice (Ibi
396 et al., 2013). Neonatal polyI:C treatment activates astrocytes and increases the expression of IFITM3
397 (Ibi et al., 2013) and major histocompatibility complex I (MHCI) in astrocytes (Sobue et al., 2018).
398 Neonatal polyI:C treatment in wild-type mice results in the development of behavioral and
399 neuropathological abnormalities whereas IFITM3 gene knockout mice show no these deficits in
400 adulthood (Ibi et al., 2013). The viral-mediated MHCI expressions in astrocytes of the prefrontal cortex
401 decreased spine density in neurons and the numbers of parvalbumin-positive interneurons, which were
402 accompanied by the development of cognitive and emotional abnormalities in mice (Sobue et al., 2018).
403 Accordingly, it is possible that alteration of ALDH1L1 and CRMP5 protein levels in the hippocampus
404 of neonatal polyI:C-treated adult mice may represent one of signature for astrocyte-neuron interaction
405 in the brain. Moreover, it should be determined whether antipsychotic treatment such as clozapine in
406 polyI:C-treated mice affects the changes in protein expressions of ALDH1L1 and CRMP5.

407 In conclusion, neonatal polyI:C-treated mice were validated as an animal model for

408 developmental psychiatric disorders to evaluate the effect of antipsychotics. Although the causal
409 relationship between changes in ALDH1L1 or CRMP5 protein expression and behavioral
410 abnormalities in neonatal polyI:C-treated adult mice remains unclear, we believe that changes in
411 ALDH1L1 and CRMP5 protein expression levels support our previous findings that astrocyte-neuron
412 interaction plays a role in the pathophysiology of neurodevelopmental disorders induced by neonatal
413 immune activation. Further studies are needed to investigate the role of ALDH1L1 or CRMP5 in the
414 neurochemical and behavioral abnormalities of neonatal polyI:C-treated adult mice.
415

Conflicts of interest

None.

Acknowledgments

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561

562 **Figure legends**

563 **Fig. 1.** Effects of clozapine and haloperidol on PPI deficit of startle response in neonatal polyI:C-
564 treated adult mice. (A) PPI (%) at four different prepulse intensities (69, 73, 77, and 81 dB). (B)
565 Acoustic startle amplitude as measured in trials without prepulse. Clozapine (5 mg/kg, p.o.) or
566 haloperidol (0.3 mg/kg, p.o.) was administered 60 min before the behavioral test. Values indicate the
567 mean ± S.E. (n=8 for each group). *p<0.05 and **p<0.01 vs. corresponding saline-treated control
568 group. #p<0.05 and ##p<0.01 vs. saline-treated polyI:C group.

569

570 **Fig. 2.** Effects of clozapine and haloperidol on performance in the open field test in neonatal polyI:C-
571 treated adult mice. Clozapine (5 mg/kg, p.o.) or haloperidol (0.3 mg/kg, p.o.) was administered 60 min
572 before the behavioral test. Individual mice were allowed to explore the open field freely for 5 min. (A
573 and B) Time spent in (A) inner and (B) outer sectors. (C) Total distance traveled. Values indicate the
574 mean ± S.E. (n=8 for each group). **p<0.01 vs. saline-treated control group (Tukey's multiple
575 comparison test). #p<0.05, ##p<0.01 vs. saline-treated polyI:C group (Tukey's multiple comparison
576 test).

577

578 **Fig. 3.** Effects of clozapine and haloperidol on performance in the novel object recognition test in
579 neonatal polyI:C-treated adult mice. (A and C) Exploratory preference in the training session (A) and
580 retention session (C). (B and D) Total exploration time in the training session (B) and retention session

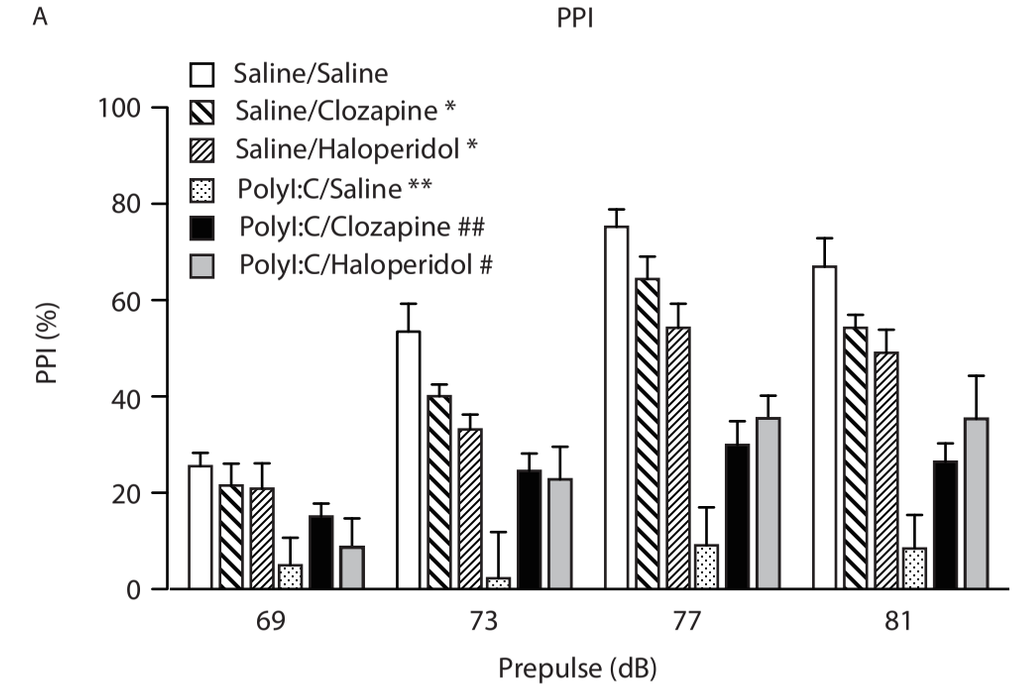
(D). Clozapine (5 mg/kg, p.o.) or haloperidol (0.3 mg/kg, p.o.) was administered 60 min before the training session. The retention session was performed 24 h after the training session. Values indicate the mean ± S.E. (n=8 saline-treated and polyI:C-treated control groups, n=7 for clozapine and haloperidol-treated groups). **p<0.01 vs. saline-treated control group. #p<0.05 and ##p<0.01 vs. saline-treated polyI:C group.

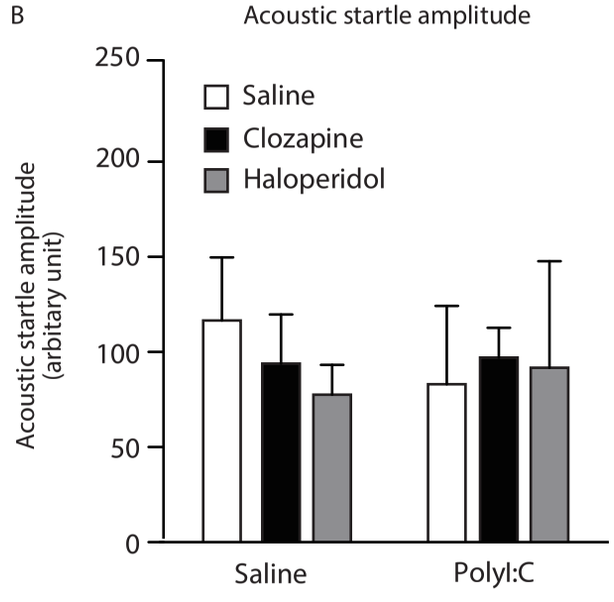
Fig. 4. Effects of clozapine and haloperidol on performance in the social interaction test in neonatal polyI:C-treated adult mice. (A) Social interaction, (B) escape behavior, and (C) aggressive behavior. Clozapine (5 mg/kg, p.o.) or haloperidol (0.3 mg/kg, p.o.) was administered 60 min before the behavioral test. Values indicate the mean ± S.E. (n=8 for each group). *p<0.05 vs. saline-treated control group. #p<0.05 vs. saline-treated polyI:C group.

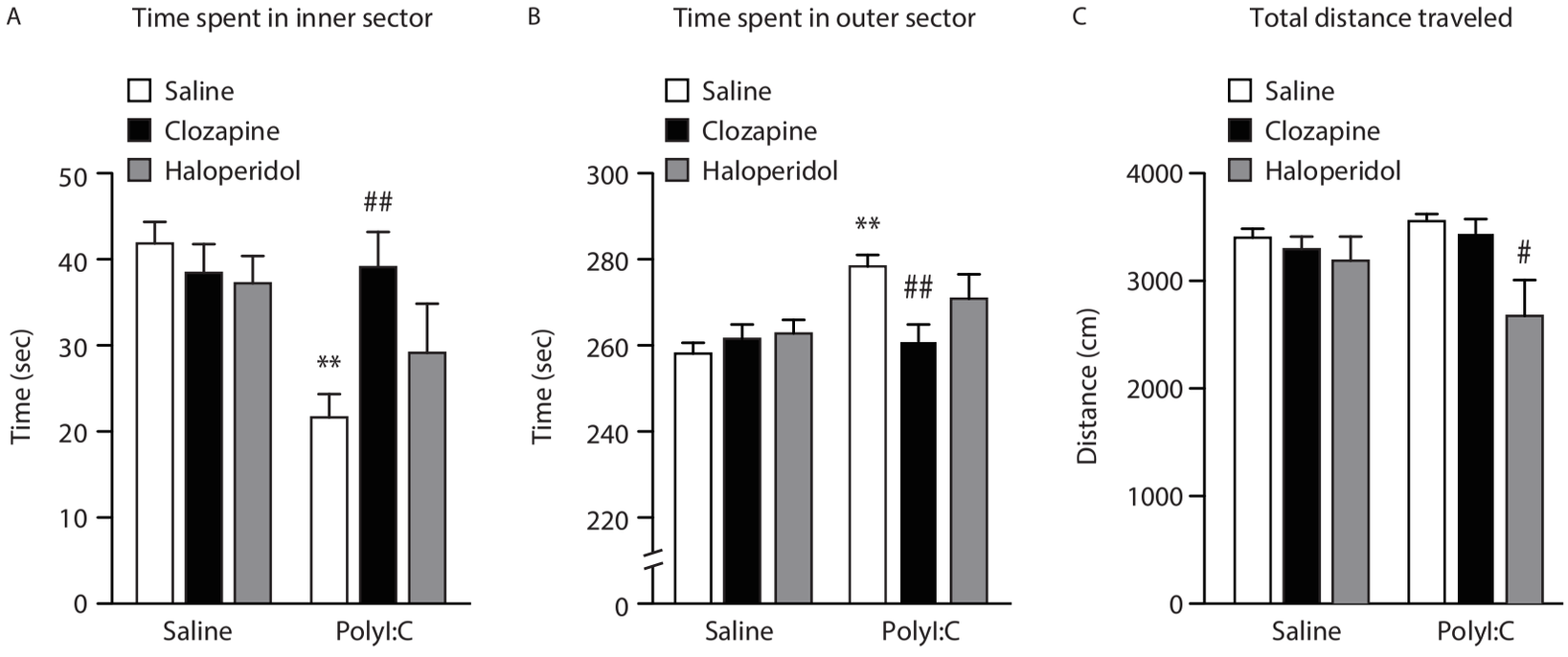
Fig. 5. 2D-DIGE analysis in the hippocampus of neonatal polyI:C-treated adult mice.
(A) Representative 2D image of CyDye-labeled hippocampal proteins of saline-treated mice (green) and polyI:C-treated mice (red). (B) Boxes I and II show areas with differentially expressed proteins that were excised and identified by LC-MS/MS. Arrows indicate identified proteins (see Table 1).

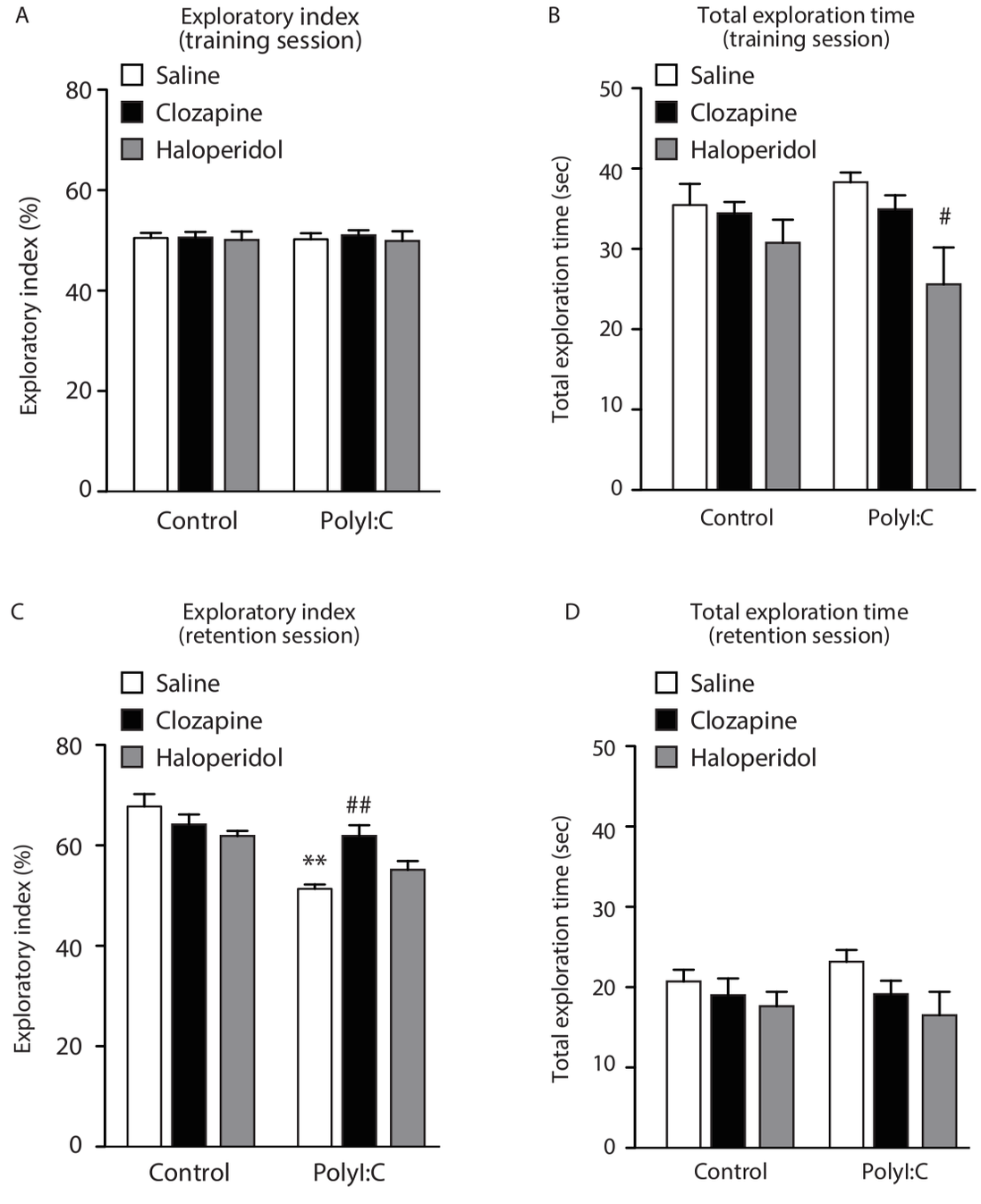
Fig. 6. Expression of ALDH1L1 and CRMP5 in the hippocampus of neonatal polyI:C-treated adult mice.

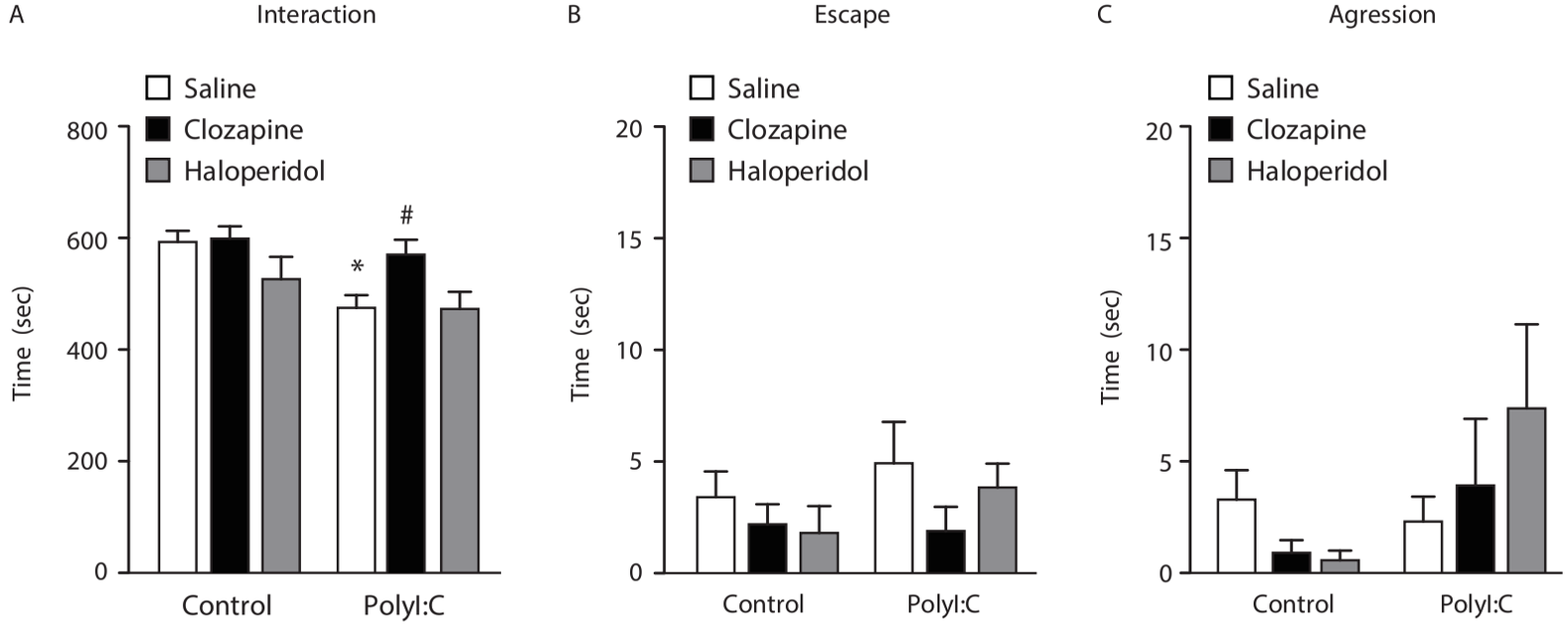
From PD 2 to 6, mice were injected s.c. daily with either pyrogen-free saline or polyI:C at a dose of 5 mg/kg. Mice were sacrificed at the age of 10 weeks. Values indicate the mean ± S.E. (n=6). *$p<0.05$ and **$p<0.01$ vs. saline-treated control group.

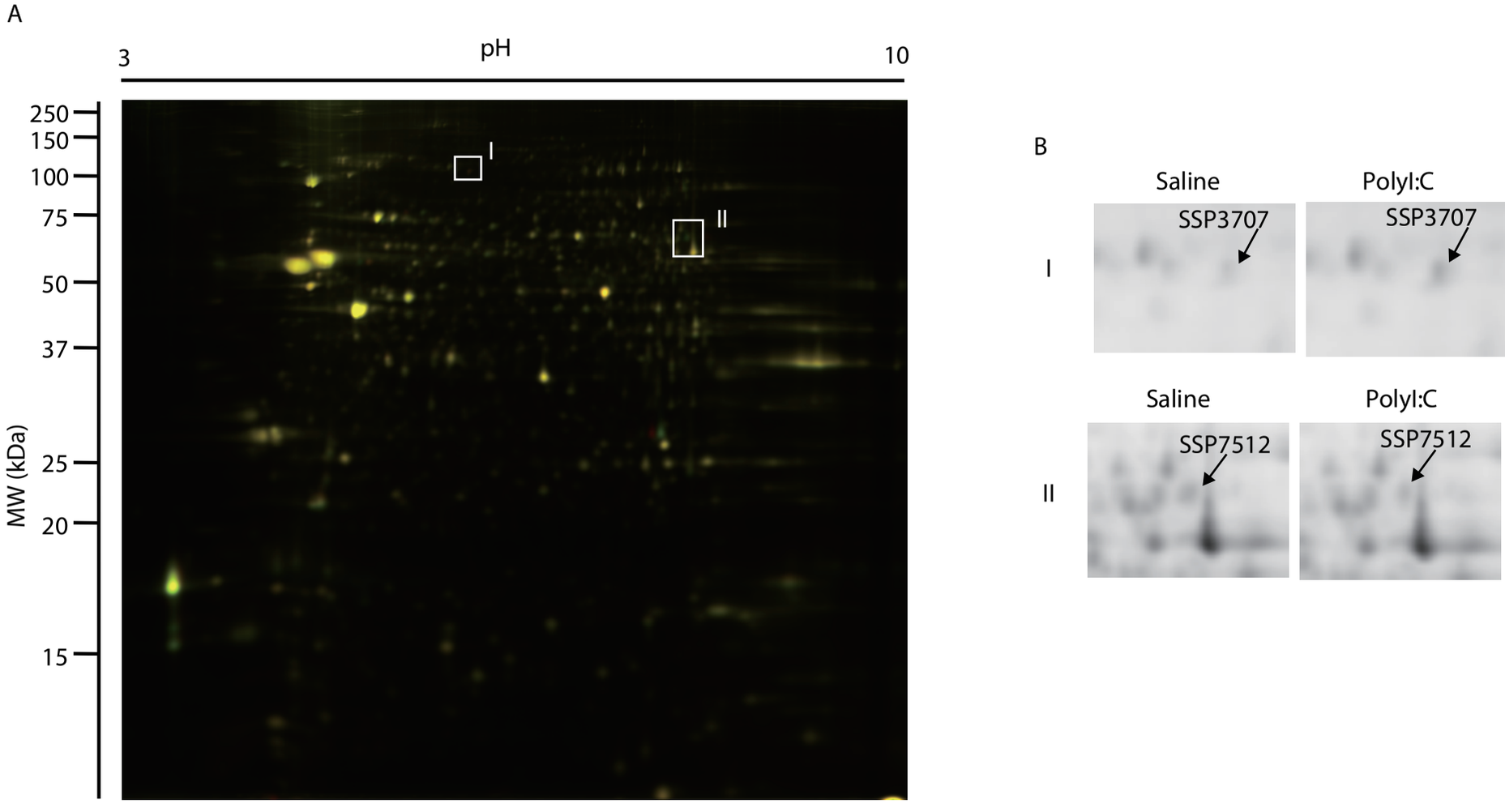

A
PPI
Saline/Saline
Saline/Clozapine *
Saline/Haloperidol *
PolyI:C/Saline **
PolyI:C/Clozapine ##
PolyI:C/Haloperidol #
PPI (%)
100
80
60
40
20
0
69
73
77
81
Prepulse (dB)


B
Acoustic startle amplitude
Saline
Clozapine
Haloperidol
Acoustic startle amplitude
(arbitary unit)
250
200
150
100
50
0
Saline
PolyI:C


A
Time spent in inner sector
Saline
Clozapine
Haloperidol
Time (sec)
50
40
30
20
10
0
**
##
Saline
PolyI:C
B
Time spent in outer sector
Saline
Clozapine
Haloperidol
Time (sec)
300
280
260
240
220
0
**
##
Saline
PolyI:C
C
Total distance traveled
Saline
Clozapine
Haloperidol
Distance (cm)
4000
3000
2000
1000
0
#
Saline
PolyI:C


A
Exploratory index
(training session)
Saline
Clozapine
Haloperidol
Exploratory index (%)
80
60
40
20
0
Control
PolyI:C
B
Total exploration time
(training session)
Saline
Clozapine
Haloperidol
Total exploration time (sec)
50
40
30
20
10
0
#
Control
PolyI:C
C
Exploratory index
(retention session)
Saline
Clozapine
Haloperidol
Exploratory index (%)
80
60
40
20
0
**
##
Control
PolyI:C
D
Total exploration time
(retention session)
Saline
Clozapine
Haloperidol
Total exploration time (sec)
50
40
30
20
10
0
Control
PolyI:C


A
Interaction
Saline
Clozapine
Haloperidol
800
600
400
200
0
Time (sec)
*
#
Control
Polyl:C
B
Escape
Saline
Clozapine
Haloperidol
20
15
10
5
0
Time (sec)
Control
Polyl:C
C
Agression
Saline
Clozapine
Haloperidol
20
15
10
5
0
Time (sec)
Control
Polyl:C


A
pH
3
10
250
150
100
75
50
37
25
20
15
MW (kDa)
I
II
B
Saline
PolyI:C
SSP3707
SSP3707
I
Saline
PolyI:C
SSP7512
SSP7512
II

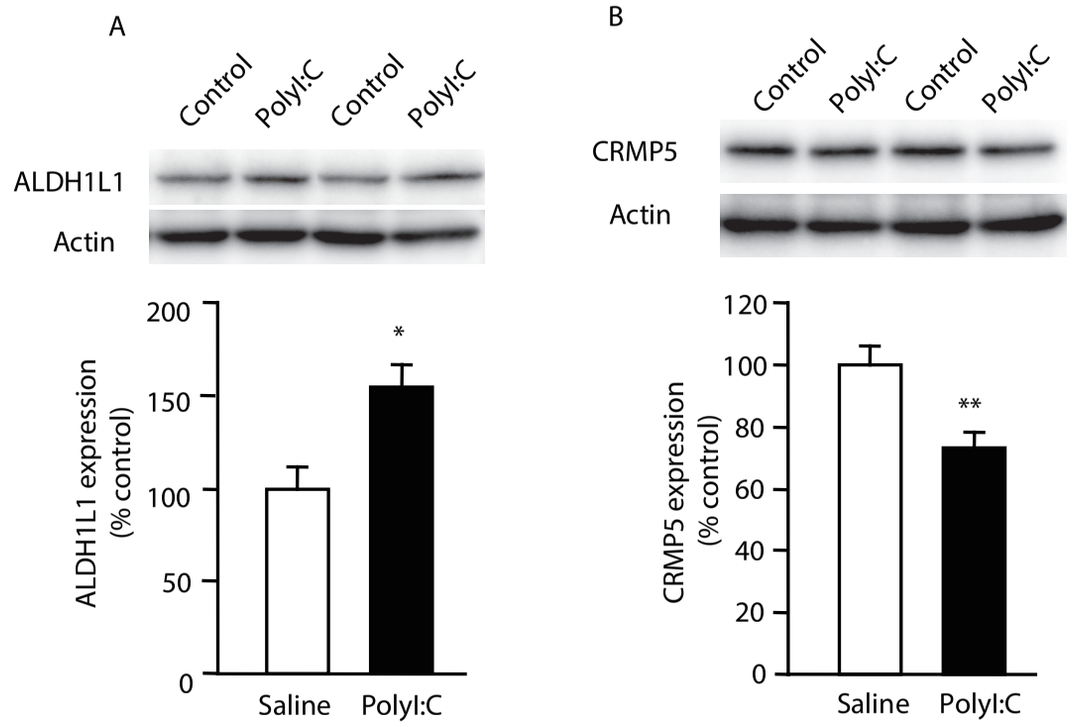

A
Control
PolyI:C
Control
PolyI:C
ALDH1L1
Actin
ALDH1L1 expression (% control)
200
150
100
50
0
*
Saline
PolyI:C
B
Control
PolyI:C
Control
PolyI:C
CRMP5
Actin
CRMP5 expression (% control)
120
100
80
60
40
20
0
**
Saline
PolyI:C

Table 1. List of identified proteins that are differentially expressed in the hippocampus of polyI:C-treated mice

Spot no.[a]	Protein name	SW/TR Acc[b]	Fold change[c]	*p*-value	MASCOT score[d]	Sequence coverage (%)[e]	Mr (kDa) /pI[f]
3707	Aldehyde dehydrogenase family 1 member L1	Q8R0Y6	1.6	<0.05	803	34	99/5.64
7512	Collapsin response mediator protein 5	Q9EQF6	0.6	<0.01	382	42	61/6.62

[a]Spot no. corresponds to those in Fig. 5. [b]Accession number is derived from the Swiss-Prot/TrEMBL database. [c]Fold changes in polyI:C-treated mice. [d]MASCOT score indicates the total of individual peptide scores. [e]Sequence coverage achieved by MS-Fit search. [f]Theoretical molecular weight (Mr)/isoelectric point (pI) of the protein.