

Maternal Influenza Viral Infection Causes Schizophrenia-Like Alterations of 5-HT_{2A} and mGlu₂ Receptors in the Adult Offspring

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Epidemiological studies indicate that maternal influenza viral infection increases the risk for schizophrenia in the adult offspring. The serotonin and glutamate systems are suspected in the etiology of schizophrenia, as well as in the mechanism of action of antipsychotic drugs. The effects of hallucinogens, such as psilocybin and mescaline, require the serotonin 5-HT_{2A} receptor, and induce schizophrenia-like psychosis in humans. In addition, metabotropic glutamate receptor mGlu_{2/3} agonists show promise as a new treatment for schizophrenia. Here, we investigated the level of expression and behavioral function of 5-HT_{2A} and mGlu₂ receptors in a mouse model of maternal influenza viral infection. We show that spontaneous locomotor activity is diminished by maternal infection with the mouse-adapted influenza A/WSN/33 (H1N1) virus. The behavioral responses to hallucinogens and glutamate antipsychotics are both affected by maternal exposure to influenza virus, with increased head-twitch response to hallucinogens and diminished antipsychotic-like effect of the glutamate agonist. In frontal cortex of mice born to influenza virus-infected mothers, the 5-HT_{2A} receptor is upregulated and the mGlu₂ receptor is downregulated, an alteration that may be involved in the behavioral changes observed. Additionally, we find that the cortical 5-HT_{2A} receptor-dependent signaling pathways are significantly altered in the offspring of infected mothers, showing higher *c-fos*, *egr-1*, and *egr-2* expression in response to the hallucinogenic drug DOI. Identifying a biochemical alteration that parallels the behavioral changes observed in a mouse model of prenatal viral infection may facilitate targeting therapies for treatment and prevention of schizophrenia.

Introduction

Maternal infection with a variety of agents, such as influenza virus, rubella virus, herpes simplex virus, and poliovirus, has been associated with development of schizophrenia in the adult offspring (Pearce, 2001; Patterson, 2009; Yudofsky, 2009; Brown and Derkits, 2010). In preclinical research, the influenza virus is among the most widely used models to study the effects of prenatal viral infection on brain development (Patterson, 2002; Meyer et al., 2009a). The offspring of mothers infected with mouse-adapted influenza virus display a series of behavioral ab-

normalities that are relevant to schizophrenia (Shi et al., 2003). As yet, the biochemical changes underlying the behavioral abnormalities induced by maternal viral infection are not definitely established.

The human psychoactive effects of drugs such as lysergic acid diethylamide (LSD) and phencyclidine (PCP) share several features with schizophrenia, including changes in time and sensory perception and in thought, speech, mood, and affect (Geyer and Vollenweider, 2008; González-Maeso and Sealfon, 2009). It has been demonstrated that the serotonin 5-HT_{2A} receptor is necessary for the effects of hallucinogenic drugs, such as LSD, mescaline, and psilocybin, in both murine and human model systems (Vollenweider et al., 1998; González-Maeso et al., 2003, 2007). Indeed, LSD effects were one of the earliest models of schizophrenia (Keeler, 1965), and are currently believed to model the symptoms that occur at the onset of this disorder (Geyer and Vollenweider, 2008).

Preclinical findings in rodents have demonstrated that metabotropic glutamate receptor mGlu_{2/3} agonists block both the locomotor hyperactivity induced by PCP-like drugs (Moghaddam and Adams, 1998) and the head-twitch behavioral response induced by LSD-like drugs (Gewirtz and Marek, 2000). In addition, in a recent clinical trial, an mGlu_{2/3} receptor agonist has shown promise as a new treatment for schizophrenia (Patil et al., 2007). It has been recently demonstrated that 5-HT_{2A} and

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mGlu₂ form a receptor heterocomplex that may be responsible for some of the effects induced by hallucinogenic 5-HT_{2A} agonists and antipsychotic mGlu_{2/3} agonists (González-Maeso et al., 2008). The effect of maternal viral infection on the level of expression and behavioral function of 5-HT_{2A} and mGlu₂ receptors remains unknown.

The natural hosts for influenza viruses are avian species, and a limited number of influenza virus subtypes infect mammalian species, including humans (Palese, 2004; Bouvier and Palese, 2008). Although mice are not natural hosts for influenza virus, following an intranasal inoculation they develop an illness that closely resembles the disease in humans. In this study, using mouse-adapted influenza A/WSN/33 (H1N1) virus, we investigated the effects of maternal viral infection on the level of expression of 5-HT_{2A} and mGlu₂ receptors in the adult offspring. We show that the cellular and behavioral responses induced by hallucinogenic 5-HT_{2A} and antipsychotic mGlu_{2/3} receptor agonists are affected in mice born to influenza virus-infected mothers. In humans, influenza virus replication is typically restricted to the respiratory track. Being aware of the differences in the primary target tissue for influenza virus infection in mouse and human (Li et al., 1993; Takahashi et al., 1995), we also considered and evaluated whether mouse-adapted influenza virus directly infects and replicates in the embryonic brain.

Materials and Methods

Cells and viruses. Madin-Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (ATCC) and were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin. Mouse-adapted A/WSN/33 (H1N1) influenza virus was routinely propagated in MDCK cells as previously reported (Martínez-Sobrido et al., 2010). Virus titers were measured by plaque assay on MDCK cells. The titer of viral stocks was calculated in plaque-forming units (pfu), and stored at -80°C until viral infections were performed. All experiments with live virus were performed under biosafety level 2 (BSL-2) containment.

Mouse viral infection. Timed pregnant CD1 mice were obtained from Charles River Laboratories. On day 9.5 of pregnancy, mice were anesthetized with ketamine/xylazine before intranasal (i.n.) infection with 5×10^3 pfu of influenza A/WSN/33 (H1N1) virus in 50 μl of PBS. Human epidemiological studies highlight a direct association between infections during the first trimester of pregnancy and schizophrenia. Infectious agents during early pregnancy that have been shown to increase the risk of schizophrenia in the adult offspring include influenza virus (Brown et al., 2004a), rubella virus (Brown et al., 2001), genital and reproductive infections (Babulas et al., 2006), and bacterial infection (Sørensen et al., 2009). We chose day 9.5 of pregnancy for infection because it is approximately midpregnancy in mouse, and this equates to the end of the first trimester of human pregnancy (Clancy et al., 2007). Mock-infected mothers were treated identically but were infused with PBS. Offspring were separated from their mothers after 3 weeks, and males and females were caged separately in groups of three to five. Subsequent experiments were performed in adult (10–12 weeks) mice unless otherwise indicated.

The sublethal dose of infection was determined by inoculating groups of three mice with serial 10-fold dilutions of virus (5×10^2 to 5×10^5 pfu). Infection of pregnant mice with 5×10^3 pfu caused sickness behavior (lethargy, sleepiness, ruffled fur, and lack of grooming) for several days, but loss of pregnancy was not common as long as mice were not disturbed (data not shown). No decrease in litter size or in the appearance or capacity of the mice to thrive was noted in mice born to influenza virus-infected mothers (5×10^3 pfu) relative to controls; infected mice gave birth to 7.2 ± 0.8 ($n = 5$), whereas control mice gave birth to 7.8 ± 0.6 pups ($n = 5$). This observation is consistent with previous results (Asp et al., 2005). The litter size was reduced in mice born to mothers infected with higher doses of influenza virus (data not shown; see also Shi et al., 2003). Animals from at least three separate litters were sex-matched and subjected to the different protocols (Meyer et al., 2009b).

Animals were fed with food and water *ad libitum*, and maintained in laminar flow racks under pathogen-free conditions for 12 h light and 12 h darkness. The Institutional Animal Use and Care Committee approved all experimental procedures at Mount Sinai School of Medicine.

Virus titration. Timed pregnant mice were infected with influenza virus (see above), and lungs were collected at 3 and 6 d after infection. Embryos from the same mother were pooled and processed as a single sample. Mock-infected mothers and embryos were used as negative controls. Tissue samples were stored at -80°C until they were analyzed. Lung samples from virus-infected mothers and embryo samples were homogenized and resuspended in sterile PBS. Virus titers were determined by plaque assay using 10-fold serial dilutions of tissue samples on MDCK cells as previously described (Martínez-Sobrido et al., 2010). Briefly, MDCK cells were infected with 100 μl of tissue homogenate for 1 h at 37°C . Cells were washed with PBS, and overlaid with MEM medium (see above) containing 0.9% agar. Plates were incubated for 3 d at 37°C , and plaques were counted by crystal violet staining (Martínez-Sobrido et al., 2010).

Detection of influenza A/WSN/33 (H1N1) virus-specific antibody from mice sera by ELISA. Blood samples were obtained from the tail vein and allowed to clot for 30 min at room temperature. Serum samples, obtained after centrifugation at $16,000 \times g$ for 10 min, were stored at -80°C until further analysis.

The presence of influenza virus-specific antibodies in mice sera was determined by an ELISA using mock- and influenza virus-infected MDCK cell extracts as antigens (Tumpey et al., 2005). Briefly, 50 μl of mock- and influenza virus-infected cell extracts (dilution 1:1000) were added to 96-well plates and incubated overnight at 4°C . Cells extracts were previously titrated with an anti-WSN polyclonal antibody (data not shown; for specificity of the antibody, see Martínez-Sobrido et al., 2010). Plates were blocked with blocking solution (1% bovine serum albumin, BSA, in PBS) for 1 h and, after, incubated with 50 μl of the indicated dilutions of mice sera in blocking solution for 1 h at 37°C . After three washes, plates were incubated with 50 μl of the goat anti-mouse (IgG, IgM, and IgA) horseradish peroxidase conjugate antibody (1:1000; Thermo Scientific) in blocking solution for 30 min at room temperature. After three washes, assay was developed with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) Liquid Substrate System for ELISA following the manufacturer's instructions (Sigma-Aldrich). The absorbance was measured at 405 nm with an ELISA microplate reader. Representative results from two separate experiments are indicated. Results obtained with a 1:100 dilution of sera from mice born to infected mothers and controls are shown in Figure 8d.

Drug administration. 1-(2,5-Dimethoxy-4-iodophenyl)-2-aminopropane (DOI; Sigma-Aldrich); (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohept-5,10-imine hydrogen maleate [dizocilpine, (+)-MK-801; Sigma-Aldrich]; and (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268; Tocris Bioscience) were dissolved in saline and injected intraperitoneally (i.p.). The injected doses were DOI, 0.5 mg/kg; LY379268, 5 mg/kg; and MK-801, 0.5 mg/kg; unless otherwise indicated. The administered doses have been shown previously to induce different behavioral responses in mice (González-Maeso et al., 2007, 2008).

Behavioral studies. Behavioral testing took place between 9:00 A.M. and 6:00 P.M. Head-twitch behavioral response was performed as follows. Animals were injected (i.p.) with appropriate treatments, and 15 min later, they were placed into the center of a Plexiglas cage ($28 \times 18 \times 15$ cm) for 30 min, during which they were videotaped at close range by a video camcorder positioned directly above the cage. Videotapes were scored for head twitches by an experienced observer blind to prenatal conditions and treatment. Testing cages were thoroughly cleaned after each animal was tested to eliminate odor cues. The differences between the head-twitch response elicited by vehicle and the hallucinogenic drug DOI (0.5 mg/kg) in mice born to influenza virus-infected mothers and controls were assessed by Bonferroni's *post hoc* test of two-way ANOVA.

Motor function was assessed using a computerized three-dimensional activity monitoring system (AccuScan Instruments). The activity monitor has 32 infrared sensor pairs with 16 along each side spaced 2.5 cm apart. The system determines motor activity based on frequency of interruptions to infrared beams traversing the x , y , and z planes. Horizontal

activity and vertical activity were automatically determined from the interruptions of beams in the horizontal and vertical planes, respectively. The differences between the locomotor response in mice born to influenza virus-infected mothers and controls were assessed by Bonferroni's *post hoc* test of two-way repeated-measures ANOVA.

Radioligand binding. Tissue samples of mouse frontal cortex were homogenized using a Teflon-glass grinder (10 up-and-down strokes at 1500 rpm) in 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.4), supplemented with 0.25 M sucrose. The crude homogenate was centrifuged at $1000 \times g$ for 5 min at 4°C, and the supernatant was recentrifuged at $40,000 \times g$ for 15 min at 4°C. The resultant pellet (P₂ fraction) was washed twice in homogenization buffer and recentrifuged in similar conditions. Aliquots were stored at -80°C until assay. Protein concentration was determined using the Bio-Rad protein assay.

[³H]Ketanserin (DuPont-NEN) binding (0.0625–10 nM; 10 concentrations) to 5-HT_{2A} receptor was measured at equilibrium in 500 μl aliquots (50 mM Tris-HCl; pH 7.4) of membrane preparations (20–40 μg of protein per tube) that were incubated at 37°C for 60 min. Nonspecific binding was determined in the presence of 10 μM methysergide (Tocris Bioscience), and ranged from 12 ± 1% to 65 ± 1% of total binding in all experimental groups.

[³H]LY341495 (American Radiolabeled Chemicals) binding (0.0625–30 nM; 11 concentrations) to mGlu_{2/3} receptors was measured at equilibrium in 500 μl aliquots (potassium phosphate buffer supplemented with 100 mM potassium bromide, pH 7.6) of membrane preparations (20–40 μg of protein per tube) that were incubated at 4°C for 60 min. Nonspecific binding was determined in the presence of 1 mM L-glutamic acid (Tocris Bioscience), and ranged from 5 ± 0% to 15 ± 1% of total binding in all experimental groups.

Incubations were terminated by dilution with 3 ml of ice-cold incubation buffer and free ligand was separated from bound ligand by rapid filtration under vacuum through GF/C glass fiber filters. The filters were then rinsed twice with 3 ml of incubation buffer, air dried, and counted for radioactivity by liquid scintillation spectrometry using a Betaplate counter (Wallac-PerkinElmer).

Quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) assays were performed in quadruplicate as previously described with minor modifications (González-Maeso et al., 2003). See González-Maeso et al. (2008) for primer sequences. Briefly, during the 7 d preceding the qRT-PCR experiments, mice were habituated to injections by daily saline administration (i.p.). The day of the experiment, mice were killed by cervical dislocation 60 min after drug or vehicle injection (i.p.). Frontal cortex was dissected and immediately frozen at -80°C until processing for total RNA isolation following the manufacturer's instructions (Stratagene).

Results

Spontaneous locomotor activity

The activity in the open-field represents a complex behavior that mimics the natural conflict in mice between their tendency to explore a novel environment and safety. Spontaneous locomotor activity in the open field is generally considered as one of the variables reflecting exploratory behavior. To investigate the effect of maternal viral infection on spontaneous locomotor activity in the offspring, we first examined horizontal activity, vertical activity, and time spent in the center of the arena in the open field exploration test. Two-way ANOVA repeated measures indicated that there was a significant effect of prenatal influenza infection on horizontal activity (Fig. 1*a*) ($F_{(1,464)} = 41.31$; $p < 0.001$). Analysis of the total locomotor activity for the entire 80 min indicated that locomotion was significantly decreased in mice born to virus-infected mothers (Fig. 1*b*) ($p < 0.05$). Two-way ANOVA repeated measures also indicated that there was a significant effect of prenatal influenza on vertical activity (Fig. 1*c*) ($F_{(1,464)} = 100.89$; $p < 0.001$). Analysis of the total vertical activity for the entire 80 min indicated that rearing was significantly decreased in mice born to virus-infected mothers (Fig. 1*d*) ($p <$

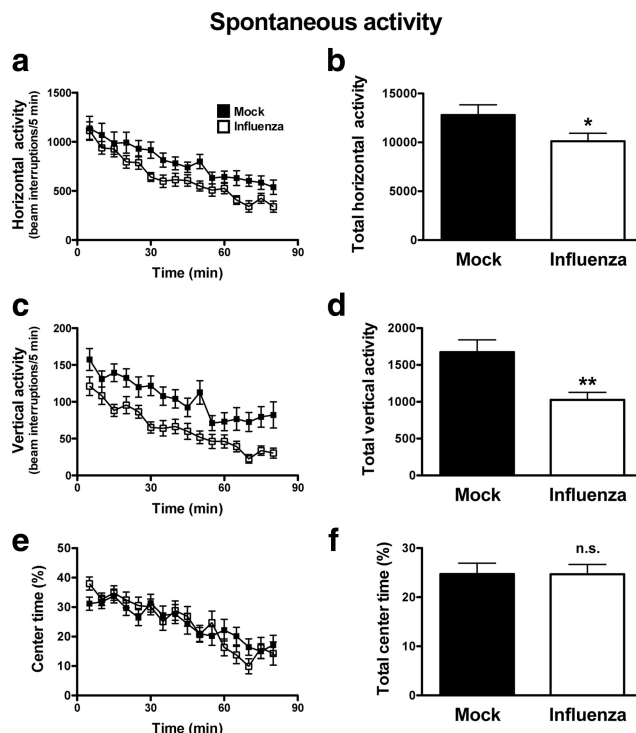


Figure 1. Mice born to influenza virus-infected mothers show decreased exploratory activity in the open field test, and unaffected locomotor activity occurring in the center of the arena. Mice born to influenza virus-infected mothers (white) and controls (black) were placed in the locomotor chamber and allowed to habituate for 5 min. After habituation, the locomotor activity was measured for 80 min. *a–f*, Horizontal activity (*a, b*), vertical activity (*c, d*), and number of entries into the center field (*e, f*). Both time course (*a, c, e*) and total counts (*b, d, f*) of the effect of maternal viral infection are shown for each measurement ($n = 30$). Data are means \pm SEM (* $p < 0.05$; ** $p < 0.01$; n.s., not significant; Student's *t* test).

0.01). Under brightly lit conditions, the center of the open field is aversive and potentially risk-laden, whereas exploration of the periphery provides a safer choice. We found that exploration of the center portion of the environment was not affected by prenatal viral infection (Fig. 1*e, f*).

Behavioral responses induced by hallucinogens

It has been demonstrated that hallucinogenic 5-HT_{2A} agonists, such as mescaline, psilocybin, and LSD, produce symptoms in normal subjects comparable to those seen in acutely ill unmedicated or first episode schizophrenics (Young, 1974; Vollenweider et al., 1998), and that hallucinogens aggravate hallucinations in schizophrenic patients (Hoch et al., 1952). We have previously demonstrated that head-twitch behavior is reliably and robustly elicited by hallucinogenic 5-HT_{2A} agonists, and is absent in 5-HT_{2A} knock-out mice (González-Maeso et al., 2007). Consistent with previous studies, two-way ANOVA indicated that there was a significant effect of the hallucinogenic drug DOI on head-twitch behavior (Fig. 2*a*) ($F_{(1,12)} = 577.03$; $p < 0.001$). *Post hoc* analysis indicated that DOI significantly increased head-twitch response in adult mice born to mock-infected mothers (Fig. 2*a*) ($p < 0.001$). *Post hoc* analysis also indicated that DOI significantly increased head-twitch response in adult mice born to influenza virus-infected mothers (Fig. 2*a*) ($p < 0.001$). Two-way ANOVA indicated that there was a significant effect of maternal viral infection on the head-twitch response to DOI (Fig. 2*a*) ($F_{(1,12)} = 51.39$; $p < 0.001$). Notably, *post hoc* analysis revealed that the hallucinogen DOI elicited significantly more head-twitch

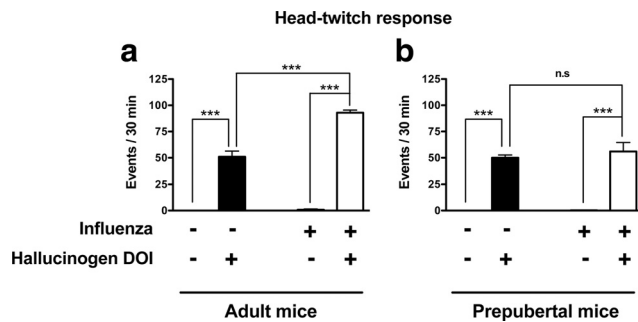


Figure 2. Head-twitch behavioral response is increased in adult mice born to influenza virus-infected mothers. Head-twitch response was determined in adult (10–12 weeks old; **a**) and prepubertal (21–28 d old; **b**) mice injected with vehicle or DOI (0.5 mg/kg). Data are means \pm SEM ($n = 4$ –6 per group). *** $p < 0.001$, Bonferroni's *post hoc* test of two-factor ANOVA.

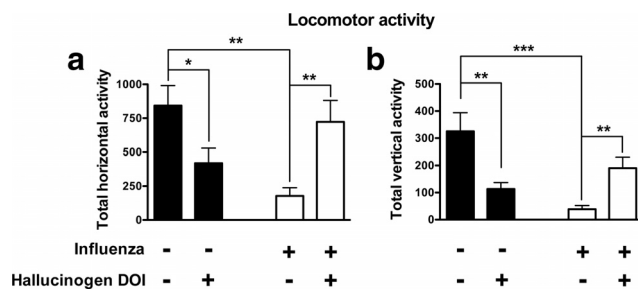


Figure 3. Effect of the hallucinogen DOI on locomotor activity in adult mice born to influenza virus-infected mothers and controls. Mice born to influenza virus-infected mothers (white) and controls (black) were placed in the locomotor chamber and allowed to habituate for 60 min. Mice were then injected with vehicle or DOI (0.5 mg/kg), and locomotor activity was measured 15 min after injection for another 60 min. Total horizontal activity (**a**) and total vertical activity (**b**) are shown for each measurement ($n = 13$ –22 per group). Data are means \pm SEM (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Bonferroni's *post hoc* test of two-factor ANOVA). Note that locomotor activity is decreased by DOI in mice born to mock-infected mothers, and increased by DOI in mice born to influenza virus-infected mothers.

behavior in adult mice born to virus-infected mothers than in mice born to mock-infected mothers (Fig. 2a) ($p < 0.001$).

In humans, the onset of schizophrenia typically occurs during late adolescence or early adulthood. Notably, our results show that alterations in the head-twitch response induced by DOI are not observed in prepubertal mice (21–28 d old). Thus, two-way ANOVA indicated that, similar to adult mice (10–12 weeks old) (Fig. 2a), there was a significant effect of DOI on head-twitch behavior in prepubertal mice (Fig. 2b) ($F_{(1,12)} = 140.85$; $p < 0.001$). *Post hoc* analysis indicated that DOI significantly increased head-twitch response in both prepubertal mice born to mock-infected mothers (Fig. 2b) ($p < 0.001$) and prepubertal mice born to influenza-infected mothers (Fig. 2b) ($p < 0.001$). However, two-way ANOVA indicated that there was not a significant effect of prenatal viral infection on the head-twitch response to DOI in prepubertal mice (Fig. 2b) ($F_{(1,12)} = 0.49$; $p = 0.49$).

We next examined the effect of treatment with DOI on locomotor and investigatory behavior in the adult offspring of influenza virus-infected mothers and control mice. As in the previous study (see Fig. 1), two-way ANOVA indicated that there was a significant effect of prenatal viral infection on horizontal (Fig. 3a) ($F_{(1,60)} = 12.07$; $p < 0.001$) and vertical (Fig. 3b) ($F_{(1,60)} = 11.59$; $p < 0.01$) spontaneous activity. Two-way ANOVA also indicated that there was a significant effect of DOI on horizontal (Fig. 3a)

($F_{(1,60)} = 18.12$; $p < 0.001$) and vertical (Fig. 3b) ($F_{(1,60)} = 11.59$; $p < 0.01$) activity in mice born to mock- and influenza virus-infected mothers. *Post hoc* analysis revealed that the hallucinogen DOI significantly decreased horizontal ($p < 0.05$) and vertical ($p < 0.01$) activity in mice born to mock-infected mothers. Notably, and opposite to what was found in mice born to mock-infected mothers, *post hoc* analysis revealed that the hallucinogen DOI significantly increased horizontal ($p < 0.01$) and vertical ($p < 0.01$) activity in mice born to influenza virus-infected mothers.

Antipsychotic-like response induced by LY379268

Noncompetitive NMDA receptor antagonists, such as PCP, ketamine, and MK-801, are used as pharmacological models for schizophrenia in animals because of their capacity to evoke positive and negative symptoms as well as sensorimotor gating deficits resembling those seen in this disease (Morris et al., 2005; Kristiansen et al., 2007). Consistent with previous studies (Geyer and Ellenbroek, 2003), MK-801 (0.5 mg/kg) induced a robust increase in locomotor activity (Fig. 4). Two-way ANOVA revealed that prenatal infection did not affect the locomotor response induced by MK-801 (Fig. 4a–c) ($F_{(1,36)} = 0.45$; $p = 0.51$). Two-way ANOVA also indicated that there was a significant effect of LY379268 (5 mg/kg) on the locomotor response induced by MK-801 (Fig. 4a,c) ($F_{(1,36)} = 8.06$; $p < 0.01$). Interestingly, *post hoc* analysis revealed that LY379268 reduced the locomotor response induced by MK-801 in mice born to mock-infected mothers (Fig. 4a,c) ($p < 0.01$), but not in mice born to influenza virus-infected mothers (Fig. 4b,c) ($p > 0.05$).

Level of expression of 5-HT_{2A} receptor and mGlu₂ receptor in frontal cortex

The alterations in the behavioral responses induced by hallucinogens and LY379268 prompted us to study the level of expression of 5-HT_{2A} receptor and mGlu_{2/3} receptor in the offspring of influenza virus-infected mothers. The specific binding of the antagonist [³H]ketanserin, a selective 5-HT_{2A} receptor ligand, to mouse frontal cortex membrane preparations was a saturable process to a single population of high-affinity sites with dissociation constant (K_D) values in the narrow range of 0.6–5.1 nM (Fig. 5a) ($F_{(2,102)} = 0.76$; $p = 0.47$) in control mice. The simultaneous analysis of multiple saturation curves indicated the existence of a significantly different [³H]ketanserin binding saturation curve in the offspring of mice born to influenza virus-infected mothers (Fig. 5a) ($F_{(2,200)} = 20.03$; $p < 0.001$). Analysis of individual maximum number of binding sites (B_{max}) values demonstrated a higher density of 5-HT_{2A} receptors in mice born to influenza virus-infected mothers (Fig. 5b) ($p < 0.05$). The affinity (K_D values) for [³H]ketanserin did not differ from those in the control group ($p > 0.05$).

The specific binding of the antagonist [³H]LY341495, a selective mGlu_{2/3} receptor ligand (Wright et al., 2001), to mouse frontal cortex membrane preparations was a saturable process to a single population of high-affinity sites with K_D values in the narrow range of 2.1–10.7 nM (Fig. 5c) ($F_{(2,84)} = 0.74$; $p = 0.29$) in control mice. The simultaneous analysis of multiple saturation curves indicated the existence of a significantly different [³H]LY341495 binding saturation curve in the offspring of mice born to influenza virus-infected mothers (Fig. 5c) ($F_{(2,171)} = 9.39$; $p < 0.001$). Analysis of individual B_{max} values demonstrated a lower density of mGlu_{2/3} receptors in mice born to influenza-infected mothers (Fig. 5d) ($p < 0.05$). The K_D values for

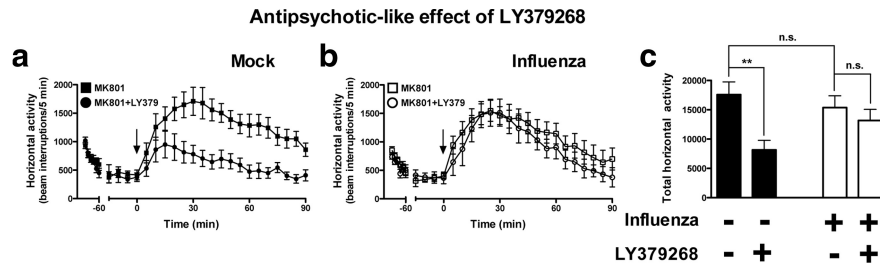


Figure 4. The MK-801-stimulated locomotor activity is attenuated by LY379268 (5 mg/kg) in control mice (black; *a, c*), but it is not affected in the offspring of infected mothers (white; *b, c*). The panels depict the time course of MK-801-induced locomotion in 5 min blocks (*a, b*). Time of injections is indicated by arrows. *c*, Data summary of the total MK-801-induced locomotion as a summation of horizontal activity from *t* = 15 to *t* = 90. Mice were administered LY379268 (circles), or vehicle (squares) followed by MK-801 (*n* = 8–12 per group). Data are mean ± SEM (***p* < 0.01; n.s., not significant; Bonferroni's *post hoc* test of two-factor ANOVA).

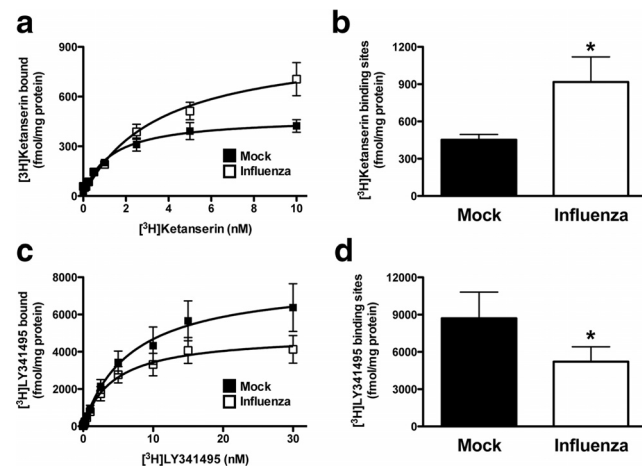


Figure 5. Prenatal viral infection affects the expression of 5-HT_{2A} and mGlu_{2/3} receptors in mouse frontal cortex. *a, c*, [³H]ketanserin (*a*) and [³H]LY341495 (*c*) binding saturation curves in frontal cortex of mice born to influenza virus-infected mothers (white) and controls (black; *n* = 12–16 per group). *b, d*, Maximum number of binding sites (*B*_{max}) for [³H]ketanserin (*b*) and [³H]LY341495 (*d*) obtained from individual saturation curves. Data are mean ± SEM (**p* < 0.05, Student's *t* test).

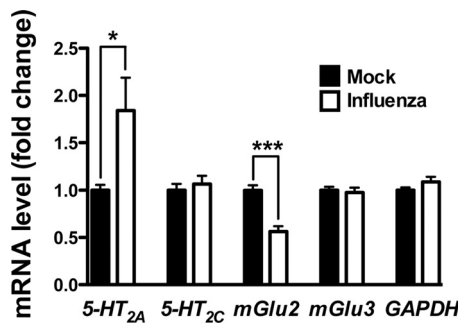


Figure 6. Expression of 5-HT_{2A}, 5-HT_{2C}, mGlu₂, and mGlu₃ mRNA in frontal cortex of adult mice born to influenza virus-infected mothers (white) and controls (black) determined by qRT-PCR assays (*n* = 11–22 per group). Data are mean ± SEM (**p* < 0.05; ****p* < 0.001). Student's *t* test.

[³H]LY341495 did not differ from those in the control group (*p* > 0.05).

We next tested the level of expression of 5-HT_{2A}, 5-HT_{2C}, mGlu₂, and mGlu₃ mRNA in mouse frontal cortex by qRT-PCR assays (Fig. 6). Expression level was significantly higher for 5-HT_{2A} (*p* < 0.05; Student's *t* test) and significantly lower for mGlu₂

(*p* < 0.001; Student's *t* test) in mice born to influenza virus-infected mothers. Neither 5-HT_{2C} nor mGlu₃ mRNA expression was significantly changed by prenatal infection (Fig. 6).

Cellular response to hallucinogens

We have previously demonstrated that hallucinogenic 5-HT_{2A} agonists cause induction of *c-fos*, *egr-1*, and *egr-2* in mouse cortical neurons (González-Maeso et al., 2003, 2007). We have also found that the induction of *c-fos* by DOI is not affected by LY379268. In contrast, the hallucinogen-specific induction of *egr-1* and *egr-2* is selectively blocked by LY379268 in both mouse cortex and primary cortical neurons

(González-Maeso et al., 2008). Here we tested the effect of maternal viral infection on the induction of *c-fos*, *egr-1*, and *egr-2* by the hallucinogen DOI in the adult offspring (Fig. 7). Consistent with previous studies, DOI elicited a dose-dependent gene response in mouse frontal cortex (*c-fos*, *F*_(3,40) = 34.99, *p* < 0.001; *egr-1*, *F*_(3,48) = 96.66, *p* < 0.001; *egr-2*, *F*_(3,56) = 102.47, *p* < 0.001). Notably, the cellular response induced by DOI was significantly different in the adult offspring of influenza virus-infected mothers (*c-fos*, *F*_(1,40) = 4.41, *p* < 0.05; *egr-1*, *F*_(1,48) = 10.24, *p* < 0.01; *egr-2*, *F*_(1,56) = 788, *p* < 0.01). *Post hoc* analysis revealed that the cellular response to DOI was significantly higher in mice born to infected mothers (Fig. 7).

Absence of replication of influenza A/WSN/33 (H1N1) virus in mouse embryos

To determine whether influenza A/WSN/33 (H1N1) virus can spread to fetuses during maternal infection, mice were infected on day 9.5 of pregnancy under identical conditions to the ones described above. On days 3 and 6 after infection, embryo samples from influenza virus- and mock-infected mothers were collected and titrated in MDCK cells. Lung samples from influenza virus- and mock-infected mothers were used as positive and negative controls, respectively. As shown in Figure 8, *a* and *b*, virus replication was detected in the lungs of infected mothers on days 3 and 6 after infection. However, virus replication was undetectable in embryo samples from infected mothers.

Detection of influenza A/WSN/33 (H1N1) virus-specific antibody in maternal and offspring serum

To further investigate the virulence of mouse-adapted influenza A/WSN/33 (H1N1) virus in mouse embryos, we assayed the detection of virus-specific antibodies in serum samples. All the mothers were bled 8–10 weeks after the birth, and the serum samples were analyzed for the presence of influenza A/WSN/33 (H1N1) virus-specific antibodies. Serial dilutions of the sera demonstrated elevated antibodies against influenza virus in all the serum samples from influenza virus-infected mothers compared to those of control mice (see Fig. 8*c* for representative examples). We next tested the presence of anti-influenza virus antibodies in adult mice born to infected mothers and controls to determine whether mouse-adapted influenza A/WSN/33 (H1N1) virus can spread to the fetuses during maternal viral infection. Blood samples were collected at the age of 10–14 weeks. Elevated antibodies against influenza A/WSN/33 (H1N1) virus were detected in 2 of 31 mice born to influenza virus-infected mothers, using mice born to mock-infected mothers as controls (Fig. 8*d*). Positive and negative controls in ELISA tests

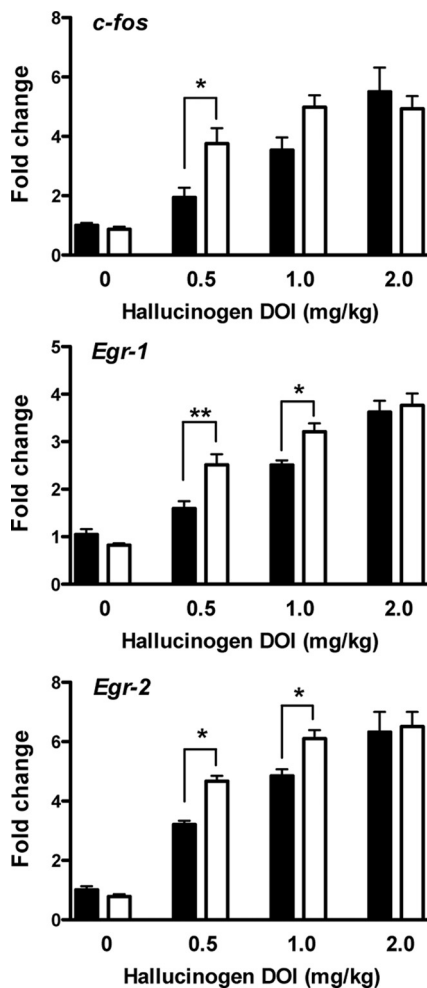


Figure 7. Cellular response induced by the hallucinogen DOI in frontal cortex of adult mice born to influenza virus-infected mothers (white) and controls (black) determined by qRT-PCR assays. Mice were injected (i.p.) with the doses indicated, and killed after 60 min. Relative fold changes of mRNA expression of *c-fos*, *egr-1*, and *egr-2* are shown ($n = 6-8$). Data are mean \pm SEM (* $p < 0.05$; ** $p < 0.01$; Bonferroni's *post hoc* test of two-factor ANOVA).

included anti-influenza virus antibody, mock- and influenza virus-infected MDCK cell extracts, and blood samples from mice exposed neither to mock nor to viral infection (data not shown).

Discussion

Our study investigates the effects of maternal influenza viral infection on the level of expression and function of 5-HT_{2A} and mGlu₂ receptors as a mouse model of schizophrenia. We found that head-twitch response, a mouse behavior induced only by hallucinogenic 5-HT_{2A} agonists, was significantly increased in adult mice born to influenza virus-infected mothers. It has been described that mGlu₂, and not mGlu₃, is the metabotropic glutamate receptor responsible for the effects of the mGlu_{2/3} agonist LY379268 on the locomotor response induced by the noncompetitive NMDA receptor antagonist MK-801 (Woolley et al., 2008); see also (Fell et al., 2008). Our data demonstrate that the antipsychotic-like behavioral effects of LY379268 are absent in the offspring of influenza virus-infected mothers. The behavioral responses were associated with biochemical alterations in frontal cortex, showing a higher and a lower level of expression of 5-HT_{2A} and mGlu₂, respectively, in mice with prenatal exposure to maternal infection. We also found that maternal viral infection leads to an increased cellular response induced by the hallucino-

genic 5-HT_{2A} agonist DOI. Thus our results demonstrate that maternal viral infection dysregulates the cellular and behavioral responses that require the 5-HT_{2A}-mGlu₂ complex in the adult offspring.

Frontal cortex presents a wide array of cognitive functions, such as learning–reward processing, decision making, attention, and social cognition. From the viewpoint of the model of psychosis paradigm, there is an association between acute psychosis episodes and hyperactivity in frontal cortical regions (Wiesel et al., 1987; Szechtman et al., 1988; Parellada et al., 1994). Several laboratories have reported alterations in frontal cortex of mouse models of maternal viral infection and/or immune challenge, including changes in gene expression (Fatemi et al., 1999, 2002a, 2005), abnormal cortical neurogenesis (De Miranda et al., 2010), pyramidal cell atrophy (Fatemi et al., 2002b; Baharnoori et al., 2009), and loss of GABA-ergic interneurons (Meyer et al., 2008a). The behavioral effects of hallucinogenic drugs result from their activity at 5-HT_{2A} receptors expressed by cortical pyramidal neurons (González-Maeso et al., 2007). It has been shown that activation of mGlu₂ receptors specifically blocks the cellular and behavioral responses induced by hallucinogenic 5-HT_{2A} agonists (Marek, 2004; González-Maeso et al., 2008). We have recently reported that, in postmortem prefrontal cortex from young untreated schizophrenic subjects, the 5-HT_{2A} receptor is upregulated, and the mGlu₂ receptor is downregulated, a pattern that could predispose to psychosis (González-Maeso et al., 2008). Since the same pattern of dysregulated expression was found in frontal cortex of mice born to influenza virus-infected mothers, these results are of considerable importance for future studies to unravel the etiology of schizophrenia and the neurochemical mechanisms responsible for the alterations observed in cortical regions in schizophrenia patients.

It is important to note that many influenza viruses do not optimally replicate or cause disease in mice (Matsuoka et al., 2009). However, some influenza viruses, including influenza A/WSN/33 (H1N1) virus (Ward et al., 1993), have been adapted to mice through serial passage, and some highly pathogenic influenza viruses can cause disease and replicate in the respiratory tract and extrapulmonary sites in mice without adaptation (Li et al., 1993; Takahashi et al., 1995; Ward, 1995). Since human influenza viruses replicate mainly in the respiratory tract, a potential factor that may question the validity of the mouse model of maternal viral infection is the presence of influenza virus in fetal brain. Discrepant results have been reported in the literature regarding detection of A/WSN/33 and A/NWS/33 influenza virus strains in the fetus (Aronsson et al., 2002; Shi et al., 2005). Our data found that mouse-adapted influenza virus is not detectable in embryo samples from infected mothers. Interestingly, our findings show that influenza A/WSN/33 (H1N1)-specific antibody is detected in ~5% of all the adult mice (10–14 weeks) whose mothers were infected with influenza virus. Although immature, the fetal immune system is still capable of responding to certain antigenic stimuli, which might account for the antibodies against influenza detected in some of the mice born to influenza virus-infected mothers. Fetuses and neonates are also deficient in several components of inflammatory, innate, and specific immune responses. In humans, maternal antibody transfer to the offspring is mostly transplacental (Leach et al., 1996). In mice, however, the offspring receive almost all the maternally derived antibody in the colostrum and milk, and this passive antibody typically declines over the first period of life. Although further investigation is necessary, the presence of anti-influenza virus antibodies that we found in some of the adult mice born to in-

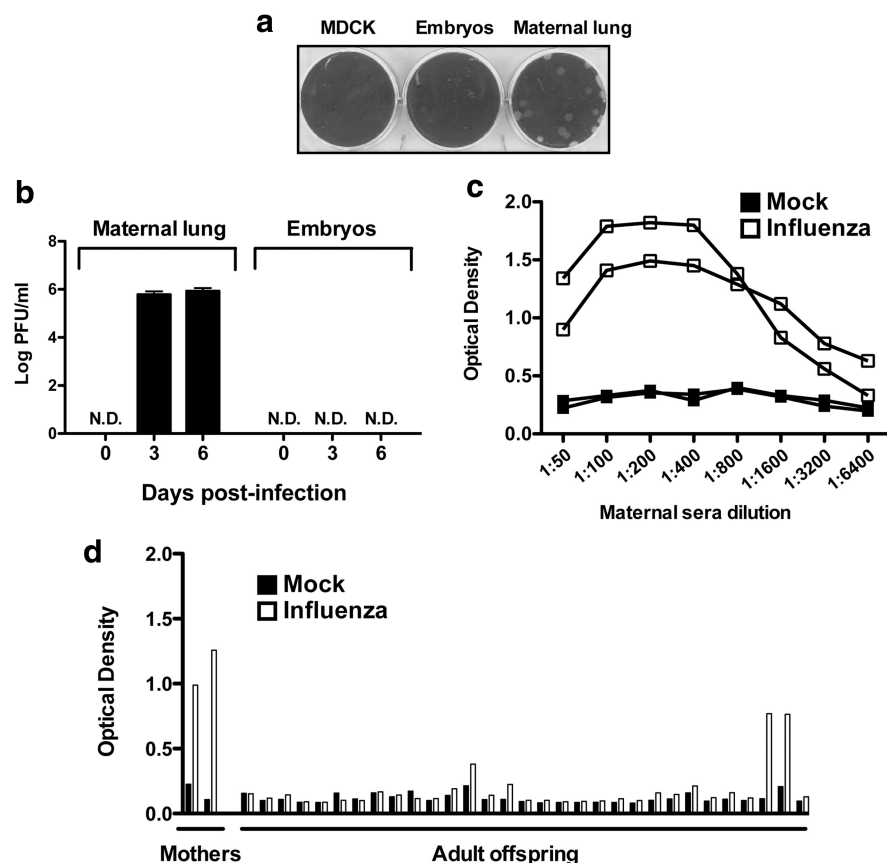


Figure 8. Influenza virus is not detected in the fetus. **a, b**, Determination of maternal lung and embryo virus titers over a 6 d period for pregnant females inoculated (i.n.) with influenza A/WSN/33 (H1N1) virus. Three pregnant females were killed at each time point. **a**, Viral titers in tissue homogenates were determined by plaque assay in MDCK cells. Forty-eight hours postinfection, cells were stained with crystal violet. **b**, Virus titers following influenza virus inoculation (i.p.). Pregnant females were killed 3 and 6 d after inoculation. Animals were also mock-infected (day after infection = 0). Viral titers of tissue homogenates were determined by plaque assay in MDCK cells. No virus could be detected in the fetus of influenza virus-infected pregnant females (N.D., not detected). **c, d**, Influenza A/WSN/33 (H1N1) virus-specific antibody response. **c**, Pregnant females were infected with influenza A/WSN/33 (H1N1) virus (white) or PBS as a negative control (black), and serum samples were collected 8–10 weeks after the birth. Antibody response in serial dilutions of the sera from four representative mice is shown. **d**, Antibody response of mice born to influenza virus-infected mothers (white) and that of mice born to mock-infected mothers (black). Sera of the individual mice were tested for the presence of virus-specific antibodies at the age of 10–14 weeks. Elevated antibodies against influenza virus were detected in 2 of 31 mice born to influenza virus-infected mothers. Influenza A/WSN/33 (H1N1) virus-specific antibodies were detected by ELISA as described in Materials and Methods.

infected mothers may be then antibodies passively acquired during lactation. Together with the absence of detectable virus in embryo samples, these data also suggest that the changes observed in the offspring are a consequence of maternal response to viral infection, and not of direct fetal infection by mouse-adapted influenza virus.

Schizophrenia patients show “positive” symptoms (e.g., hallucinations, delusions, and other thought disorders) and negative symptoms (e.g., social withdrawal, apathy, and abnormal emotional responses). In addition to the positive and negative symptoms of schizophrenia, the importance of anxiety, depression, and cognitive deficits has gained increasing attention. Significant correlations between anxiety symptoms and positive symptoms in schizophrenia have been replicated in several studies (Muller et al., 2004). Behavior in the open field is used as an anxiety test (Nestler et al., 2002). In the open field, mice face a conflict between avoidance and exploration of the center, which is more aversive than the peripheral area proximal to the walls. It has been shown that measures of behavior in the open field fall into two

main categories: (1) time and distance traveled in the center, which may be related to anxiety; and (2) total distance traveled and rearing, which reflect general behavioral activation or exploration. We found that mice born to influenza virus-infected mothers displayed strong deficits in locomotor and exploratory activity, whereas anxiety-like behavior was unchanged. This reduction of locomotor activity suggests abnormal habituation to a novel environment in mice born to influenza virus-infected mothers. A subset of schizophrenic patients exhibits psychomotor agitation, which includes hyperactivity or increased stereotypic movements (Powell and Miyakawa, 2006). Locomotor response to novelty has been shown to be higher (Rojas et al., 2007) and lower (Mukai et al., 2004) in different knock-out mouse models of schizophrenia. However, previous studies have repeatedly demonstrated lower exploratory behavior score in mouse schizophrenia models of perinatal insults (Shi et al., 2003; Smith et al., 2007; Meyer et al., 2008b; Lodge et al., 2009). Speculatively, the decreased activity that we observed in the adult offspring of influenza virus-infected mothers could be related to negative symptoms of schizophrenia. Earlier reports have shown that mice born to influenza virus-infected mothers spend significantly less time in the center of the squares (Shi et al., 2003). Here we found that the amount of time spent in the center of the open field was not affected by maternal influenza viral infection. These divergent results may be explicable by strain differences in exploratory behavior between CD1 and BALB/c mice.

Antenatal and postnatal environmental factors have profound implications on the behavior of the adult offspring (Meyer et al., 2009b). Maternal behavior also alters the offspring epigenome (Weaver et al., 2004). Thus, litter effects are substantial, and their outcome might not always be independent of the effects of other factors, including maternal viral infection. To avoid litter effects, our experiments included animals originating from at least three different litters (see Materials and Methods). Although the possibility of litter effects cannot be firmly excluded (Meyer et al., 2009b), the use of offspring from different mothers, together with the association between biochemical and behavioral results obtained in mice from different litters, suggests that the results described here are consistent with the hypothesis that maternal viral infection affects the level of expression and function of 5-HT_{2A} and mGlu₂ receptors.

It has been suggested that prenatal exposure to maternal stress increases the risk of schizophrenia in the offspring (Khashan et al., 2008; Malaspina et al., 2008). In rodents, prenatal stress has been shown to induce learning deficits (Lemaire et al., 2000), as well as alterations in the immune system (Vanbesien-Mailliot et al., 2007) and feeding behavior (Pankevich et al., 2009). During shipping, animals are likely to be exposed to noise and vibrations,

as well as temperature, humidity, and light–dark changes. Shipping might be then a stressful experience because many of these factors are actually used as independent variables in stress research. In our experiments, timed pregnant mice were shipped on the fifth day of gestation. Although confounding effects from other sources cannot be excluded, mice to be mock- and influenza virus-infected were shipped under the same conditions to minimize a possible bias due to maternal stress.

Some researchers have suggested that maternal infection during pregnancy produces behavioral changes in the offspring that include increased MK-801-induced locomotion (Meyer and Feldon, 2009). We found that the locomotor activity elicited by MK-801 in mice born to influenza virus-infected mothers and control mice was indistinguishable. These differences may be due to the dose of MK-801 used. The MK-801-stimulated locomotor activity was significantly attenuated by the glutamate antipsychotic LY379268 in control mice, but it was not affected in the offspring of influenza virus-infected mothers. In concordance with these effects of maternal viral infection in our mouse behavior models, the density of mGlu₂ was reduced in frontal cortex of mice born to influenza virus-infected mothers.

It has been shown that a low dose of the hallucinogen DOI increases locomotor activity, an effect that is absent in 5-HT_{2A} knock-out mice (Halberstadt et al., 2009). In contrast, a high dose of DOI decreases the locomotor activity, an effect that is blocked by a 5-HT_{2C} selective antagonist (Halberstadt et al., 2009). These findings suggest that 5-HT_{2A} and 5-HT_{2C} receptors exert opposite effects on locomotor activity in mice, and they propose the involvement of 5-HT_{2A} receptor in the locomotor-stimulating effects of DOI. We examined the locomotor activity induced by DOI in mice born to influenza virus-infected mothers and controls. We found that the locomotor activity was decreased by DOI in mice born to mock-infected mothers, whereas it was increased by DOI in mice with prenatal exposure to maternal influenza viral infection. Our data also demonstrate an increased head-twitch response to DOI in mice born to influenza virus-infected mothers (see above). Regulation of *c-fos*, *egr-1*, and *egr-2* robustly predicts the head-twitch behavioral response induced by hallucinogenic drugs (González-Maeso et al., 2007). In the present study, we found that the dose-dependent cellular response induced by the hallucinogenic 5-HT_{2A} receptor agonist DOI was significantly different in the adult offspring, with a higher cellular response to DOI in mice born to influenza virus-infected mothers. Concurrently, these cellular and behavioral responses correlate with the higher density of 5-HT_{2A} receptors in mice prenatally exposed to maternal viral infection.

Several laboratories suggest that schizophrenia is a result of neurodevelopmental defects triggered by cytokine-related inflammatory events (Kronfol and Remick, 2000; Brown et al., 2004b; Meyer et al., 2009a; Patterson, 2009). Thus, cytokines and chemokines such as IL-6, CXCL8 (IL-8), IL-10, and TNF- α (Brown et al., 2004b; Meyer et al., 2006; Smith et al., 2007; Meyer et al., 2008b) have been proposed as responsible for pathways whereby maternal immune activation induces schizophrenia-like behaviors in the adult offspring. Influenza virus infections trigger a profound cytokine and chemokine response at both local and systemic levels, more pronounced with virus strains that appear to be more pathogenic (Kobasa et al., 2007). Further investigation will be necessary to unravel the role, if any, of cytokines and chemokines on the abnormal behavioral function of the 5-HT_{2A}-mGlu₂ receptor complex in schizophrenia murine models of maternal viral infection.

In humans, the onset of schizophrenia typically occurs during late adolescence or early adulthood. Notably, our results demonstrate that alterations in the head-twitch behavioral response are only observed after puberty in prenatally infected mice—a finding that parallels the adult onset of the disease in humans and supports our mouse schizophrenia model of perinatal insult. The antipsychotics currently available are designed only to treat the symptoms of schizophrenia, and not the underlying causes of the disease. Because of this, schizophrenia remains an incurable illness, and treatment of the symptoms with the available antipsychotics typically continues for life. The prevention of psychotic disorders is therefore a worthwhile goal. Remarkably, a prodromal state commonly precedes the acute phase of a first psychotic episode. Recent findings suggest that it is possible to identify young patients at imminent risk of psychosis. Unfortunately, results of several clinical trials that have been published recently demonstrate that the available anti-schizophrenia drugs do not delay or prevent conversion to psychosis in subjects with prodromal symptoms of schizophrenia (McGlashan et al., 2006; Cannon et al., 2008; Phillips et al., 2009). On the other hand, experiments in rodents suggest that prepubertal treatment with atypical antipsychotics such as clozapine and risperidone prevents postpubertal schizophrenia-like alterations induced by prenatal exposure to maternal immune challenge (Piontkewitz et al., 2009; Meyer et al., 2010; Piontkewitz et al., 2010). Our findings may provide a better understanding of the biochemical alterations responsible for the behavioral abnormalities observed in mouse models of prenatal insults, and may lead to the identification of new therapeutic approaches for not only treatment, but also prevention of schizophrenia and other neurodevelopmental psychiatric conditions.

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