Behavioral/Cognitive

Respiratory Viral Infection in Neonatal Piglets Causes Marked Microglia Activation in the Hippocampus and Deficits in Spatial Learning

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Environmental insults during sensitive periods can affect hippocampal development and function, but little is known about peripheral infection, especially in humans and other animals whose brain is gyrencephalic and experiences major perinatal growth. Using a piglet model, the present study showed that inoculation on postnatal day 7 with the porcine reproductive and respiratory syndrome virus (PRRSV) caused microglial activation within the hippocampus with 82% and 43% of isolated microglia being MHC II $^{+}$ 13 and 20 d after inoculation, respectively. In control piglets, <5% of microglia isolated from the hippocampus were MHC II $^{+}$. PRRSV piglets were febrile ($p < 0.0001$), anorectic ($p < 0.0001$), and weighed less at the end of the study ($p = 0.002$) compared with control piglets. Increased inflammatory gene expression (e.g., IL-1β, IL-6, TNF-α, and IFN-γ) was seen across multiple brain regions, including the hippocampus, whereas reductions in CD200, NGF, and MBP were evident. In a test of spatial learning, PRRSV piglets took longer to acquire the task, had a longer latency to choice, and had a higher total distance moved. Overall, these data demonstrate that viral respiratory infection is associated with a marked increase in activated microglia in the hippocampus, neuroinflammation, and impaired performance in a spatial cognitive task. As respiratory infections are common in human neonates and infants, approaches to regulate microglial cell activity are likely to be important.

Key words: **brain; cognition; inflammation; PRRSV; sickness behavior**

Introduction

Infectious disease remains the most common cause of illness in children, with acute respiratory infection constituting the most prevalent reason for medical intervention in children under one year of age (Hall et al., 2009). Given the well-characterized immune-to-brain signaling pathways that lead to neuroinflammation (Dantzer et al., 2008), serious peripheral infection in the neonatal period is a concern. Although brain areas are formed prenatally, the brain growth spurt continues for the first 2 years after birth due to dendritic growth, synaptogenesis, and glial cell proliferation. Neurogenesis in the hippocampal dentate gyrus, as well as different cortical regions, persists in the neonatal period in infants (Sanai et al., 2011; Feliciano and Bordey, 2013). Developing and mature neurons, as well as glia, have numerous cytokine receptors, and the hippocampus is especially rich with receptors for proinflammatory cytokines (Parnet et al., 2002). Overall, studies on the effects of neonatal infection on neuroinflammation are lacking.

Rodent studies designed to mimic prenatal infections in humans suggest that untimely neuroinflammation can produce structural and functional changes to the brain that are measurable in adulthood (Fatemi et al., 1998, 1999, 2002, 2008; Shi et al., 2003; Boissé et al., 2004; Ikeda et al., 2005; Bilbo et al., 2006; Meyer et al., 2006; Kohman et al., 2008). However, little is known about peripheral infection in the neonatal period, especially in humans and other animals whose brain is gyrencephalic and experience major perinatal growth. Furthermore, because rodent models are not amenable to behavioral testing as neonates, the impact of infection on hippocampal function during this period is largely unknown. To overcome several of these obstacles, the present study used a neonatal piglet model. Several attributes make the piglet an excellent translational model for psychoneuroimmunological studies. For example, pigs and humans experience a major brain growth spurt from the late prenatal to early postnatal period, which differs from other common animal models (Dobbing and Sands, 1979). Gross brain anatomical features, including gyral pattern and distribution of gray and white matter, are similar in piglets and human infants (Dickerson

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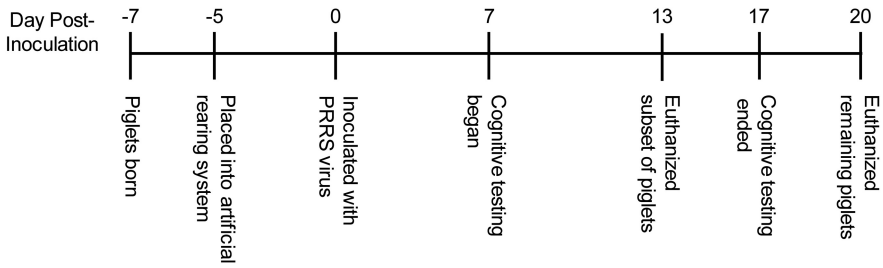

Figure 1. Experimental timeline.

and Dobbing, 1967; Thibault and Margulies, 1998). Additionally, piglets can undergo behavioral testing at a young age (Dilger and Johnson, 2008; Elmore et al., 2012) and show far more overlap with humans concerning genes involved in immunity compared with rodents (Dawson, 2011; Meurens et al., 2012).

In the present study, we tested the hypothesis that peripheral infection with porcine reproductive and respiratory syndrome virus (PRRSV) in the neonatal period activates hippocampal microglia and induces deficits in spatial learning in piglets. In young pigs, PRRSV preferentially infects mononuclear myeloid cells in the lungs inducing interstitial pneumonia, reduced appetite and growth, and leucopenia (Escobar et al., 2004; Liu et al., 2009; Miguel et al., 2010). PRRSV was selected over other infectious agents or mimetics (e.g., *Escherichia coli* or LPS) due to its ability to cause chronic respiratory tract infection.

Materials and Methods

Animals, housing, handling, and feeding. Naturally farrowed crossbred piglets (16 females and 17 males) were obtained from nine separate litters from the University of Illinois swine herd. Piglets were killed at two different time points after inoculation (see details below), creating a 2 × 2 factorial design with the variables of treatment and time after inoculation (control, d 13: $n = 6$; PRRSV, d 13: $n = 8$; control, d 20: $n = 8$; PRRSV, d 20: $n = 11$; Fig. 1). Piglets were brought to the biomedical animal facility 48 h after birth (to allow for colostrum consumption from the dam) and housed individually in cages (0.87 m × 0.87 m × 0.49 m; length × width × height). Control and PRRSV piglets were housed in separate biomedical containment chambers, and a strict protocol of handling and cleaning was followed to prevent cross-contamination. Each cage was positioned in a rack, with stainless steel perforated wall partitions and clear acrylic front and rear doors. In addition, each cage was fitted with flooring designed for neonatal animals (Tenderfoot/NSR, Tandem Products). A toy (plastic Jingle Ball, Bio-Serv) 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). Piglets were maintained on a 12 h light/dark cycle; however, during the dark cycle, minimal lighting was provided.

Piglets were handled by the experimenters multiple times per day during cleaning/feeding and daily observations. In addition, for several days before inoculation (d −4 to d −1), each piglet was permitted an exploration/exercise period in an adjacent hallway for 5 min daily. In a previous experiment (Elmore et al., 2012), we found that this habituation period reduced noncompliance (i.e., piglets failing to choose a reward arm in the given time) during cognitive behavioral testing.

Piglets were fed a nutritionally complete commercial piglet milk replacer (Advance Liqui-Wean, Milk Specialties). Milk was reconstituted fresh each morning to a final concentration of 206 g/L using tap water and supplied at a rate of 285 ml/kg body weight (BW; based on daily recorded weights). This level of feeding allowed for maintenance and growth but prevented complete satiation to ensure that the piglets remained motivated for food rewards in the behavioral task. Water was not provided separately from that used in the milk replacer. Milk replacer was delivered from a reservoir to a stainless-steel bowl (secured to the side of each cage) via a peristaltic pump (Control). Using this automated delivery system similar to that described previously (Dilger and Johnson, 2008), piglets received their daily allotted milk over 18 meals (once per hour), followed by a 6 h fasting period before behavioral testing where no milk was provided. All animal care and experimental procedures were in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals* and approved by the University of Illinois Institutional Animal Care and Use Committee.

Inoculation with PRRSV and sickness measures. At 7 d of age, piglets were inoculated intranasally with either 1 ml of 1×10^5 50% tissue culture infected dose (TCID 50) of live PRRSV (strain P129-BV), obtained from the School of Veterinary Medicine at Purdue University (West Lafayette, Indiana) or sterile PBS. Daily recorded body weights acted as an important indicator of health status throughout the study. In addition, before inoculation (d −1) and throughout the remainder of the study, daily rectal temperatures were obtained each morning. Last, the willingness of the piglets to consume their first daily meal after behavioral testing was determined using a feeding score (0 = no attempt to consume the milk; 1 = attempted to consume the milk, but did not finish within 1 min; 2 = consumed all of the milk within 1 min), which was collected from d −1 to d 12 or 19 after inoculation.

Cognitive testing using a spatial T-maze task. Piglet spatial learning and memory were assessed using a clear plastic plus-shaped maze (essentially a double T-maze) with extra maze cues, which has been previously described and validated (Elmore et al., 2012). The maze consisted of two start arms (north and south) and two reward arms (east and west). Start arm location was alternated throughout testing, which ensured that the piglet did not solve the task using an egocentric mechanism (i.e., turn body left or right, striatum-dependent), and instead was forced to adopt an allocentric mechanism (i.e., used extra-maze visual cues to create a spatial map of the room, hippocampus-dependent) for solving the task (Fitz et al., 2008). This task is similar to that used in rodents to assess "place" and/or "direction" learning (Tolman et al., 1946; Stringer et al., 2005; Zurkovsky et al., 2006; Walsh et al., 2008). Starting on d 7 after inoculation (d 14 of age), piglets were tested daily between 08:00 h and 12:00 h by one trained experimenter. Piglets completed 10 trials per day (60 s per trial), for a total of 11 d. The first 8 d of testing constituted the acquisition phase, where piglets learned to locate the chocolate milk reward (3 ml of the same milk replacer used for regular feedings with the addition of Nesquik cocoa powder, supplied according to the manufacturer's directions) in a constant place in space, as well as direction (e.g., west reward arm), using the extra-maze visual cues. Chocolate milk was provided in both reward arms to balance for olfactory cues but was only accessible in the correct reward arm. Although PRRSV infection can reduce food intake, the combined effect of a 6 h deprivation period before testing, as well as small portions of a desired food source (i.e., 3 ml of chocolate milk) ensured that the piglets were motivated to perform the behavioral task. A performance criterion of 80% correct was applied, which, when reached, would indicate that the piglets had successfully acquired the task. Acquisition was followed by a reversal phase of testing, where the previously incorrect arm (e.g., east), was now rewarded. A video camera was mounted from the ceiling above the arena and used to record piglet movement within the maze. Piglet movement was tracked live using commercially available software (EthoVision 3.1; Noldus Information Technology), providing data on latency to reward arm choice (seconds) and total distance moved (centimeters).

Death and tissue collection. The first subset of piglets was killed 13 d after inoculation (d 20 of age) after completing 5 d of acquisition training. This time point was determined from previous pilot work (data not shown), which showed peak performance differences between control and PRRSV piglets in the spatial T-maze task. The remaining piglets were killed after the completion of cognitive testing (20 d after inoculation, d 27 of age). All piglets were anesthetized using a telazol:ketamine:xylazine solution (50.0 mg of tiletamine, plus 50.0 mg of zolazepam, reconstituted

with 2.50 ml ketamine [100 g/L] and 2.50 ml xylazine [100 g/L]; Fort Dodge Animal Health); the anesthetic combination was administered intramuscularly (i.m.) at 0.03 ml/kg BW. After verifying anesthetic induction, piglets were killed via intracardiac (i.c.) administration of sodium pentobarbital (86 mg/kg BW; Fatal Plus, Vortech Pharmaceuticals). Blood serum (~3 ml) was collected and stored frozen (−80°C). Peripheral tissues (lung tissue and tracheobronchial lymph nodes) and brain tissue (hippocampus, prefrontal cortex, and striatum) were collected, rinsed in PBS, and stored at −80°C in RNA later (Ambion) until processing.

Peripheral PRRSV detection and cytokines. 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). This assay has 98.8% sensitivity and 99.9% specificity, with an S/P ratio of >0.4 indicating a positive sample. Serum proinflammatory cytokine (IL-1β, IL-6, and TNF-α) levels at the end of the study were analyzed using porcine-specific sandwich enzyme immunoassays (R&D Systems). Samples were assayed according to the manufacturer's instructions, and mean minimum detectable concentrations were as follows: IL-1β, 6.72 pg/ml; IL-6, 2.03 pg/ml; TNF-α, 3.7 pg/ml. Interassay and intra-assay coefficients of variation were <7.2%, 5.1%, and 6.9%, respectively.

Microglia isolation and cell sorting using flow cytometry. Microglia were isolated using a method adapted from Henry et al. (2009). Approximately 500 mg of hippocampal tissue from each hemisphere (i.e., the majority of each hemisphere was collected; however, the ventral region of the hippocampus was removed for additional analyses) was collected from each piglet and rinsed in PBS. Each hemisphere was treated as its own sample for the first several steps of the protocol to maximize the number of isolated cells. Tissue was pushed through a 70 μm nylon mesh cell strainer (BD Biosciences) for homogenization using Dulbecco's PBS (Sigma) with 0.2% glucose and spun at 600 × *g* for 6 min to form a pellet. The supernatant was removed, and the pellet was resuspended in a 70% isotonic Percoll (GE Healthcare) solution. Layers of 50%, 35%, and 0% isotonic Percoll were overlaid to form a discontinuous gradient, which was then spun for 20 min at 2000 × *g*. The microglia layer, located at the interface of the 50% and 70% layers, was removed (Frank et al., 2006). At this point, isolated microglial cells from both hemispheres of each piglet were combined and spun at 600 × *g* for 6 min to form a pellet. This pellet was resuspended in flow buffer (PBS with 1% BSA; Fisher Scientific), 0.1% sodium azide (Sigma), and 20 mM glucose (Sigma). Purified CD16/CD32 antibodies (eBioscience) were used to block competitive binding of the Fc receptor on microglia. The cells were incubated with antibodies for receptors used to identify microglia cells: CD11b (BioLegend) and CD45 (AbD Serotec), as well as MHC II (Antibodies Online), as a marker of cell activation. Using the FACS Aria II flow cytometer (BD Biosciences), the viable microglia cell population from a massed unstained control sample (consisting of a subsample of isolated cells from each piglet) was gated according to size and granularity based on forward and side scatter properties, as well as autofluorescence. Similar to mice, cells were identified by the expression of CD11b and CD45 surface receptors. However, the expression pattern of these receptors differs from mouse data in that porcine microglia express lower levels of CD11b and higher levels of CD45 (Gregerson and Yang, 2003), identified as $CD11b^{+}$/$CD45^{int}$ (Gregerson and Yang, 2003) versus $CD11b^{+high}$/$CD45^{low}$ in mice (Ford et al., 1995). Cells with high CD45 expression were excluded from the analysis, as this distinct population is likely other myeloid cell types (e.g., peripheral macrophages) (Gregerson and Yang, 2003). Cells were then quantified according to MHC II positivity, resulting in a proportion of $MHC\ II^{-}$ and $MHC\ II^{+}$ cells for each piglet.

Real-time PCR. Total RNA from ~75 mg of the lungs, tracheobronchial lymph nodes, hippocampus, prefrontal cortex, and striatum of each piglet was isolated using the Tri Reagent protocol (Sigma). A QuantiTect Reverse Transcription Kit (QIAGEN) was used for cDNA synthesis with integrated removal of genomic DNA contamination according to the manufacturer's protocol. Quantitative real-time PCR was performed using the Applied Biosystems TaqMan Gene Expression Assay protocol. A custom TaqMan Low Density Array card was designed, which contained a reference gene (ribosomal protein L19, RPL19) and 22 genes of interest (Table 1), including PRRSV (Chen et al., 2009), which were run in duplicate. In brief, cDNA was amplified by PCR where a target cDNA (Table 1) and reference cDNA (RPL19) were amplified simultaneously using an oligonucleotide probe with a 5′ fluorescent reporter dye (6-FAM) and a 3′ quencher dye (NFQ). Conditions for the PCR procedure included: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Fluorescence was determined on an ABI PRISM 7900HT-sequence detection system (PerkinElmer). Data were excluded from analysis for the following reasons: (1) both wells did not run; or (2) high SE between the wells (>0.5). Data were analyzed using the comparative threshold cycle (C_t) method (Livak and Schmittgen, 2001), and results are expressed as fold change to the standardized relative quantification (RQ) baseline (RQ = 1), whereby a value >1 would represent a fold increase in mRNA expression compared with control for a particular gene and a value between 0 and 1 would represent a fold decrease in mRNA expression compared with controls.

Statistical analysis. Data analysis was conducted using the MIXED procedure of the Statistical Analysis Systems software (SAS Institute). Data were transformed as necessary to meet the assumptions of the test (e.g., homogeneity of variance). Transformations include weight (log), latency to choice (log), and total distance moved (log). For sickness measures, data were analyzed as a three-way (treatment × sex × day) repeated-measures ANOVA, where the variable pig was treated as a random effect and nested within treatment and sex. Cognitive performance data were analyzed similarly, with the addition of day nested within phase to ac-

Table 1. Quantitative real-time PCR primer information

Gene	Classification	Accession number[a]	Assay identification[b]
RPL19, ribosomal protein L19	Reference	AF435591	Ss03375624_g1
IL-10	Anti-inflammatory	NM_214041	Ss03382372_u1
IL-13	Anti-inflammatory	NM_213803	Ss03392351_m1
TGF-β1	Anti-inflammatory	NM_214015	Ss03382325_u1
CD200r, cluster of differentiation 200 receptor[c]	Microglial	NA	NA
CX3CL1, fraktalkine	Neuronal	DQ991100	Ss03377157_u1
BDNF, brain-derived neurotrophic factor	Neuronal	NM_214259	Ss03822335_s1
CD200, cluster of differentiation 200	Neuronal	AJ584612	Ss03375826_u1
MBP	Neuronal	NM_001001546	Ss03385047_u1
NGF[d]	Neuronal	NA	NA
SOD1, superoxide dismutase 1	Neuronal	NM_001190422	Ss03375614_u1
SYP, synaptophysin[e]	Neuronal	NA	NA
HMOX1, heme oxygenase (decycling) 1	Oxidative Stress	NM_001004027	Ss03378516_u1
NOS2, nitrous oxide synthase	Oxidative Stress	NM_001143690	Ss03374608_u1
NQ01, NAD(P)H dehydrogenase, quinone 1	Oxidative Stress	NM_001159613	Ss03226763_g1
IFN-γ	Pro-inflammatory	NM_213948	Ss03391054_m1
IL-1β	Pro-inflammatory	NM_214055	Ss03393804_m1
IL-6	Pro-inflammatory	NM_214399	Ss03384604_u1
TNF-α	Pro-inflammatory	NM_214022	Ss03391318_g1
PRRSV[f]	Viral	NA	NA
TLR-3	Viral	NM_001097444	Ss03388861_m1
TLR-4	Viral/Bacterial	NM_001113039	Ss03389780_m1
TLR-7	Viral	NM_001097434	Ss03385429_u1

[a]NCBI GenBank accession number.

[b]Applied Biosystems TaqMan Gene Expression Assay identification number.

[c]CD200r custom probe: forward primer, TGTTCCAAGTTACTAATCAGGCTGAA; reverse primer, AGCCCATTAGCAACATGATACTCTTT; probe, ACATAGAATTGAAGGAAGGG.

[d]NGF custom probe: forward primer, TCAACAGGACTCACAGGAGCAA; reverse primer, ACTCCCCCCGGTGGAAA; probe, CGGTCGTCATCCC.

[e]SYP custom probe: forward primer, GGCCAAGGACGGCTCAT; reverse primer, TTTTCCGCCCTTAGCATGTAG; probe, CAAGATTAAATGGTACGTAGGAC.

[f]PRRSV custom probe (Chen et al., 2009): forward primer, CGCACCAGATGGGACCTACTT; reverse primer, ACGGTGTTCAGTGAGGGCTTT; probe, CGCTGCGTTGACTGG.

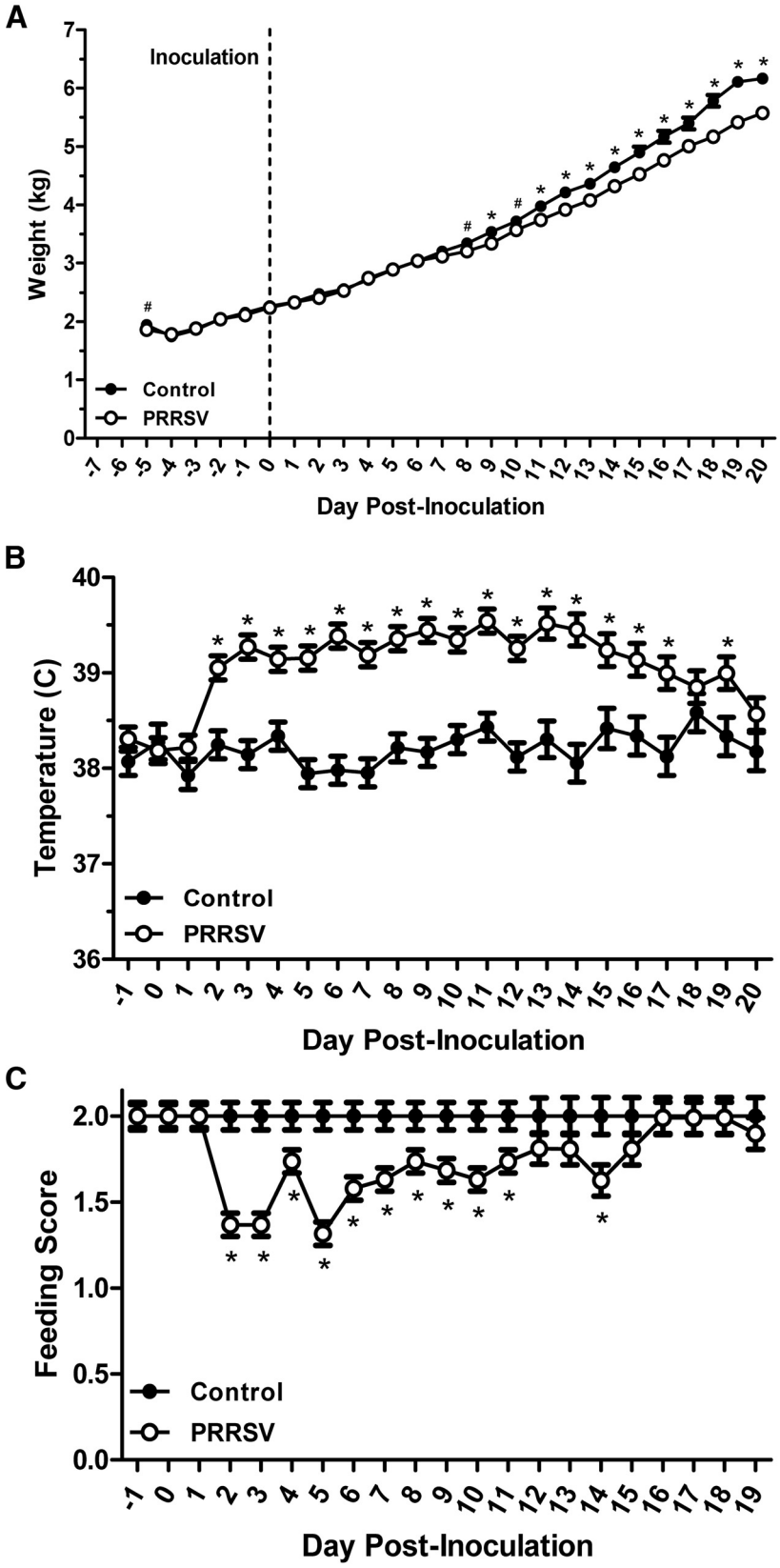

Figure 2. Daily recorded sickness measures (***A***, body weight; ***B***, rectal temperature; ***C***, feeding score) for control (13 and 20 d after inoculation combined, $n = 14$ total) and PRRSV (13 and 20 d after inoculation combined, $n = 19$ total) piglets throughout the course of the study. Body weight was measured from the start of the experiment, and rectal temperature and feeding score were measured beginning 1 d before inoculation. Data are LSM ± SEM. Contrasts were used to separate treatment means on each day $^{*}p < 0.05$. $^{\#}p < 0.10$.

count for both testing phases of the spatial T-maze task (i.e., acquisition and reversal). Performance during the reversal phase of cognitive testing was analyzed in two ways: (1) with all animals or (2) with only those animals that reached learning criterion (80% correct) during acquisition. As the statistical analysis revealed similar outcomes for both approaches (data not shown), cognitive performance data for both testing phases are shown with all animals included in the dataset. Gene expression data were first analyzed as treatment × sex × time. However, because very few interactions showed statistical significance, both time points were combined and the effect of treatment alone was also determined. For all other measures, which were only sampled once at the end of the study, data were analyzed as a three-way (treatment × sex × time after inoculation) ANOVA. The parameter sex was removed from the statistical model if not significant as a main effect or as part of an interaction. *Post hoc* paired contrasts were used to further examine significant effects. Correlations were determined using Proc Corr in SAS, and the best fit line was found using the Linear Regression function in GraphPad Prism 5 (GraphPad Software). Statistical significance was accepted at $p < 0.05$, statistical trends at $p \leq 0.10$. Unless otherwise stated, data are presented as untransformed Least Square Means (LSM) ± SEM.

Results

PRRSV infection and measures of sickness

Serum ELISA results confirmed that all control piglets tested negative for PRRSV antibodies (S/P ratio range: 0.00–0.02) at the end of the study, whereas all PRRSV-infected piglets tested positive (S/P ratio range: 0.88–2.36). Changes in body weight, temperature, and willingness to consume the first daily meal were used to measure the sickness response of piglets infected with PRRSV. Body weight data showed a significant effect of treatment ($F_{(1,585)} = 26.55$), day ($F_{(25,585)} = 915.18$), and treatment × day ($F_{(25,585)} = 3.24$; all effects, $p < 0.001$), indicating that, although all piglets gained weight throughout the course of the study, PRRSV piglets had lower average body weights at the end of the study compared with control piglets (Fig. 2*A*). In addition, there was an effect of sex ($F_{(1,585)} = 7.75$; $p = 0.006$) on body weight, as female piglets (3.42 ± 0.03) weighed less than males (3.50 ± 0.03) overall. There was also a treatment × sex ($F_{(1,585)} = 5.46$; $p = 0.02$) and treatment × sex × day interaction ($F_{(25,585)} = 1.86$; $p = 0.007$), where PRRSV females (3.39 ± 0.04) weighed less than control females (3.45 ± 0.05; $p = 0.051$) and control males (3.66 ± 0.05; $p < 0.001$), but not PRRSV males (3.34 ± 0.04; $p = 0.714$), which was exacerbated over time

Table 2. Serum proinflammatory cytokine concentrations[a]

	CON (pg/ml)	PRRS (pg/ml)	*p*
IL-1β	1045 ± 13.6	1105 ± 11.7	0.0024
IL-6	362 ± 1.50	375 ± 1.30	< 0.001
TNF-α	827 ± 495	4335 ± 426	< 0.001

[a]Data from CON (13 and 20 d after inoculation combined, $n = 14$ total) and PRRS (13 and 20 d after inoculation combined, $n = 19$ total) piglets at the end of the study. Data are LSM ± SEM.

(data not shown). Rectal temperature data showed similar results, with significant effects of treatment ($F_{(1,529)} = 156.10$), day ($F_{(21,529)} = 4.28$), and treatment × day ($F_{(21,529)} = 2.77$; all effects, $p < 0.001$), revealing that PRRSV piglets had elevated temperatures after infection, which increased at ~2 d after inoculation and returned to baseline by the end of the study (Fig. 2*B*). Last, the same pattern seen for body temperature was also observed for the feeding score (Fig. 2*C*), with treatment ($F_{(1,508)} = 57.46$), day ($F_{(20,508)} = 3.39$), and the interaction of these two variables ($F_{(20,508)} = 3.39$) all showing statistical significance (all, $p < 0.001$). Interestingly, although the piglets showed reduced motivation to consume their standard milk replacer in the home cage after behavioral testing, many of the piglets willingly drank the chocolate milk in the behavioral task, which is either due to the 6 h deprivation period before testing or a preference for the chocolate milk compared with the standard milk replacer. Combined, these data reveal that sickness behavior in PRRSV piglets was evident from 2 to 14 d after inoculation, which then returned to baseline levels by the end of the study (d 20). However, body weight in PRRSV piglets remained lower than control from d 8 through d 20 after inoculation.

Serum proinflammatory cytokines

Data in Table 2 show that serum proinflammatory cytokines IL-1β ($F_{(1,29)} = 11.04$; $p = 0.002$), IL-6 ($F_{(1,29)} = 38.93$; $p < 0.001$), and TNF-α ($F_{(1,29)} = 28.79$; $p < 0.001$) were elevated at the end of the study in piglets infected with PRRSV. Interestingly, there was no effect of time after inoculation (IL-1β: $F_{(1,29)} = 0.00$; $p = 0.987$; IL-6: $F_{(1,29)} = 0.54$; $p = 0.468$; and TNF-α: $F_{(1,29)} = 0.82$; $p = 0.373$) or treatment × time after inoculation (IL-1β: $F_{(1,29)} = 0.11$; $p = 0.747$; IL-6: $F_{(1,29)} = 0.57$; $p = 0.456$; and TNF-α: $F_{(1,29)} = 0.82$; $p = 0.374$) on any of these measures. These data demonstrate that PRRSV-infected piglets exhibited similar serum proinflammatory cytokine expression at 13 and 20 d after inoculation.

Performance in a spatial T-maze task

PRRSV infection altered piglet performance in a spatial T-maze task ($F_{(1,226)} = 11.18$; $p = 0.001$), which varied according to phase of testing ($F_{(1,2926)} = 64.22$; $p < 0.001$), where PRRSV piglets performed more poorly, as indicated by the proportion of correct choices in the maze, than control piglets during acquisition (control: 0.78 ± 0.02 vs PRRSV: 0.61 ± 0.02; $p < 0.001$), but not reversal testing (control: 0.47 ± 0.04 vs PRRSV: 0.46 ± 0.04; $p = 0.77$). However, treatment × day (phase), which accounts for the effects of treatment on each day of testing within a certain phase (i.e., acquisition or reversal), was not significant ($F_{(9,226)} = 0.89$; $p = 0.532$; Fig. 3*A*). In addition, piglets infected with PRRSV exhibited a longer latency to make a reward arm choice in the maze (control: 2.18 ± 0.78 s vs PRRSV: 10.42 ± 0.66 s; $F_{(1,226)} = 166.10$; $p < 0.001$), but infection status did not alter performance in either phase of testing ($F_{(1,226)} = 2.65$; $p = 0.105$) or the interactions of treatment × phase ($F_{(1,226)} = 0.00$; $p = 0.992$) and treatment × day (phase) ($F_{(9,226)} = 1.37$; $p = 0.201$; Fig. 3*B*).

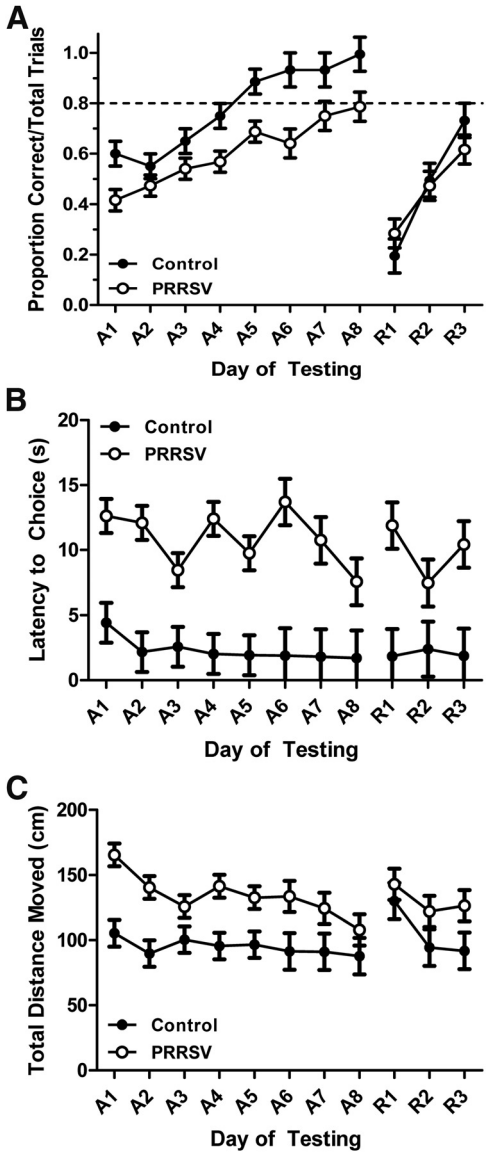

Figure 3. Performance of control (13 and 20 d after inoculation combined, $n = 14$ total) and PRRSV (13 and 20 d after inoculation combined, $n = 19$ total) piglets during the acquisition phase (A1–A8) and reversal phase (R1–R3) of cognitive testing in a spatial T-maze task (***A***, proportion of correct reward arm choices/total trials) and locomotor readouts (***B***, latency to choice; ***C***, total distance moved). Data are LSM ± SEM.

Total distance moved in the maze was greater in PRRSV piglets (control: 100.05 ± 5.16 cm vs PRRSV: 132.23 ± 4.42 cm; $F_{(1,206)} = 31.07$; $p < 0.001$) and greater in males compared with females regardless of treatment (males: 123.92 ± 4.70 cm vs females: 108.37 ± 4.91 cm; $F_{(1,206)} = 7.07$; $p = 0.009$). In addition, there was a trend for a treatment × phase effect ($F_{(1,206)} = 3.32$; $p = 0.07$), where PRRSV piglets traveled a greater distance in the maze than control piglets during both acquisition (control: 94.70 ± 4.34 cm vs PRRSV: 133.96 ± 3.72 cm; $p < 0.001$) and reversal testing (control: 105.40 ± 8.73 cm vs PRRSV: 130.50 ± 7.47 cm; $p = 0.035$). There was a sex × phase effect ($F_{(1,206)} = 4.08$; $p = 0.045$) as well, where in reversal testing, females traveled a shorter total distance (103.34 ± 8.29 cm) than males (132.57 ± 7.95 cm; $p = 0.009$). Last, there was a treatment × sex × phase effect ($F_{(1,206)} = 5.65$; $p = 0.018$), where PRRSV females moved a greater distance during acquisition than control females (control: 91.46 ± 6.14 cm vs PRRSV: 135.33 ± 5.47 cm; $p < 0.001$), but

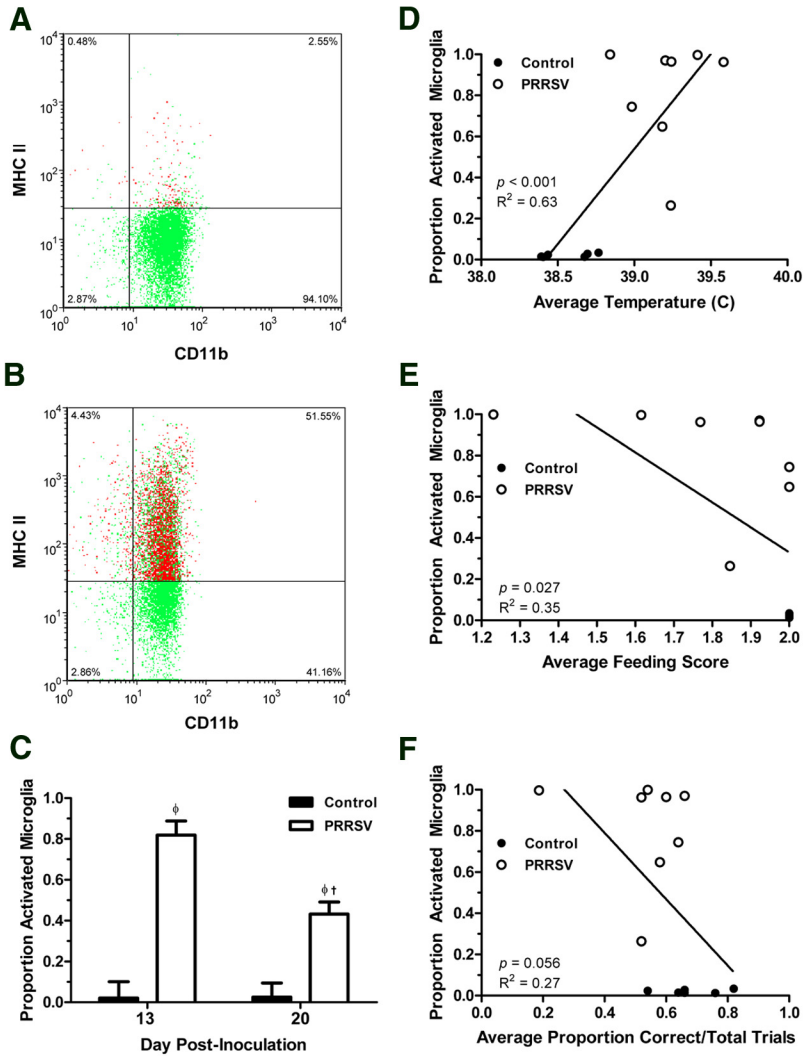

Figure 4. A representative two-color dot plot for a control (***A***) and a PRRSV (***B***) piglet is shown; cells were incubated with antibodies for receptors used to identify microglia cells (CD11b and CD45, data not shown), as well as a marker of cell activation (MHC II, shown). The proportion of activated microglia cells at the end of the study in piglets killed 13 d after inoculation (control 1: $n = 6$; PRRSV 1: $n = 8$) and 20 d after inoculation (control 2: $n = 8$; PRRSV 2: $n = 11$; ***C***). In addition, the correlation of this measure to the average rectal temperature (***D***), the average feeding score (***E***), and performance in the cognitive behavioral task (***F***) for piglets killed at 13 d after inoculation are shown. ***A–C***, Data are LSM ± SEM. Contrasts were used to separate treatment means within each time point (control vs PRRSV at 13 d after inoculation; control vs PRRSV at 20 d after inoculation; $^{\phi}p < 0.05$) and between cohorts (control 13 d vs control 20 d after inoculation; PRRSV 13 d vs PRRSV 20 d after inoculation; $^{\dagger}p < 0.05$).

PRRSV females moved a shorter distance during reversal than PRRSV males (females: 105.37 ± 11.08 cm vs males: 155.63 ± 10.01 cm; $p < 0.001$). However, there was not a treatment × sex ($F_{(1,206)} = 1.24$; $p = 0.266$), treatment × day (phase) ($F_{(9,206)} = 0.60$; $p = 0.798$; Fig. 3*C*), or a treatment × sex × day (phase) effect ($F_{(9,206)} = 1.13$; $p = 0.343$) for total distance moved in the maze. Combined, these data reveal that PRRSV piglets showed cognitive deficits in learning a spatial T-maze task, as evidenced by a reduction in the proportion of correct reward arm choices, longer latency to make a choice, and a greater total distance moved in the maze. The finding that PRRSV piglets moved a greater total distance in the maze eliminates two potential explanations for reduced cognitive performance in the maze: decreased motor ability or lethargy resulting from infection.

Microglial activation

Isolated cells from the hippocampus were incubated with antibodies for receptors used to identify microglia cells (CD11b and CD45), as well as a marker of cell activation (MHC II; Fig. 4*A*,*B*), and sorted using flow cytometry. The presence of peripheral macrophages (characterized as CD11b^{+}/CD45^{high}) in the samples of isolated microglia cells was very low (control: 1.47 ± 0.47%; PRRSV: 1.68 ± 0.38%) and did not differ according to treatment ($F_{(1,24)} = 0.11$; $p = 0.738$), time after inoculation (data not shown; $F_{(1,24)} = 1.03$; $p = 0.32$), or sex (data not shown; $F_{(1,24)} = 0.62$; $p = 0.439$). The proportion of activated microglia cells showed a significant effect of treatment ($F_{(1,29)} = 73.68$; $p < 0.001$), time after inoculation ($F_{(1,29)} = 7.45$; $p = 0.012$), and treatment × time after inoculation ($F_{(1,29)} = 7.76$; $p = 0.009$), demonstrating that PRRSV infection resulted in a dramatic increase in activated microglial cells in the hippocampus, which was more pronounced at 13 d after inoculation than 20 d after inoculation (Fig. 4*C*). The proportion of activated microglia correlated with the S/P ratio obtained in the PRRSV ELISA at both 13 d ($R^2 = 0.35$; $p = 0.027$) and 20 d after inoculation ($R^2 = 0.67$; $p < 0.001$), demonstrating that piglets with a higher proportion of activated microglia had higher levels of PRRSV antigen in their serum. In addition, the proportion of activated microglia correlated strongly with average daily temperature in piglets killed at 13 d after inoculation (Fig. 4*D*) and 20 d after inoculation ($R^2 = 0.59$; $p < 0.001$) and with average feeding score for piglets at these time points (d 13: Fig. 4*E*; d 20: $R^2 = 0.67$; $p < 0.001$). Last, the proportion of activated microglia showed a trend to correlate with performance in the cognitive behavioral task at 13 d after inoculation (acquisition only; Fig. 4*F*) and 20 d after inoculation (acquisition and reversal combined; $R^2 = 0.15$; $p = 0.10$), demonstrating that piglets with increased activated microglia in the hippocampus performed more poorly in the spatial T-maze task. Overall, PRRSV infection caused neuroinflammation in piglets, as indicated by levels of microglial activation, which correlated with markers of peripheral inflammation, sickness behavior, and reduced cognitive performance.

Gene expression

PRRSV was detected in both the lung (control: 1.00 ± 0.00 vs PRRSV: 3414.10 ± 1055.80; raw data; $F_{(1,31)} = 8.06$; $p = 0.008$) and the tracheobronchial lymph node (control: 1.00 ± 0.00 vs PRRSV: 1437.56 ± 385.72; raw data; $F_{(1,25)} = 7.58$; $p = 0.011$) of PRRSV-infected piglets but was not present in control piglets. In the lung (Fig. 5*A*), PRRSV piglets showed an increase in IL-10, but a decrease in TGF-β1, both of which are anti-inflammatory genes. PRRSV piglets showed a decrease in neuronal factors (BDNF, CD200, NGF, and SYP) and a marker of oxidative stress (NOS2), but an increase in CD200r (a microglial receptor), pro-

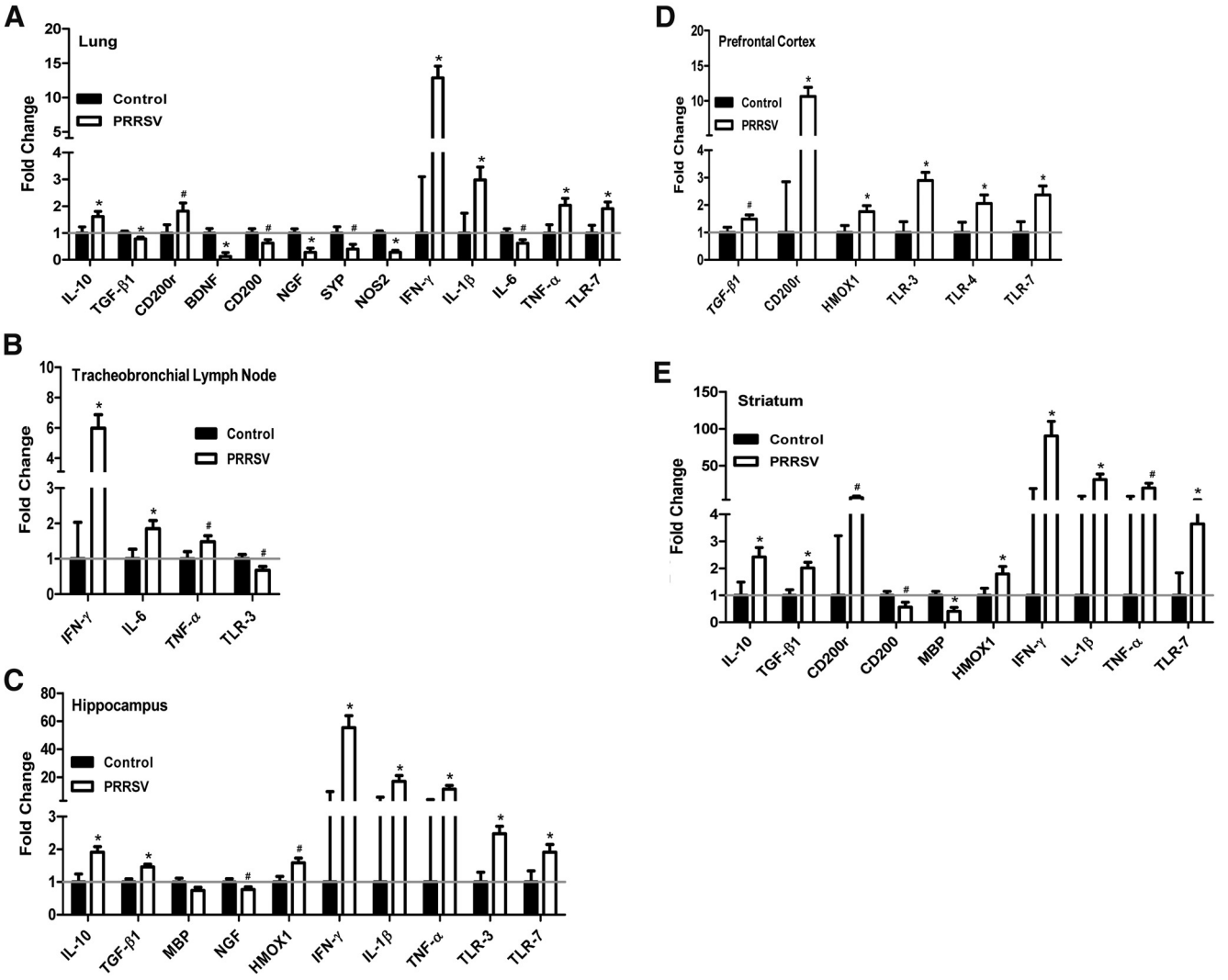

Figure 5. Relative abundance of mRNA in peripheral tissues (***A***, lung; ***B***, tracheobronchial lymph node) and brain tissues (***C***, hippocampus; ***D***, prefrontal cortex; ***E***, striatum) for control (13 and 20 d after inoculation combined, $n = 14$ total) and PRRSV (13 and 20 d after inoculation combined, $n = 19$ total) piglets. Only significant treatment effects are shown. Data are LSM ± SEM. The gray line indicates the standardized Relative Quantification (RQ) baseline (RQ = 1); all data are expressed as relative fold change to the baseline. Contrasts were used to separate treatment means for each gene. $^{*}p < 0.05$. $^{\#}p < 0.10$.

inflammatory cytokines (IFN-γ, IL-1β, and TNF-α), and a viral marker (TLR-7) in the lungs. The lymph node (Fig. 5*B*) revealed fewer significant differences between control and PRRSV piglets than the lung, with an increase in proinflammatory genes (IFN-γ, IL-6, and TNF-α) and a decrease in a single viral marker (TLR-3).

Within the brain tissues (Fig. 5*C–E*), PRRSV was detected in the hippocampus (control: 1.00 ± 0.00 vs PRRSV: 32.93 ± 10.82; raw data; $F_{(1,24)} = 6.82$; $p = 0.015$), prefrontal cortex (control: 1.00 ± 0.00 vs PRRSV: 240.87 ± 115.02; raw data; $F_{(1,22)} = 3.17$; $p = 0.089$), and striatum (control: 1.00 ± 0.00 vs PRRSV: 129.35 ± 61.85; raw data; $F_{(1,24)} = 3.37$; $p = 0.079$) of PRRSV-infected piglets, but was not present in control piglets. IL-13 was not detected in the hippocampus or striatum of either PRRSV or control piglets and, therefore, could not be analyzed. PRRSV piglets showed an increase in anti-inflammatory genes (IL-10 and/or TGF-β1) in all brain tissues, but only a significant increase in proinflammatory genes (IFN-γ, IL-1β, and TNF-α) in the hippocampus and striatum, but not the prefrontal cortex. CD200r expression was increased in the prefrontal cortex and the striatum of PRRSV piglets, whereas the hippocampus and the striatum showed a decrease in neuronal factors (CD200, MBP, and/or NGF). All three brain tissues showed an increase in a marker of oxidative stress (HMOX1) and in at least one of the viral markers (TLR-3, TLR-4, and/or TLR-7). Gene expression data for the interaction of treatment × time after inoculation for peripheral and central tissues are shown in Table 3.

Discussion

Very little is known about the effects of peripheral infection in the neonatal period on neuroinflammation and cognitive behavior, especially in humans and other animals whose brain is gyrencephalic and undergoes major perinatal growth. Therefore, in the present study, we inoculated neonatal piglets with a virus that induced pneumonia and examined neuroinflammation, behavior, and spatial learning. A unique aspect of the study is that due to the size of the piglet brain, it was possible to isolate microglia from the hippocampus instead of the whole homogenized brain. We used Percoll gradient centrifugation, coupled with antibody staining and flow cytometry, to identify $CD11b^{+}/CD45^{int}$ cells, which are considered to be microglia. Further staining allowed us to show substantial MHC II expression by microglia in the hippocampus due to infection, which was still evident in microglia

Table 3. Relative abundance of mRNA in peripheral and central tissues[a]

		Control		PRRSV	
Tissue	Gene	13 d after inoculation	20 d after inoculation	13 d after inoculation	20 d after inoculation
Lung	IL-13	1.10 ± 0.59	2.24 ± 0.68	3.17 ± 0.68[c]	0.48 ± 0.45[b,c]
	CD200r	1.41 ± 0.59	1.41 ± 0.51	3.66 ± 0.51[c]	1.31 ± 0.55[b]
	BDNF	1.11 ± 0.58	3.85 ± 0.50[b]	0.32 ± 0.50	0.33 ± 0.45[c]
	IL-6	1.16 ± 0.44	2.72 ± 0.38[b]	1.69 ± 0.38	0.96 ± 0.32[c]
	TLR-7	1.19 ± 0.67	1.97 ± 0.58	4.24 ± 0.58[c]	2.32 ± 0.49[b]
Lymph node	SOD1	1.01 ± 0.27	1.55 ± 0.23	2.07 ± 0.23[c]	1.69 ± 0.20
Prefrontal cortex	TGF-β1	1.26 ± 0.27	0.78 ± 0.21	1.15 ± 0.21	1.58 ± 0.18[c]
	NGF	1.89 ± 0.44	2.40 ± 0.34	3.35 ± 0.39[c]	2.24 ± 0.29[b]
	NOS2	1.16 ± 0.16	0.78 ± 0.12	0.68 ± 0.12[c]	0.79 ± 0.11
Striatum	BDNF	1.00 ± 276	26.1 ± 175	1004 ± 225[c]	138 ± 148[b]
	IFN-γ	1.00 ± 23.5	1.00 ± 20.4	1.89 ± 33.2[c]	57.6 ± 19.2[b,c]
	TLR-3	1.17 ± 1.28	1.50 ± 0.99	6.42 ± 1.28[c]	2.14 ± 0.74[b]

[a]Significant interactions of treatment × time after inoculation.

[b]Control 13 d versus Control 20 d after inoculation; PRRSV 13 d versus PRRSV 20 d after inoculation; $p < 0.05$.

[c]Control versus PRRSV at 13 d after inoculation; Control versus PRRSV at 20 d after inoculation; $p < 0.05$.

isolated nearly 3 weeks after inoculation. Microglial MHC II expression was positively correlated with other markers of neuroinflammation, sickness behavior, and deficits in spatial learning. We believe this to be the first study to report in a neonatal gyrencephalic species a relationship between peripheral infection, microglial cell activation in the hippocampus, and deficits in spatial learning.

The dramatic effect of neonatal infection on microglial cell expression of MHC II is notable because of its role in formation, refinement, maintenance, and plasticity of the brain. The period from birth to age 2 is a critical phase of postnatal brain development in humans. Total brain volume doubles the first year and reaches 80–90% of adult volume by age 2 (Knickmeyer et al., 2008). This phase of rapid growth represents a sensitive period, wherein environmental insults may alter neurodevelopment (McEwen, 1999). Indications that neuroinflammation in the neonatal period can affect brain development and have a role in psychopathology are emerging from studies of autism spectrum disorder (ASD). A population-based cohort study of children born in Denmark from 1980 to 2002 reported that children admitted to the hospital for an infectious disease displayed a significantly higher rate of ASD diagnosis later (Atladóttir et al., 2010). The increased diagnosis of ASD was independent of the type of infectious pathogen (viral vs bacterial), indicating the involvement of a generalized immune response (e.g., neuroinflammation). In another large population-based case–control study among children born in Northern California from 1995 to 1999, children diagnosed with an infection in the first month of life were more likely to be diagnosed later with ASD (Rosen et al., 2007). An emerging hypothesis is that disruptions in MHC expression in the developing brain due to infection and immune dysregulation contribute to the altered brain connectivity and function seen in individuals with ASD (Needleman and McAllister, 2012). Although the present study did not focus on brain development *per se*, it did show that peripheral infection affected expression of MHC II by hippocampal microglia for a significant period of time. This was associated with reduced expression of NGF in the hippocampus, which is consistent with what others have found in rodents after peripheral respiratory infection (Jurgens et al., 2012). Expression of a gene necessary for myelination (i.e., MBP) was also reduced in the hippocampus and striatum of PRRSV piglets. The expression of MHC II was related to poorer performance in a spatial T-maze task, which is consistent with the view that neuroinflammation underlies infection-related deficits in cognition (Yirmiya and Goshen, 2011). Similar to human infants, in piglets the first month after birth (i.e., the period studied here) is characterized by a large expansion in total brain volume, including the hippocampus (Conrad et al., 2012). The present study focused on microglial activation, neuroinflammation, and hippocampal function within a narrow developmental window, but the results clearly warrant further investigation into the long-term structural and functional effects of infection on the hippocampus and other brain regions.

We think that the increased MHC II expression in microglia from PRRSV piglets was likely the result of stimulation of neural and humoral communication pathways from the peripheral immune system to the brain (Dantzer et al., 2008), which are known to lead to microglial cell activation and neuroinflammation. We cannot discount, however, the potential influence of PRRSV entering the CNS. The ability of PRRSV to enter the brain (Rossow et al., 1999; Shin and Molitor, 2002a, b; Tian et al., 2007) and microglia (Rossow et al., 1999) has been reported previously. In the present study, PRRSV was present in the lung and tracheobronchial lymph node, but to a much lesser extent in the hippocampus, prefrontal cortex, and striatum. Although uncertain, the very low levels detected by RT-PCR in brain tissues may have been due to contamination by peripheral blood because piglets were not perfused before sample collection. This seems likely because PRRSV, which preferentially infects mononuclear myeloid cells (e.g., microglia), was not detected in isolated hippocampal microglia from PRRSV-infected piglets (data not shown). In any case, consistent with prior reports (Escobar et al., 2004; Klinge et al., 2009; Liu et al., 2009; Miguel et al., 2010), in both peripheral and central tissues, the general effect of PRRSV infection was to increase expression of genes encoding for proinflammatory cytokines. For example, PRRSV piglets showed an increase in IFN-γ in nearly all tissues, including the brain. IFN-γ is an antiviral cytokine that upregulates MHC II (Kettenmann et al., 2011) and potentiates further cytokine production after infection (De Simoni et al., 1997). In brain tissues, this tended to be associated with increased expression of several receptors that recognize pathogen-associated molecular patterns (TLR-3, TLR-4, and TLR-7).

PRRSV also caused an increase in the anti-inflammatory genes IL-10 and/or TGF-β1 in all brain tissues, as well as an increase in a gene involved in oxidative stress, HMOX1. It is known that HMOX1 is antioxidative, is involved with the anti-inflammatory response, and is protective during infection (Courtney and Maxwell, 2008; Piantadosi et al., 2011). Therefore, it is not surprising that both of these subsets of genes would be upregulated after PRRSV infection. Immunomodulatory factor CD200 is broadly distributed throughout the brain (Wright et al., 2003) and is known to play an important role in downregulating inflammation and microglial activation (Hoek et al., 2000; Jurgens and Johnson, 2012). In this study, CD200 was reduced in the hippocampus and striatum of PRRSV-infected pigs (previously observed in mice with influenza infection) (Jurgens et al., 2012), whereas its receptor CD200r, which is located on myeloid cells and is involved in regulation of macrophage function (Wright et al., 2003), was increased in the prefrontal cortex and the striatum. Similar to influenza infection in mice (Rygiel et al., 2009), loss of CD200-CD200r signaling regulation could lead to excessive production of antiviral responses, leading to increased immune pathology in the PRRSV infection model. It appears that, at least within the striatum, microglia were attempting to compensate for a reduction in the CD200 ligand caused by PRRSV infection by upregulating the inhibitory immune receptor CD200r.

In response to PPRSV infection, the number of MHC II-positive microglia in the hippocampus increased dramatically to ~80%. Differential expression of MHC II in response to a pathogen is not unique to our neonatal infection model, as this finding has been observed in other models of infection (murine acquired immunodeficiency syndrome) (Mutnal et al., 2013), neurodegenerative disease and aging (Jurgens and Johnson, 2012), as well as brain injury (Nagamoto-Combs et al., 2010). Although beyond the scope of the present study, to understand why some microglia undergo a phenotypic shift in response to insult whereas others do not is a significant issue with major clinical ramifications. However, an important point is that, although MHC II is an accepted marker of microglial activation (Saijo and Glass, 2011), it is now clear that resting microglia can transition between M1 (considered "classically activated," proinflammatory) and M2 (considered "alternatively activated," anti-inflammatory and neuroprotective) phenotypes (Mosser and Edwards, 2008; Kigerl et al., 2009; Michelucci et al., 2009). It is important to note that MHC II positivity cannot be categorized as entirely M1 or M2, as both classical and alternative activation involve presentation of MHC II (Gordon, 2003). In future studies, it will be critical to determine how M1 and M2 microglia are regulated, especially because our data suggest that MHC II activation is important for the expression of sickness behaviors and cognitive performance in neonates with respiratory infection.

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