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Gene expression is altered in hippocampus following prenatal viral infection in mouse at E16

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Abstract

The hippocampus governs memory formation and emotional regulation and there is widespread evidence of hippocampal dysfunction in psychiatric disorders including schizophrenia and autism. There is abundant evidence that prenatal viral infection may play a role in the development of these two disorders. In the current study we have examined gene expression and structural changes of the hippocampi of exposed neonates following maternal infection at E16 (middle second trimester). We observed significant changes in gene expression at P0 (birth), P14 (childhood), and P56 (adulthood) including a number of candidate genes for autism and schizophrenia. qRT-PCR, verified the direction and magnitude of change for 5 of the genes from the microarray data set revealed mRNA changes for additional genes associated with schizophrenia and autism. MRI revealed a decrease in hippocampal volume at P35 (adolescence). Our results demonstrate altered gene expression and reduced hippocampal volume following prenatal viral infection at E16.

Keywords

schizophrenia; autism; viral model; mouse; DNA microarray; hippocampus

1. Introduction

The hippocampus governs memory formation and emotional regulation and there is widespread evidence of hippocampal dysfunction in psychiatric disorders including schizophrenia and autism. Hippocampal abnormalities in subjects with schizophrenia include disturbed cytoarchitecture, biochemical changes, and an overall reduction in volume (Arnold and Trojanowski, 1996; Harrison and Owen, 2003; Weiss et al., 2005). A greater prevalence of hippocampal shape anomaly (HAS), characterized by a rounded shape, medial location, and a

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deep collateral sulcus, has been found in familial schizophrenia patients (Connor et al., 2004). Likewise, abnormalities in hippocampus have been observed in subjects with autism including a reduction in neuronal cell size and increased cell density (Bauman and Kemper 1994). MRI volumetric studies have found alterations in hippocampal volume, with Aylward et al. (1999) and Herbert et al. (2003) finding a reduction in volume for adults and children, respectively, while, in contrast, Sparks et al. (2002) found an increase in volume in autistic children.

There is robust epidemiologic evidence indicating that environmental contributions, including prenatal infections, may lead to genesis of schizophrenia (Fatemi, 2005, 2008; Brown, 2004, 2006) and autism (Arndt et al., 2006; Libbey et al., 2005). Animal models, developed by our laboratory and others, have demonstrated that viral infections and/or immune challenges during pregnancy lead to abnormal brain structure and function in the exposed offspring that replicate abnormalities observed in brains of subjects with schizophrenia and autism (Fatemi et al., 2005, 2008a; Meyer et al, 2006, 2007; Shi et al., 2003).

Our laboratory has previously described abnormal changes following infection at E9, which corresponds to middle first trimester (Fatemi et al., 2005; Shi et al., 2003), and E18, which corresponds to late second trimester (Fatemi et al., 2008a). Embryonic day 16 (E16) in mice corresponds to middle second trimester in humans, a period when prenatal infection has been linked to the development of schizophrenia later in life (reviewed in Brown et al., 2004; Susser et al., 1997). Additionally, E16 immediately follows the period of neurogenesis of hippocampal pyramidal cells (E11-E15.5; Rodier, 1980).

We hypothesized that middle second trimester infection (E16) in mice would alter brain gene expression in hippocampus including candidate genes for autism and schizophrenia. Moreover, we hypothesized that prenatal viral infection at E16 would reduce hippocampal volume in exposed offspring. Here, we present genetic and MRI data showing that a similar sublethal dose of human influenza virus (H1N1) in C57BL6J mice at E16, leads to altered expression of many brain genes in the hippocampi of the exposed mouse offspring.

2. Experimental procedures

2.1. Viral infection

All experimental protocols used in this study were approved by the Institute for Animal Care and Use and Institutional Biosafety Committees at the University of Minnesota. Influenza A/NWS/33 (H1N1) virus was obtained from R.W. Cochran, University of Michigan (Ann Arbor). A virus pool was prepared in Maden Darby canine kidney (MDCK) cells; the virus was ampuled and frozen at -80°C until used. Data were expressed as $\log_{10}$ cell culture infectious doses ($CCID_{50}$)/ml by the method of Reed and Muench (Reed and Muench, 1938). By this titration, it was determined that at a dilution of $10^{-4.5}$, none of the mice died of the infection but displayed a mean lung consolidation scores and mean lung weights similar to those obtained by Fatemi et al (2002b) and had a mean virus titer of $10^{5.25}$ $CCID_{50}$/ml, indicating that a moderate but sublethal infection had been induced. This was the virus dose selected for use in the pregnant mouse study. On day 16 of pregnancy, C57BL6J mice (Charles River, Wilmington, MA) were anesthetized using 200 μl isoflurane, and intranasally (i.n.) administered a dilution of $10^{-4.5}$ of 6.5 log 10 ($CCID_{50}$) per 0.1 ml human influenza virus A/NWS/33 in 90 μl of minimum essential medium (MEM). Sham infected mothers were treated identically but administered i.n. 90 μl MEM. After being infected, their drinking water contained 0.006% oxytetracycline (Pfizer, New York, NY) to control possible bacterial infections. Pregnant mice were allowed to deliver pups. The day of delivery was considered day 0. Groups of infected and sham-infected neonates were deeply anesthetized and killed on P0, P14, P35 and P56. Offspring were weaned from mothers at P21, and males and females were caged separately in groups of 2-4 littermates.

2.2. Brain collection and dissection

Pregnant mice were allowed to deliver pups. Day of delivery was considered as postnatal day P0. Male litter mates from P0, 14, 35, and 56 were obtained. Groups of infected (N=10 litters) and sham-infected male neonates (N=10 litters) were deeply anesthetized. The choice of ten animals per group was based on earlier calculations that yield an average effect size of 1.45sd between infected and control animals. With alpha = 0.05, and a sample size of 10 per group, power is = 0.798. Brains were removed from skull cavities following perfusion with phosphate-buffered 4% paraformaldehyde (pH 7.4) with later immersion in the same fixative for 14 days at 4°C and used for all imaging studies. Alternatively, unfixed cryopreserved brains were snap-frozen by immersion in liquid nitrogen and stored at -85°C for all gene array studies. Whole hippocampus was dissected using the method of Zapala et al. (2005), provided in step-by-step diagrams in their online supporting Appendix 2.

2.3. Microarray

Tissues (N=3 control and N=3 infected for hippocampus at each of three time points (P0 (birth), P14 (childhood), P56 (adulthood)) were prepared and microarray performed as previously described (Fatemi et al., 2008a). (For a detailed protocol please see Supplemental Experimental Procedures).

2.4. qRT-PCR

Quantitative RT-PCR for selected genes from our microarray data set that had an association with autism or schizophrenia was performed as previously described (Fatemi et al., 2005b, 2008) using the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and a GAPDH endogenous control assay. Single strand cDNA was synthesized from 0.02 to 2.0 μg of each total RNA sample using the High Capacity cDNA Synthesis Kit (Applied Biosystems) according to the manufacturer's instructions. Approximately 20 ng of each cDNA (9 μL) was mixed with 10 μL of TaqMan® Gene Expression 2X PCR Master Mix (Applied Biosystems, Roche), and 1 μL of each indicated TaqMan® Gene Expression Assay (Applied Biosystems) in 384-well plates and analyzed on the 7900HT Fast Real Time PCR system according to the manufacturer's instructions (Applied Biosystems). Primary analysis of the acquired signal data was performed in SDS 2.3 and RQ Manager 1.2 (Applied Biosystems). Outlier reactions were removed after Grubb's test identification and differential expression was calculated using the $\Delta\Delta C_T$ method.

2.5. MRI and DTI scanning

2.5.1. Brain tissue acquisition for MR scanning—Brains from C57BL/6 male neonates born to infected and sham infected E16 mothers at P0, P14, P35 and P56 (N=4 infected, N=3 control, 1 male/litter per group) were perfusion fixed in 4% paraformaldehyde in PBS buffer (pH 7.4) and subjected to DTI and MR scanning in PBS at room temperature as previously described (Fatemi et al., 2008a; Mori et al., 2001). (For a detailed protocol please see Supplemental Experimental Procedures).

2.6. Statistical Analysis

All statistical analyses were performed using SPSS. Differences of the normalized mRNA expression levels of selected genes between infected and control mice were assayed using two-tailed student's t-test. Significant differences are defined as those with at least a 1.5 fold change and a p value < 0.05.

3. Results

We used Affymetrix microarrays, qRT-PCR, and MRI-based imaging to evaluate our results. Gene expression data showed a significant ($p<0.05$) at least 1.5 fold up- or downregulation of genes in hippocampus (299 upregulated and 191 downregulated at P0; 34 upregulated and 46 downregulated at P14; and 87 upregulated and 30 downregulated at P56) of mouse offspring (Supporting Table 1 online). Several genes, which have been previously implicated in etiopathology of autism and schizophrenia, were shown to be affected significantly ($p<0.05$) by DNA microarray including: Aquaporin 4 (Aqp4), Gamma-aminobutyric acid receptor gamma 1 (Gabrg1), forkhead box P2 (Foxp2), myelin basic protein (Mbp), neurotensin (Nts), and neural cell adhesion molecule (Nrcam) (Table 1). The direction and magnitude of change for Aqp4, Foxp2, Gabrg1, Mbp, and Nts were verified by qRT-PCR (Table 1).

Additionally, qRT-PCR showed significant changes in mRNA for other schizophrenia and autism susceptibility genes including autism susceptibility candidate 2 (Auts2), regulator of G Protein 4 (Rgs4), protein phosphatase 1, regulatory subunit 1b (Ppp1r1b), and glial fibrillary associated protein (Gfap) (Table 2). Interestingly, a number of genes related to myelination displayed significant downregulation of mRNA at P0 including Mbp, Myelin and lymphocyte-associated protein (Mal), Myelin-associated glycoprotein (Mag), myelin-associated oligodendrocytic basic protein (Mobp), Myelin oligodendrocyte protein (Mog), Proteolipid protein (myelin) 1 (Plp1) (Tables 1 and 2).

Morphometric analysis of brain following infection of C57BL/6 mice at E16 revealed an approximately 7% reduction in overall brain volume at P14 ($p<0.019$) (Table 3) and a 6% reduction in area for hippocampus at P35 ($p<0.014$) (Table 3). Fractional anisotropy revealed no significant changes in white matter in hippocampus although there was a nearly significant increase in white matter at P14 ($p<0.057$;Table 4).

4. Discussion

Prenatal viral infection led to altered gene expression in hippocampus at P0, P14, and P56 including schizophrenia and autism candidate genes such as Aqp4, Mbp, Nts, Foxp2, Nrcam, and Gabrg1. qRT-PCR verified the direction and magnitude of change for all of the above genes except for Nrcam. Additionally, qRT-PCR revealed significant changes in a number of other genes including downregulation of myelination genes Mag, Mog, Mobp, Mal, and Plp1 at P0. Morphometric analysis showed a reduction in hippocampal volume at P35. Table 5 compares gene expression and morphological changes following infection at E9, E16, and E18.

Interestingly, Foxp2, Mbp and Aqp4 also display altered expression following infection at E9 (Table 5; Fatemi et al., 2005,2008c). Additionally, we have observed an upregulation of Foxp2 protein in hippocampus at P14 and P56 following infection at E18 (Table 5;Fatemi et al., 2008a). Foxp2 is a putative transcription factor containing a polyglutamine tract and a forkhead DNA binding domain. Foxp2 is associated with language and speech deficits (Hurst et al., 1990) that are commonly observed with autism. Aqp4 is localized to astrocytes and ependymal cells in brain and is involved with water transport (Papadopoulos et al., 2002; Verkman et al., 2006). Aqp4 is also reduced in cerebella of subjects with autism (Fatemi et al., 2008b). Mbp comprises about 30% of the myelin membrane and is believed to be involved in myelin compaction (Chambers and Perrone-Bizzozero, 2004). Mbp immunoreactivity is reduced in hippocampi of female subjects with schizophrenia (Chambers and Perrone-Bizzozero, 2004). Moreover, shiverer mice, which lack Mbp, display delayed action potentials (Lehman and Harrison, 2002) suggesting a role for Mbp in synaptic transmission. The recurrence of these genes in our data sets at multiple time points and multiple brain regions suggest they may be key to the changes observed following prenatal viral infection.

We demonstrated a 6% reduction in hippocampal volume ($p<0.014$) at P35 following infection at E16, and have previously demonstrated a 14% reduction in hippocampal volume ($p<0.00005$) at P35 following infection at E18 (Fatemi et al., 2008), consistent with the observed reductions in subjects with schizophrenia and autism. We do not have microarray data for this time point following infection at E16 or E18 so it is difficult to determine what genetic factors may play a role in hippocampal atrophy at P35. It is likely, however, that genes related to myelination, growth factors, and synaptic plasticity would be involved. Future studies should include microarray at P35 following infection at these dates. While infection at E18 resulted in morphological changes at P35, infection at E16 resulted in a greater number of changes at various time points as well as a greater number of changes in gene expression (Table 5) and it is likely that the two linked.

In conclusion, we observed that infection at E16 led to gene expression changes in hippocampi of exposed offspring. There were some similarities in gene expression when compared with infection at E9 and E18 although the total number of genes altered was greater following infection at E16. Moreover, there were a greater number of morphological changes. The greater magnitude of changes at E16 suggests that infection during middle second trimester leads to more deleterious effects in the exposed offspring than during middle first or late second trimester, consistent with findings that infection during middle second trimester carries a greater risk for the development of schizophrenia and autism in humans. These changes are in many cases similar to what has been observed in neuropsychiatric disorders, specifically autism and schizophrenia. Due to a low N, replication of our work is needed to examine the impact of these hippocampal changes in the exposed offspring.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Microarray and qRT-PCR Results for Selected Affected Genes in E16 Infected Mouse Hippocampus

Gene	Symbol	Disorder	Day	Microarray fold change	Microarray p-value	Gene relative to normalizer (qRT-PCR)	QPCR p-value
Aquaporin 4	Aqp4	Aut[1]	P0	-2.44	**0.012**	-2.48	**0.008**
Forkhead Box P2	Foxp2	Aut[2], Scz[3]	P0	-6.383	**0.046**	-4.68	**0.0038**
Gamma-aminobutyric acid receptor gamma 1	Gabrg1	Aut[4]	P0	-2.17	**0.049**	-2.78	**0.015**
Myelin basic protein	Mbp	Scz[5,6]	P0	-2.70	**0.019**	-3.94	**0.0007**
Neuronal cell adhesion molecule	Nrcam	Aut[7]	P0	1.85	**0.006**	-2.38	**9.10E-06**
Neurotensin	Nts	Scz[8]	P0	3.08	**0.009**	1.97	**4.75E-05**

*Not altered in microarray analysis; Aut, autism; Scz, schizophrenia;

[1]Fatemi et al., 2008b;

[2]Gong et al., 2004;

[3]Sanjuan et al., 2006;

[4]Kakinuma et al., 2008;

[5]Tkachev et al., 2003;

[6]Chambers and Perrone-Bizzozero, 2004;

[7]Marui et al., 2008;

[8]Hamid et al., 2002

Table 2

qRT-PCR Results for Selected Affected Genes in E16 Infected Mouse Hippocampus

Gene	Symbol	Disorder	Day	Gene relative to normalizer (qRT-PCR)	QPCR p-value
Aquaporin 4	Aqp4	Aut[1]	P0	-2.48	**0.008**
			P56	-1.24	**0.007**
Autism susceptibility candidate 2	Auts2	Aut[2]	P56	-1.35	**0.026**
Forkhead Box P2	Foxp2	Aut[3], Scz[4]	P0	-4.68	**0.0038**
			P14	8.81	**0.001**
Gamma-aminobutyric acid receptor gamma 1	Gabrg1	Aut[5]	P0	-2.78	**0.015**
Glial fibrillary acidic protein	Gfap	Aut[6], Scz[7]	P56	-1.37	**0.023**
Myelin-associated glycoprotein	Mag	Scz[8]	P0	-22.58	**0.0002**
			P14	1.86	**0.022**
Myelin and lymphocyte-associated protein	Mal	Scz[8,9]	P0	-4.63	**0.0009**
Myelin basic protein	Mbp	Scz[10,11]	P0	-3.94	**0.0007**
Myelin-associated oligodendrocytic basic protein	Mobp	Scz[12]	P0	-57.9	**0.00017**
Myelin oligodendrocyte protein	Mog	Scz[10]	P0	-29.60	**0.00046**
Neuronal cell adhesion molecule	Nrcam	Aut[13]	P0	-2.38	**9.10E-06**
Neurotensin	Nts	Scz[14]	P0	1.97	**4.75E-05**
Proteolipid protein (myelin) 1	Plp1	Scz[8]	P0	-6.52	**0.00019**
			P14	2.64	**0.0024**
Protein phosphatase 1, regulatory subunit 1b	Ppp1r1b	Scz[15]	P0	-17.07	**0.002592**
Regulator of G-protein signaling 4	Rgs4	Scz[16]	P0	-2.88	**0.0001**
			P14	-2.46	**3.72E-07**

*Not altered in microarray analysis; Aut, autism; Scz, schizophrenia;

[1]Fatemi et al., 2008b;

[2]Sultana et al., 2002;

[3]Gong et al., 2004;

[4]Sanjuan et al., 2006;

[5]Kakinuma et al., 2008;

[6] Laurence and Fatemi, 2005;

[7] Steffek et al., 2008;

[8] Hakak et al., 2001;

[9] LeNiculescu et al., 2007;

[10] Tkachev et al., 2003;

[11] Chambers and Perrone-Bizzozero, 2004;

[12] Lewis et al., 2003;

[13] Marui et al., 2008;

[14] Hamid et al., 2002;

[15] Feldcamp et al., 2008;

[16] So et al., 2008

Table 3

Changes in hippocampus area following infection at E16

PD	Brain Area	Control	Infected	Δ	P
P0	Hippocampus	2.29 ± 0.37	2.27 ± 0.12	—	0.916
	Total Brain	88.03 ± 4.03	89.67 ± 2.87	—	0.532
P14	Hippocampus	15.24 ± 0.60	14.26 ± 0.77	—	0.116
	Total Brain	359.94 ± 11.28	332.92 ± 8.55	↓7.5	**0.019**
P35	Hippocampus	19.43 ± 0.60	18.24 ± 0.34	↓6%	**0.014**
	Total Brain	416.56 ± 18.28	408.14 ± 16.67	—	0.521
P56	Hippocampus	18.81 ± 0.81	18.52 ± 0.32	—	0.534
	Total Brain	436.55 ± 14.82	423.44 ± 15.95	—	0.274

Δ, change; ↓, decrease; PD, postnatal date

Table 4

Changes in hippocampus white matter following infection at E16

PD	Control	Infected	Δ	P
P0	0.69 ± 0.037	0.70 ± 0.075	—	0.69
P14	0.62 ± 0.046	0.73 ± 0.070	—	0.057
P35	0.58 ± 0.040	0.57 ± 0.075	—	0.82
P56	0.77 ± 0.121	0.78 ± 0.079	—	0.95

Δ, change; ↓, decrease; PD, postnatal date

Table 5

Comparative Effects of Prenatal Viral Infection at E9, E16, and E18 on Gene Expression and Brain Morphology

Date of Infection	E9				E16[b]				E18[b]			
Microarray	P0	WB	↑21[b]	↓18[b]	P0	PFC	↑151	↓35	P0	PFC	↑43	↓29
	P35	NC	↑50[b]	↓21 [b]		Hipp	↑299	↓191		Hipp	↑129	↓46
		Cer	↑103[b]	↓102[b]		Cer	↑26	↓72		Cer	↑120	↓37
	P56	NC	↑13[b]	↓11[b]	P14	PFC	↑106	↓86	P14	PFC	↑16	↓17
		Cer	↑27[b]	↓23 [b]		Hipp	↑34	↓46		Hipp	↑9	↓12
						Cer	↑204	↓15		Cer	↑11	↓5
					P56	PFC	↑107	↓118	P56	PFC	↑86	↓24
						Hipp	↑87	↓30		Hipp	↑45	↓17
						Cer	↑449	↓204		Cer	↑74	↓22
Genes/Proteins (qRT-PCR and/or western blotting)	P0	↑nNos[a], ↑ Snap25[a], ↓Reelin[a], ↓Aqp4[a], ↓Mbp[a], ↑Gfap[b]			P0	↓Foxp2, ↓Aqp4, ↓Gabrg1, ↑Nrcam			P0	↑Erbb4, ↑Ivns1abp, ↑Myt1l, ↑Sema3a, ↑Sox2, ↑Trfr2, ↑Foxp2		
	P14	↑Gfap[a], ↑Gad65[b]										
	P35	↑nNos[a], ↑Gfap[a], ↑Gad65[b], ↑Gad67[b], ↑Foxp2[b]			P14	↓Nrcam, ↓Auts2, ↓Mbp, ↓Mag			P35	↑Foxp2		
	P56	↓nNos[a], ↑Gad67[b]			P56	↓Mbp, ↓Plp1, ↓Rgs4, ↑Auts2, ↓Gabrg1			P56	↑Dby, ↑Uty, ↑Foxp2		
Brain Morphology	P0	↑PC density [a], ↓PC nuclear size[a]			P0	↓VA; ↓FA in IC			P35	↓Brain volume, ↓Cer volume, ↓Hipp volume; ↓FA in CC		
	P98	↑PC density [a], ↑Non-PC density [a], ↓PC nuclear size [a], ↑Brain area[a], ↓VA[a]			P14	↓Cer volume, ↓ Brain volume; ↑FA in CC						
					P35	↓Hipp volume						
					P56	↑FA in MCP						

[a] C57BL6J mice;

[b] Balb/c mice;

CC, corpus callosum; Cer, cerebellum; FA, fractional anisotropy; Hipp, hippocampus; MCP, middle cerebellar peduncle; NC, neocortex; PC, pyramidal cell; VA, ventricular area; WB, whole brain. Data from Fatemi et al, 1998a, b, 1999, 2000, 2002a,b, 2004, 2005, 2008a, unpublished observations.