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The Effects of Prenatal H1N1 Infection at E16 on FMRP, Glutamate, GABA, and Reelin Signaling Systems in Developing Murine Cerebellum

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Abstract

Prenatal viral infection has been identified as a potential risk factor for the development of neurodevelopmental disorders such as schizophrenia and autism. Additionally, dysfunction in gamma-aminobutyric acid (GABA), Reelin, fragile X mental retardation protein (FMRP)-metabotropic glutamate receptor 5 (mGluR5) signaling systems has also been demonstrated in these two disorders. In the current report, we have characterized the developmental profiles of selected markers for these systems in cerebella of mice born to pregnant mice infected with human influenza (H1N1) virus on embryonic day 16 (E16) or sham-infected controls using SDS-PAGE and western blotting techniques and evaluated the presence of abnormalities in the above-mentioned markers during brain development. The cerebellum was selected due to emerging evidence that it plays roles in learning, memory, and emotional processing – all of which are disrupted in autism and schizophrenia. We identified unique patterns of gene and protein expression at birth [postnatal day 0 (P0)] childhood (P14), adolescence (P35), and young adulthood (P56) in both exposed and control mouse progeny. We also identified significant differences in protein expression for FMRP, very low density lipoprotein receptor (VLDLR), and glutamic acid decarboxylase 65 and 67 kDa proteins at specific postnatal timepoints in cerebella of the offspring of exposed mice. Our results provide evidence of disrupted FMRP, glutamatergic,

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Conflict of Interest

All authors declare that they have no conflicts of interest.

Role of Authors

Author SHF designed the study and supervised all aspects of the experiments and writing of the manuscript. Authors SBL, REK, and MKY performed western blotting experiments and analyzed data. Author TDF performed mouse infections and brain dissections, performed literature searches, and contributed to the writing of the manuscript.

and Reelin signaling in the exposed mouse offspring which *explains* the multiple brain abnormalities observed in this animal model.

Graphical Abstract

We investigated expression of Reelin, FMRP, and GABAergic signaling molecules in the offspring of mice exposed to influenza virus on day 16 of pregnancy vs. those who were sham infected. We identified changes for four proteins (FMRP, VLDLR, GAD65, and GAD67) in cerebella of exposed offspring on specific postnatal dates.

Keywords

FMRP; Reelin; GABA; cerebellum; mouse; influenza

Introduction

Brain disorders such as schizophrenia and autism are hypothesized to have genetic and environmental etiologies (Atladóttir et al., 2010; Fatemi, 2005a; Kneeland and Fatemi, 2013; Nielsen et al., 2015, 2016). An important environmental risk factor for the development of brain dysfunctions relevant to these neurodevelopmental disorders is maternal infection during critical stages of pregnancy (Boksa, 2003; Brown, 2006; Brown and Susser, 2002; Fatemi, 2008; Fatemi et al., 2002a; Patterson, 2007). Our laboratory has demonstrated that infection of pregnant mice with a mouse-adapted human influenza virus [A/NWS/33 (H1N1)] on embryonic days 7 (E7), E9, E16, and E18 results in abnormal expression of brain genes, altered brain structure, neurochemical changes, and behavioral deficits in the offspring of exposed mice (Fatemi et al., 1999, 2002a, 2005a, 2008a,b,c, 2009a,b, 2012a; Shi et al., 2003; Winter et al., 2008). These results show concordance with some of the brain biochemical, structural, and behavioral indices observed in subjects with schizophrenia or autism (Fatemi, 2005a; Meyer et al., 2009, 2011; Palmen et al., 2004).

Prenatal viral infection may lead to disturbances in key signaling systems associated with brain development. In autism, schizophrenia and mood disorders, there is ample evidence of dysfunction of multiple signaling systems including gamma-aminobutyric acid (GABA)ergic (Beneyto et al., 2011; Blatt et al., 2001; Bullock et al., 2008; Fatemi et al., 2009c,d, 2010a; 2011a; 2013a,b; Glausier and Lewis, 2011; Guptill et al., 2007; Ohnuma et al., 1999; Samaco et al., 2005; Volk et al., 2002), glutamatergic (Breese et al., 1995; Fatemi and Folsom, 2011; Fatemi et al., 2011b, 2013a), fragile X mental retardation (FMRP)-metabotropic glutamate receptor 5 (mGluR5) (Fatemi and Folsom, 2011; Fatemi et al., 2010, 2011, 2013a,c; Folsom et al., 2015; Kovács et al., 2013; Kelemen et al., 2013), and Reelin (Fatemi et al., 2001, 2005b,c; Guidotti et al., 2000; Impagnatiello et al., 1998; Keller et al., 2000; Lugli et al., 2003). These signaling systems are interconnected with each other, playing important roles during development including formation of correct lamination of the

brain and neuronal migration (Reelin), proper excitatory/inhibitory signaling (glutamatergic/GABAergic), and regulation of protein translation (FMRP). Impairments in one system could impact other systems ultimately resulting in deficits associated with major psychiatric disorders (Estes and McAllister, 2016).

Previous data from our laboratory demonstrated that Reelin immunoreactivity was reduced in brains of the offspring of exposed mice (Fatemi et al., 1999). Additionally, we demonstrated reduced Reelin expression in brains of subjects with autism and schizophrenia (Fatemi et al., 2000, 2001, 2005b,c), two neurodevelopmental disorders which are successfully modeled by this mouse model of prenatal viral infection. Similar to Reelin, previous data from our laboratory demonstrated altered expression of GAD65/67 kDa and GABA_A and GABA_B receptor subunits in brains of subjects with autism and schizophrenia (Fatemi et al., 2002b, 2005c, 2009c,d, 2010a, 2013a,b, 2014), prompting us to determine whether these molecules also display an altered expression in the brains of the offspring of exposed mice. The GABAergic and FMRP-mGluR5 signaling systems are also intertwined. FMRP downregulation in animal models of fragile X syndrome has been accompanied by reduced expression of multiple GABA_A receptor subunits (Adusei et al., 2010; D'Hulst et al., 2006; El Idrissi et al., 2005; Gantois et al., 2006; Hong et al., 2012) and FMRP has been shown to co-localize with GABA_A receptors (Frederikse et al., 2015).

An important but neglected site of synaptic and memory-related tasks is the cerebellum. Several positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) studies have demonstrated activation of the cerebellum during tasks that measure attention, spoken and written language, verbal working memory, spatial and episodic memory, associative learning, and problem solving, providing strong evidence that cerebellum is involved in a wide variety of cognitive processes (reviewed by Cabeza and Nyberg, 2000; Segarra et al., 2008; Strata, 2015). It is therefore not surprising that the cerebellum has been implicated in multiple psychiatric disorders including schizophrenia and autism. Indeed, multiple brain morphological abnormalities of the cerebellum have been identified in both disorders (Bailey et al., 1998a; Courchesne et al., 1988; Fatemi et al., 2012b; Kyriakopoulos et al., 2008; Segarra et al., 2008; Varnäs et al., 2007; Whitney et al., 2008).

In the current paper-review hybrid, we characterized postnatal developmental profiles of markers for GABAergic, FMRP, and Reelin signaling in postnatal mouse cerebella and review the model of prenatal viral infection that has been developed in our laboratory. Previous reports from multiple laboratories have evaluated mRNA profiles for Reelin, Fmr1, glutamate and GABA_A receptor subunits in normal rodent brain (Catania et al., 1994; Chang et al., 1995; Laurie et al., 1992; Liu and Wong-Ridley, 2006; Lu et al., 2004; Muñoz et al., 1999; Popp et al., 2009; Richard et al., 2008; Singh and Prasad, 2008; Temple and Wray, 2005; Zdilar et al., 1992). We sought to verify and correlate these earlier reports with protein data. We also evaluated the effects of prenatal human influenza viral infection on pregnant mice infected on embryonic day 16 (E16) on cerebellar protein expression. We hypothesized that prenatal human influenza viral infection on E16 would result in altered expression of GABA_A receptor subunits, FMRP, mGluR5, and Reelin markers in the offspring of infected dams vs. controls.

Materials and Methods

Viral Infection

All study procedures involving animals were approved by the University of Minnesota Institutional Animal Care Use Committee (IACUC). Timed pregnant C57BL/6 mice (6–8 week old, Charles River Laboratories) were randomized [1:1 H1N1 vs. minimum essential medium (MEM)] and either infected on E16 with a sublethal dose of influenza A/NWS/33 (H1N1) virus or sham-infected using vehicle solution (MEM) intranasally as previously described (Fatemi et al., 2009a,b). The average weight for the infected mice was 31.2 ± 3.10 grams ($n=16$) and the average weight for the sham-infected mice was 32.4 ± 2.37 grams ($n=16$). An A/NWS/33 virus pool was prepared in Maden Darby canine kidney (MDCK) cells. Virus was contained in an ampule and stored in a -80°C freezer until the time of infection. Using the method of Reed and Muench (1938), data were expressed as $\log_{10}$ cell culture infectious doses (CCID_{50})/ml. Previous work by our laboratory has determined that a dilution of $10^{-4.5}$ did not cause death in mice as a result of infection but did produce lung consolidation scores and mean lung weights that are consistent with infection. The lungs had a mean virus titer of $10^{5.25}$ CCID_{50} /ml, which showed that a moderate, sublethal infection had been induced (Fatemi et al., 2002a, 2008a). Thus, this viral dose was employed for the present mouse study. Pregnant mice were allowed to deliver pups and the day of delivery was considered postnatal day zero (P0).

Brain Collection and Dissection

Male offspring (only male mice were used to avoid hormonal effects which could confound the experimental data) of the infected and sham-infected mice were collected at P0, P14, P35, and P56, deeply anesthetized with ketamine (200 mg/kg) and nembutal (60 mg/kg), and sacrificed. Cerebella were identified and dissected as previously described (Fatemi et al., 2008a,b,c, 2009a,b, 2012a) using coronal images from a stereotaxic mouse brain atlas (Franklin and Paxinos, 1997). The following coordinates were used: starting at -1.40 mm to -3.68 mm from interaural line and from -5.20 mm to -7.48 mm from bregma. Sagittally, the cerebellum was identified using the boundary between the brain stem and lateral recess of the 4th ventricle to the anterolateral edge. Whole cerebellum was dissected and flash frozen in liquid nitrogen and stored at -80°C for future assays.

Antibody Characterization

The antibodies used in the current study are listed in Table 1, which summarizes their immunogenicity, antibody information (company, product number, and source), the concentration used, and a reference, where possible. With the exception of anti-very low density lipoprotein receptor (VLDLR) and anti-GABA_A beta 2/3, we have published results for all of the antibodies used in the current study that establish their specificity (Fatemi et al., 2002b, 2005b,c, 2009c, 2010a,b, 2011b, 2013b,c; Folsom et al., 2015). Four antibodies that were used are currently listed in the Journal of Comparative Neurology (JCN) database volume 14: anti-GABA_A receptor alpha 1, anti-Reelin, anti-glutamic acid decarboxylase 65 and 67 kDa, and β -actin antibody.

SDS-PAGE and Western Blotting

Brain tissue was prepared and SDS-PAGE and western blotting was carried out as previously described (Fatemi et al., 2008a,b,c, 2009a,b, 2011b). The blots were incubated overnight at 4°C with the primary antibodies listed in Table 1. Blots were subsequently washed using phosphate buffered saline with 0.03% Tween-20 (PBST), then incubated in a medium containing anti-mouse (Sigma Aldrich, Cat# A9044, RRID: AB_258431) or anti-rabbit (Sigma Aldrich, Cat# A9169, RRID: AB_258434) secondary antibodies for one hour at room temperature (IgG, Sigma Aldrich, St. Louis, MO, 1:80 000). Later, blots were again washed twice using PBST and the immune complexes were visualized using the ECL Plus detection system (G.E. Healthcare, Little Chalfont, Buckinghamshire, UK). All experiments were performed blind to the nature of the experimental group.

Sample densities were also analyzed blind to the nature of treatment using a BioRad densitometer and the BioRad Multi Analyst software. The molecular weights of approximately 410 kDa, 330 kDa, 180 kDa (Reelin); 224 kDa and 112 kDa (dimer and monomer species of mGluR5, respectively); 143 kDa (VLDLR); 71–76 kDa (FMRP); 67 kDa (GAD67); 65 kDa (GAD65); 54 kDa GABR β 2/3 (upper band), GABR β 3); 52 kDa (GABR α 5); 51 kDa (GABR α 1, GABA α 2, GABR β 2/3 (lower band) and GABR δ); 46 kDa (NSE); 45 kDa (homer 1); 42 kDa (β -actin); and 21 kDa (RAC1) immunoreactive bands were quantified with background subtraction. Results obtained are based on at least two independent experiments with at least N = 3–4 infected and N = 3–4 control mice per gel. For each experiment, samples from exposed and control offspring were run on the same gel and processed simultaneously to avoid variability due to intragel differences.

Statistical Analysis

All protein measurements for infected and control mice were normalized against β -actin and NSE. We used SPSS package for statistical analyses. Sample size for each group varied from 3–8 depending on postnatal time point. A series of 4×2 (time by group) analysis of variance were conducted to test for time, group and group by time effects. In the event of significant findings, follow-up student's t-tests were performed with significance set at $p < 0.05$.

Results

The viral hypothesis of schizophrenia and autism is based on both epidemiologic and experimental evidence (Estes and McAllister, 2016; Kneeland and Fatemi, 2013; Meyer et al. 2009, 2011) that immunologic and inflammatory mechanisms may be responsible for some of the behavioral deficits present in both disorders. As previous work by us and others have pointed to several brain markers such as Reelin (Fatemi et al., 2001, 2005b,c; Guidotti et al., 2000; Impagnatiello et al., 1998; Keller et al., 2000; Lugli et al., 2003), GABA receptors (Beneyto et al., 2011; Blatt et al., 2001; Bullock et al., 2008; Fatemi et al., 2009c,d, 2010a; 2011a; 2013a,b; Glausier and Lewis, 2011; Guptill et al., 2007; Ohnuma et al., 1999; Samaco et al., 2005; Volk et al., 2002), and FMRP (Fatemi and Folsom, 2011; Fatemi et al., 2010b, 2011b, 2013a,c; Folsom et al., 2015; Kóvacs et al., 2013; Kelemen et al., 2013) we hypothesized that these proteins may also be involved in E16 infected

offspring. This premise is the main justification for our choice of these markers in the current study.

All protein values discussed in this study were normalized against β -actin and are shown as ratios of the various proteins to β -actin in all four time points: P0, P14, P35, P56. Additionally, all protein values were also normalized against NSE, with the results mirroring those obtained against β -actin (data not shown). NSE was chosen because it is specific for neuronal cells and significant differences in NSE expression would indicate changes in neuronal cell population.

A series of 4×2 (time by group) analysis of variance (ANOVA) were conducted to test for time, group and group by time effects. Significant group effects were found for FMRP/ β -actin [$F_{1,23}=35.29$, $n=24$, $P=0.001$], GAD67/ β -actin [$F_{1,20}=6.67$, $n=21$, $P=0.017$] and GAD65/ β -actin [$F_{1,20}=4.41$, $n=21$, $P=0.049$]. There was also a significant group by time effect for VLDLR/ β -actin [$F_{3,25}=3.67$, $n=26$, $P=0.032$] and FRMP/ β -actin [$F_{3,23}=4.87$, $n=24$, $P=0.009$].

Follow up student's t-tests were performed for FMRP/ β -actin, GAD67/ β -actin, GAD65/ β -actin, and VLDLR/ β -actin. FMRP levels were significantly higher in cerebella of the offspring of exposed mice at P0 [$t_6=3.47$, $P=0.013$] and P14 [$t_5=7.12$, $P=0.001$] when compared with sham-infected mice (Figures 1 and 2). FMRP levels were not significantly different at P35 or P56. GAD65 and GAD67 are the rate-limiting enzymes converting glutamate to GABA. At P35 there were significantly higher levels of GAD65/ β -actin [$t_5=4.30$, $P=0.008$] as well as for GAD67/ β -actin [$t_5=4.00$, $P=0.01$] in cerebella of the offspring of exposed mice (Figures 1 and 3). Finally, we identified significantly lower values of VLDLR/ β -actin in cerebella of the offspring of exposed mice at P14 [$t_4=1.90$, $P=0.013$] while at P56 higher values of VLDLR/ β -actin were observed in cerebella of the offspring of exposed mice [$t_5=2.71$, $P=0.042$] (Figures 1 and 3).

Additionally, protein values for mGluR5, homer 1, Reelin, and GABA_A receptors did not vary significantly between the two experimental groups (Figures 2, 3, and 4). Finally, neither β -actin, nor NSE showed significant differences between infected and sham-infected offspring at any of the postnatal time points (Figures 2 and 4).

Microarray experimental results involving cerebella from the offspring of exposed and control mothers have previously been published (Fatemi et al., 2009b). Table 2 summarizes important significantly altered genes (fold change of at least 1.5, $p<0.05$) associated with glutamatergic and GABAergic signaling, synaptic transmission, cell migration and myelination processes..

Discussion

Prenatal human influenza viral infection with a sublethal dose of H1N1 on embryonic day 16 resulted in several significant changes in levels of multiple excitatory and inhibitory genes and proteins in cerebellum of H1N1 exposed progeny as follows: 1) FMRP protein levels increased significantly at P0 and P14; 2) GAD65 and 67 kDa proteins increased significantly at P35; 3) several glutamatergic receptor genes, i.e., glutamate receptor,

ionotropic, AMPA1 (alpha 1) (Gria1), glutamate receptor, ionotropic, AMPA3 (alpha 3) (Gria3), glutamate receptor, ionotropic, AMPA4 (alpha 4) (Gria4), glutamate receptor, ionotropic, delta 2 (Grid2), glutamate receptor, ionotropic, kainate 2 (beta 2) (Grik2) increased significantly at P56 (Table 2; Fatemi et al., 2009b); 4) glutamate receptor, ionotropic, kainate 5 (gamma 2) (Grik5) and glutamate receptor, ionotropic, kainate 1 (Grik1) genes were significantly downregulated at P0 and P56 periods, respectively (Table 2; Fatemi et al., 2009b); 5) level of GABA_A receptor alpha 1 gene increased significantly at P56 (Table 2; Fatemi et al., 2009b); 6) protein level of Reelin receptor VLDLR decreased significantly at P14; and finally, 7) gene and protein levels for VLDLR increased significantly and concordantly at P56 (Table 2; Fatemi et al., 2009b). Additionally, the lack of statistical differences for levels of β -actin and NSE proteins at all postnatal dates in both experimental groups is suggestive of absence of neuronal or glial cell number changes in cerebella of the offspring of exposed mice.

FMRP is an mRNA binding protein that regulates protein translation, usually as a suppressor. It is estimated to bind 5% of mRNA in the mammalian brain (Darnell and Klann, 2013) and is known for its role in synaptic plasticity, learning, and memory (Deng et al., 2011). The loss of FMRP expression results in the cognitive and behavioral deficits associated with fragile X syndrome (FXS) (Oostra and Willemsen, 2009). FXS and autism share several commonalities including reduced cerebellar volume, altered dendritic spine morphology, presence of seizures and mental retardation (Bailey et al., 1998b; Gothelf et al., 2008; Hallahan et al., 2009; Hatton et al., 2006; Hutsler and Zhang, 2010; Irwin et al., 2002). The rate of FXS in subjects with autism varies from 2–8% (Chudley et al., 1998; Wassink et al., 2001). The prevalence for autism in subjects that have FXS has been estimated at anywhere from 25–47% (Bailey et al., 1998b; Hatton et al., 2006; Kauffman et al., 2004). Our laboratory has recently documented reductions in FMRP protein levels in frontal cortex and cerebellar vermis of adults with autism (Fatemi and Folsom, 2011; Fatemi et al., 2011b), in lateral cerebella of adults with schizophrenia, bipolar disorder, and major depression and superior frontal cortex of subjects with schizophrenia and bipolar disorder (Fatemi et al., 2010b; 2013a) (Table 3). The observed significant increases in FMRP expression at P0 and P14 in cerebella of the offspring of H1N1-exposed mice do not correlate with levels of FMRP in autism, although we have identified reduced expression of phosphorylated FMRP in cerebellar vermis of children with autism (Rustan et al., 2013). Interestingly, GABA_B receptor activation in cultured mouse cerebellar granule neurons increased the levels of FMRP protein (Zhang et al., 2015). The authors suggested two mechanisms for this increase in FMRP levels: 1) via transactivation of insulin-like growth factor 1 receptor; and 2) via activation of protein kinase C. Additionally, Lugo et al. (2013) reported increased levels of total and phosphorylated FMRP in hippocampus of neuron subset-specific phosphatase and tensin homolog detected on chromosome 10 (PTEN) conditional knockout mice, presumably via involvement of phosphatidylinositol 3-kinase/AKT pathway. This increase in FMRP expression appears to be part of a cellular survival mechanism in response to cellular stress (Jeon et al., 2011). Indeed, as shown by Jeon et al. (2012) the provisional role of AKT-dependent upregulation of FMRP in glutamate stimulated cultured neurons as well as in ischemic brain may have relevance to traumatic brain injury, neurodegenerative disorders, and especially to brain developmental

abnormalities following prenatal exposure to H1N1 or other viruses. Finally, microarray data following infection at E16 demonstrated that two downstream FMRP targets – amyloid beta precursor protein (APP) and homer 1 – displayed significant changes in expression (Fatemi et al., 2009a). APP displayed reduced expression at P0 in hippocampus while homer 1 displayed reduced expression in hippocampus at P56 and at P14 in prefrontal cortex (PFC) of the offspring of exposed mice (Fatemi et al., 2009a).

Glutamic acid decarboxylase 65 and 67 kDa proteins (GAD65/67) are the rate limiting enzymes for the conversion of glutamate to GABA. Similar to what is reported here, infection of BALB/c mice at E9, resulted in significantly increased protein expression of both GAD65 and GAD67 at P35; increased expression of GAD65 protein at P14; and increased expression of GAD67 protein at P56 in the offspring of exposed mice (Fatemi et al., 2004). A previous study employing qRT-PCR, similarly found a pattern of increased expression of GAD65/67 in rat cerebella through postnatal development (Popp et al., 2009). Further evidence of glutamatergic dysfunction is suggested by the number of glutamate receptor genes that are upregulated at P56, i.e., Gria1, Gria3, Gria4, Grid2 and Grik2 in the cerebella of offspring of exposed mice (Table 2). Interestingly, only a single GABA_A receptor (α 1) is upregulated significantly at P56 (Table 2). Moreover, several glutamate receptor genes are downregulated such as Grik5 at P0 and Grik1 at P56, respectively (Table 2).

GABA is the major inhibitory neurotransmitter in the brain. GABA_A receptors are ligand-gated chloride channels consisting of five subunits and are responsible for mediating fast synaptic inhibition (Farrant and Nusser, 2005). The expression patterns we observed for GABA_A receptor subunits α 1, α 2, β 2, β 3, and δ in the control mouse cerebellum are supported by previous results in various normal rodent brain regions (Chang et al., 1995; Laurie et al., 1992; Liu and Wong-Riley, 2006; Temple and Wray, 2005; Zdilar et al., 1992). However, in contrast to our data, Laurie et al. (1992) found little to no signal for the α 5 subunit in rat cerebellum. While we did not observe significant changes in protein levels in the cerebella of the offspring of exposed mice, we have observed changes in mRNA expression for some of the receptors as measured by microarray following prenatal viral infection on E9, E16, and E18 (Fatemi et al., 2008a,b,c, 2009a,b). Following infection at E9, we observed increased mRNA expression of GABR α 2 at P0 in PFC of the offspring of exposed mice and increased mRNA for GABR α 5 at P56 in cerebellum of the offspring of exposed mice (Fatemi et al., 2008b,c). Infection at E16 resulted in reduced expression of mRNA for GABR α 2 at P0 in hippocampus and increased mRNA expression of GABR α 1 in cerebellum of the offspring of exposed mice (Fatemi et al., 2009a,b). Finally, infection at E18 resulted in increased mRNA expression of GABR α 1 in hippocampi of exposed mice at P14. These changes indicate that time of infection may impact which GABA receptors display altered expression and at which brain site, e.g. frontal vs. hippocampal vs. cerebellar cortices.

Reelin glycoprotein is a large extracellular matrix protein composed of several proteolytic fragments and is present in brain and other locations including pituitary, liver, and adrenal chromaffin cells (Fatemi, 2005a). During brain development, Reelin glycoprotein plays key roles in guiding neuronal migration and mediating cortical lamination in an orderly fashion

(Fatemi, 2005b). Changes in the level of this protein, its receptors (such as VLDLR), or downstream signaling molecules are likely to result in abnormal corticogenesis. In adult brain, Reelin helps to mediate synaptogenesis and also plays a direct role on enhancement of long-term potentiation (LTP) in hippocampus (Weeber et al., 2002). Decreases in Reelin expression have previously been shown using this model following infection on E9 (Fatemi et al., 1999). Similar results have been observed in brains of the offspring of exposed mice treated with the viral mimic polyriboinosinic-polyribocytidilic acid (PolyI:C) at E9 and E17 (Meyer et al. 2008), suggesting that Reelin reduction results from a variety of prenatal insults at various time points. While we did not observe a significant group effect for Reelin at E16 (despite a significant decrease in Reelin 180 which did not survive the post hoc analysis), the increased expression of VLDLR at P56 provides further evidence of disruption of the Reelin signaling system following prenatal viral infection. Interestingly, at E16, microarray data revealed increased expression of disabled 1 (Dab1), a downstream Reelin signaling molecule, in hippocampus of the offspring of exposed mice (Fatemi et al., 2009a). In BA9 and cerebella of subjects with autism, we have previously observed reduced expression of Reelin mRNA, accompanied by increased expression of VLDLR mRNA (Fatemi et al., 2005c) (Table 3), which may be a compensatory response to reduced presence of its ligand, Reelin (Fatemi et al., 2005c) (Table 3).

Prenatal viral infection provides an important model for studying neurodevelopmental disorders, particularly autism and schizophrenia. Many of these changes are dependent on the time point of infection. Our model has identified behavioral abnormalities relevant to schizophrenia and autism including reduced prepulse inhibition of the acoustic startle response (PPI) and impaired social interaction in the offspring of dams infected at E9 (Shi et al., 2003). Dysregulation of neurotransmitter levels has also been established. When compared to controls, we identified reduced serotonin levels in the cerebella of virally-exposed mice at P14 (Winter et al., 2008). Following infection at E18, the offspring of exposed mice displayed significant reductions in serotonin and its primary metabolite 5-hydroxyindoleacetic acid (5-HIAA) at P14 and significant reductions in serotonin and taurine at P35 when compared with controls (Fatemi et al., 2008a).

Brain structural alterations have also been observed including overall changes in brain volume and ventricular area, altered volume of cerebellum and hippocampus, increased pyramidal cell density, and disorganized white matter as measured by fractional anisotropy (Fatemi et al., 1999; 2008a, 2009a,b, 2012a). Specifically, with regard to the cerebellum, overall reductions in cerebellar volume were identified at P14 following infection at E16 and at P35 following infection at E18 (Fatemi et al., 2008a, 2009a).

Microarray analyses have revealed gene expression changes that are dependent on the date of prenatal viral infection. In Balb/c mice that were infected on E9, there was significant at least two-fold change in 205 genes at P35 and 50 genes at P56 in cerebella of the offspring of exposed mice when compared with controls (Fatemi et al., 2008c). There was also a more pronounced effect of prenatal viral infection on cerebellar gene expression at E16 in C57BL/6J mice with 98 genes at P0, 219 at P14, and 653 at P56 displaying altered expression (Fatemi et al., 2009a,b). Infection at E18 resulted in changes in 157 genes at P0, 16 genes at P14, 96 genes at P56 (Fatemi et al., 2008a). Similar patterns of gene expression

changes were also observed in PFC and hippocampus of offspring of exposed mice (Figure 5).

This study contains a few limitations. First, the sample size is low (N ranging from 3–8 per group). Increasing sample size would have increased the power of our study. A second limitation is the use of only one brain area. Including multiple brain regions important to the pathology of neurodevelopmental disorders such as frontal cortex or hippocampus would have given a more accurate picture of potential protein expression abnormalities resulting from exposure to prenatal viral infection.

In conclusion, the prenatal viral infection model studied over a period of two decades in our laboratory and confirmed by a number of investigators recapitulates some of the neurochemical, brain structural, and behavioral deficits associated with schizophrenia and autism. While a direct role for transmission of viral effects directly through the placenta cannot be ascertained currently, it appears that maternal immune system, once activated due to viral infections, can have deleterious effects on fetal brain structure and function. Unfortunately, the implications of this effect can be identified through emergence of another neurotropic virus, e.g., Zika virus which has had a devastating impact on fetal brain development.

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Significance Statement

Our laboratory has developed an animal model of prenatal human influenza viral infection that recreates some of the behavioral, brain morphological, gene/protein expression, and neurochemical deficits observed in schizophrenia and autism. In the current manuscript, we investigated the effects of prenatal human influenza viral infection of pregnant mice at embryonic day 16 (E16) on GABAergic, Reelin, and FMRP signaling systems. We analyze our findings in the context of previous results using this model as well as findings by other laboratories.

Figure 1.

Representative samples of FMRP, GAD65/67, VLDLR, β -actin and NSE from cerebella of the offspring of control (C), sham-infected mice vs. the offspring of infected (I) mice at P0, P14, P35, and P56.

Figure 2. Developmental expression of FMRP (A), mGluR5 224 kDa (B), mGluR5 112 kDa (C), RAC1 (D), homer 1 (E), and (F) β-actin in cerebella of exposed and control mice from birth (P0) through young adulthood (P56). Data expressed as mean ± standard deviation. *, p < 0.05.

Figure 3. Developmental expression of Reelin 410 kDa (A), Reelin 330 kDa (B), Reelin 180 kDa (C), VLDLR (D), GAD65 (E), and GAD67 (F) in cerebella of exposed and control mice from birth (P0) through young adulthood (P56). Data expressed as mean $\pm$ standard deviation. *, $p < 0.05$.

Figure 4. Developmental expression of GABRA1 (A), GABRA2 (B), GABRA5 (C), GABRB3 (D), GABRB2/β3 51 kDa (E), GABRB2/β3 54 kDa (F), GABRD (G), and NSE (H) in cerebella of exposed and control mice from birth (P0) through young adulthood (P56). Data expressed as mean ± standard deviation.

Figure 5.

A comparison of the total number of brain genes that are significantly altered ($p < 0.05$, fold change of at least 1.5) in hippocampus and PFC of the offspring of exposed mice at P0 and P56 following prenatal H1N1 infection at E7, E9, E16, E18 (Fatemi et al., 2005, 2008a,b, 2009a, unpublished observations). (Note, value for E9-P0 is for whole brain, and value for E9-P56 is for PFC only). Reprinted from *Neuropharmacology*, 62(3), Fatemi SH, Folsom TD, Rooney RJ, Mori S, Kornfield TE, Reutiman TJ, Kneeland RE, Liesch SB, Hua K, Hsu J, Patel DH, The viral theory of schizophrenia revisited: abnormal placental gene expression and structural changes with lack of evidence for H1N1 viral presence in placentae of infected mice or brains of exposed offspring, pages 1290–1298, Copyright (2012) with permission from Elsevier.

Table 1

Primary Antibodies Used in the Current Study

Name	Immunogen/Epitope	Antibody information	Concentration	Band size	Reference
anti-GABA _A alpha 1	KLH-conjugated linear peptide corresponding to the topological domain of rat GABA _A Receptor α 1/Topological domain	06-868, EMD Millipore, rabbit, polyclonal, AB_310272	1:1000	51 kDa	Fatemi et al., 2009c, 2013b
anti-GABA _A alpha 2	12-aa peptide from rat GAA2; Designation (GAA21-P, control peptide/blocking peptide) conjugated to KLH	GAA21-A, Alpha Diagnostics International, rabbit, polyclonal, AB_1616429	1:1000	51 kDa	Fatemi et al., 2009c
anti-GABA _A alpha 5	Synthetic peptide corresponding to the middle region of Mouse GABA _A Receptor alpha 5 (NP_795916.1)	ab10098, Abcam, rabbit, polyclonal, AB_296840	1:500	52 kDa	Fatemi et al., 2010a
anti-GABA _A beta 2/3	Purified GABA/benzodiazepine receptor from bovine cortex/ Extracellular domain of the beta2 & beta3 subunits of the GABA _A receptor	MAB341, EMD Millipore, mouse, monoclonal, AB_2109419	1:1000	54 kDa and 51 kDa	
anti-GABA _A beta 3	Fusion protein: APMDVHNEMN EVAGSVGDTR NSAISFDNSG IQYRKQSMKP EGHGRYMGDR SIPHKTHLR RRSS, corresponding to amino acids 370–433 of Mouse GABA _A Receptor beta 3	ab98968, Abcam, mouse, monoclonal, AB_10670809	1:500	54 kDa	Fatemi et al., 2013b
anti-GABA _A delta	A synthesized peptide derived from an internal sequence of human GABA _A receptor delta	ab110014, Abcam, rabbit, polyclonal, AB_10861508	1:1000	51 kDa	Fatemi et al., 2013b
anti-FMRP	Fusion protein with a full length FMRP (human)	MAB2160, EMD Millipore, mouse, monoclonal, AB_2283007	1:500	71 kDa to 76 kDa	Fatemi et al., 2010b, 2011b
Anti-mGluR5	N-terminal synthetic peptide derived from human Metabotropic Glutamate Receptor 5	Ab53090, Abcam, rabbit, polyclonal, AB_881222	1:300	224 kDa and 112 kDa	Fatemi et al., 2011b, 2013a
anti-Reelin	Recombinant reelin amino acids 40–189/a.a. 164–189	MAB5366, EMD Millipore, mouse, monoclonal, AB_2285132	1:1000	410 kDa, 330 kDa, 180 kDa	Fatemi et al., 2005b,c
anti-GAD65/67	A synthetic peptide with the amino acid sequence [C]DFLIEEIERLGQDL from rat glutamate decarboxylase (GAD65; C-terminus residues [Cys] + 572–585)	AB1511, EMD Millipore, rabbit, polyclonal, AB_90715	1:3333	67 kDa and 65 kDa	Fatemi et al., 2002b, 2005b
anti-VLDLR	KLH-conjugated linear peptide corresponding to the C-terminus of human VLDL-R/C-terminus	MABS25, EMD Millipore, mouse, monoclonal, AB_10807542	1:1000	143 kDa	
anti-RAC1	Human RAC1	610651, BD Biosciences, mouse, monoclonal, AB_397978	1:500	21 kDa	Fatemi et al., 2013c; Folsom et al., 2015
anti-homer 1	Recombinant protein corresponding to full length human HOMER1	MAB4202, Abnova, mouse, monoclonal, AB_10554030	1:750	45 kDa	Fatemi et al., 2013c; Folsom et al., 2015
anti- β -actin	slightly modified β -cytoplasmic actin N-terminal peptide, Ac-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, conjugated to KLH	A5441, Sigma Aldrich, mouse, monoclonal, AB_476744	1:5000	42 kDa	Fatemi et al., 2013c; Folsom et al., 2015
anti-NSE	Recombinant full length protein purified from E. coli (Human)	ab16808, Abcam, mouse, monoclonal, AB_2099196	1:2000	46 kDa	Fatemi et al., 2013c; Folsom et al., 2015

Table 2

Classification of selected significantly altered cerebellar genes of interest following E16 infection

Symbol	Name	Date	Change	Function
Auts2	Autism 2	P56	↑	Cell migration
Dtna	dystrobrevin alpha	P56	↑	Cell communication; signal transduction
Dtnb	dystrobrevin, beta	P56	↑	Cell growth and/or maintenance
Gabra1	gamma-aminobutyric acid (GABA-A) receptor, subunit alpha 1	P56	↑	GABAergic signaling
Gfap	Glial fibrillary acidic protein	P14	↑	Cell growth and/or maintenance
Gria1	glutamate receptor, ionotropic, AMPA1 (alpha 1)	P56	↑	Glutamatergic signaling
Gria3	glutamate receptor, ionotropic, AMPA3 (alpha 3)	P56	↑	Glutamatergic signaling
Gria4	glutamate receptor, ionotropic, AMPA4 (alpha 4)	P56	↑	Glutamatergic signaling
Grid2	glutamate receptor, ionotropic, delta 2	P56	↑	Glutamatergic signaling
Grik1	glutamate receptor, ionotropic, kainate 1	P56	↓	Glutamatergic signaling
Grik2	glutamate receptor, ionotropic, kainate 2 (beta 2)	P56	↑	Glutamatergic signaling
Grik5	glutamate receptor, ionotropic, kainate 5 (gamma 2)	P0	↓	Glutamatergic signaling
Mbp	Myelin basic protein	P14, P56	↑, ↓	Myelination
Mobp	myelin-associated oligodendrocytic basic protein	P56	↓	Myelination
Mog	myelin oligodendrocyte glycoprotein	P56	↓	Myelination
Ncam1	neural cell adhesion molecule 1	P56	↓	Cell communication; signal transduction
Nrxn1	neurexin I	P56	↑	Synaptic transmission
Nrxn2	neurexin II	P56	↑	Synaptic transmission
Negr1	neuronal growth regulator 1	P56	↑	Cell growth and/or maintenance
Nos1	nitric oxide synthase 1, neuronal	P56	↑	Metabolism; Energy Pathways
Ptp1	proteolipid protein 1	P56	↓	Myelination
Rgs4	regulator of G-protein signaling 4	P56	↓	Cell communication; signal transduction
Sdc1	syndecan 1	P56	↑	Synaptic transmission
Sdc2	syndecan 2	P56	↓	Synaptic transmission
Sdcbp	syndecan binding protein	P56	↑	Cell communication; signal transduction
Sstr4	somatostatin receptor 4	P56	↓	Cell communication; signal transduction
Stx18	Syntaxin 18	P56	↓	Synaptic transmission
Stxbp2	syntaxin binding protein 2	P14, P56	↑, ↑	Synaptic transmission
Syne1	synaptic nuclear envelope 1	P56	↑	Cell growth and/or maintenance
Synj2	synaptojanin 2	P14	↑	Synaptic transmission
Sypl	synaptophysin-like protein	P14	↑	Synaptic transmission
Syt12	synaptotagmin-like 2	P14	↑	Synaptic transmission
Vldlr	Very low density lipoprotein receptor	P56	↑	Cell migration (Reelin receptor)

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Table 3

Comparison of cerebellar protein findings following viral model on E16 vs. major psychiatric disorders

	Viral Model	Schizophrenia	Bipolar Disorder	Major Depression	Autism
Reelin 410 kDa	--	--	↓	--	↓
Reelin 330 kDa	--	--	--	--	↓
Reelin 180 kDa	--	↓	↓	--	↓
VLDLR	↓P14, ↑P56	NA	NA	NA	↑ mRNA
GAD 65 kDa	↑P35	↓	↓	↓	↓
GAD 67 kDa	↑P35	↓	↓	↓	--
FMRP	↑P0, P14	↓	↓	↓	↓
mGluR5	--	↓	↓	--	↑
RAC1	--	--	↑	--	↑
Homer 1	--	--	--	--	↓
GABRα1	--	--	--	↑	↓
GABRα2	--	↑	↑	↑	--
GABRα5	--	--	--	--	--
GABRβ2/3	--	NA	NA	NA	N/A
GABRβ3	--	--	--	--	↓
GABRδ	--	--	--	--	--

NA, not available; ↑, increase; ↓ decrease; --, no change