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MAJOR ARTICLE

# Hepatitis E Virus Infects Neurons and Brains

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Hepatitis E virus (HEV), as a hepatotropic virus, is supposed to exclusively infect the liver and only cause hepatitis. However, a broad range of extrahepatic manifestations (in particular, idiopathic neurological disorders) have been recently reported in association with its infection. In this study, we have demonstrated that various human neural cell lines (embryonic stem cell–derived neural lineage cells) induced pluripotent stem cell–derived human neurons and primary mouse neurons are highly susceptible to HEV infection. Treatment with interferon-α or ribavirin, the off-label antiviral drugs for chronic hepatitis E, exerted potent antiviral activities against HEV infection in neural cells. More importantly, in mice and monkey peripherally inoculated with HEV particles, viral RNA and protein were detected in brain tissues. Finally, patients with HEV-associated neurological disorders shed the virus into cerebrospinal fluid, indicating a direct infection of their nervous system. Thus, HEV is neurotropic in vitro, and in mice, monkeys, and possibly humans. These results challenge the dogma of HEV as a pure hepatotropic virus and suggest that HEV infection should be considered in the differential diagnosis of idiopathic neurological disorders.

**Keywords.** central nervous system; cerebrospinal fluid; hepatitis E virus; peripheral nervous system.

Hepatitis viruses, classified as A, B, C, D, and E, share the common feature of infecting the human liver, resulting in hepatocellular inflammation. Hepatitis E virus (HEV) containing a positive-sense, single-stranded RNA genome belongs to the *Hepeviridae* family, and is the most common cause of acute hepatitis worldwide [1]. Although it was initially thought to cause acute infection only, chronic infection has been well documented in immunocompromised individuals (including those with HIV infection or undergoing chemotherapy for cancer), but is most common in organ transplantation recipients [2]. Compared to other hepatotropic viruses, HEV genotypes 3 and 4 are now recognized as zoonotic in developed countries with a broad spectrum of animals serving as reservoirs (the primary host being the pig) [3]. HEV infection is surprisingly common in developed countries [4]. Infections are normally asymptomatic or unrecognized and, as a result, HEV has found its way into the human blood supply. For example, recent data show that 1 in 600 blood donors in the South-East of England are viremic at the time of donation [5]. Notably however, the health risks following viremic blood transfusion remain unclear.

Previously, HEV was thought to exclusively infect hepatocytes, resulting in hepatocellular necrosis and hepatitis. However, a number of recent studies have shown that HEV infection is associated with a broad range of idiopathic extrahepatic manifestations, including renal injury, acute pancreatitis, hematological diseases, and a variety of neurological disorders [6]. Among these, neurological disorders have been described as a relatively common but under-recognized extrahepatic manifestation related to HEV infection [4, 7, 8]. An increasing number of central or peripheral nervous system diseases have been documented in patients associated with acute or chronic HEV infection [4, 7, 9–11], including Guillain-Barré syndrome (GBS) and neuralgic amyotrophy (NA) [4, 9, 12, 13].

The etiology of HEV-associated neurological injury is unknown. In the existing paradigm, HEV is considered to be primarily a hepatotropic virus. In this context, the most widely held neuropathogenic hypothesis posits that HEV causes neurological injury by immune mechanisms related to molecular mimicry, as has been well described for GBS associated with a variety of infectious triggers [9, 14]. The alternative hypothesis, however, that HEV causes direct injury via neurotropism, has not been sufficiently evaluated. The aim of the present study

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was to investigate if HEV can directly infect the nervous system. Our results demonstrate that HEV is capable of central nervous system infection in multiple species and therefore its neurotropic property should be recognized.

## METHODS

### HEV Plasmids

HEV genomic RNA was generated from a plasmid construct containing the full-length HEV genome (genotype 3 Kernow-C1 p6 clone, GenBank accession number JQ679013) or a construct containing subgenomic HEV sequence coupled with a *Gaussia* luciferase reporter gene (p6-Luc), using the Ambion mMESSAGE nMACHINE in vitro RNA transcription kit (Life Technologies, CA) [15, 16]. The plasmid p6-Luc-GAD with a mutation in the HEV polymerase of the p6-Luc, resulting in defect of viral replication, was also used.

### Reagents and Cell Culture

Human interferon-α (IFN-α; Thermo Scientific, The Netherlands) was dissolved in PBS. Ribavirin (Sigma-Aldrich, MO) and rapamycin (Bio-Connect, TE Huissen, The Netherlands) were dissolved in dimethyl sulfoxide (Sigma). Mycophenolic acid (MPA) (Sigma) was dissolved in methanol. A primary anti-HEV capsid protein ORF2 antibody (aa 434–457, clone 1E6, Millipore, Amsterdam, The Netherlands) was used for immunoblotting and staining.

Human hepatoma cell line HuH7 was cultured in Dulbecco's Modified Eagle medium (DMEM) (GIBCO Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) fetal calf serum (Hyclone, Lonan, Utah), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Invitrogen-Gibco). All neural cell lines were kind gifts: glioblastoma U87 cell line from the Department of Viroscience, Erasmus Medical Centre; glioblastoma U343 cell line from Dr. Elly Hol (Dept of Translational Neuroscience, Brain Center Rudolf Magnus, University Medical Center Utrecht); neuroepithelioma SK-N-MC cell line from Dr Thomas Pietschmann (Institute for Experimental Virology, Twincore, Centre for Experimental and Clinical Infection Research); and neuroblastoma SH-SH5Y cell line from Dr Marcel E. Meima (Dept of Internal Medicine, Erasmus Medical Center). They were cultured in DMEM supplemented with 10% volume/volume (v/v) fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine. Human embryonic stem cell (H9)–derived neural progenitor cells (hES-NPCs) were cultured as previously described [17]. Induced pluripotent stem (iPS) cell–derived iCell Neurons (Cellular Dynamics, WI), a highly pure population of human neurons, were cultured in the complete iCell Neurons Maintenance Medium (Cellular Dynamics) according to the manufacturer's guidelines (https://cellulardynamics.com/assets/CDI_iCellNeuronsUsersGuide.pdf). All cells were cultured in an incubator at 37°C with 5% $CO_2$.

### Electroporation of HEV RNA

The glioblastoma U87 cells were collected and centrifuged for 5 minutes at 1500 rpm, 4°C. The supernatant was removed and the cells were washed with 4 mL Opti-MEM (Thermo Scientific, The Netherlands) by centrifuging for 5 minutes at 1500 rpm, 4°C. The cell pellet was resuspended in 100 µL Opti-MEM and mixed with p6 full-length HEV RNA or p6-Luc subgenomic RNA. Electroporation was performed with Bio-Rad's electroporation systems using the protocol of a designed program (400 volts, pulse length 0.5, number 1, 4 mm cuvette) [15]. The supernatant of the cultured p6 full-length HEV RNA electroporated cells were collected to perform viral infection.

### Isolation and Culture of Primary Mouse Neurons

The primary mouse cerebellum and hippocampus were isolated from E17-E19 C57/BL6 mouse (Charles River Laboratories, MA) embryos. In brief, the cerebellum and hippocampus were dissected in ice-cold Hank's balanced salt solution (HBSS) supplemented with 20 µg/mL gentamicin (both from Life Technologies), then incubated with 10 U/mL papain (Sigma), 2.5 U/mL DNAse I (Roche), and 4 mM $MgCl_2$ (Sigma) at 33°C for 30 minutes. The cerebella were titrated in HBSS with 2.5 U/mL DNAse I and 4 mM $MgCl_2$, and were filtered with 200 µM nylon mesh. After washing in HBSS twice, the cells were plated on poly-ornithine (500 µg/mL, Sigma) coated #1.5H glass-bottomed slides (Ibidi, Germany) at a density of $1.2 \times 10^6$ cells/$cm^2$. For electroporation, these primary mouse neurons were transfected with p6 full-length HEV RNA using a 4D-Nucleofector system (Lonza, Switzerland) according to manufacturer's protocol before plating. The culture medium contains primary neuron growth medium (PNGM; Lonza, Switzerland), GS-21 supplement (GlobalStemCells), 5 µg/mL gentamicin, and 2 mM glutamax (Life Technologies). Half of the medium was changed once a week, and 2 µM Ara-C (Sigma-Aldrich) was added to prevent glia growth.

### Measurement of Luciferase Activity

For *Gaussia* luciferase (Luc), the activity of secreted luciferase in the cell culture medium was measured by the BioLux Gaussia Luciferase Flex Assay Kit (New England Biolabs, MA) and quantified with a LumiStar Optima luminescence counter (BMG LabTech, Offenburg, Germany).

### Animal Models of HEV Infection

The Animal Care and Use Committee of the Kunming University of Science and Technology approved the study protocol and provided the guidelines for this study. Specific pathogen-free (SPF) male Balb/c nude mice were purchased from the National Rodent Laboratory Animal Resources, Shanghai Branch (China) and maintained in a pathogen-free animal facility. All mice were female and 6 weeks old. Prior to their inoculation with HEV, mice were tested negative for HEV immunoglobulin G (IgG) and immunoglobulin M (IgM) antibodies in serum

samples and HEV RNA in both their serum and feces samples. Swine HEV (Genotype 4, KM01 strain) isolated from the feces was intravenously injected into each mouse with a viral load of $1 \times 10^5$ copies/mL, as calculated by the viral genomic titer determined by quantitative real-time polymerase chain reaction (qRT-PCR). Mice were humanely euthanized at 14 days postinoculation, following the guidelines of the Care and Use of Laboratory Animals.

Rhesus macaques were obtained from Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. Prior to their inoculation with HEV, they were tested negative for HEV IgG and IgM antibodies and HEV RNA. Two 2-year-old male rhesus macaques were intravenously injected with 2 mL fecal supernatant from swine infected with HEV (genotype 4, KM01, $2 \times 10^4$ copies) as previously established [18]. One macaque without inoculation served as a control. One monkey that died unexpectedly after over 600 days of chronic infection was subjected for analysis. For the mice and monkeys, serum samples, liver tissues, and brain tissues were collected for qRT-PCR quantification of HEV RNA. The brain tissues were collected for immunohistochemistry staining of ORF2 viral protein.

Additional details can be found in Supplementary Methods.

## RESULTS

### HEV Infects Neurons in Cell Culture

To investigate whether HEV can directly infect human neural cells, neuroblastoma SH-SH5Y, neuroepithelioma SK-N-MC, and glioblastoma U87 and U343 cell lines were employed. A human liver cell line, human hepatoma 7 (HuH7), was used as a positive control because of its high permissiveness for HEV infection. After inoculation with cell culture–derived HEV particles for 24, 48, and 96 hours, intracellular viral RNA was quantified by qRT-PCR. All of the neural cell lines were permissive to HEV infection. U87 and SH-SH5Y cells exerted a similar susceptibility compared to HuH7 cells; U343 and SK-N-MC cells were less susceptible than HuH7 cells to HEV infection (Figure 1A). Moreover, hES-NPCs (Supplementary Figure 1A) exhibited a particularly high susceptibility to HEV infection, compared to HuH7 liver cells (Figure 1A). We next investigated HEV infection of cultures in which neural differentiation of human iPS cell-derived neural progenitor cells was induced. Notably, these cultures appeared remarkably permissive for HEV infection, as evidenced by the abundance of intracellular HEV RNA quantified 4 days after inoculation of HEV particles (Supplementary Figure 1B). Subsequently, human iPS cell–derived neuronal cultures, consisting of a pure population of postmitotic neurons (iCell

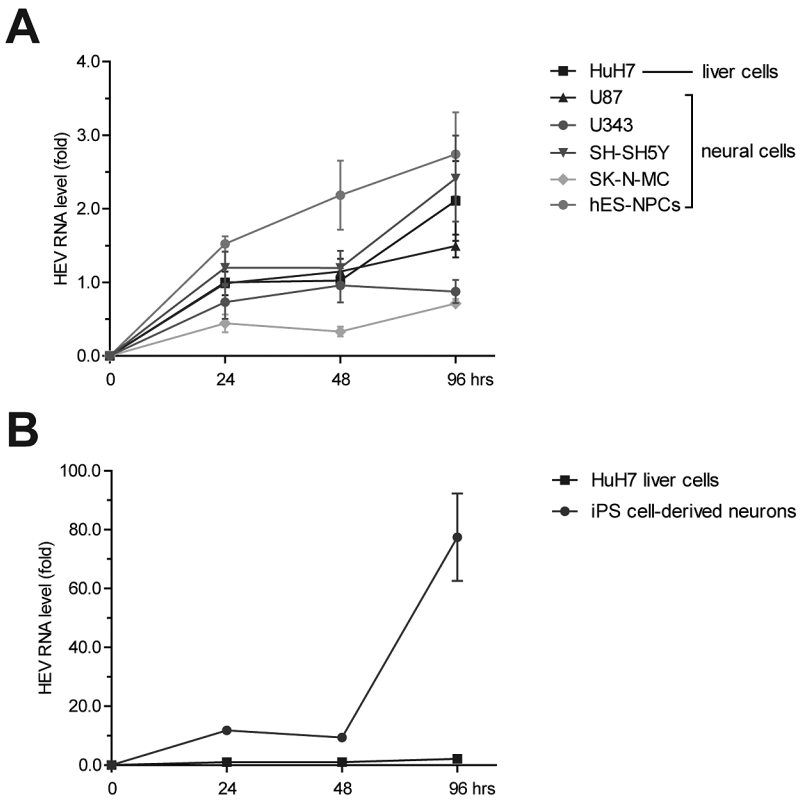


**Figure 1.** Neural cells are permissive for HEV infection in vitro. Cellular HEV RNA levels of various human neural cell lines and hES-NPCs (*A*), and cellular HEV RNA levels of iPS cell–derived differentiated human neural cultures (*B*) were analyzed with inoculation of cell culture–derived HEV particles for 24, 48, and 96 hours, compared to human liver HuH7 cells, which are considered the canonical model for HEV infection (mean ± SD; n = 3–4). The level of HEV infection in HuH7 cells for 24 hours serves as 1. Abbreviations: hES-NPCs, human embryonic stem cell–derived neural progenitor cells; HEV, hepatitis E virus; HuH7, human hepatoma 7 cells; iPS, induced pluripotent stem.

neurons), as observed under the white light microscopy analysis (Supplementary Figure 1C) were also tested. These cells were also highly permissive for HEV infection, as shown by the replication level of cellular HEV RNA (especially at 96 hours of virus infection) compared to HuH7 liver cells (Figure 1B). HEV copy numbers presented a time-dependent increase in the secreted medium (Supplementary Figure 1D). These results demonstrate that HEV is able to directly infect neural cells in vitro.

#### Long-term Replication and Production of HEV in Neural Cells

Further detailed study of HEV infection was performed using the glioblastoma U87 cell line, a well-established and appropriate model for investigating the molecular details of virus-related neuropathology [19]. Upon electroporation of the full-length genomic HEV RNA, viral protein ORF2 was subsequently expressed in U87-HEV cells, as detected by immunofluorescent and immunohistochemical staining (Figure 2A and Supplementary Figure 2). We further investigated whether these U87-HEV cells can secret viral particles into the supernatant. To this end, an HEV plasmid–based standard curve was constructed as a reference to assess and quantify the production of infectious virion (Supplementary Figure 3A). We observed that the HEV load was $1.7 \times 10^6$ copies/mL in supernatant of U87-HEV cells and $1.3 \times 10^9$ copies/mL in ultra-centrifuged medium (Supplementary Figure 3B). Viral protein was detected in the ultra-centrifuged medium by Western blot analysis (Figure 2B). Thus human glioblastoma cells appear capable of supporting the HEV life cycle.

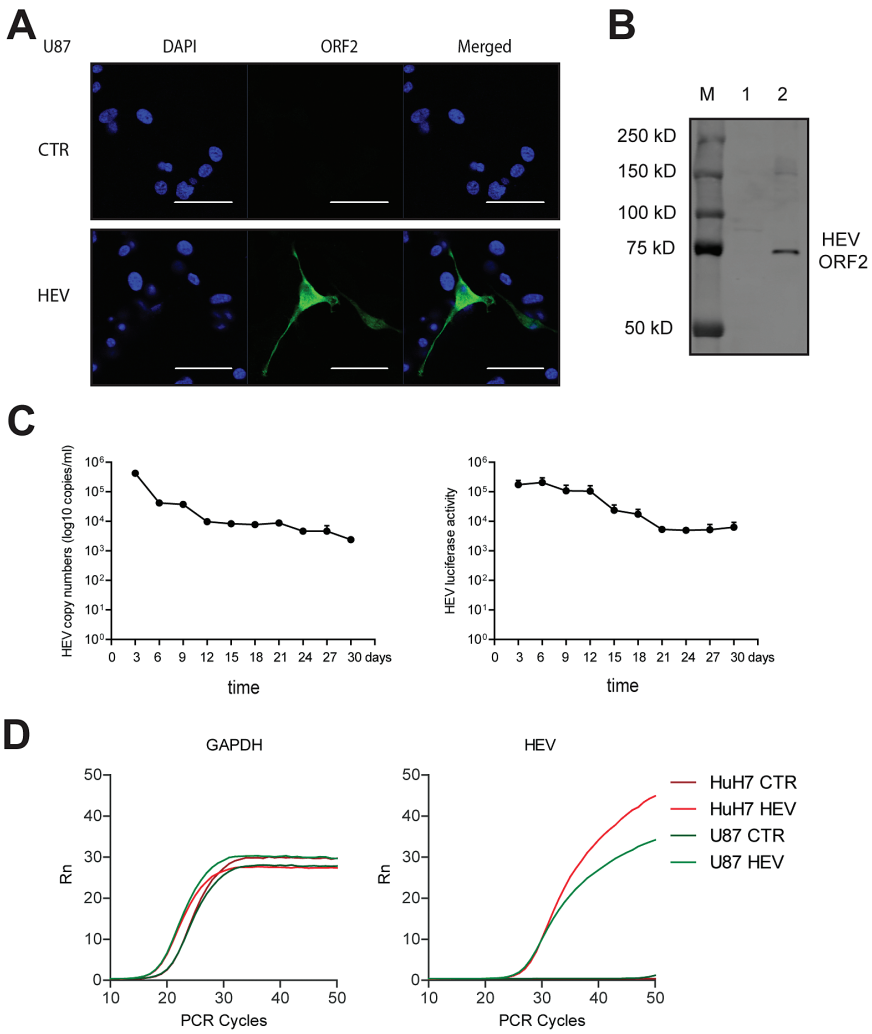


**Figure 2.** Neuroglial cells support long-term HEV replication and production. *A*, Immunofluorescence staining of viral protein ORF2 (green) in neuroglial U87 cells upon electroporation of the full-length genomic HEV RNA. DAPI (blue) was applied to visualize nuclei. Shown confocal images are representative for 3 independent experiments. (Scale bar, 50 µm; 40× oil immersion objective). *B*, In ultra-centrifuged supernatant produced by U87-HEV cells, viral protein ORF2 is observed by Western blot assay. *C*, U87 supports long-term production of HEV as tested by copy numbers in the medium for 30 days (mean ± SD; n = 2, each with 2 replicate experiments). In U87 cells upon electroporation of the subgenomic HEV RNA with luciferase reporter, HEV luciferase activity (representing virus replication level) was detected maintaining for 30 days (mean ± SD; n = 2, each with 2 replicate experiments). *D*, qRT-PCR results indicate that HuH7 and U87 cells are permissive for secondary infection with inoculation of U87-HEV produced viral particles for 96 hours. GAPDH serves as a reference gene. One representative experiment of 3 is shown. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEV, hepatitis E virus; qRT-PCR, quantitative real-time polymerase chain reaction.

To ascertain whether glioblastoma cells also can support long-term production of HEV, the viral load of extracellular U87-HEV medium was monitored over 30 consecutive days, and high viral loads were consistently secreted over the entire time period (Figure 2C). For U87-HEV-Luc cells with electroporation of subgenomic HEV RNA in which ORF2 was replaced by a *Gaussia* luciferase reporter gene (p6-Luc), secreted luciferase activity directly served as a pseudomarker for the level of HEV replication. Glioblastoma cells apparently supported long-term HEV replication, as luciferase activity could be readily detected for 30 days (Figure 2C). Of note, introducing a mutation in the viral polymerase of the p6-Luc model resulted in a defect of viral replication and very limited secretion of *Gaussia* luciferase (Supplementary Figure 4), demonstrating the efficiency of viral replication in our models.

Furthermore, comparable levels of HEV infection were detected upon inoculation of HuH7 liver cells and U87 cells with U87-produced HEV particles for 96 hours, demonstrating that these cells produce infectious particles (Figure 2D), a hallmark of bona fide infectivity. These data show that human glioblastoma cells are capable of supporting long-term replication and production of infectious HEV.

### Effects of Clinically Relevant Drugs on HEV Replication in Neuroglial Cells

HEV-associated neurological manifestations are documented in both immunocompetent patients and immunocompromised patients in particular organ transplant recipients. If managed/treated adequately, viral load can be reduced in most of the patients and neurological symptoms can be resolved [20]. Reducing the dose of immunosuppressants is often the first line of therapeutic intervention for immunosuppressed patients [21]. Although no approved HEV medication is available, ribavirin or IFN-α has been used as an off-label treatment for some cases; in particular, chronically infected patients. We previously demonstrated the antiviral effects of ribavirin [22] and IFN-α [23], the antiviral effect of the immunosuppressant MPA [22], and the proviral effect of the immunosuppressant rapamycin [24] in liver cell lines infected with HEV, suggesting that such drugs are relevant for managing HEV-associated neurological disorders as well. Demonstration of these hypotheses came from experiments in which we studied the effects of clinically relevant concentrations of these medications in neuroglial U87 cells, employing the subgenomic HEV replication model U87-HEV-Luc cells and infectious

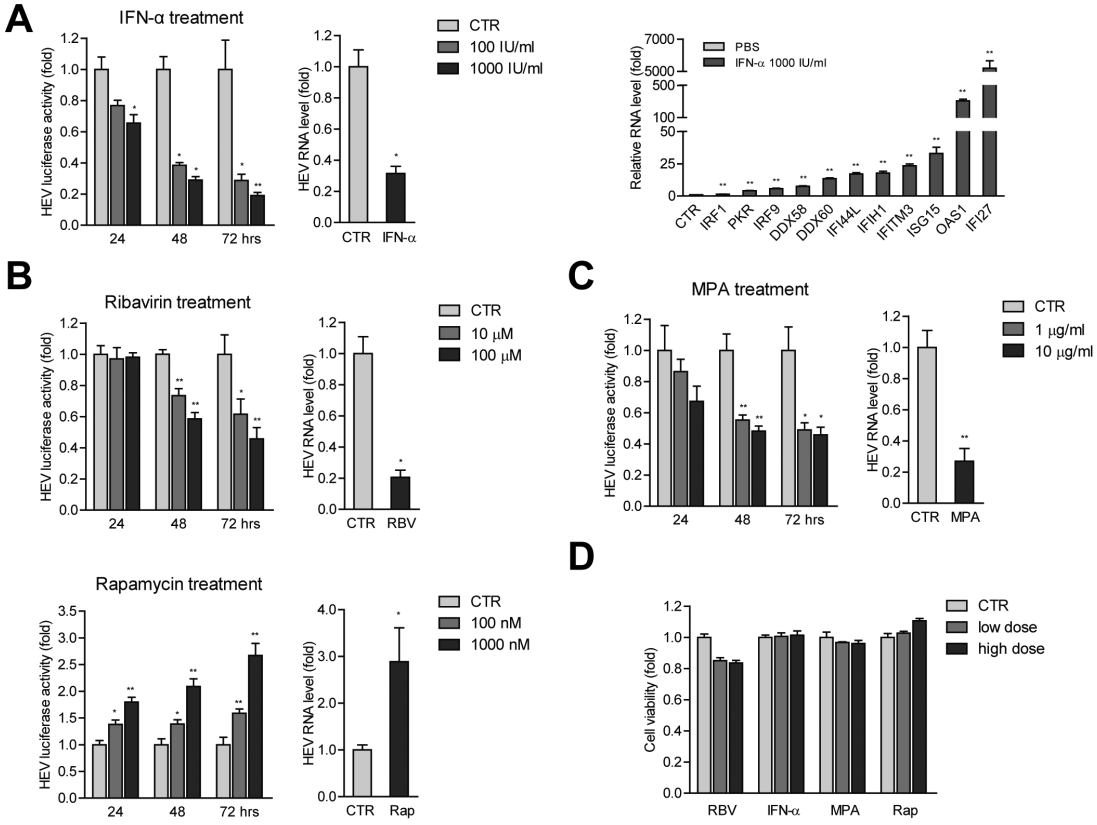


**Figure 3.** Effects of relevant drugs on HEV infection in neuroglial cells. U87-HEV-Luc cells were treated with 100 (low)/1000 (high) IU/mL IFN-α (*A*), 10 (low)/100 (high) μM RBV (*B*), 1 (low)/10 (high) μg/mL MPA (*C*), and 100 (low)/1000 (high) nM rapamycin (*D*) for 24, 48, and 72 hours, and in U87-HEV cells for 48 hours. HEV luciferase activity and cellular RNA level were analyzed respectively. *A*, 1000 IU/mL IFN-α significantly induced a subset of interferon-stimulated genes. *D*, MTT assay shows no clear cytotoxicity of these drugs after 3 days of treatment in U87 cells. Abbreviations: HEV, hepatitis E virus; Luc, luciferase; IFN-α, interferon-α; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RBV, ribavirin.

U87-HEV cells. IFN-α exerted significant antiviral effects in U87-HEV-Luc cells, and a high dose (1000 IU/mL) potently reduced the level of cellular HEV RNA level in U87-HEV cells (Figure 3A). A subset of interferon-stimulated genes was significantly induced following treatment with 1000 IU/mL IFN-α, indicating that canonical interferon signaling mediates these anti-HEV effects in neuroglial cells (Figure 3A). Analogously, anti-HEV effects of ribavirin in these neuroglial cell cultures were observed, supporting the use of this compound for treating HEV infection complicated by neurological manifestations (Figure 3B). Interestingly, the immunosuppressive medication MPA (often used to suppress organ rejection) counteracted HEV infection, but a proviral effect of the immunosuppressant rapamycin was observed (Figure 3C). Results for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay showed no clear cytotoxicity in U87 cells treated with these drugs (Figure 3D). Thus, our data indicate that the use of MPA may be indicated as an immunosuppressive regimen for counteracting rejection in organ transplant recipients at risk for HEV infection; ribavirin appears to be a rational choice for combating an established HEV infection complicated by neurological manifestations.

#### Primary Mouse Neurons Efficiently Support HEV Infection

Further support for the results obtained with respect to the neurotropism of HEV came from experiments in which we infected primary isolated mouse neurons with HEV. Primary cerebellar and hippocampal neurons cultured from mouse embryos were incubated with cell culture–derived HEV particles (Figure 4A). HEV replicated efficiently in both neuronal cultures, as judged by cellular levels of HEV RNA. Compared with the canonical model for HEV replication (Huh7 cells), HEV infection in cerebellar cultures was 7.09 ± 4.51-fold and 11.16 ± 18.09-fold (mean ± standard error of the mean [SEM], n = 5; $P < .05$) more efficient at 48 and 72 hours, respectively. In hippocampal cultures, when compared with the Huh7 benchmark, HEV infection was 6.03 ± 3.92-fold and 4.91 ± 10.14-fold (mean ± SEM, n = 5; $P < .05$) more efficient at 48 and 72 hours, respectively (Figure 4B). We next examined whether these mouse neurons can produce infectious HEV particles. Full-length HEV genome RNA was electroporated in cerebellar cells with detection of the viral ORF2 protein, using immunofluorescent staining for this protein at 72 hours (Figure 4C). HEV RNA was present both in the cells and in the supernatant of these cultures. Inoculation of naive cells with the supernatant resulted in HEV infection (Figure 4C). Similar results were observed in hippocampal cells (Figure 4D). Together, these data demonstrate that both primary cerebellar and hippocampal cultures are capable of the production of infectious HEV particles in vitro and show that primary neurons are more susceptible to HEV infection compared with the canonical model for HEV infectivity (in human HuH7 liver cells).

#### HEV Infects the Brains of Mouse and Monkey

To prove the hypothesis that the central nervous system is an important target for infection by HEV would be to demonstrate such infection in vivo. Thus, mice were intravenously inoculated with fecal samples contacting HEV particles via tail vein injection. As expected, high titers of HEV genomic RNA were detected in serum samples and mouse liver at 14 days postinoculation. Importantly, in parallel, the measurement of HEV levels in the mouse brain revealed a high level of infection in this organ (Figure 5A). Consistently, in the cerebellum of the mouse brain tissue, viral protein ORF2 was expressed in the granule layer by immunohistochemistry analysis (Figure 5B). Analogously, in a monkey (rhesus macaque) model for persistent HEV infection [18] in an animal dying unexpectedly after over 600 days of chronic infection, viral RNA was detected in the serum sample and liver and brain tissues of this monkey (Figure 5A). Again, viral protein ORF2 was expressed in the granule layer of cerebellum (Figure 5B). These data show that HEV infects the brains of mouse and monkey following experimental inoculation of HEV.

#### Human Patients with HEV-Associated Neurological Disorders Shed Virus into Cerebrospinal Fluid

Our data that HEV can infect brain tissue suggest that the neurological manifestations associated with HEV do not necessarily relate to autoimmunity but may relate to direct HEV infection. In order to test this hypothesis directly, cerebrospinal fluid (CSF) from patients with HEV-associated neurological disorders was collected and tested for the presence of HEV RNA by polymerase chain reaction, employing patients with bona fide acute or chronic HEV infection, as evident from testing the serum for HEV antibodies IgM, IgG, and HEV RNA. We thus analyzed 3 cohorts of patients (some patients have been described in our previous publications) with HEV-associated neurological disorders [9, 25–27]. Over half of the patients were positive for HEV RNA in the serum samples, indicating an active infection in these patients (Table 1). Among these patients, 42.9% (6 out of 14) shed virus into the CSF. These results suggest that HEV may directly infect the peripheral nerve roots or central nervous system and/or peripheral nerve roots in humans.

## DISCUSSION

The data in the present study show that HEV can efficiently infect neural cell types and tissues at efficacies that exceed that of canonical liver-derived models. Thus, the aspect of HEV appears also neurotropic, substantially challenging the current dogma. The data appear clinically relevant in that HEV can be recovered from the CSF of patients with acute HEV infection complicated by neurological manifestations. As HEV infection is often undiagnosed, it has been suggested that HEV may be involved in at least some cases of "idiopathic" neurological disease. Taken together, these findings challenge the conventional

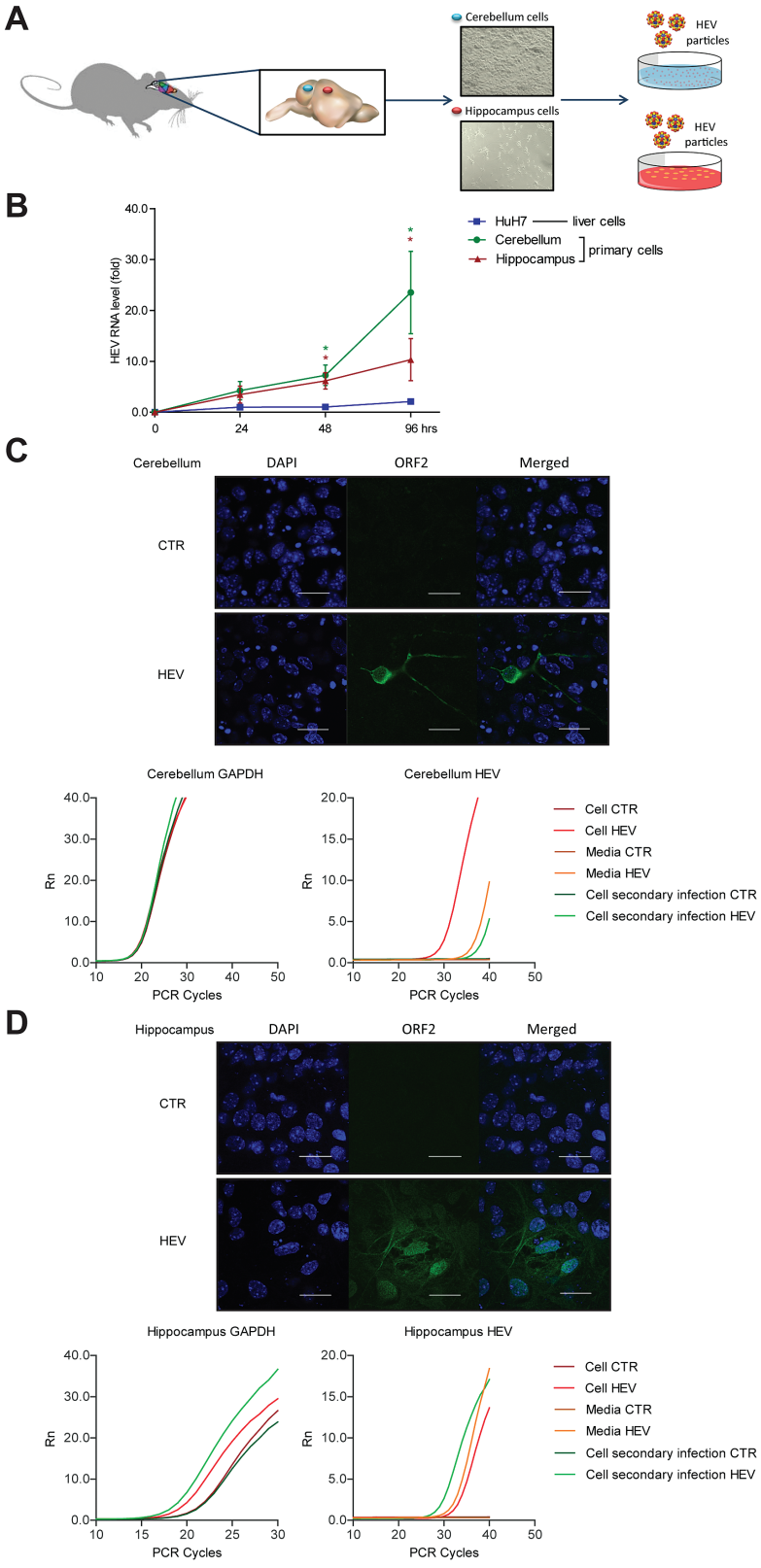


**Figure 4.** HEV infects primary mouse neurons. *A*, Cerebellar and hippocampal neurons were cultured and inoculated with HEV particles for 96 hours. *B*, Results of qRT-PCR show a time-dependent increase of HEV replication in both neurons (mean ± SEM, n = 5; $P < .05$). Immunofluorescence staining of viral protein ORF2 (green) was detected 3 days postelectroporation of HEV RNA in primary cerebellar (*C*) and hippocampal (*D*) neurons. DAPI was applied to visualize nuclei (scale bar, 50 µm.) Results from qRT-PCR indicate that primary cerebellar (*C*) and hippocampal (*D*) neurons are permissive for secondary infection with inoculation of viral particles for 96 hours. GAPDH served as a reference gene. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEV, hepatitis E virus; qRT-PCR, quantitative real-time polymerase chain reaction.

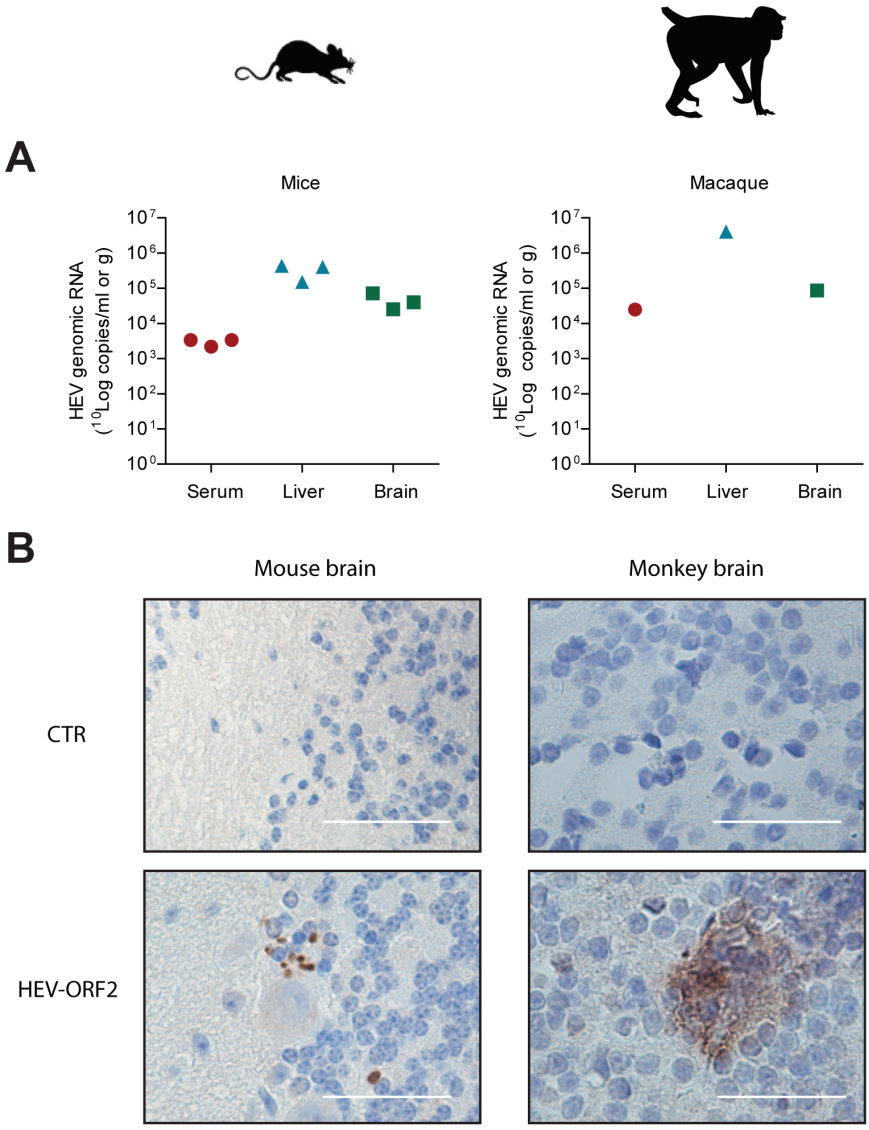


**Figure 5.** HEV infection in the brains of mouse and monkey. *A*, Viral RNA has been observed in serum samples, liver tissues, and brain tissues of infected mice and monkeys. *B*, Viral HEV ORF2 protein is observed in brain tissues of infected mice and chronically infected monkeys by immunohistochemical assay. Brain tissues of uninfected mice served as a control. For monkey brain tissues, staining without inoculating viral antibody samples served as a control. The brain cerebellum tissues were observed, and HEV positivity presented in the cerebellum granule cell layer. Hematoxylin was applied to visualize nuclei, and DAB solution was used to visualize cytoplasm (scale bar: 50 μm). Abbreviations: DAB, 3,3′-diaminobenzidine; HEV, hepatitis E virus.

wisdom that HEV is primarily a hepatotropic virus and helps our understanding of the potential pathogenic mechanism of HEV-associated neurological injury.

Recent studies show that HEV demonstrates tropism to a range of tissues of nonhepatic origin. Although liver cells are commonly used for modeling HEV infection in vitro, a human lung epithelial cell line A549 [28] and some ectodermally derived cell lines [29, 30] have been reported to support HEV replication in cell culture. We have recently demonstrated that mouse embryonic fibroblasts are also susceptible to HEV infection [31]. In nude mice experimentally infected with HEV, viral RNA was detected in the liver, spleen, kidney, jejunum, ileum, and colon [32]. In the tree shrew (*Tupaia belangeri chinensis*), HEV capsid protein was expressed in the liver, spleen, and kidney [33]. In specific pathogen-free rabbits infected with rabbit HEV strain, both positive- and negative-stranded viral RNA and antigen expression were detected in the liver, brain, stomach, duodenum, and kidney, suggesting its active replication in these tissues [34]. In Mongolian gerbils infected with a genotype 4 strain of swine HEV, the virus could be detected in the liver, kidney, spleen, small intestine [35], and brain [36]. In rhesus macaques infected with human [37] or swine [18] HEV strains, viral infection in the kidney and dissemination of viruses into the urine were observed. Thus, our data add to the gathering momentum of thought that HEV is not an exclusively hepatotropic virus. Furthermore, our studies also demonstrate

**Table 1. Characteristics of Patients With HEV-Associated Neurological Disorders**

| Patient | Country | Gender | Age | Immunocompetence | IgG HEV | IgM HEV | PCR Serum | PCR Feces | PCR CSF | Type ofNeurologic Disorder |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | NL | M | 55 | + | + | + | – | NT | – | GBS |
| 2 | NL | F | 60 | + | + | + | – | NT | – | GBS |
| 3 | NL | M | 67 | + | + | + | – | NT | – | GBS |
| 4 | NL | M | 44 | + | + | + | – | NT | – | GBS |
| 5 | UK | M | 58 | + | + | + | + | NT | – | NA |
| 6 | UK | M | 52 | + | + | + | + | NT | – | NA |
| 7 | UK | M | 49 | – | + | + | + | + | + | Peripheral neuropathy |
| 8 | UK | M | 34 | + | + | + | + | NT | – | Peripheral neuropathy |
| 9 | UK | M | 42 | + | + | + | + | NT | - | GBS |
| 10 | FR | M | 74 | + | + | + | + | + | + | GBS |
| 11 | FR | M | 33 | + | - | + | + | + | - | GBS |
| 12 | FR | M | 59 | – | + | + | + | + | + | Ataxia |
| 13 | FR | M | 32 | – | + | + | + | + | + | Cognitive dysfunction |
| 14 | FR | M | 44 | – | + | + | + | + | + | Polyradiculoneuropathy |
| 15 | FR | M | 66 | – | + | + | + | + | - | GBS |
| 16 | FR | F | 33 | + | - | + | + | + | - | Meningitis |
| 17 | FR | M | 56 | + | + | + | + | + | - | NA |
| 18 | FR | M | 57 | – | + | + | + | NT | + | GBS |

Abbreviations: +, positive; –, negative; CSF, cerebrospinal fluid; FR, France; GBS, Guillain-Barré syndrome; HEV, hepatitis E virus; Ig, immunoglobulin; NL, The Netherlands; NT, not tested; PCR, polymerase chain reaction; UK, United Kingdom; NA, neuralgic amyotrophy.

the clinical relevance of extra-hepatic infection. Finally, our studies suggest that the use of MPA for preventing rejection in organ transplant recipients might be accompanied by a lower incidence of HEV-associated neurological manifestations, and that ribavirin is suitable for managing such complications.

The strain of human HEV used in the current study deserves comment. Kernow C1p6 was isolated from a United Kingdom patient with HIV-1 chronically infected with HEV genotype 3 (case 7, Table 1) [16, 38]. The patient had neurological symptoms in the legs, which resolved with viral clearance following therapy with ribavirin/Peg-interferon [39]. The strain of HEV isolated from the patient was shown to have a novel virus–host recombination [16]. Thus, this strain of HEV was unusual and had the documented ability to produce clinically relevant neurological injury in humans. For the in vivo mouse and monkey experiments, however, we used genotype 4 HEV of porcine origin in which we previously demonstrated infectivity in both species. This suggests that multiple HEV stains share high neurotropism. The clinical observation that HEV-associated neurological injury is found worldwide and caused by HEV of differing genotypes would support this notion [4, 13].

In conclusion, HEV is neurotropic in vitro and in vivo in mice, monkeys, and possibly humans. These findings challenge the notion that HEV is primarily a hepatotropic virus and suggest that HEV infection is a possibility in idiopathic neurological disease.

## Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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