



BASIC SCIENCE ARTICLE

Impairment in neurocognitive function following experimental neonatal guinea pig cytomegalovirus infection

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BACKGROUND: Cytomegalovirus (CMV) is a leading infectious cause of neurologic deficits, both in the settings of congenital and perinatal infection, but few animal models exist to study neurodevelopmental outcomes. This study examined the impact of neonatal guinea pig CMV (GPCMV) infection on spatial learning and memory in a Morris water maze (MWM) model.

METHODS: Newborn pups were challenged intraperitoneally (i.p.) with a pathogenic red fluorescent protein-tagged GPCMV, or sham inoculated. On days 15–19 post infection (p.i.), pups were tested in the MWM. Viral loads were measured in blood and tissue by quantitative PCR (qPCR), and brain samples collected at necropsy were examined by histology and immunohistochemistry.

RESULTS: Viremia (DNAemia) was detected at day 3 p.i. in 7/8 challenged animals. End-organ dissemination was observed, by qPCR, in the lung, liver, and spleen. CD4-positive (CD4⁺) and CD8-positive (CD8⁺) T cell infiltrates were present in brains of challenged animals, particularly in periventricular and hippocampal regions. Reactive gliosis and microglial nodules were observed. Statistically significant spatial learning and memory deficits were observed by MWM, particularly for total maze distance traveled ($p < 0.0001$).

CONCLUSION: Neonatal GPCMV infection in guinea pigs results in cognitive defects demonstrable by the MWM. This neonatal guinea pig challenge model can be exploited for studying antiviral interventions.

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IMPACT:

- CMV impairs neonatal neurocognition and memory in the setting of postnatal infection.
- The MWM can be used to examine memory and learning in a guinea pig model of neonatal CMV infection.
- CD4⁺ and CD8⁺ T cells infiltrate the brain following neonatal CMV challenge.
- This article demonstrates that the MWM can be used to evaluate memory and learning after neonatal GPCMV challenge.
- The guinea pig can be used to examine central nervous system pathology caused by neonatal CMV infection and this attribute may facilitate the study of vaccines and antivirals.

INTRODUCTION

Human cytomegalovirus (HCMV) is a common congenital viral infection known to damage the neonatal central nervous system (CNS). CNS manifestations in newborns can include microcephaly, cerebellar atrophy, polymicrogyria, ventriculomegaly, periventricular calcifications, focal necrosis, lissencephaly, and sensorineural hearing loss (SNHL; refs. 1–4). Long-term neurological sequelae are observed in 16.8–18.8% of children with congenital CMV.⁵ Recently, it has also been suggested that postnatally acquired CMV infections can produce long-term neurodevelopmental sequelae in very-low-birthweight premature infants.^{6,7}

Little is known about the mechanisms of long-term neurologic injury following perinatal viral infections. Since human CMV cannot be studied in animals, rodent models of perinatal viral challenge have been described using species-specific CMVs, such as the guinea pig CMV (GPCMV; refs. 8,9) and murine CMV (MCMV;

refs. 10–13). Following intraperitoneal (i.p.) MCMV inoculation of newborn mice, virus disseminates to the CNS, and infection is coupled with recruitment of immunocytes and expression of pro-inflammatory cytokines. Perinatal MCMV infection also results in long-term motor and sensory disabilities, including cerebellar pathology¹² and SNHL.^{14,15} We therefore sought to extend these neuropathogenesis studies to a neonatal viral challenge model in the guinea pig.

The studies we describe in the current report used the Morris water maze (MWM) to examine memory and cognition following neonatal infection with GPCMV. There have been limited studies of the MWM in rodent models of infectious diseases. The MWM was first described in the context of hippocampal-dependent learning and memory in rats.¹⁶ Previous rodent MWM studies observed that damage to the striatum, basal forebrain, cerebellum, and cerebral cortex disrupted spatial learning and maze

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performance.¹⁷ Armien et al.¹⁸ showed that mice latently infected with herpes simplex virus-1 demonstrated inflammation and neuronal loss, associated with severe spatial memory deficits. Buenz et al.,¹⁹ using C57BL/6J mice infected with Theiler's murine encephalomyelitis virus, showed that memory impairment correlated with hippocampal injury. Maze performance in guinea pigs is impaired after ethanol exposure,^{20,21} prenatal iron deficiency,^{22,23} and exposure to the pesticide chlorpyrifos.²⁴ To our knowledge, the impact of viral CNS infection (in particular with CMV) on MWM performance has not been evaluated in guinea pigs. We therefore examined the neuropathogenesis of GPCMV infection, following neonatal challenge, using the MWM.

METHODS

Virus, cells, and animals

Cell culture amplification of clonal, BAC-derived viral stocks was carried out in guinea pig lung fibroblast cells (ATCC-CCL158) as previously described.²⁵ Pups were challenged with a virulent, red fluorescent protein (RFP)-tagged variant of GPCMV, N13R10r129-RFP, a strain expressing TurboRFP635.^{25,26} Hartley neonatal guinea pigs born to dams purchased from Elm Hill Laboratories (Chelmsford, MA, USA) were inoculated i.p. within 72 h post delivery with 5×10^6 plaque-forming units (PFU) of N13R10r129-RFP (8 pups), or sham inoculated with an equal volume of phosphate-buffered saline (6 pups). Approval was obtained from the UMN Institutional Animal Care and Use Committee.

Real-time quantitative PCR (qPCR) analysis

DNA was isolated and viral loads were determined by qPCR as previously described.²⁵ DNA was extracted from either 100 μ l citrated blood collected at 0, 3, 7, 14, 21, and 35 days post challenge or from 0.05 g of homogenized liver, lung, spleen, or brain (as described below).

Histology and immunohistochemistry

Two sham-inoculated and seven GPCMV-challenged guinea pigs were necropsied at day 35 p.i. One GPCMV-challenged guinea pig was necropsied on day 14 p.i. Brain, liver, and spleen tissues were fixed overnight with 4% paraformaldehyde. Tissue sections were stained with hematoxylin and eosin, mounted using Permount (Fisher), and examined by light microscopy. Immunofluorescence staining was performed as previously described.²⁵ Supplementary Table S1 specifies the antibodies used in these experiments.

Morris water maze

Guinea pig pups were tested in the MWM beginning at day 15 p.i. The rationale for selecting this time point was based on published data demonstrating resolution of guinea pig CNS infection by day 14 post-GPCMV challenge;²⁷ hence, any neurocognitive abnormalities should represent sequelae of perinatal infection, and not be related to acute viremia/active infection of the CNS. Indeed, our data (described below in "Results" section) demonstrated clearance of viremia by day 14. The MWM consisted of a circular (160 cm in diameter, 63.5 cm in height) metal tank filled with water (~42 cm deep, temperature 20–21 °C). The water was tinted black with the addition of nontoxic black latex paint. A hidden circular escape platform (20 cm in diameter, 40 cm in height) was placed in the center of one of the four tank quadrants, submerged ~2–3 cm. A visual cue (bright blue geometric shape on a white background) was placed on each of the four walls above the tank to adapt the guinea pigs to their surroundings.

All pups were tested for five consecutive days. The five days were divided into two phases: the acquisition phase (days 1–4), followed by the probe phase (day 5). During the acquisition phase, the escape platform remained in the same position in the tank, whereas during the probe phase, the escape platform was removed. Each animal was tested four times daily for a maximum

of 90 s, or until the animal found the escape platform. To begin a trial, the animal was placed into the maze facing the wall of the tank at a specified point. The entry point was changed between trials. The order of the entry point was the same for each animal in the group, but was pseudo-randomized among training days. Once the animal found the platform or was guided there, it stayed for 10–15 s to observe its surroundings. The animals were studied in groups of five, and completed their entire day of training before the next five animals began. On the fifth day of testing, the platform was removed and each animal completed one 60 s free swim. The path of each guinea pig was recorded by a Fire-i™ board video camera outfitted with a 2.1 mm lens (Unibrain, Inc., San Ramon, CA, USA) and movements analyzed using ANY-maze software (Version 4.70, Stoelting Co., Wood Dale, IL, USA). Recorded data included (1) total distance traveled; (2) swim speed; (3) time to first target zone entry (escape latency); and (4) number of platform crossings. Data obtained during the acquisition phase was averaged across the four trials each day to quantify and compare the metrics of task acquisition/learning.

Statistical methods

Statistical analysis was performed using Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Two-sample *t* test comparisons were performed between the experimental groups at each data point sequentially, so there were multiple comparisons that were being made within a single dataset. The probe test was analyzed by quantifying platform crosses, a single number for each animal and group. Learning trials such as escape latency, swim speed, and distance were analyzed with one-way analysis of variance (ANOVA), with post hoc *t* tests correcting for multiple comparisons. Differences between the two groups during the singular probe test were analyzed by Student's *t* test. Pup weights were compared with Dunn's multiple comparisons.

RESULTS

Disseminated viral infection occurs, with clearance of DNAemia by day 14 following challenge with a virulent, RFP-tagged GPCMV. Stocks of a virulent, RFP-tagged GPCMV strain, N13R10r129-RFP, were generated as previously described.^{25,26} Animal weights in sham-inoculated (6/6) and virus-challenged animals (8/8) were recorded daily as a measure of pathogenesis. Sham-inoculated animals showed significantly greater weight gain compared to GPCMV-challenged animals ($p < 0.05$) (Fig. 1a). Viral load analyses demonstrated that DNAemia was present in 7/8 challenged animals at day 3 p.i. and in 8/8 animals at day 7 (mean viral load, 1.4×10^4 GPCMV genome copies/ml of blood). At day 14 p.i., one challenged animal was necropsied; this animal had a blood viral load of 7.9×10^6 GPCMV genome copies/ml. DNAemia cleared in the remaining challenged animals by day 35 p.i. (Fig. 1b). To examine visceral viral load, tissues from the animal sacrificed at day 14 were harvested, and the seven remaining challenged animals (and two sham-inoculated animals, data not shown) were sacrificed at day 35 p.i. and viral load was quantified in the lung, liver, spleen, and brain. Viral DNA was detected in the spleen (mean, 2.47×10^2 genomes/mg) and liver (mean, 4.9×10^1 genomes/mg) at day 35 in 6/7 challenged animals, but less frequently in the lung (1 of 7, 1.3×10^1 genomes/mg). No viral DNA was identified in brain tissue in day 35 samples (Fig. 1c), but DNA was detected in the day 14 brain sample (closed circle). Immunoperoxidase staining with a GP83-specific rabbit monoclonal antibody (moab; ref. 28) also identified GPCMV antigen in brain tissue (brown cells, Fig. 1d). Immunostaining with neuronal nuclear protein (NeuN) and glial fibrillary acidic protein (GFAP) antibodies demonstrated co-localization with viral antigen (Fig. 1d; yellow cells, white arrows), confirming bona fide infection of brain cells, and not simply leukocytic trafficking of antigen into

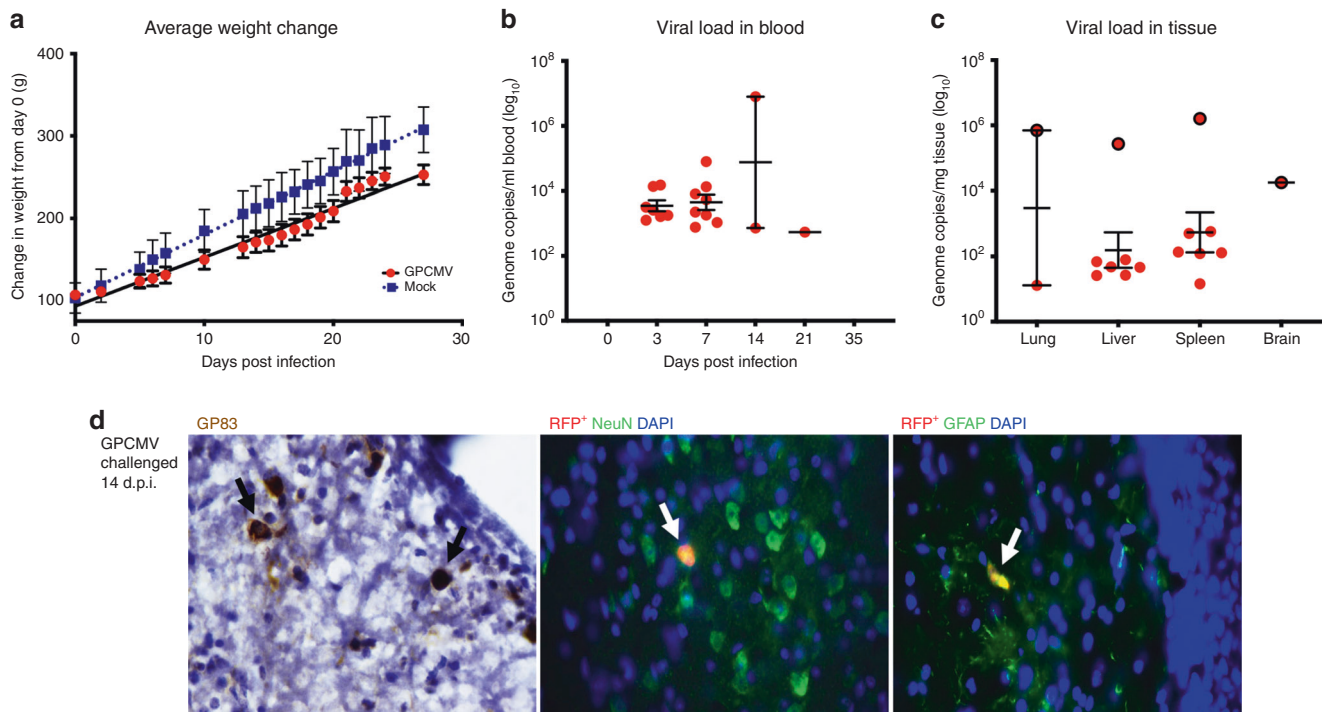


Fig. 1 Dissemination of GPCMV in a neonatal challenge model. **a** Mean percentages of changes in weight demonstrating significantly slower weight gain ($p < 0.05$) over time in challenged vs. sham-inoculated animals (mock). **b** DNAemia was measured at the indicated time points post infection. **c** End-organ dissemination following examination of tissues at the time of necropsy (day 35), following completion of MWM studies. Viral DNA was noted in the brain (1/8), lung (2/8), liver (7/8), and spleen (7/8). All data are from day 35, except for viral DNA detected in brain and visceral organs from the animal that necropsied at day 14 (closed circles); no viral DNA was noted in brains in animals necropsied at day 35. Data are represented as mean \pm SEM. **d** RFP⁺ (N13R10r129-RFP-infected) cells were observed in brain sections from animals described in **c** above. Immunoperoxidase staining was performed using a GP83-specific rabbit moab,²⁸ followed by secondary staining with HRP-conjugated horse anti-rabbit IgG (Vector Laboratories MP-7401) antibody and DAB substrate (SK-4105 Vector Labs, brown cells), and this demonstrated GPCMV GP83 antigen in the brain (left panel). Staining with either a rabbit anti-NeuN moab (middle panel) or a polyclonal rabbit anti-GFAP antibody (right panel) was also performed; secondary antibody, Alexa Fluor® 488 donkey anti-rabbit IgG (Supplementary Table S1). This identified infected neurons and astrocytes, respectively (merged images, arrows), that were also expressing GPCMV antigen (as evidenced by RFP signal). All sections were counterstained with DAPI. $\times 40$ Objective.

the brain. No RFP-positive (RFP⁺) cells were observed in animals sacrificed on day 35 p.i. (data not shown).

Visceral dissemination was also confirmed by visualization of N13R10r129-RFP⁺ cells in tissue sections. RFP⁺ cells (red cells) were observed in spleen and liver sections in a pup necropsied at day 14 p.i. Supplementary Figure S1 demonstrates RFP fluorescence of N13R10r129-RFP⁺ cells (red cells, white arrow) in brain and liver sections. Supplementary Figure S2 demonstrates spleen sections: (a) stained with a mouse anti-GPCMV gB 29.29 moab; (b) unstained cryosection exhibiting RFP expression from GPCMV-positive cells; and (c, d) merged images with counterstaining with 4',6-diamidino-2-phenylindole (DAPI) at low and high magnification. Confocal fluorescence images demonstrated RFP⁺ (N13R10r129-RFP-infected) cells in brain sections from the animal necropsied at day 14 p.i., and Z-stack images of RFP⁺ cells were recorded, processed, and analyzed for three-dimensional (3D) reconstruction (Supplementary Video S1). Fluorescence intensity in the 3D reconstitutions was quantified using the IMARIS software (version 7.4). The fluorescence intensity of the red channel corresponded to infected cells expressing RFP, while the intensity of the blue channel reflected nuclear fluorescence (DAPI). Non-infected cell nuclei had higher mean fluorescence intensities (400 AU) compared to infected cells (200 AU; yellow arrow, Supplementary Fig. S3).

Histopathological abnormalities in GPCMV-infected brain
Histological examinations of coronal brain sections from the challenged animals were compared to uninfected controls. Gross

disruption of brain histology, including bilateral ventricular dilatation with reduced thickness of cerebral cortex, was observed in two animals (Fig. 2a). We analyzed levels of NeuN immunoreactivity in damaged brains. In the challenged group, decreased NeuN staining was observed in the cerebral cortex and hippocampus, compared to controls where a high NeuN immunoreactivity was observed (Fig. 2b, c).

Inflammation and reactive gliosis in GPCMV-infected pups
To investigate the nature of CNS inflammation following neonatal GPCMV challenge, immunohistochemical staining for CD4-positive (CD4⁺) and CD8-positive (CD8⁺) T lymphocyte cell markers was performed on brain sections. No CD4⁺ or CD8⁺ lymphocytes were observed in brains of sham-inoculated animals (Fig. 3a). CD4⁺ and CD8⁺ T cell infiltration were identified in all brains of GPCMV-challenged pups (8/8). Figure 3b demonstrates a representative coronal section from a brain collected at day 14 p.i. with inflammatory cell infiltrates localized in the cortical region (black arrows). By immunofluorescence staining, these cells were confirmed to be CD8⁺ lymphocytes. The brain sections from the challenged animals collected at 35 days p.i. exhibited scattered inflammatory infiltrates localized in the periventricular and hippocampal regions. Focal aggregates of T lymphocytes were also identified in the thalamus, hypothalamus, and third ventricle regions of these brains (Fig. 3c).

We next examined the immunoreactivity of reactive astrocytes and activated microglia in brain sections using GFAP and ionizing calcium-binding adaptor molecule 1 (Iba1) abs, respectively.

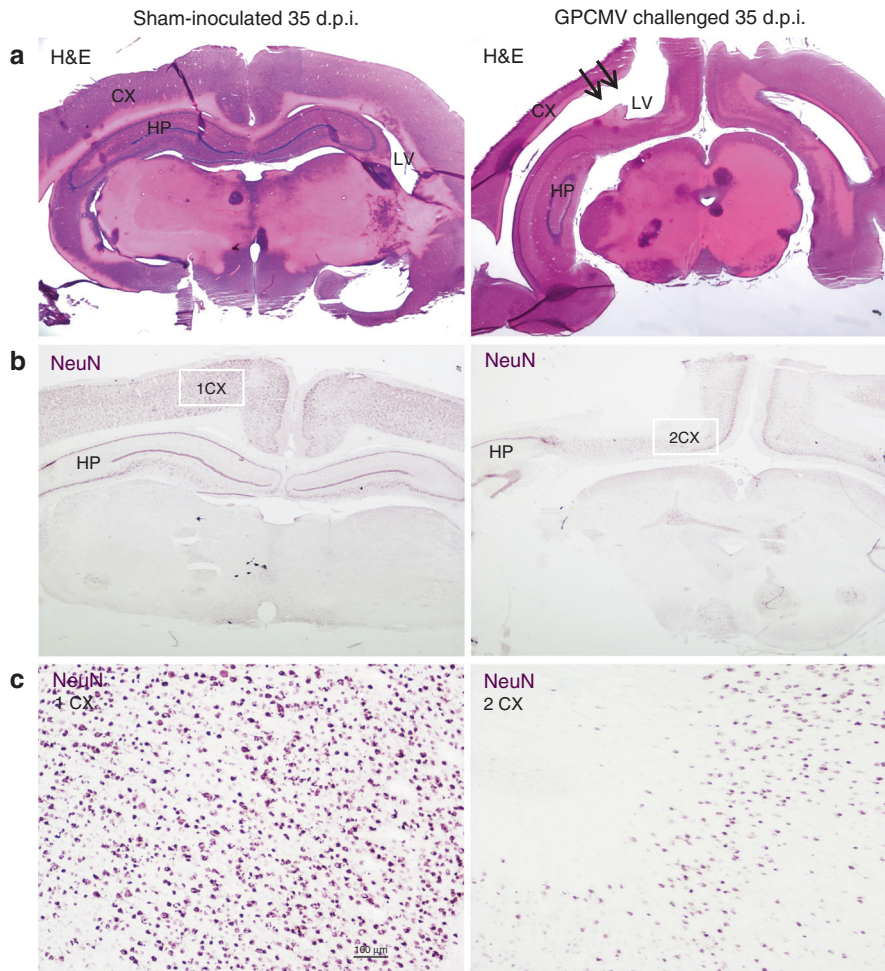


Fig. 2 Representative coronal brain sections from N13R10r129-RFP- and sham-inoculated guinea pigs. **a** Histological examination revealed extensive bilateral dilatation of lateral ventricles (LVs) and reduced thickness of cerebral cortex (black arrows) in the challenged animal compared to the control. Magnification $\times 10$. **b** Coronal sections ($\times 10$) demonstrating neuronal necrosis in N13R10r129-RFP-challenged guinea pig (left panel) and control (right panel) at 35 days p.i. Immunostaining was performed with rabbit NeuN moab and HRP-conjugate horse anti-rabbit IgG (Supplementary Table S1), using VIP substrate (purple cells, SK 4605 Vector Labs). Note the differences in intensity of NeuN immunoreactivity in uninfected control in the cerebral cortex (CX) and hippocampus (HP) regions compared to those in the GPCMV-infected animal. **c** represents higher magnification images of the indicated areas in **b**. In the uninfected control brain section, numerous NeuN-positive cells were observed. In contrast, fewer NeuN-positive cells were visualized in the brain section of the GPCMV-challenged guinea pig.

Immunostaining for GFAP revealed reactive astrogliosis in the brains of challenged guinea pigs, including the hippocampus and ventricular regions. The cell bodies of these astrocytes were enlarged, and their processes were thickened, and increased GFAP immunoreactivity was observed compared to sham-inoculated animals (Fig. 4a–c). 3D reconstructions of astrocyte filaments were generated from confocal Z-stacks using Imaris (Bitplane). Dual staining sections for T lymphocytes and astrocyte activation markers revealed that areas of increased inflammatory cell infiltration were associated with areas of reactive gliosis (Fig. 4b, d). Evidence of astrocyte activation and persistent chronic inflammation around the hippocampus in N13R10r129-RFP-challenged guinea pigs at 35 days p.i. is noted in Supplementary Fig. S4, demonstrating brain sections double-stained for GFAP⁺ (red cells) and CD8⁺ inflammatory T cells (green cells) and counterstained with DAPI, with strongly positive GFAP astrocytes with multiple elongated processes in challenged animals noted compared to controls. Scattered CD8⁺ infiltrates were also observed (see also Supplementary Video S2 online). Dense accumulations of GFAP⁺ end-feet and processes of reactive astrocytes wrapping around blood vessels were observed (Fig. 4c and Supplementary Video S3 online). Figure 5 shows representative microscopic images

of coronal brain sections stained with Iba1 antibody. Strongly staining cells with highly branched processes were observed in brain sections from five GPCMV-challenged animals, but not controls, suggesting that these immunopositive cells were activated microglia (Fig. 5a, b). Moreover, microglial nodules were also observed throughout the brain in three guinea pigs in the challenged group (Fig. 5c).

GPCMV infection impairs spatial learning and memory
In order to study the effect of neonatal GPCMV challenge on spatial learning and memory, guinea pigs challenged with N13R10r129-RFP (7/8, day 15 p.i.) and sham-inoculated animals (6/6) were tested using the MWM for 5 consecutive days using previously described methods.^{21–23} From days 1 to 4 of the experiment (acquisition phase), the escape platform remained in the same location in the pool, and on day 5 (probe phase) the platform was removed. On the last day of the acquisition phase, there was a significant decrease in total distance traveled between the two groups: GPCMV-challenged animals swam an average of 11.7 m, compared to the 5.0 m traveled in sham-inoculated animals ($p < 0.0001$ by one-way ANOVA, Fig. 6a). A statistically significant increase in the time to find the hidden platform was

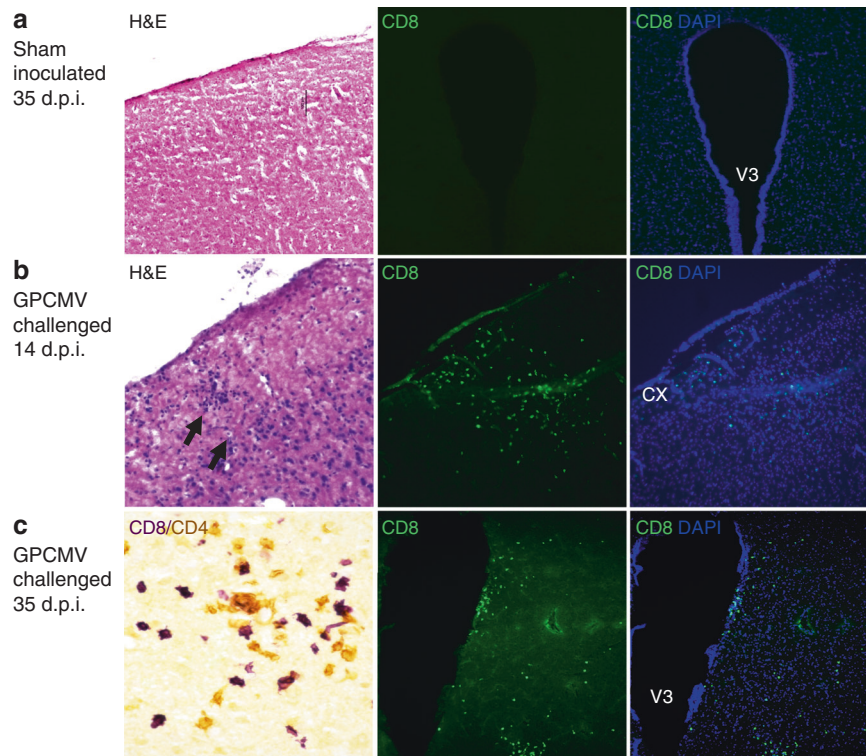


Fig. 3 Evidence of inflammatory cell infiltration in N13R10r129-RFP-infected guinea pig brain. a No CD8⁺ T lymphocytes were observed in sham-inoculated brains. **b** Inflammatory cell infiltrates were localized in the brain cortical region (CX) and visualized by H&E (black arrows). T cells were visualized by staining with anti-guinea pig CD8⁺ moab and secondarily incubated with Alexa Fluor® 488 polyclonal anti-mouse IgG ab (green cells) at 14 days p.i. **c** T lymphocytes in brain sections were visualized in tissue obtained at 35 days p.i. by immunoperoxidase staining (double label): ImmPRESS anti-mouse IgG using CD4⁺ moab with DAB (brown cells) substrate, and ImmPRESS anti-mouse IgG using CD8⁺ moab with VIP (purple cells) substrate. CD8⁺ cells were also visualized in day 35 tissue using CD8⁺ moab (middle panel, CD8⁺; right panel, IFL with DAPI). Focal aggregates of CD8⁺ T lymphocytes were localized around the region of the 3rd ventricle (V3). $\times 10$ Objective.

noted (escape latency, a measure used as an early indicator of learning [22]) in the challenged compared to sham-inoculated animals ($p < 0.001$ by one-way ANOVA, Fig. 6b). During this phase, swim speed was also measured, and the sham-inoculated animals swam significantly faster than the challenged animals ($p < 0.05$ by one-way ANOVA, Fig. 6c). Overall, these results indicate that the challenged guinea pigs had more difficulty finding the hidden platform compared to controls. A representation of these results is observed in the occupancy plots in Fig. 6d. On day 4 of training (final training day), the animals in the GPCMV-challenged group spent a significant amount of their time exploring the maze and were unable to locate the platform, while the control animals quickly found their way to the platform.

After learning was complete, reference memory was assessed by measuring the number of passes over the target quadrant where the platform used to be. The challenged group performed fewer platform crosses than the sham-inoculated animals. During the first 30 s of the probe test, challenged animals crossed the target platform zone on average 0.86 times, compared to controls crossing 2.17 times ($p < 0.05$ by unpaired *t* test; Fig. 6e). The lack of preference to the target quadrant seen in the challenged group is evidence of significant memory deficit, further demonstrated by occupancy plots (Fig. 6d), where the challenged group displayed a fairly distributed search of all quadrants, whereas controls targeted their search to where the quadrant was previously located. Additionally, it is noteworthy that the search strategy employed by the virus-challenged groups was to swim around the periphery of the tank. This serial search strategy, looking for an escape, further demonstrates cognitive impairment, insofar as the animals failed to acquire a spatial strategy of search based upon visual cues in the maze.

DISCUSSION

Neurodevelopmental impairment, commonly including learning and memory deficits, has long been associated with perinatally acquired herpesvirus infections, including CMV.^{1,5} We sought to develop a small animal model to assess neurobehavioral sequelae resulting from experimental neonatal CMV infection, using the MWM. In the MWM, spatial learning and memory is evaluated by a decrease in time (latency) to find the hidden platform and a reduction in path length (distance) traveled to the platform. The MWM has proven useful in other rodent viral challenge models. Espinoza et al.²⁹ demonstrated that intranasal respiratory syncytial virus infection in mice and rats can spread from the airways to the CNS, causing functional alterations in the brain and resulting in poor MWM performance. Impaired cognition using the MWM was also shown by Jurgens et al.³⁰ in a study of 4–6-month-old male BALB/C mice following intranasal infection with live influenza virus. For MCMV, pregnant BALB/C mice were subjected to intraplacental inoculation of virus (1 μ L, 1×10^6 PFU), and learning and memory in the 70-day-old offspring were analyzed by MWM test, which demonstrated that the infected groups exhibited longer latency times and increased swimming distance compared to controls.³¹ Similar results were observed by Lokensgard et al.,³² who reported that MCMV infection induced long-term neurological deficits (examined using a dry-land Barnes maze) in spatial learning and memory following regulatory T cell (Treg) depletion. In the current study, we extended previous studies of spatial learning and memory in guinea pigs^{20–24,33} to the study of GPCMV infection. We found that neonatal guinea pigs challenged with a virulent, RFP-tagged GPCMV demonstrated significant impairment in MWM test performance, providing evidence of impairment of learning and memory associated with perinatal viral CNS infection.

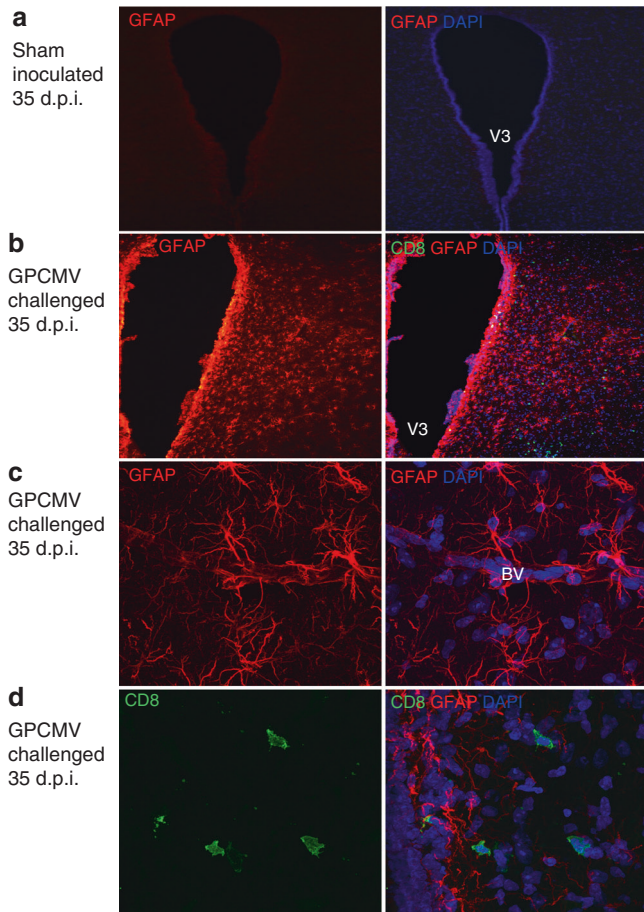


Fig. 4 Evidence of astrocyte activation and persistent chronic inflammation in N13R10r129-RFP-infected guinea pig (35 days p.i.) compared to uninfected control. a, c Brain sections from infected and control animals were stained with polyclonal rabbit GFAP astrocyte marker antibody Z0334 (red cells) and Alexa Fluor® 594 donkey anti-rabbit secondary ab. Note the strongly positive GFAP-staining astrocytes with multiple elongated cell processes in tissues from infected animals in contrast to sham-inoculated animals. Confocal microscopy fluorescence of dense accumulation of GFAP⁺ end-feet and processes of reactive astrocytes wrapping around blood vessels (BVs) is noted. Note the dense staining of the enlarged cell bodies and the highlighted cell processes. **b, d** In addition to GFAP, anti-CD8⁺ antibody staining with secondary ab (Alexa Fluor® 488 polyclonal anti-mouse, green cells) staining is shown, with sections counterstained with DAPI. A reactive phenotype was visualized around the ventricular region (V3) and focal aggregates of CD8⁺ T lymphocytes were noted around these reactive areas. CD8⁺ cells are associated with a prominent reactive astrogliosis as shown. **a, b** ×10 Objective; **c, d** ×100 objective.

One potential limitation of our model is that we did not perform direct intracerebral (i.c.) inoculation to unequivocally ensure CNS infection in all animals. Booss et al.³⁴ inoculated young guinea pig i. c. with GPCMV. These authors reported that after i.c. inoculation with 10^{3.5} TCID₅₀ (median tissue culture infectious dose) of GPCMV, levels of virus were found to peak in the brain during the first week post infection, and were reduced or cleared by week 3 or 4 p.i. Glial nodule encephalitis was described in this direct CNS inoculation model. Other studies, however, have demonstrated that brain infection with GPCMV does not require direct i.c. inoculation, and that extension of GPCMV infection to the CNS can occur following systemic routes of infection, both in pregnant (with subsequent infection of the fetus) and non-pregnant animals.^{9,27,35} A hematogenous route of seeding of the CNS, as opposed to a direct

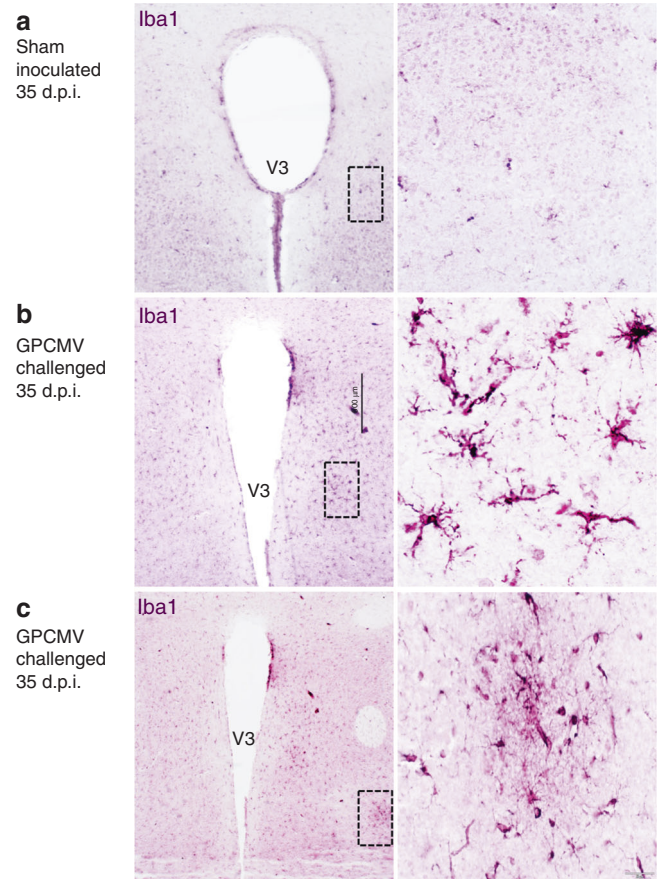


Fig. 5 Microglial cell reactivity and persistent chronic inflammation in N13R10r129-RFP-challenged guinea pigs at 35 days p.i. Brain sections were stained with rabbit moab to the microglial cell marker Iba1 and an HRP-conjugated horse anti-rabbit IgG (MP-7401) with the addition of VIP (purple) substrate. **a, b** Positive microglial staining demonstrating a more reactive phenotype in challenged animals (**b**) in contrast with sham-inoculated animals (**a**). **c** Iba1⁺ staining demonstrating discrete microglial nodule formation dispersed throughout the brain in the same guinea pig shown in **b**, necropsied at day 35. Left, low-power magnification (×40); right panels, enlargement of boxed regions designated in left panels.

inoculation route, is more likely to recapitulate the pathology of brain infection in newborn infants with perinatally acquired CMV infections. Bravo et al.⁹ described a neonatal GPCMV challenge model, with viral dissemination to multiple organs, including brain, by day 10 post challenge. Griffith et al.³⁵ described brain and visceral infection in the context of congenitally transmitted CMV infection in guinea pigs, and showed that GPCMV was recovered in cell cultures from salivary glands (18/24) and the spleen (8/24), but less commonly in the brain (1/24).

In the current study, we used qPCR analysis to demonstrate that, after i.p. inoculation, high viral loads were detected in all organs, including brain from an animal that was necropsied at day 14 p.i. In this challenge model, viremia essentially cleared after day 14. Growth velocities were also similar in infected and control groups after this day 14 time point, concomitant with the clearance of viremia (Fig. 1a). Although evidence of active viral infection in the CNS was observed in one animal necropsied at day 14 (as evidenced by PCR and viral antigen expression), no evidence of active viral replication was present in the brains at the time of necropsy of other challenged animals at day 35. These findings are compatible with the observations made by Booss et al.,³⁴ which indicated that GPCMV leptomeningitis and encephalitis had run their course and resolved by day 14 p.i.

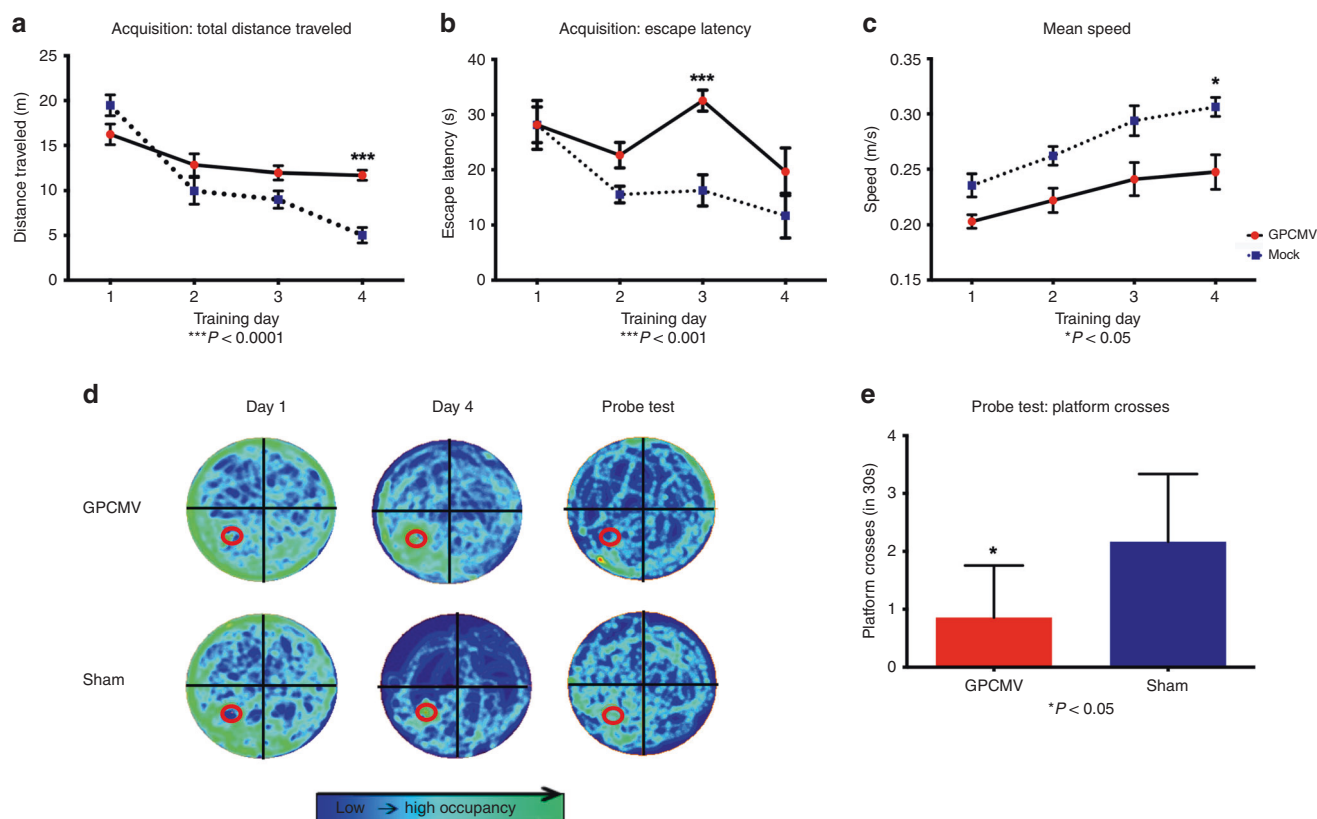


Fig. 6 Spatial learning and memory deficits in the challenged animals during the Morris water maze test. **a** On the first day of acquisition training, there was no difference in total distance traveled between the sham-inoculated (mock) and the N13R10r129-RFP-challenged group. By day 4, the challenged animals swam an average of 11.7 m compared to the 5.0 m traveled by the sham-inoculated group ($***p < 0.0001$). **b** Statistically significant decrease in the escape latency (time to find platform) in the sham-inoculated animal group during acquisition phase ($***p < 0.001$). **c** Graph shows swim speed of the N13R10r129-RFP-challenged group vs. sham-inoculated guinea pig. Statistical analysis showed a significant increase in the mean speed of the sham-inoculated group compared to challenge group ($*p < 0.05$). **d** Occupancy plots showing combined travel path of all animals on acquisition days 1 and 4 and during the probe phase on day 5. Sham-inoculated animals learned where the hidden platform was faster than N13R10r129-RFP-challenged group. Red circle represents the hidden escape platform. Blue represent areas of low occupancy vs. green regions representing areas of high occupancy. **e** Reference memory tested during the probe phase. Challenged group performed fewer platform crosses (0.86) than the sham-inoculated animals (2.17; $*p < 0.05$). Data are expressed as mean \pm SEM.

MWM performance was assessed in our study beginning on day 15 p.i., minimizing any concern that impaired MWM performance was due to an acute, symptomatic viremic illness.

Notably, both CD4⁺ and CD8⁺ T cell infiltrates could be detected in brains from virus-challenged, but not sham-inoculated controls (Fig. 3). Similar patterns of brain infection have been described with MCMV. Bantug et al.³⁶ detected virus in the liver, spleen, and CNS following i.p. inoculation of neonatal BALB/c mice with MCMV, with virus peaking in the CNS between postnatal days 10–14. The activation of microglia we observed with Iba1 staining was of interest, given that CMV infection in human fetuses (20–29 weeks gestation) demonstrates a tropism for stem cells and radial glial cells, with concomitant focal microglial activation.^{2,3} Although newborn mice are developmentally immature compared to newborn guinea pigs, our findings nonetheless suggest that GPCMV, similar to MCMV, disseminates to the CNS following neonatal challenge, eliciting histopathology and altering neurocognitive function. By analogy with MCMV studies, it is likely that eventual control of GPCMV infection in the guinea pig CNS requires both a T cell and a humoral immune response,^{13,37,38} although our observations demonstrate that neurocognitive sequelae occur even after clearance of infection is achieved.

Additionally, we postulate that neonatal brain damage induced by GPCMV in this model is not only due to virally mediated damage to specific cell types, but also the attendant inflammatory

response, which has been described in human fetal brain at the time of necropsy.^{2,3} Gabrielli et al.³ demonstrated that in brains with severe cerebral damage, microglial nodules included cytomegalic cells surrounded by activated CD8⁺ T lymphocytes and apoptotic cells. Observations in MCMV demonstrated chronic neuroinflammation, mediated by B cells, microglial cell activation, and retention of virus-specific memory CD8⁺ T cells, persisting in the absence of detectable viral antigens. Interferon- γ secretion by brain-infiltrating T cells contributed to microglial activation and tumor necrosis factor- α production following MCMV infection of Fox3p-DTR-GRP mice, was exacerbated by Treg depletion, and was associated with reduced cognitive performance in spatial learning and memory tasks in the Barnes maze.³² We similarly noted that GPCMV-challenged guinea pigs demonstrated prominent reactive gliosis with inflammatory cell infiltrates, even in the absence of viral replication, at 35 days p.i.

In summary, this is also to our knowledge the first functional assessment of memory and neurocognition in guinea pigs challenged with a neurotropic virus, GPCMV, utilizing the MWM. The MWM provides useful experimental endpoints to evaluate the impact of neonatal GPCMV infection and, potentially, the therapeutic benefits of antivirals and vaccines. The findings in this study provide evidence that neonatal GPCMV infection in guinea pigs produces cognitive and learning defects, demonstrable histologically by immunostaining, and functionally by assessment

of performance in the MWM. This model may be useful in studying therapies relevant to perinatally acquired CMV infections in infants. Future MWM studies of pups with congenital GPCMV infection would also be of translational relevance in the examination of the benefits of vaccine strategies targeting prevention of the sequelae of congenital HCMV infection.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data: C.F.-A., L.E.M., M.A.M., J.R.L., S.H., M.A.B., K.M.A., B.C.J., and M.R.S. Drafting the article or revising it critically for important intellectual content: C.F.-A., L.E.M., M.A.M., J.R.L., S.H., M.A.B., K.M.A., and M.R.S. Final approval of the version to be published: C.F.-A., L.E.M., M.A.M., J.R.L., S.H., M.A.B., K.M.A., B.C.J., and M.R.S.

ADDITIONAL INFORMATION

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