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Early Postnatal Respiratory Viral Infection Induces Structural and Neurochemical Changes in the Neonatal Piglet Brain

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Abstract

Infections that cause inflammation during the postnatal period are common, yet little is known about their impact on brain development in gyrencephalic species. To address this issue, we investigated brain development in domestic piglets which have brain growth and morphology similar to human infants, after experimentally infecting them with porcine reproductive and respiratory syndrome virus (PRRSV) to induce an interstitial pneumonia. Piglets were inoculated with PRRSV on postnatal day (PD) 7 and magnetic resonance imaging (MRI) was used to assess brain macrostructure (voxel-based morphometry), microstructure (diffusion tensor imaging) and neurochemistry (MR-spectroscopy) at PD 29 or 30. PRRSV piglets exhibited signs of infection throughout the post-inoculation period and had elevated plasma levels of TNF α at the end of the study. PRRSV infection increased the volume of several components of the ventricular system including the cerebral aqueduct, fourth ventricle, and the lateral ventricles. Group comparisons between control and PRRSV piglets defined 8 areas where PRRSV piglets had less gray matter volume; 5 areas where PRRSV piglets had less white matter volume; and 4 relatively small areas

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Conflicts of Interest

The authors declare no conflicts of interest.

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where PRRSV piglets had more white matter. Of particular interest was a bilateral reduction in gray and white matter in the primary visual cortex. PRRSV piglets tended to have reduced fractional anisotropy in the corpus callosum. Additionally, *N*-acetylaspartate, creatine, and myo-inositol were decreased in the hippocampus of PRRSV piglets suggesting disrupted neuronal and glial health and energy imbalances. These findings show in a gyrencephalic species that early-life infection can affect brain growth and development.

Keywords

Magnetic Resonance Imaging; Infection; Neonatal; Pig; Development; Brain

1. Introduction

Infectious disease is the most common cause of illness in children, with acute respiratory infection constituting the most prevalent reason for hospitalization in children under one year of age (Hall et al., 2009). This is a concern for neurodevelopment because the phase of rapid brain growth that begins in the late prenatal period continues into the early postnatal period due to dendritic growth, synaptogenesis, and intense glial cell proliferation (Dietrich et al., 1988; Huttenlocher, 1979; Rice and Barone, 2000). During infection, immune-to-brain signaling pathways activate microglia, causing neuroinflammation (Dantzer and Kelley, 2007). Developing and mature neurons, as well as glia, have numerous pro-inflammatory cytokine receptors (Sawada et al., 1993), and untimely inflammation in rodent models, especially in the prenatal period, is known to affect neural development and increase risk for behavioral disorders that manifest later (Meyer et al., 2011). Little is known, however, about peripheral infection in the neonatal period, especially in humans and other animals whose brain is gyrencephalic and experience major perinatal growth.

Quantitative magnetic resonance imaging (MRI) can provide important information on brain development in early childhood and adolescence but few studies have focused on the period from birth to 2 years of age when dramatic brain development occurs. Progress on understanding the influence of infection on brain development in human infants has been especially slow because separating the impact of infection from the many other factors impacting brain development is difficult. Furthermore, while studies in rodents have been critical in establishing the neuroimmune hypothesis of developmental disorders, translating the results to human infants can be challenging due to the differences in brain development and morphology. In this regard, the domestic piglet (*Sus scrofa domestica*) may be a useful translational model. Similar to humans, the major brain growth spurt in pigs extends from the late prenatal to the postnatal period (Dobbing and Sands, 1979). Gross anatomical features, including gyral pattern and distribution of gray and white matter of the neonatal piglet brain are similar to that of human infants (Dickerson and Dobbing, 1967; Thibault and Margulies, 1998). Moreover, their physical size allows neuroimaging instruments designed for humans to be used with piglets. Indeed, structural MRI, functional MRI, and positron emission tomography have all been conducted in pigs (Conrad et al., 2012b; Fang et al., 2005; Jakobsen et al., 2006). Additionally, piglets can undergo cognitive testing at a young age and show greater overlap with humans in genes involved in immunity compared to

rodents (Dawson, 2011; Dilger and Johnson, 2010; Meurens et al., 2012). Thus, piglets represent a gyrencephalic species with brain growth similar to humans that can be used in highly controlled experiments to explore how infection and inflammation affects brain structure and function.

In the present study, we used MRI to assess macrostructure (voxel-based morphometry), white matter microstructure (diffusion-tensor imaging) and metabolites (spectroscopy) to test the hypothesis that neuroinflammation in the early postnatal period affects brain development in piglets. For this study piglets were given porcine reproductive and respiratory syndrome virus (PRRSV), which infects mononuclear myeloid cells in lungs inducing interstitial pneumonia (Done et al., 1996). PRRSV is a useful model for investigating neuroinflammation as it was recently reported to activate brain microglial cells (Elmore et al., 2014).

2. Materials and Methods

2.1 Animals, Housing, and Feeding

Naturally farrowed crossbred piglets from three separate litters from the University of Illinois swine herd were used. Piglets remained with the sow for 24–48h in order to receive colostrum which confers postnatal passive immunity. Importantly, sows were PRRSV-free and not vaccinated for PRRSV. Piglets were brought to the biomedical animal facility on PD 2, where upon arrival they were assigned to either the control group (3 males and 3 females) or the PRRSV infection group (4 males and 4 females) based on sex, litter of origin, and body weight. They were housed individually in cages (0.76 m L × 0.58 m W × 0.47 m H) designed for neonatal piglets (Elmore et al., 2014). Each cage was positioned in a rack, with stainless steel perforated side walls and clear acrylic front and rear doors within one of two separate but identical disease containment chambers that have been described (Elmore et al., 2014). Each cage was fitted with flooring designed for neonatal animals (Tenderfoot/NSR, Tandem Products, Inc., Minneapolis, MN, USA). A toy (plastic Jingle Ball™, Bio-Serv, Frenchtown, NJ, USA) was provided to each piglet. Room temperature was maintained at 27°C and each cage was equipped with an electric heat pad (K&H Lectro-Kennel™ Heat Pad, K&H Manufacturing, LLC, Colorado Springs, CO, USA). Piglets were maintained on a 12-h light/dark cycle; however, during the dark cycle minimal lighting was provided.

Piglets were fed a commercial sow milk replacer (Advance Liqui-Wean, Milk Specialties Co., Dundee, IL, USA). Milk was reconstituted daily to a final concentration of 206 g/L using tap water and supplied at a rate of 285 mL/kg BW (based on daily recorded weights) to a stainless steel bowl via a peristaltic pump (Control Company, Friendswood, TX). Using this automated feeding system (similar to that described previously (Dilger and Johnson, 2010)), piglets received their daily allotted milk over 18 meals (once per hour from 0600 h to 2400 h).

Two of the PRRSV females developed severe diarrhea and were removed from the study. All animal experiments were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

2.2 Experimental Design and Assessment of Sickness

At PD 7, piglets were inoculated intranasally with either 1mL of 1×10^5 50% tissue culture infected dose (TCID₅₀) of live PRRSV (strain P129-BV, obtained from the School of Veterinary Medicine at Purdue University, West Lafayette, IN, USA) or sterile phosphate buffered saline (PBS). Daily body weights (kg) were obtained to monitor piglets' growth. In addition, daily rectal temperatures were obtained PD 7 through PD 28. The willingness of the piglets to consume their first daily meal was determined from PD 7 to PD 28 using a feeding score (1 = no attempt to consume the milk; 2 = attempted to consume the milk, but did not finish within 1 min; 3 = consumed all of the milk within 1 min). Body weight, rectal temperature, and feeding score data were analyzed as a two-way (treatment x day) repeated measures ANOVA using the MIXED procedure in SAS (SAS Institute Inc, Cary, NC, USA). There were no significant differences due to sex, therefore it was not included in final analysis of body weight, rectal temperature, and feeding score data. Significance was accepted at $p < 0.05$.

The presence of PRRSV antibodies in the serum of all piglets at the end of the study was analyzed by the Veterinary Diagnostic Laboratory (University of Illinois, Urbana, Illinois) using a PRRSV-specific ELISA kit (IDEXX Laboratories, Westbrook, MD). Serum tumor necrosis factor alpha (TNF- α) levels at the end of the study were determined using porcine-specific sandwich enzyme immunoassays (R&D Systems, Minneapolis, MN). Data analysis was conducted using the MIXED procedure in SAS. Serum TNF- α concentrations were analyzed as a two-way (treatment x sex) ANOVA. Sex was not significant so it was removed from the model and data were reanalyzed as a one-way ANOVA to determine the effects of treatment. Significance was accepted at $p < 0.05$.

At PD 29 and PD30 control and PRRSV piglets were transported to the Biomedical Imaging Center at the Beckman Institute and MRI was conducted using a Siemens MAGNETOM Trio 3 T imager and a 32-channel head coil (Siemens, Erlangen, Germany). The MRI sequences have been previously described (Radlowski et al., 2014), and are briefly explained below. The total scan time per pig was roughly 50 minutes.

2.3 Structural MRI Acquisition and Analysis

For brain structure analysis, anatomic images were acquired using a 3D T1-weighted magnetization-prepared rapid gradient-echo (MPRAGE) sequence and registered using Diffeomorphic Anatomical Registration using Exponentiated Lie Algebra (DARTEL). Before scanning, piglets were anesthetized using a telazol:ketamine:xylazine (100/50/50 mg/kg/BW; Fort Dodge Animal Health). Anesthesia was maintained by inhalation of isoflurane (98% oxygen/2% isoflurane). A MRI compatible pulse oximeter was used to monitor heart rate and oxygen saturation throughout the scanning procedure. After piglets entered a sustainable plane of deep anesthesia, they were placed in the MRI machine. Piglets were restrained and remained immobilized throughout the MRI scans to prevent motion artifact. After conclusion of the scans, the piglets were euthanized via intracardiac injection of sodium pentobarbital (Fatal Plus, 72 mg/kg BW).

Three repetitions were acquired using the following parameters: repetition time = 1,900 ms; echo time = 2.49 ms; inversion time = 900 ms; flip angle = 9°; matrix = 256 × 256, 224 slices with thickness = 0.7 mm. The final voxel size was 0.7 mm isotropic across the entire head from the tip of the snout to the cervical/thoracic spinal cord junction as described previously (Conrad et al., 2012a).

The three MPRAGE image sets were averaged and the brains were manually extracted using FSL (Analysis Group, FMRIB, Oxford, UK) (Jenkinson et al., 2012). The extracted brains were brought in to a common space by 12 parameter affine registration to a digital piglet brain atlas (<http://pigmri.illinois.edu> and <http://doi.org/10.5061/dryad.9mj12>) (Conrad et al., 2014). The remainder of the voxel-based morphometry technique was conducted using SPM8 (Wellcome Department of Clinical Neurology, London; <http://www.fil.ion.cul.ac.uk>) in MATLAB version 8.0.0.783 (Mathworks). The brains were segmented into gray matter, white matter, and CSF classifications using the prior probability maps available in the piglet brain atlas dataset (<http://pigmri.illinois.edu> and <http://doi.org/10.5061/dryad.9mj12>). Next, the Diffeomorphic Anatomical Registration using Exponentiated Lie Algebra (DARTEL) package of SPM8 was used to create study specific templates (Ashburner, 2007).

Changes from the default DARTEL procedures include a bounding box of −30.1 to 30.1, −35 to 44.8, −28 to 31.5; and a voxel size of 0.7 mm³. A linear bend energy was used as the cost function. Flow fields generated from the DARTEL procedure were converted to warp fields which represent the expansion/shrinkage from the subject to atlas space. The modulated data were smoothed with a 4 mm full-width half maximum (FWHM).

The statistical non-parametric methods (SnPM) toolbox was used to compare the modulated gray and white matter data between the control and PRRSV groups (<http://go.warwick.ac.uk/tenichols/snpm>). SnPM uses non-parametric permutation tests which is advantageous for small numbers per treatment (Nichols and Holmes, 2002). A maximum number of permutations were used with a FWHM of 7 mm for variance smoothing. ANCOVA was used for global normalization and a mean voxel value was used for Global Calculation. All other options were default. No covariates were used. Two-sample permutation *t* tests were performed voxel-wise for both gray and white matter to detect differences between the control and PRRSV treatments. An uncorrected *p* < 0.01 was used. A threshold of at least 20 edge-connected voxels (forming a cluster) was set and clusters that appeared on the edges of the brain were excluded. The differences between control and PRRSV piglets are displayed in pseudo-*t* statistic images. The cortical regions of the significant voxel clusters were estimated using an MRI-based atlas for adult domestic pigs (Saikali et al., 2010).

In addition to the VBM, brain region volume estimations were calculated. Using the warp fields from the DARTEL procedure, inverse warps were created for each subject. The inverse warp represents the expansion/shrinkage from the atlas to the subject space. The inverse warps were then applied to the regions of interest (ROI) maps found in the piglet brain atlas (Conrad et al., 2014). The volumes were then estimated for each ROI for each subject using FSLstats. Data analysis was conducted using the MIXED procedure in SAS. ROI volumes were analyzed as a two-way (treatment x sex) ANOVA. Significance was

accepted at $p < 0.05$. Unless otherwise stated, data are presented as Least Square Means (LSM) $\pm$ Standard Error of the Means (SEM).

2.4 Diffusion Tensor Imaging and Analysis

Analysis of white matter microstructure was conducted using a diffusion tensor imaging (DTI) sequence to visualize fiber tracts non-invasively. A diffusion-weighted echo-planar-imaging (DW-EPI) sequence was used with the following parameters: repetition time = 5000 ms; echo time = 91 ms; averages = 3; diffusion weightings = 2; b-value of 1000 s/mm² across 30 directions; two images with a b-value of 0 s/mm². Forty slices with a 2.0 mm thickness were collected with a matrix size of 100 $\times$ 100 for a final voxel size of 2.0 mm isotropic. Masks of the corpus callosum and white matter from the piglet brain atlas were nonlinearly transformed to the subject's MPRAGE space and then linearly transformed into the DTI space. The corpus callosum had a threshold of 0.15 applied to compensate for expansion caused by interpolation. The white matter ROI had a threshold of 0.5 applied and was dilated twice. Averages were taken in the regions of interest with a FA > 0.2 mask to select only white matter tracts. MD values were averaged over the same region of interest.

The DW-EPI images were analyzed using the diffusion toolbox in the FSL software package to create fractional anisotropy (FA) and mean diffusivity (MD) images with ROI analysis for white matter and the whole corpus callosum. Due to limitations of the piglet brain atlas, subregion analysis of the corpus callosum was not conducted. Data analysis was conducted using the T-TEST procedure of SAS software. A two-sample two-sided t-test design was used to compare control versus PRRSV animals. Significance was accepted at $p < 0.05$, statistical trends at $p < 0.10$.

2.5 MR Spectroscopy Parameters and Analysis

Magnetic resonance spectroscopy (MRS) was performed using a single voxel PRESS sequence (repetition time = 3000 ms; echo time = 30 ms; 128 averages, weak water suppression). The voxel size was 12 $\times$ 25 $\times$ 12 mm and was placed over the left and right dorsal hippocampus. Six regional saturation bands were placed around the volume of interest. Shimming was performed before each spectroscopic scan to ensure a FWHM of less than 18 Hz. Water-suppressed spectra were analyzed between 0.2 and 4.0 ppm without further T₁ or T₂ correction. Absolute concentrations were calculated for each metabolite. Cramer-Rao lower bounds (S.D.%) were calculated by LC Model to estimate the uncertainty of the concentration values. Only those levels that were less than 20% were considered reliable and were included for further statistical analysis. For quantification of the metabolite signal, a second scan without water suppression and 8 averages was obtained immediately after the water-suppressed scan. Spectral analysis and quantification was performed using LC Model, version 6.3 (Provencher). Absolute metabolite concentrations, in institutional units, were obtained by using eddy current correction and water scaling. Data analysis was conducted using the MIXED procedure in SAS. Metabolite concentrations were analyzed as a two-way (treatment $\times$ sex) ANOVA. Sex was not a significant main effect for any metabolite so it was removed from the model and data were reanalyzed as a one-way ANOVA to determine the effects of treatment. Significance was accepted at $p < 0.05$.

3. Results

3.1 PRRSV Infection and Measures of Sickness

Serum ELISA results indicated all control piglets were negative for PRRSV at the end of the study, whereas all piglets inoculated were positive for PRRSV. Body weight was recorded and rectal temperature and the willingness to consume the first daily meal (feeding score) were determined to provide an indication of the sickness response of piglets infected with PRRSV (Figure 1). Analysis of body weight data showed a significant effect of day ($F(28, 275) = 31.51, p < 0.001$), but neither treatment ($F(1, 10) = 0.53$) nor the day $\times$ treatment interaction ($F(27, 275) = 1.25$) were significant indicating control and PRRSV piglets gained similar weight over the course of the study, probably owing to the fact that control and PRRSV piglets were limit-fed. Nonetheless, analysis of feeding score data revealed a significant effect of treatment ($F(1, 10) = 10.21, p < 0.01$), day ($F(22, 214) = 2804.45, p < 0.001$), and a trend toward a treatment $\times$ day interaction ($F(21, 214) = 1.58, p = 0.056$), indicating PRRSV piglets' motivation to consume the first meal of the day was reduced towards the last half of the study. Analysis of rectal temperature data showed a significant effect of treatment ($F(1, 10) = 43.19$), day ($F(22, 215) = 3.56$), and a treatment $\times$ day interaction ($F(21, 215) = 2.88$) (all, $p < 0.0001$). PRRSV piglets became febrile 3 d after inoculation and remained so during most of the experimental period. The proinflammatory cytokine TNF- α was significantly increased in PRRSV piglets ($F(1, 20) = 98.87, p < 0.001$) with an average serum concentration of 135.6 ± 13.0 pg/mL compared to 17.1 ± 1.7 pg/mL in controls. Collectively, these data indicate infection with PRRSV in the neonatal period induced clinical signs of illness that persisted throughout the study period.

3.2 Brain Region Volume Estimation

Volume estimations were made for 19 regions of interest (ROI's) with regions chosen from the piglet brain atlas (Conrad et al., 2014) (Table 1). Using the deformation maps created during the DARTEL procedure, ROI's from the piglet brain atlas were warped to the individual subject's brain. In addition, gray and white matter volumes were estimated by the segmented data from the DARTEL procedure and whole brain volume was estimated using the extracted brain. Overall, there were very few differences in brain region volumes between control and PRRSV piglets or between male and female piglets (Table 1). An exception was that PRRSV infection increased the volume of several components of the ventricular system including the cerebral aqueduct, fourth ventricle, and the lateral ventricles. In the case of the cerebral aqueduct and fourth ventricle, the effects of PRRSV were greater in females compared to males.

3.3 Voxel-based Morphometry

Although there were few effects of PRRSV on brain region volumes, the VBM analysis (Table 2) revealed eight clusters where PRRSV piglets had significantly less gray matter compared to control piglets. The local maxima coordinates were mapped to the piglet brain atlas and histological atlas (Felix et al., 1999) to estimate the anatomic region. Of particular interest are two clusters located in the left and right primary visual cortex which had the highest pseudo-t value. Pseudo-t maps for areas that have reduced gray matter volume in

PRRSV piglets are shown in Figure 2. There were no areas where the PRRSV piglets had increased gray matter volumes compared to controls.

Analysis of white matter volumes revealed there were five clusters where PRRSV piglets had less white matter volume than controls (Table 2). Pseudo t-maps for areas that have reduced white matter volume in PRRSV piglets are shown in Figure 3. We also found four regions where PRRSV piglets had increased white matter compared to controls (Table 2). These regions had smaller clusters and lower pseudo-t values compared to clusters where controls had more white matter volume.

3.4 Diffusion Tensor Imaging

Fractional anisotropy maps were compared between control and PRRSV piglets to evaluate white matter tract differences. An example FA map is shown in Figure 4. Two areas of interest were evaluated for FA and MD differences including white matter and the corpus callosum (Figure 4). There were no differences in white matter FA ($p > 0.10$). However, when the region of interest was restricted to the corpus callosum, control piglets tended to have a larger FA than PRRSV piglets ($p = 0.06$). There were no significant differences in corpus callosum or white matter for MD ($p > 0.10$ in each case).

3.5 Magnetic Resonance Spectroscopy

Differences in absolute metabolite concentrations between control and PRRSV piglets were evaluated in the hippocampus using MRS. The hippocampus was chosen because PRRSV infection (a) activates microglia in this brain region; and (b) inhibits performance in a hippocampal-dependent spatial T-maze task (Elmore et al., 2014). A single voxel was placed over the left and right dorsal hippocampus. This location and a representative MR spectrum are shown in Figure 5. Six metabolites/metabolite combinations reached Cramer-Rao lower bounds of $SD < 20\%$ in all subjects. These metabolites and their absolute concentrations are shown in Table 3. Four of the metabolites were significantly reduced in the PRRSV piglets including creatine/phosphocreatine (Cr+PCr), N-Acetylaspartate (NAA), NAA/N-Acetylaspartylglutamate (NAAG), and Myo-inositol (Ins). There were no differences in water signal between control and PRRSV animals.

4. Discussion

Infections that cause inflammation during the early postnatal period are common, yet little is known about their impact on brain development. Progress in this area has been slow because studies in human infants are difficult to conduct and are often confounded by comorbidities. Furthermore, results from rodent models commonly used to investigate neurodevelopment can be difficult to translate to human infants due to the differences in brain development and morphology. To overcome some of these barriers, the present study was conducted using a translational piglet model.

In a recent study, several pro-inflammatory cytokines were elevated in serum 20 d after inoculation with PRRSV, and a number of pro-inflammatory genes, including interferon- γ , TNF α , and IL-1 β , were up regulated in several brain regions at the same time post inoculation (Elmore et al., 2014). Furthermore, the percentage of activated microglia, as

indicated by expression of MHC class II, was markedly increased in piglets with PRRSV infection (Elmore et al., 2014). Microglial activation was positively correlated with fever, and negatively correlated with food motivation and learning and memory (Elmore et al., 2014). In the present study, piglets were febrile throughout the study and circulating TNF α was elevated in samples collected after MRI (i.e., 21 d after inoculation). This suggests that the viral infection was either unresolved or the presence of a secondary infection. PRRSV causes leukopenia and in production settings where other infectious agents are present, secondary infections often occur. However, in this study piglets were maintained in a well sanitized (but not sterile) and highly controlled environment making a secondary infection less likely. Nonetheless, we cannot rule this out; and a secondary infection could contribute to the high circulating levels of TNF α . In any case, it is reasonable to conclude that PRRSV infected piglets in this study experienced a sustained neuroinflammatory response throughout the study period.

We employed multiple MRI-based techniques to assess the effects of PRRSV infection on piglet brain macrostructure (VBM), microstructure (DTI), and biochemistry (MRS). We recently used a similar strategy to explore brain development in piglets born small-for-gestational-age (i.e., a model of intrauterine growth restriction; (Radlowski et al., 2014)). In the present study, VBM revealed multiple brain areas in PRRSV piglets with less gray matter volume. The two most significant clusters were located bilaterally in the primary visual cortex. White matter volume was also reduced in these regions. Although not much is known about primary visual cortex development in piglets, in primates, the primary visual cortex has a high rate of synaptogenesis after birth and, therefore, may be sensitive to environmental insults at this time (Bourgeois and Rakic, 1993; Gogtay et al., 2004; Johnson, 1990). Microglia are vital for synaptogenesis in the primary visual cortex and activation may disrupt normal development (Rocheffort et al., 2002). We want to be cautious in interpretation of these findings in neonatal piglets because despite many similarities in brain development for pigs and humans, clearly there are important differences. For example, piglets have well developed motor and sensory systems at birth compared to humans. In any case, it is worth noting that a neuroimmune-based developmental hypothesis has been proposed for several psychiatric diseases in humans that are associated with deficiencies in the primary visual cortex (Meyer et al., 2011). The reduced volumes seen in the PRRSV piglets may be indicative of the sensitivity to inflammation and may result in long term consequences. Either delays in synaptogenesis or degeneration of established connections could explain the volume differences. It is possible that these changes could recover after complete resolution of the infection and long-term studies tracking this would be beneficial.

In addition to voxel-based morphometry, region of interest analysis was used to estimate volumes of 19 brain regions. Changes were found in the ventricular system, including the cerebral aqueduct, lateral, and fourth ventricle, where PRRSV piglets had an increased volume, which may reflect hydrocephalus and/or global cellular loss. Increased lateral ventricle volumes have been shown in both autism and schizophrenia (Chua and McKenna, 1995; Palmen et al., 2005; Piven et al., 1995; Rosa et al., 2010). We also found a sexual dimorphism in the corpus callosum where males had a smaller corpus callosum than females, similar to humans (Ardekani et al., 2012). Although significant differences were

found in the cortical regions with VBM, we did not find differences in left or right cortical volumes with ROI analysis. This is likely due to the insensitivity of ROI analysis.

Myelination begins in late gestation, has its highest rate of formation in early-life, and is susceptible to inflammation (Abraham et al., 2010; Favrais et al., 2011; Lenroot and Giedd, 2006; Nakagawa et al., 1998). Disruptions to white matter tracts, including the corpus callosum, have been found in psychiatric disease (Alexander et al., 2007; Cheung et al., 2009; Cheung et al., 2008). Here, we found no differences in the FA values across the whole brain and white matter. There was a trend in the whole corpus callosum where the PRRSV piglets had decreased FA values which may indicate a disruption in myelination. Previous studies in rodents have found that inflammatory cytokines block oligodendrocytes maturation and cause axonopathy including reduced myelinated axon diameter (Favrais et al., 2011). The inflammatory response that is seen in the PRRSV piglets includes an increase in pro-inflammatory cytokines and this may be a mechanism for the changes seen (Elmore et al., 2014).

MR-spectroscopy is a powerful tool to quantify chemical metabolite concentrations non-invasively (Blüml et al., 2013; Soares and Law, 2009). Creatine/phosphocreatine, myo-inositol, NAA, and NAAG were reduced in PRRSV piglets. Reductions in creatine and myo-inositol suggest an energy imbalance and decrease in glial cells or delayed gliogenesis compared to controls. NAA is a marker of the density and viability of neurons and is reduced in many neuroinflammatory conditions (Soares and Law, 2009). The reduced NAA in the PRRSV piglets may be a sign of neuronal loss or developmental impairment. Similar findings have been shown in hypoxia ischemia models using piglets suggesting that energy-dysregulation, including secondary energy failure, may be present after early-life infection (Munkeby et al., 2008).

5. Conclusion

The present study found that PRRSV infection causes structural changes in gray and white matter, as well as neurochemical changes. Due to the small number of animals used for this study, we were limited in the analysis of covariates in VBM including sex. Further, histological analysis of the regions shown to have changes indicated by MRI methods (primary visual cortex and corpus callosum) would be beneficial to characterize possible cellular changes. Regardless, results presented herein suggest that postnatal infection and inflammation can affect normal brain development. In future studies, it will be important to examine long-term endpoints to determine the lasting impact of early-life infection.

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Highlights

- Early-life respiratory viral infection impacts brain development in piglets.
- Infected piglets had region specific reductions in grey and white matter volume.
- Infected piglets had a trend for reduced FA in the corpus callosum.
- Infected piglets had a disrupted neurochemical balance in the hippocampus.

Figure 1.

PRRSV piglets show a febrile response and reduced feeding score (A to C). There were no differences in weight between the two treatments (A). PRRSV piglets had significantly higher temperatures starting on the third day that lasted throughout the study. PRRSV piglets also had a significant reduction in feeding score. Data are shown as the mean $\pm$ SEM (* p <0.05, # p <0.01; n =6 per treatment).

Figure 2. VBM analysis showing clusters where PRRSV pigs had significantly less gray matter than controls. Axial slices are shown with a 1 mm slice thickness. The color bar indicates significance level (pseudo t-statistic).

Figure 3. VBM analysis showing areas where PRRSV pigs had significantly less white matter than controls. Axial slices are shown with a 1 mm slice thickness. The color bar indicates significance level (pseudo t-statistic).

Figure 4.

PRRSV piglets have a trend towards reduced FA in the corpus callosum (A to E). There was a trend that the controls had a higher FA than PRRSV in the corpus callosum (A; $p=0.0637$). There were no differences in FA found in white matter (B). There were no differences in MD in corpus callosum (C), or white matter (D). A representative FA map in a axial, sagittal, and coronal slice from left to right is shown (E). Data are shown as the mean $\pm$ SEM ($n=6$ per treatment). Units for mean diffusivity are $10^{-3} \text{ mm}^2/\text{s}$.

Figure 5. Representative proton MR spectroscopy spectrum and location of the voxel placement. The single voxel was placed across the left and right dorsal hippocampus for each animal.

Table 1

Brain region volume estimations.

Brain Region	Control Male		Control Female		PRRSV Male		PRRSV Female		P-values		
	Mean (mm ³)	Std Error	Mean (mm ³)	Std Error	Mean (mm ³)	Std Error	Mean (mm ³)	Std Error	Treatment	Sex	Treatment x Sex
Whole Brain	81536	1292	84256	4375	76821	4249	83167	1792	0.2814	0.4808	0.6565
White Matter	18623	213	18191	1123	17079	797	19270	1429	0.3722	0.809	0.1962
Gray Matter	38894	1907	39678	3335	37955	2703	38024	463	0.8825	0.6552	0.9015
Caudate	557	7	591	41	561	40	579	14	0.4893	0.9088	0.8302
Cerebellum	5137	253	4925	306	4906	208	4745	19	0.4849	0.4427	0.9223
Cerebral Aqueduct	121	3	119	5	114	6	155	19	0.036	0.089	0.0239
Corpus Callosum	950	27	1018	55	904	43	1074	87	0.9193	0.049	0.3509
Fourth Ventricle	137	7	133	6	130	4	166	11	0.0318	0.077	0.0118
Hypothalamus	552	9	549	29	506	22	581	1	0.1555	0.7449	0.1277
Internal Capsule	4855	87	4909	257	4767	281	4910	36	0.703	0.8661	0.862
Lateral Ventricle	982	60	1137	90	1049	38	1282	178	0.0422	0.2246	0.6378
Left Cortex	17572	412	17961	1466	16718	1141	17206	210	0.7121	0.5022	0.9665
Left Hippocampus	567	12	588	30	592	23	587	6	0.742	0.6318	0.6039
Medulla	2641	144	2509	111	2424	127	2581	61	0.9271	0.5992	0.3085
Midbrain	2757	333	3158	91	2939	129	3158	32	0.1622	0.6657	0.6634
Olfactory Bulb	2990	193	3067	96	2902	218	3175	388	0.4615	0.9676	0.6741
Pons	866	16	877	27	847	22	914	24	0.1381	0.7183	0.2703
Putamen	1189	37	1219	66	1207	97	1095	89	0.6418	0.5438	0.4298
Right Cortex	16526	389	16860	1235	15709	1022	16106	23	0.7246	0.4547	0.9758
Right Hippocampus	633	8	644	28	651	29	647	34	0.894	0.7089	0.7698
Thalamus	3125	53	3148	98	3049	172	3119	50	0.747	0.7142	0.8667
Third Ventricle	197	4	203	17	203	13	202	14	0.8663	0.8711	0.7669

Volume averages for 22 regions are reported by sex and treatment. Significance due to sex, treatment, and the interaction are shown.

Table 2

Voxel-based morphometry analysis of gray and white matter differences.

Comparison	Anatomic Region	Cluster (voxels)	Cluster level (P-value)	Local Maxima Coordinates*				Pseudo-t
				x	y	z		
Gray Matter	Control>PRRSV	Left Primary Visual Ctx	.0032	-9	2	16		4.82
		Right Primary Visual Ctx	.0022	10	1	17		4.44
		Left Insular Ctx	.0032	-8	25	4		3.32
		Prefrontal Ctx	.0043	0	22	-2		3.04
		Left Auditory Ctx	.0087	-18	6	8		2.58
	PRRSV>Control	Right Fusiform Gyrus	.0087	15	-11	1		2.54
		Left Fusiform Gyrus	.0054	-13	-10	3		2.51
		Right Primary Visual Ctx	.0065	6	-3	20		2.39
		No significant findings						
White Matter	Control>PRRSV	Left Primary Visual Ctx	.0022	-6	1	17		4.60
		Right Primary Visual Ctx	.0032	8	-1	17		4.51
		Right Primary Somatosensory Ctx	.0097	12	15	15		3.94
		Left Primary Somatosensory Ctx	.0032	-11	9	17		3.61
			.0032	-13	2	14		3.48
	PRRSV>Control	Left Primary Somatosensory Ctx	.0087	-8	22	15		2.35
		Prefrontal Cortex	.0097	3	31	1		2.59
		Right Internal Capsule	.0065	8	9	10		1.75
		Left Hippocampus	.0097	-6	-6	2		1.73
		Left Internal Capsule	.0097	-7	9	10		1.73

Significant clusters of at least 20 voxels are shown with the cluster size (voxels), cluster significance, location, and pseudo-t value.

Table 3

Absolute metabolite concentration in the hippocampus.

Metabolite	Control (SEM)	PRRSV (SEM)	P-value
Creatine + Phosphocreatine (Cr + PCr)	4.3395 (0.0915)	3.6992 (0.1163)	0.0015
N-Acetylaspartate (NAA)	5.3473 (0.0579)	4.6953 (0.1977)	0.0101
NAA + N-Acetylaspartylglutamate (NAAG)	5.7728 (0.0286)	5.0563 (0.1845)	0.0033
Myoinositol (Ins)	9.5028 (0.4302)	8.2737 (0.3395)	0.0488
Phosphocholine + Glycerophosphocholine (GPC + PCh)	1.6775 (0.0506)	1.6253 (0.0561)	0.5056
Glutamate + Glutamine (Glu + Gln)	6.6178 (0.3099)	7.4558 (0.6084)	0.2478

Single voxel MRS for six metabolites that met criteria for analysis (%S.D.<20). Data are shown as the mean $\pm$ SEM (n=6 per treatment).