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Viral regulation of aquaporin 4, connexin 43, microcephalin and nucleolin

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Abstract

The current study investigated whether human influenza viral infection in midpregnancy leads to alterations in proteins involved in brain development. Human influenza viral infection was administered to E9 pregnant Balb/c mice. Brains of control and virally exposed littermates were subjected to microarray analysis, SDS-PAGE and western blotting at three postnatal stages. Microarray analysis of virally-exposed mouse brains showed significant, two-fold change in expression of multiple genes in both neocortex and cerebellum when compared to sham-infected controls. Levels of mRNA and protein levels of four selected genes were examined in brains of exposed mice. Nucleolin mRNA was significantly decreased in day 0 and day 35 neocortex and significantly increased in day 35 cerebellum. Protein levels were significantly upregulated at days 35 and 56 in neocortex and at day 56 in cerebellum. Connexin 43 protein levels were significantly decreased at day 56 in neocortex. Aquaporin 4 mRNA was significantly decreased in day 0 neocortex. Aquaporin 4 protein levels decreased in neocortex significantly at day 35. Finally, microcephalin mRNA was significantly decreased in day 56 neocortex and protein levels were significantly decreased at 56 cerebellum. These data suggest that influenza viral infection in midpregnancy in mice leads to long term changes in brain markers for enhanced ribosome genesis (nucleolin), increased production of immature neurons (microcephalin), and abnormal glial-neuronal communication (connexin 43 and aquaporin 4).

Keywords

human influenza; mouse; brain; schizophrenia; autism; microarray

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All authors have contributed to and approved the final manuscript.

Conflict of Interest

None.

1. Introduction

Schizophrenia and autism are major neurodevelopmental disorders with prevalences of 1% and 16 per 10,000, respectively (American Psychiatric Association, 1994; Fombonne, 2003). Genetic and environmental etiologies have been cited as being responsible for the genesis of both disorders (Andreasen, 1999; Acosta and Pearl, 2003). While both disorders are neurodevelopmental, the behavioral phenotype varies between the two disorders. Schizophrenia manifests itself in adolescence with signs of thought disorganization and psychosis. In autism, overt behavioral symptoms peak at the thirtieth month postnatally and symptoms include regression in speech, social deficits, communication deficits, and an increase in stereotypic behavior. There is significant epidemiological support for a viral etiology of schizophrenia with some support for an infectious etiology of autism (Brown et al, 2004; Chess, 1971; Chess et al. 1978; Mednick et al. 1994; Stubbs et al. 1984; Susser et al. 1999). New serologic data provide evidence for an infectious origin of some forms of schizophrenia (Brown et al. 2004).

Recent emergence of experimental data show that administration of human influenza virus (H1N1) or a viral mimic – polyriboinosinic polyribocytidilic acid (PolyI:C), at critical stages during pregnancy in mice, can cause deleterious effects in brains of offspring (Fatemi et al. 1999; Fatemi et al. 2002a; Shi et al. 2003a). We have previously shown infection of day E9 pregnant mice by a neurotrophic strain of human (H1N1) influenza virus causes significant biochemical, behavioral, genetic, and structural brain disorders in the offspring (Fatemi et al. 1999; Fatemi et al. 2002a; Shi et al. 2003a). Recent reports by different groups have supported our published data showing that viral mimic PolyI:C at critical stages during pregnancy in rodents can cause similar deleterious effects in the brains of offspring (Shi et al. 2003b; Meyer et al. 2005; Meyer et al. 2006a; Meyer et al 2006b; Meyer et al. 2007a; Meyer et al. 2007b; Nyffeller et al. 2007). The major difference between the new supportive data and ours is that they posit that the intrauterine insult is due to maternal cytokine upregulation and not the direct effects of a viral antigen (Shi et al. 2003b, Meyer et al. 2006). We now show that there is evidence of a potential insult by the virus on fetal brains' neuronal and glial synthetic machinery leading to perturbed gene expression in the affected offspring (Aronson et al. 2001; Aronson et al. 2002; Fatemi et al. 2007a; Fatemi, 2007). Despite these data we cannot dismiss the idea that fetal or maternal cytokine production could not be responsible for the altered mRNA and protein levels.

A previous microarray study of virally-exposed mouse brains by our laboratory has shown significantly decreased expression of nucleolin and aquaporin 4 in the brains of Day 0 mice (Fatemi et al. 2005). Nucleolin, a major protein of the nucleolus, is involved in regulating the transcription of ribosomal RNA genes by RNA polymerase I, ribosome maturation and assembly, and nucleocytoplasmic transportation of ribosomal components (Srivastava et al. 1989) and may be involved in viral replication (Hiscox, 2002). Aquaporin 4 is localized to astrocytes and ependymal cells in brain and is involved with water transport (Papadopoulos et al. 2002; Verkman et al. 2006). Our laboratory has demonstrated that aquaporin 4 and another glial marker, connexin 43 (Cx43), are significantly altered in brains of autistic subjects (Fatemi et al. unpublished observations).

In the current communication we further investigate changes in mRNA and protein levels of nucleolin, aquaporin 4, and connexin 43 at P35 and P56 in offspring of mice infected with human influenza virus, integrating our data with a microarray study by our laboratory of cerebellum from the same mice at the same two postnatal dates (Fatemi et al, 2007b). Based on our microarray results we also added microcephalin as a previous report form our laboratory has demonstrated macrocephaly using this animal model (Fatemi et al. 2002a). We propose

that viral infection in midpregnancy in mice leads to long-term changes in these four important brain markers.

2. Methods

2.1. Animals and Infection

Balb/c male and female 12-14 week old mice were used for the induction of pregnancy and all infection protocol using human influenza virus following previous protocol (Fatemi et al. 1999). Female and male 12-14 week old specific pathogen-free Balb/c mice were obtained from Simonsen laboratories (Gilroy, CA) and were used for breeding. For virus titration, the animals were quarantined 24 hours prior to use, and maintained on Wayne Lab Blox and tap water. After being infected, their drinking water contained 0.006% oxytetracycline (Pfizer, N.Y.) to control possible bacterial infections. Pregnant mice were infected intranasally on day 9 of pregnancy. Pups born to infected and sham infected mothers (n=10 each) were culled and used for blotting studies (n=3 per group) on postnatal days 0, 35 and 56.

The choice of E9 for our infection protocol was based on supportive literature showing that this day preceded the timetable for proliferation, differentiation and migration of neurons and glia in embryonic neocortex, hippocampus and cerebellum (Susser et al. 1999) and initiation of migration of neural crest derived cells to craniofacial structures (Yamagishi et al. 1999) and production of Cajal-Retzius cells (D'Arcangelo et al. 1997). The timetables for brain development vary based on various regions of the brain and are clearly based on species of interest. Thus, extrapolation from mouse to man may not be possible on specific days of pregnancy, but can be construed from prenatal and postnatal peaks of neurogenesis, gliogenesis and neuron/glia differentiation and migration (Susser et al. 1999). The choice of postnatal days 35 and 56 in our studies are based on a large group of supportive literature (Avishai-Eliner et al. 2002; Boksa et al. 2003; Kaufmann, 2000; Morgane et al. 2002; Romijn et al. 1991; Spear, 2000; Susser et al. 1999) denoting E0-E10 in rodents as equal to first trimester in humans, E11-E19 as equal to 2nd trimester, P0-P7 as equal to third trimester (Morgane et al. 2002; Stead et al. 2006), P35 as equal to adolescence (Spear, 2000) and P56 as equal to early adulthood (Spear, 2000).

The protocol for infection reported previously was used (Fatemi et al, 1999). All animal experiments were performed following IRB approval in accordance with institutional animal care and use committee guidelines at Utah State University and University of Minnesota. Briefly, day 9 pregnant Balb/c mice were exposed to influenza A/NWS/33 (H1N1) or vehicle, following determination of viral dosage, causing sublethal lung and upper respiratory infection. The virus was diluted to 10^{-5} CCID50/ml based on previous titrations using this species (Fatemi et al. 1999; Fatemi et al. 2005). Pregnant mice survived the infection protocol and were allowed to deliver pups. The day of delivery was considered day 0. Groups of exposed (n=3) and sham infected pups (n=3) were deeply anesthetized using ketamine (167mg/kg, intraperitoneally) and sacrificed on postnatal days 0, 35 or 56.

2.2. DNA microarray

DNA microarray was performed as described previously (Fatemi et al. 2005). Cerebellum and neocortex (whole brains minus brain stem and cerebellum) were homogenized by standard procedures in Trizol (Gibco BRL) for total RNA extraction. 10μg of total RNA from each sample was used to generate a high fidelity cDNA, which is modified at the 3' end to contain an initiation site for T7 RNA polymerase as per the manufacturer protocol (SuperChoice, Gibco BRL). Upon completion of cDNA synthesis, 1μg of product was used in an in vitro transcription (IVT) reaction that contained biotinylated UTP and CTP which was utilized for detection following hybridization to the microarray as per the manufacturer's protocol (ENZO). 20μg of

full length IVT product was subsequently fragmented in 200mM Tris-acetate (pH 8.1), 500 mM KOAc and 150 mM MgOAc at 94°C for 35 minutes. Following fragmentation, all components generated throughout the processing procedure (cDNA, full-length cRNA, and fragmented cRNA) were analyzed by gel electrophoresis to assess the appropriate size distribution prior to microarray analysis. All samples generated were subjected to gene expression analysis by the Affymetrix murine 430 high-density oligonucleotide array set which contained sequences from approximately 20 000 known mouse genes and EST's at the University of Minnesota BioMedical Genomics Center. Each gene on the array was represented by 16-20 pairs of 25mer oligonucleotides that spanned the coding region for each gene represented. Each probe pair consisted of a perfect match (PM) sequence that was complementary to the cRNA target and a miss match (MM) sequence that had a single base pair mutation in a region critical for target hybridization; this sequence served as a control for non-specific hybridization. Hybridization, staining, and washing of all arrays were performed in the Affymetrix fluidics module as per the manufacturer's protocol. Streptavidin phycoerythrin stain (SAPE, Molecular Probes) was the florescent conjugate used to detect hybridized target sequences. The detection and quantitation of target hybridization was performed with a GeneArray Scanner (Hewlett Packard/Affymetrix) set to scan each array twice at a factory set PMT level and resolution. In addition, all arrays were scanned pre- and post- amplification to address potential issues with respect to the dynamic range of the scanner.

Multiple data analysis approaches were used to identify genes of interest. The Microarray Analysis Suite 5.0 (Affymetrix) was employed to generate one approach to comparative analysis presented in this study. Distinct algorithms were used to determine the detection signal, which distinguished the presence or absence of a transcript, the differential change in gene expression (increase (I), decrease (D), marginal increase (MI), marginal decrease (MD), and no change (NC)), and the magnitude of change, which is represented as fold change. The fold change of any transcript between the baseline and experimental is calculated following global scaling. All data represented from this first approach were from pairwise comparison analysis. The second approach to identifying differentially expressed genes utilized Li Wong which fits a model to the probe set data from multiple microarrays. This method excludes cross hybridizing probes, single outliers and arrays with image contaminations at certain probe sets (Harr and Schlötterer, 2006). The third method was Robust Multi-array Analysis (RMA) which provides probe specific background to correct for nonspecific binding, probe level multichip quantile normalization and robust probe set summary of the probe level data (Irizarry et al. 2003). (Please see Supplemental Tables 1 (P35) and 2 (P56) for the complete data set.) Following each normalization approach, all genes differentially expressed were clustered. Genes were assessed based on primary biological function and grouped accordingly.

2.3. Western blotting

SDS-PAGE and western blotting details followed previous protocol (Fatemi et al. 2006). Sixty μg of protein per lane was loaded onto the gel and electrophoresed. The immune complexes were visualized using the ECL Plus detection system (Amersham Pharmacia Biotech) and exposed to CL-Xposure film (Pierce). Sample densities were analyzed using a Bio-Rad densitometer and Quantity One software.

Connexin 43 and β-actin were analyzed using a modification of a previous protocol (9) using mouse β-actin (Sigma A5441, 1:5000) or Connexin 43 (Fred Hutchinson Cancer Research Center, 1:10 000). Aquaporin 4, Nucleolin and microcephalin were analyzed using the previous protocol, with the following changes: either an 8% (microcephalin or nucleolin) or a 12% (aquaporin 4) SDS-polyacrylamide gel was used; after transfer, the blot was blocked at RT for 1 hour, followed by overnight incubation with primary antibody (rabbit anti-aquaporin 4, Chemicon AB3594, 1:1000; rabbit anti-microcephalin, AbCam ab2612, 1:1000 or rabbit anti-

nucleolin, NB 600-241, 1:4000) at 4°C. Secondary antibody was (goat-anti-rabbit IgG, HRP conjugated, 1:80 000, from Sigma) applied for 1 hour at RT, and all washes and visualization took place as described above.

The densities of approximately 34 kDa (aquaporin 4), 106 kDa (nucleolin), 105 kDa (microcephalin), 43 kDa (connexin 43), and 42 kDa (β-actin), immunoreactive bands were quantified with background subtraction. Results obtained are based on between two and eight independent experiments with n=3 mice per gel. For each experiment, control and infected samples were run on the same gel and processed simultaneously to avoid variability due to intragel differences.

2.4. Statistical analysis

All statistical analyses were performed using SPSS. Differences of the normalized mRNA expression levels of selected genes between virally-infected and sham-infected mice were assayed using student's t-test. Significant differences are defined as those with a p value < 0.05. For western blots, differences of the protein levels of selected genes between virally-infected and sham-infected mice were normalized against β-actin and assayed using a student's t-test. Significant differences are defined as those with a p value < 0.05.

3. Results

3.1. Microarray results

Microarray analysis of virally-exposed mouse brains showed significant ($p<0.05$) at least two-fold upregulation of 50 genes (Table 1) and downregulation of 21 genes (Table 2) in neocortex vs. control in day 35 mice. At day 56, microarray analysis showed significant ($p<0.05$) upregulation of 13 genes (Table 1) and downregulation of 11 genes (Table 2) in neocortex vs. control. A concurrent microarray study of cerebellum by our laboratory using the same virally-exposed mouse brains has shown significant ($p<0.05$) at least two-fold upregulation of 103 genes and downregulation of 102 genes in at P35 and at least two-fold upregulation of 27 genes and downregulation of 23 genes at P56 (Fatemi et al. 2007b). Previously, our laboratory has shown significantly decreased expression of nucleolin and aquaporin 4 in the brains of Day 0 mice (Fatemi et al. 2005). Interestingly we found that nucleolin expression at day 35 was significantly upregulated in cerebellum (Fatemi et al. 2007b) while it was significantly downregulated in neocortex (Table 2). Finally, our data set showed downregulation of microcephalin expression at day 56 in the neocortex (Table 2).

3.2. Changes in Protein Levels at Day 35

Day 35 neocortex showed a 22% significant decrease ($p<0.041$) in AQP4/β-actin values (Table 3; Figure 1A). Nucleolin/β-actin values increased by 40% ($p<0.032$) in the neocortex of exposed mice (Table 3; Figure 2A). In cerebellar tissues, none of the protein values were significantly different between sham-infected and virally-exposed offspring.

3.3. Changes in Protein Levels at Day 56

At day 56, CX43/β-actin values decreased significantly ($p<0.025$) by 20% in the exposed mice in neocortex (Table 3; Figure 3B). Nucleolin/β-actin values increased by 53% ($p<0.014$) in the neocortex of exposed mice (Table 3; Figure 2 B). At day 56, the exposed microcephalin/β-actin values decreased significantly ($p<0.016$) by 32% in cerebellar tissue when compared to controls (Table 3; Figure 4D). In cerebellar tissues, nucleolin/β-actin values rose by 138% ($p<0.031$) on day 56 in the exposed offspring (Table 3; Figure 2D).

β-actin values did not differ significantly between exposed and control neocortex and cerebellar tissues (Table 1; Figure 5 A, B, C, D).

4. Discussion

The current investigation provides further evidence that human influenza viral infection on day 9 of pregnancy in Balb/c mice causes significant alterations in gene expression and protein synthesis involving four major brain markers (nucleolin, microcephalin, aquaporin 4 and connexin 43) in the exposed offspring from postnatal day 0 through days 35 and 56. The presence of changes on days 35 and 56 suggest that long after the viral insult and long after any changes due to maternal-fetal cytokine production changes in brain gene expression persist. Absence of any significant change between β-actin protein product indicated the specificity of our protein levels of interest.

We present evidence that nucleolin protein levels are altered significantly at days 35 and 56 in neocortex of exposed animals. Nucleolin mRNA levels were significantly upregulated at day 35 in cerebellum while it was reduced at day 35 in neocortex. Additionally, there is also evidence that nucleolin levels continue to rise in the infected cerebellar tissues beyond day 35 with a significant increase at day 56, most likely due to the delay in cerebellar growth observed postnatally compared to the neocortex (Morgane et al. 1992).

Nucleolin is a major protein of nucleolus (Scheer and Hock, 1999) and is involved in ribosome biogenesis. It encompasses about 10% of the total nucleolar protein content and is highly phosphorylated, methylated and susceptible to ADP-ribosylation (Hiscox, 2002; Ginisty et al. 1999). Nucleolin may be involved in cleavage of rRNA, acts as a chaperone in correct folding of pre-rRNA processing, and in repression of transcription (Allain et al. 2000; Hiscox, 2002; Yang et al. 1994). The amount of nucleolin is stable in proliferative cells, but it undergoes self-cleavage in quiescent cells (Chen et al. 1991; Hiscox, 2002) and is also involved in process of cell growth and proliferation (Hiscox, 2002; Srivastava and Pollard, 1999).

Several viruses have been known to alter the distribution of nucleolin such as adenovirus, poliovirus, hepatitis delta virus, coxsackie B viruses, Maloney murine leukemia virus, and HIV (Hiscox, 2002). Occasionally, viral proteins may be anchored to nucleolin and the complex can be exchanged between nucleoplasm and nucleolus (Hiscox, 2002). Thus, interaction between viruses and nucleolin can lead to enhancement of viral replication via alterations in host cell transcription, translation, and cell cycle kinetics and contribute to autoimmune disease development (Hiscox, 2002). Increased expression of nucleolin in the brains of mice exposed to prenatal viral insult observed in the current report denotes a potential effect of the influenza virus on fetal brain cells or is due to maternal-fetal cytokine production.

Microcephalin protein levels were significantly reduced on day 56 in cerebellum while mRNA was reduced significantly at day 56 in neocortex. Microcephalin (MCPH1 or BRIT1) is a large, 835 amino acid protein which is expressed in the cerebral cortex of the fetal brain (Tang, 2006). Mutations of MCPH1 and/or related genes result in abnormalities in brain size, such as seen in primary microcephaly (Tang, 2006) where brain size is reduced to 1/3 of its normal size (Tang, 2006). Additionally, mental retardation is a major clinical finding in primary microcephaly (Tang, 2006). Thus, microcephalin has multiple important biological functions that implicate this protein in brain size regulation, cognition, and cancer (Bartek, 2006; Tang, 2006). Recent reports show that microcephalin is involved in regulation of unperturbed mitotic cell cycles such as proper execution of the intra-S phase and G2/M checkpoints in response to genotoxic stress such as ionizing radiation (Lin et al. 2005; Xu et al. 2004). Microcephalin-deficient cells exhibit abnormal G2-like lymphocytes that show elevated number of cells with prematurely-condensed chromosomes (Neitzel et al. 2002) which have not progressed normally into mitosis. Microcephalin has also recently been shown to be involved in centrosomal function potentially modulating the number of neurons generated by neuronal precursor cells (Zhong et al. 2006). Our current results show that human influenza viral

infection either directly or indirectly caused a reduction of microcephalin mRNA in neocortex and protein in cerebellum on P56 which may lead to an increase in the number of highly immature neurons that might contribute to the macrocephaly observed in the adult virally exposed mouse offspring (Fatemi et al. 2002a) similar to same scenario in brains of subjects with autism (Casanova et al. 2002). Pyramidal cells in virally exposed adult brains exhibit cell atrophy and are increased numerically compared to sham-infected mice (Fatemi et al. 2002a), potentially due to reductions in microcephalin gene expression.

The association of macrocephaly with autism has been replicated in a number of studies, showing an average rate of macrocephaly of 20% (Fombonne et al. 1999). Recent data from 10 sites of the NICHD/NIDCD Collaborative Programs of Excellence in Autism showed a rate of absolute macrocephaly of 17.3% for autistics vs. 3% for normal controls ($p<0.0004$; Lainhart et al. 2006). In light of these findings along with our finding of macrocephaly in the offspring of prenatally infected mice (Fatemi et al. 2002a), the observed alterations in microcephalin expression have the potential to be important for future studies investigating microcephalin and autism.

AQP4 protein values showed a significant reduction at day 35. In the brain, AQP4 is strongly expressed in astrocytes and ependymal cells (Yang et al. 1995). Expression is strongest at sites of fluid transport including the pial and ependymal surfaces in contact with the cerebrospinal fluid (CSF), in the subarachnoid space, and the ventricular system (Rash et al. 1998) suggesting a role for AQP4 in movement of water between brain and CSF compartments (Verkman et al. 2006). Additionally, a role for AQP4 in the movement of water between blood and brain is suggested by polarized AQP4 expression found in astrocytic foot processes in direct contact with blood vessels (Verkman et al. 2006).

Increased AQP4 expression has been demonstrated in response to a number of pathological conditions marked by astrocytic activation and/or blood-brain barrier changes including gliomas (Papadopoulos et al. 2002), stroke (Papadopoulos et al. 2002), and HIV-related dementia (St Hilarie et al. 2005). Overexpression of AQP4 may cause an increased propensity for cerebral edema. Experiments using AQP4 knockout mice revealed that AQP4 deletion offered protection from cytotoxic brain edema (Papadopoulos et al. 2005) and vasogenic (non-cellular) brain edema (Papadopoulos et al. 2004). Our laboratory has observed a significant decrease in AQP4 in the cerebellum of patients with autism (Fatemi et al. unpublished observations).

We observed that AQP4 was initially increased at day 0 in neocortex but then decreased precipitously in postnatal days 35 and 56. Changes observed in AQP4 in the virally exposed animals may reflect brain edema causing alterations in cell morphology, and potential instability in F-actin molecules leading ultimately to downregulation in CX43 and cell-cell coupling (Nicchia et al. 2005).

CX43 protein levels in neocortex showed a significant drop in day 56 animals. Glial connexins have important functions in the brain (Theis et al. 2004), including long range signaling by Ca^{++} waves (Sohl et al. 2005), gap-junction-mediated intercellular communication between astrocytes, transport of nutrients from blood to neurons, K^{+} spatial buffering, glutamate uptake, and dissipation (Sohl et al. 2005; Theis et al. 2004). Additionally, connexins - especially CX43 - help in several other important functions, such as regulation of cell growth, interaction with β-catenin, localization to nucleus and affecting gene expression, and cell-cell adhesion (Theis et al. 2004). CX43 is widely present in the astrocytes of the adult brain (Sohl et al. 2005).

Connexin 43 upregulation has been identified in several neurological abnormalities, such as Parkinson's, Huntington's, Alzheimer's, seizure/epilepsy, transient brain ischemia, and facial nerve lesion (Rouach et al. 2002). Interestingly, our laboratory has observed a significant

increase in CX43 levels in frontal cortex of autistic subjects (Fatemi et al. unpublished observations). In contrast, CX43 downregulation has been seen with severe brain ischemia, prolonged exposure to IL-1, mechanical lesions of the visual cortex, exposure to NMDA agonists, and gliomas (Rouach et al. 2002). Examination of the neocortex of CX43 null mutant mice showed delays in neuronal migration (Fushiki et al. 2003). CX43 expression was correlated inversely with cell proliferation activity during postnatal development (Miragall et al. 1996). Activated microglial cells release cytokines like IL-Iβ, and TNF-α and downregulate CX43 in cultures of astrocytes exposed to bacterial lipoplysaccarides (Meme et al. 2006). Finally, mice with astrocyte-directed inactivation of CX43 exhibit increased exploratory behavior, impaired motor capacities, and change in brain acetylcholine levels (Frisch et al. 2003). Our observed decrease in CX43 protein levels of exposed mice suggests increased cell proliferation and decreased cell-cell communication, and cell-cell coupling in the growing brains of the virally exposed mice.

Previous work in our laboratory (Fatemi et al. 1999; Fatemi et al. 2002a) has shown a deleterious viral effect during E9 which leads to abnormal corticogenesis and changes in levels of several neuroregulatory proteins in postnatal mice. E9 in the mouse coincides with the start of several important events in brain development, i.e., early neural crest cell migration to the presumptive craniofacial structures, onset of neurogenesis and neuronal migration, gliogenesis, and early synaptogenesis (Acuff-Smith and Vorhees, 1999). Moreover, E9 in mouse is equal to late 1st trimester in man (Rodier, 1980). Recent reports by Brown et al. (2004) and Buka et al. (2001) have demonstrated serological evidence that human influenza virus and herpes virus infection, respectively, during pregnancy are linked to the development of schizophrenia in offspring. Brown's study is particularly important as the infection occurred during the first trimester of pregnancy (similar to E9 in mouse) with no increased risk of schizophrenia with influenza during the second and third trimesters (Brown et al. 2004). More recently, Lloyd et al (2007) reported on minor physical abnormalities (MPAs) associated with individuals with first-episode psychosis (schizophrenia, affective psychosis, drug-induced psychosis). Lloyd found that overall facial asymmetry, asymmetry of the orbital landmarks, as well as other abnormalities distinguished patients with schizophrenia and affective psychosis from controls, suggesting an insult during organogenesis in the first trimester of pregnancy (Lloyd et al., 2007). Despite these findings most of the epidemiological evidence points to infection during the 2nd trimester to be responsible for the development of schizophrenia in offspring (Mednick et al. 1988; Barr et al. 1990; Mednick et al. 1994; Kungi et al. 1995).

It has previously been demonstrated in this model (Shi et al. 2003a) that prenatal viral infection on E9 leads to increased anxiety and deficits in PPI in the acoustic startle response similar to what has been observed in schizophrenics and autistic subjects (Geyer and Braff, 1982; Perry et al. 2007). Treatment of pregnant mice with a viral mimic – polyriboinosinic polyribocytidilic acid (PolyI:C), at critical stages during pregnancy in mice, can cause deleterious effects in brains of offspring and behavioral pathology (Meyer et al. 2005; Meyer et al. 2006a; Meyer et al 2006b; Meyer et al. 2007a; Meyer et al. 2007b) similar to what we have observed in our model. These investigators have argued that these results are due to maternal cytokine upregulation (Meyer et al. 2006; Shi et al. 2003b). We believe that the changes in nucleolin expression may indicate a potential direct viral effect on the exposed offspring. However, did not detect the presence of the pathogen in the offspring's brain and we do not exclude the possibility that it may be due to maternal-fetal cytokine production.

In conclusion, we have presented evidence that prenatal infection on day 9 of pregnancy (Figure 6) causes significant changes in four key proteins involved in cell-cell coupling, cell communication, and cell proliferation. Two of these proteins, AQP4 and CX43, are likewise altered in autistic brain (Fatemi et al. unpublished observations) and a third may be implicated in the macrocephaly, which is commonly observed in autistic subjects (Fombonne et al.

1999;Lainhart et al. 2006) as well as in our animal model (Fatemi et al. 2002a). Most importantly, the weight of accumulated evidence so far showing presence of gliosis (Fatemi et al. 2002b), pyramidal cell atrophy (Fatemi et al. 2002a), and upregulation of nucleolin, as well as previous supportive evidence (Aronsson et al. 2001;Aronsson et al. 2002), showing persistence of influenza viral RNA in brain implicate a potential viral effect on the developing fetal brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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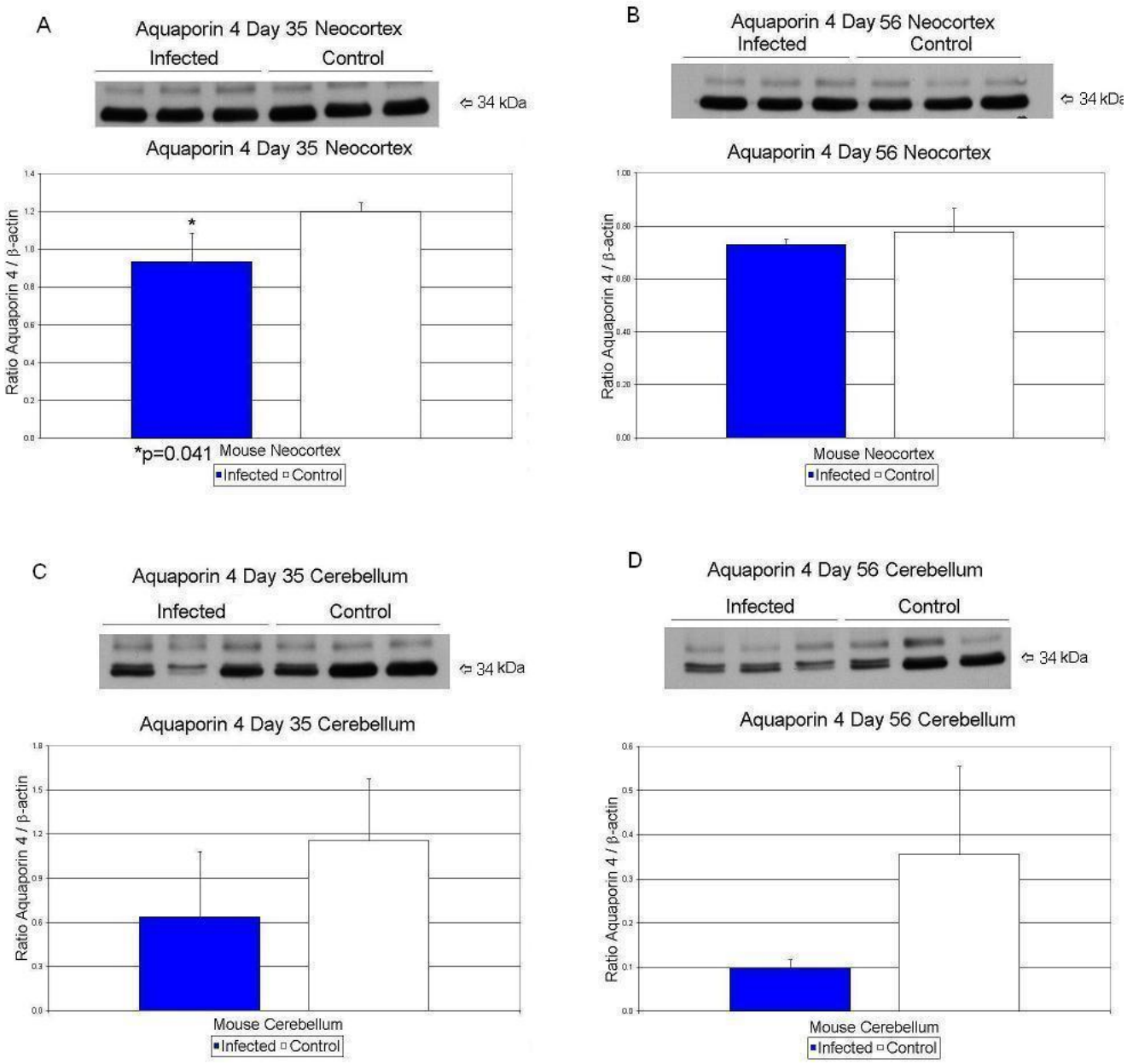

Figure 1.

Effects of prenatal human influenza viral infection on expression of aquaporin 4 protein levels in neocortex (A, B) and cerebella (C, D) of mouse progeny on postnatal days 35 (A, C) and 56 (B, D).

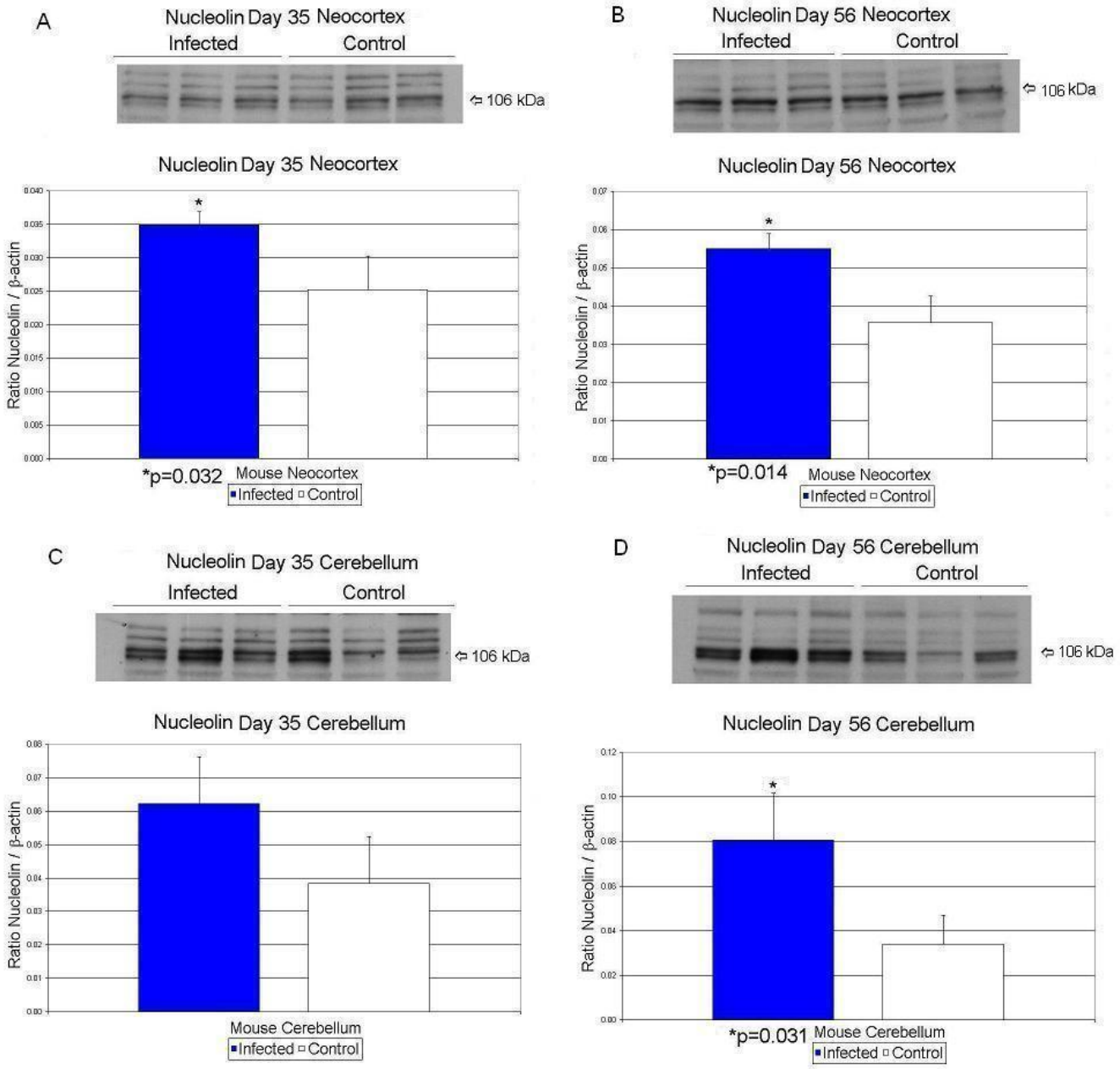

Figure 2.

Effects of prenatal human influenza viral infection on expression of nucleolin protein levels in neocortex (A, B) and cerebella (C, D) of mouse progeny on postnatal days 35 (A, C) and 56 (B, D).

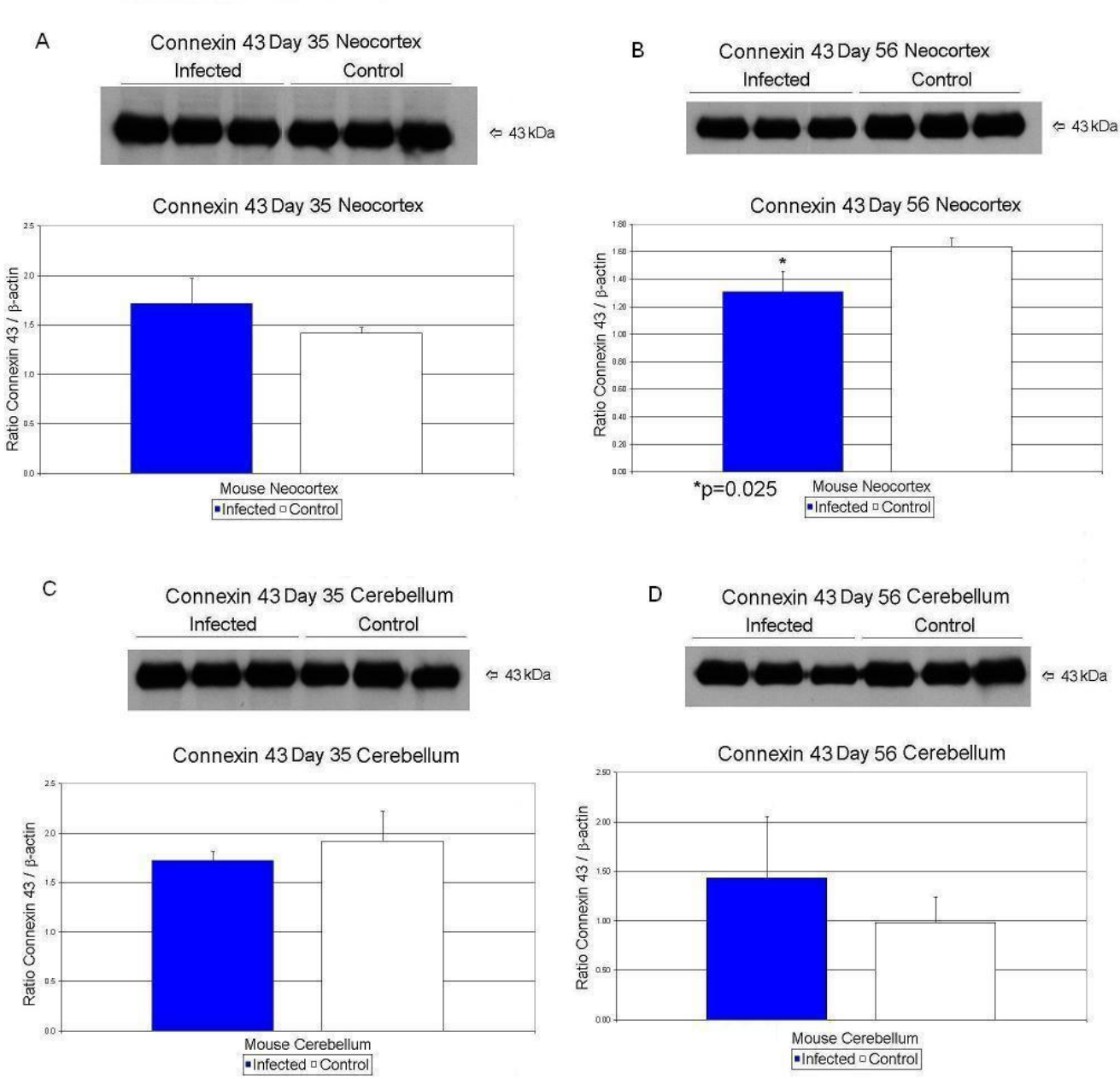

Figure 3.

Effects of prenatal human influenza viral infection on expression of connexin 43 protein levels in neocortex (A, B) and cerebella (C, D) of mouse progeny on postnatal days 35 (A, C) and 56 (B, D).

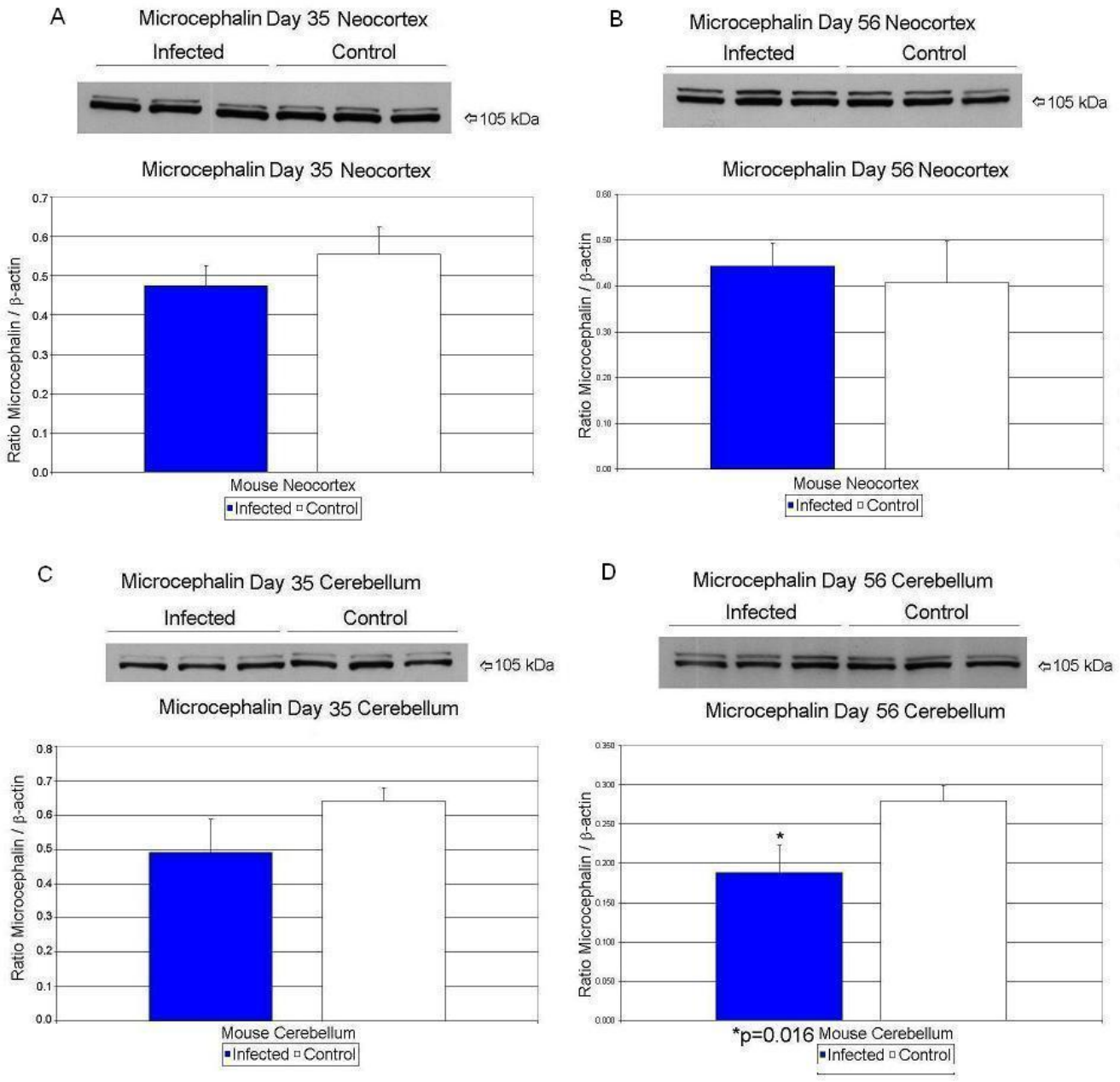

Figure 4.

Effects of prenatal human influenza viral infection on expression of microcephalin protein levels in neocortex (A, B) and cerebella (C, D) of mouse progeny on postnatal days 35 (A, C) and 56 (B, D).

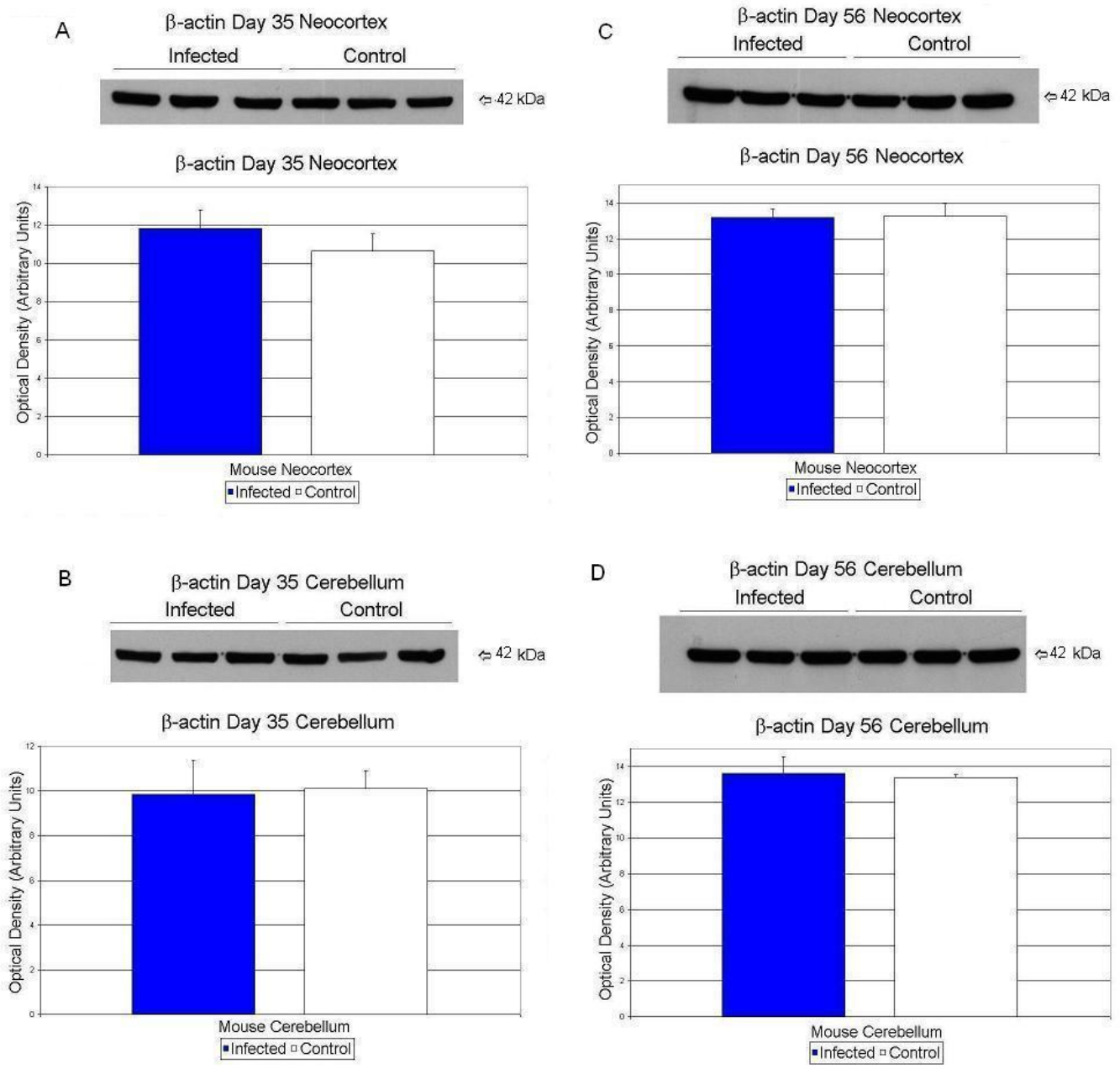

Figure 5.

Effects of prenatal human influenza viral infection on expression of β-actin protein levels in neocortex (A, B) and cerebella (C, D) of mouse progeny on postnatal days 35 (A, C) and 56 (B, D).

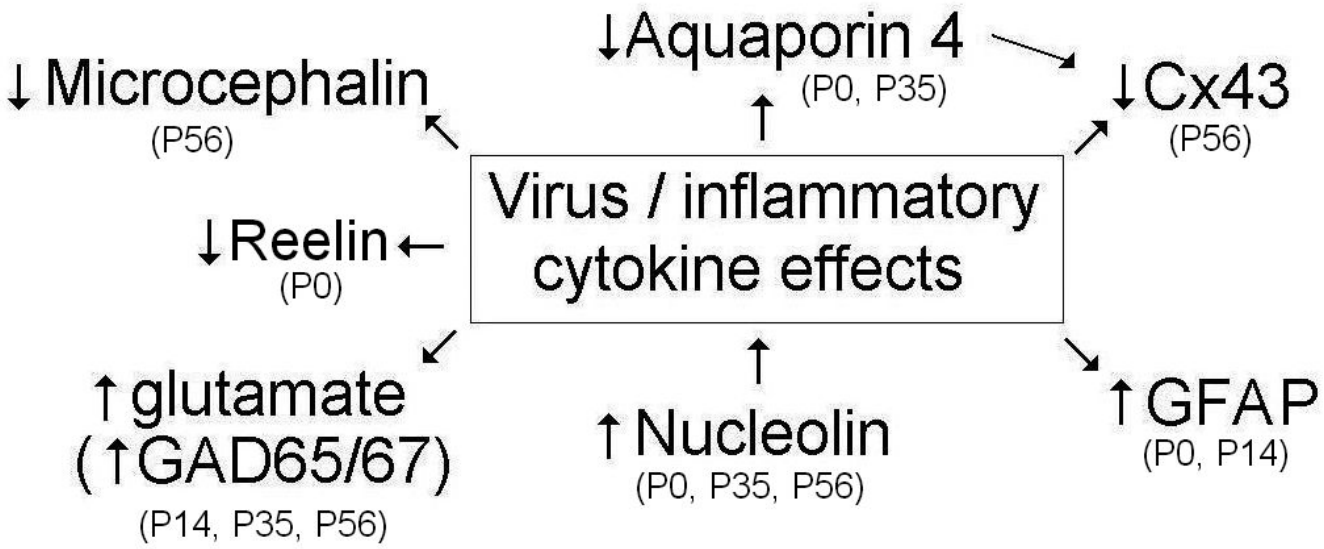

Figure 6.

Prenatal viral infection on day 9 of pregnancy causes abnormal brain structure and function in adult Balb/c mice including: 1) apoptotic change in exposed mice as seen by increased pyramidal cell atrophy and increased neuronal density (Fatemi et al. 2002a); 2) migrational abnormalities as seen by increased GFAP, decreased Reelin early thinning and enlargement of brain; and 3) abnormal cell (neurons and astrocytes) proliferation as seen by decreased microcephalin and connexin 43 expression and by increased nucleolin and GFAP expression. Please note that postnatal dates denote significant changes in levels of various mRNAs or proteins.

Table 1

Neocortical genes upregulated by prenatal viral infection on postnatal days 35 and 56

Day 35			
Function	**Name**	**Fold Change**	**Unigene**
Apoptosis	Suppression of tumorigenicity 18	2.18	Mm.234612
Cell Communication; Signal Transduction	cerebellin 1 precursor protein	2.91	Mm.4880
	chimerin (chimaerin) 2	2.27	Mm.257073
	frizzled homolog 7 (Drosophila)	2.15	Mm.297906
	G substrate	7.08	Mm.42096
	Glutamate receptor, ionotropic, delta 2	2.63	Mm.321227
	growth differentiation factor 10	3.06	Mm.40323
	nudix (nucleoside diphosphate linked moiety X)-type motif 11	2.11	Mm.41198
	Parvalbumin	2.23	Mm.422866
Cell Growth and/or Maintenance	tubulin, beta 2c	2.13	Mm.227260
	zinc finger protein 521 (AKA Evi3)	2.07	Mm.40325
Kinases and phosphatases	Mitogen activated protein kinase 12	2.31	Mm.38343
	NIMA (never in mitosis gene a)-related expressed kinase 2	2.05	Mm.33773
Metabolism; Energy Pathways	4-hydroxyphenylpyruvic acid dioxygenase	2.29	Mm.6584
	betaine-homocysteine methyltransferase	2.00	Mm.329582
	carbamoyl-phosphate synthetase 1	2.13	Mm.343942
	Carbonic anhydrase 3	2.67	Mm.300
	carbonic anhydrase 8	10.49	Mm.119320
	esterase 31	2.00	Mm.295534
	malate dehydrogenase 2, NAD (mitochondrial)	5.19	Mm.297096
Protein Metabolism	aspartate-beta-hydroxylase	2.06	Mm.239247
	coagulation factor II	2.85	Mm.89048
	fibrinogen, alpha polypeptide	2.57	Mm.88793
	protein kinase inhibitor beta, cAMP dependent, testis specific	2.23	Mm.262135
	WW domain containing E3 ubiquitin protein ligase 1	2.26	Mm.78312
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	atonal homolog 7 (Drosophila) (AKA Math5)	2.13	Mm.228661
	myelin transcription factor 1	2.30	Mm.130005
	Myocyte maintenance	2.68	NM_010134[a]
	nescient helix loop helix 2	2.31	Mm.137286
	neurogenic differentiation 1	4.56	Mm.4636
	RB1-inducible coiled-coil 1 (AKA Fip200)	4.08	Mm.293811
	synaptonemal complex protein 1	2.55	Mm.243849
	zinc finger protein of the cerebellum 1	3.09	Mm.335350
	zinc finger, matrin-like	2.11	Mm.132392
Synthesis and Degradation	cytochrome P450, family 2, subfamily c, polypeptide 29	2.34	Mm.20764
	cytochrome P450, family 2, subfamily e, polypeptide 1	2.99	Mm.21758
	cytochrome P450, family 3, subfamily a, polypeptide 11	2.32	Mm.358586

Function	Name	Fold Change	Unigene
	Day 35		
Transport	albumin 1	3.87	Mm.1673
	apolipoprotein A-II	2.10	Mm.389209
	aquaporin 6	3.41	Mm.202309
	ATPase, Ca++ transporting, ubiquitous	2.39	Mm.6306
	fatty acid binding protein 1, liver	3.64	Mm.22126
	potassium voltage-gated channel, subfamily G, member 4	2.11	Mm.358699
	transient receptor potential cation channel, subfamily M, member 4	2.22	Mm.349430
Miscellaneous	ankyrin repeat domain 25	2.19	Mm.257371
	serine (or cysteine) proteinase inhibitor, clade A, member 1a	5.67	Mm.358636
	Serine (or cysteine) proteinase inhibitor, clade A, member 3m	2.40	Mm.291569
	Serine (or cysteine) proteinase inhibitor, clade C (antithrombin), member 1	2.08	Mm.260770
	Tumor-suppressing subchromosomal transferable fragment 8	2.47	BB233597[a]
	urate oxidase	2.25	Mm.10865
	Day 56		
Cell Growth and/or Maintenance	Lipin 2	2.07	Mm.227924
Growth Factors and Hormones	insulin-like growth factor binding protein 5	2.15	Mm.309617
Metabolism; Energy Pathways	mannosidase 2, alpha B2	2.05	Mm.761
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	speckle-type POZ protein	2.19	Mm.285454
Signaling Intermediates	peptidylglycine alpha-amidating monooxygenase	2.98	Mm.5121
	Ribosomal protein L37a	2.06	Mm.379003
Transcription Regulators	SRY-box containing gene 8	2.09	Mm.258220
Transport and Trafficing	ATPase, H+ transporting, V1 subunit E isoform 1	3.13	Mm.29045
	D site albumin promoter binding protein	2.02	Mm.3459
Miscellaneous	deaminase domain containing 1	2.49	Mm.331841
	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	2.24	Mm.298256
	NECAP endocytosis associated 1	2.00	Mm.288114
	Tumor-suppressing subchromosomal transferable fragment 8	2.22	BB233597[a]

[a] All accession numbers from Unigene except for Entrez[A].

Table 2

Neocortical genes downregulated by prenatal viral infection on postnatal days 35 and 56

Day 35			
Function	**Name**	**Fold Change**	**Unigene**
Apoptosis	nuclear receptor subfamily 4, group A, member 1	−2.34	Mm.119
Cell Communication; Signal Transduction	cyclin D2	−2.06	Mm.333406
	Discs, large homolog 2 (Drosophila) (AKA PSD93)	−2.22	Mm.323861
	hematological and neurological expressed sequence 1	−2.40	Mm.1775
	Homer homolog 1 (Drosophila)	−2.51	Mm.37533
	suppression of tumorigenicity 13	−2.03	Mm.180337
Cell Growth and/or Maintenance	actin, beta, cytoplasmic	−2.22	Mm.297
Immune Response	sialophorin (AKA CD43)	−2.38	Mm.283714
Kinases and phosphatases	protein kinase C, zeta	−2.09	Mm.28561
Protein Metabolism	Heat shock protein 1A	−2.18	Mm.6388
	peptidylglycine alpha-amidating monooxygenase	−3.50	Mm.5121
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	CCAAT/enhancer binding protein (C/EBP), delta	−2.79	Mm.347407
	early growth response 2 (AKA Krox20)	−2.92	Mm.290421
	FBJ osteosarcoma related oncogene	−2.66	Mm.246513
	fos-like antigen 2 (AKA Fra2)	−2.24	Mm.24684
	Nucleolin	−2.16	Mm.154378
	THO complex 1	−2.18	Mm.219648
Miscellaneous	mRNA fragment for heat shock cognate hsc73	−2.50	AK004608[a]
	transmembrane and coiled-coil domains	−2.20	Mm.131623
	BAT2 domain containing 1	−2.13	Mm.245446
	tubulin cofactor a	−2.37	Mm.379025
Day 56			
Function	**Name**	**Fold Change**	**Unigene**
Cell Communication; Signal Transduction	Membrane-associated protein 17	−2.02	Mm.30181
	nudix (nucleoside diphosphate linked moiety X)-type motif 11	−2.07	Mm.41198
	Ras homolog enriched in brain like 1	−2.15	Mm.259708
Membrane receptors	chemokine (C-X-C motif) receptor 3	−2.09	Mm.12876
Signaling Intermediates	Ribosomal protein S12	−2.32	Mm.353923
	Sjogren syndrome antigen B	−2.72	Mm.10508
Transport and Trafficing	apolipoprotein A-I	−2.68	Mm.26743
	transient receptor potential cation channel, subfamily M, member 4	−2.04	Mm.349430

Day 35			
Function	**Name**	**Fold Change**	**Unigene**
Miscellaneous	metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA) gene	−2.15	Mm.298256
	Microcephaly, primary autosomal recessive 1	−6.99	Mm.235296
	Serine (or cysteine) proteinase inhibitor, clade A, member 3M	−2.11	Mm.379018

[a] All accession numbers from Unigene except for Entrez[A].

Table 3

Descriptive Data for Aquaporin 4, Connexin 43, Microcephalin, Nucleolin, and β-Actin Protein Levels

Area	Protein Level	Control (n=3)	Infected (n=3)	% Δ	*p* Value
Neocortex	Aquaporin/β-Actin	0.107 ± .089	0.198 ± .12	85% ↑	p < 0.34
Day 0	Microcephalin/β-Actin	0.29 ± .12	0.16 ± .03	45% ↓	p < 0.15
	Nucleolin/β-Actin	0.032 ± .021	0.049 ± .005	53% ↑	p < 0.25
	β-Actin	10.13 ± 1.57	10.25 ± 0.88	1% ↑	p < 0.92
Cerebellum	Aquaporin/β-Actin	1.15 ± .42	0.64 ± .44	44% ↓	p < 0.21
Day 35	Connexin 43/β-Actin	1.92 ± .30	1.72 ± .09	10% ↓	p < 0.33
	Microcephalin/β-Actin	0.64 ± .038	0.49 ± .097	23% ↓	p < 0.067
	Nucleolin/β-Actin	0.038 ± .014	0.062 ± .014	63% ↑	p < 0.11
	β-Actin	10.11 ± .81	9.85 ± 1.53	3% ↓	p < 0.86
Neocortex	Aquaporin/β-Actin	1.20 ± .052	0.94 ± .15	22% ↓	**p < 0.041**
Day 35	Connexin 43/β-Actin	1.42 ± .06	1.72 ± .25	21% ↑	p < 0.11
	Microcephalin/β-Actin	0.56 ± .069	0.47 ± .052	16% ↓	p < 0.18
	Nucleolin/β-Actin	0.025 ± .005	0.035 ± .001	40% ↑	**p < 0.032**
	β-Actin	10.63 ± .91	11.82 ± .96	11% ↑	p < 0.19
Cerebellum	Aquaporin/β-Actin	0.35 ± .20	0.10 ± .02	71% ↓	p < 0.085
Day 56	Connexin 43/β-Actin	0.98 ± .26	1.44 ± .62	47% ↑	p < 0.31
	Microcephalin/β-Actin	0.28 ± .02	0.19 ± .035	32% ↓	**p < 0.016**
	Nucleolin/β-Actin	0.034 ± .013	0.081 ± .021	138% ↑	**p < 0.031**
	β-Actin	13.37 ± .17	13.62 ± .92	2% ↑	p < 0.67
Neocortex	Aquaporin/β-Actin	0.78 ± .087	0.73 ± .02	6% ↓	p < 0.42
Day 56	Connexin 43/β-Actin	1.64 ± .06	1.31 ± .15	20% ↓	**p < 0.025**
	Microcephalin/β-Actin	0.41 ± .09	0.44 ± .05	7% ↑	p < 0.59
	Nucleolin/β-Actin	0.036 ± .007	0.055 ± .004	53% ↑	**p < 0.014**
	β-Actin	13.25 ± .70	13.20 ± .49	0.4% ↓	p < 0.91