

Hepatitis C Virus Core Protein Induces Neuroimmune Activation and Potentiates Human Immunodeficiency Virus-1 Neurotoxicity

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Abstract

Background: Hepatitis C virus (HCV) genomes and proteins are present in human brain tissues although the impact of HIV/HCV co-infection on neuropathogenesis remains unclear. Herein, we investigate HCV infectivity and effects on neuronal survival and neuroinflammation in conjunction with HIV infection.

Methodology: Human microglia, astrocyte and neuron cultures were infected with cell culture-derived HCV or exposed to HCV core protein with or without HIV-1 infection or HIV-1 Viral Protein R (Vpr) exposure. Host immune gene expression and cell viability were measured. Patch-clamp studies of human neurons were performed in the presence or absence of HCV core protein. Neurobehavioral performance and neuropathology were examined in HIV-1 Vpr-transgenic mice in which stereotaxic intrastriatal implants of HCV core protein were performed.

Principal Findings: HCV-encoded RNA as well as HCV core and non-structural 3 (NS3) proteins were detectable in human microglia and astrocytes infected with HCV. HCV core protein exposure induced expression of pro-inflammatory cytokines including interleukin-1 β , interleukin-6 and tumor necrosis factor- α in microglia ($p < 0.05$) but not in astrocytes while increased chemokine (e.g. CXCL10 and interleukin-8) expression was observed in both microglia and astrocytes ($p < 0.05$). HCV core protein modulated neuronal membrane currents and reduced both β -III-tubulin and lipidated LC3-II expression ($p < 0.05$). Neurons exposed to supernatants from HCV core-activated microglia exhibited reduced β -III-tubulin expression ($p < 0.05$). HCV core protein neurotoxicity and interleukin-6 induction were potentiated by HIV-1 Vpr protein ($p < 0.05$). HIV-1 Vpr transgenic mice implanted with HCV core protein showed gliosis, reduced neuronal counts together with diminished LC3 immunoreactivity. HCV core-implanted animals displayed neurobehavioral deficits at days 7 and 14 post-implantation ($p < 0.05$).

Conclusions: HCV core protein exposure caused neuronal injury through suppression of neuronal autophagy in addition to neuroimmune activation. The additive neurotoxic effects of HCV- and HIV-encoded proteins highlight extrahepatic mechanisms by which HCV infection worsens the disease course of HIV infection.

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Introduction

Hepatitis C virus (HCV) infects approximately 180 million people worldwide [1] while 30% of individuals infected with human immunodeficiency virus type 1 (HIV-1) are co-infected with HCV due to similar routes of transmission [2]. Epidemiological studies suggest that HCV co-infection is associated with accelerated HIV disease progression, worsened clinical outcomes and increased mortality [2,3]. HIV/HCV co-infected patients have higher HCV levels and lower likelihood of spontaneous HCV

clearance, together with faster progression to liver cirrhosis [1,2]. HCV is a member of the *Flaviviridae* family, which consists of several neurotropic viruses including St Louis encephalitis virus, Dengue and West Nile virus [4,5]. HCV mono-infected and HIV/HCV co-infected individuals display neuropsychological deficits indicative of impaired cognition [5,6,7]. Magnetic resonance spectroscopy studies report alterations in cerebral metabolites among HCV-infected individuals correlated with neurocognitive impairment, including suppression of the neuronal marker, *N*-acetyl aspartate [8].

HCV transcripts and proteins have also been detected in brains from HIV/HCV co-infected patients, indicating that HCV is neuroinvasive [9,10,11]. The negative strand of HCV RNA, a viral replication intermediate, has been detected in the brain and cerebrospinal fluid [10,12]. Several studies showed that HCV RNA sequences isolated from the central nervous system (CNS) were closely related to those found in peripheral blood mononuclear cells (PBMC) but were phylogenetically different from serum- or liver-derived sequences [10,11,12,13], leading to the postulation that HCV enters the brain through the ‘Trojan horse’ mechanism similar to HIV-1 [4]. However, the mechanisms by which HCV exerts neuropathogenic effects remain unknown and the understanding of combined neuropathogenesis of HCV and HIV-1 is limited.

HIV-1 exerts its direct neurotoxic effects through several secreted proteins including gp120, Tat, Nef and Vpr [14]. HIV-1 Viral Protein R (Vpr) triggers neuronal apoptosis and transgenic mice expressing Vpr in brain monocytoic cells display neuronal injury as well as neurobehavioral deficits [15]. Resident brain macrophages or microglia infected with HIV-1 or exposed to HIV-encoded proteins secrete proinflammatory cytokines e.g. interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) and chemokines e.g. CXCL10 and CXCL12, which cause neuronal death and pathogenic immune responses in the brain [14]. Suppression of neuronal autophagy by retroviral infections has been highlighted as a putative mechanism leading to neuronal cell death and neurodegeneration. The increased level of p62 transcript and the reduction of light chain 3 type II (LC3-II), markers of autophagy inhibition, were detected in brain tissues of patients with HIV-associated dementia [16,17].

Recently, HCV proteins including core, non-structural protein 3 (NS3) and NS5A were detected in macrophages/microglia and astrocytes but not in neurons nor oligodendrocytes of patients with HIV/HCV co-infection [9,18]. Nevertheless, the underlying mechanisms by which HCV infects macrophages/microglia or astrocytes remain unclear. The HCV JFH1 clone, derived from a Japanese individual with fulminant hepatitis, replicates and produces infectious virus in Huh 7.5 hepatocytes [19,20]. The entry of HCV into cells involves several membrane receptors including the scavenger receptor class B type I (SR-BI), the tetraspanin CD81, and the tight-junction molecules, claudin-1 and occludin [21]. Given these receptors are expressed by microglia and astrocytes in human and mouse brains [22,23,24,25,26], these cells are potentially permissive to infection by HCV.

The most abundantly expressed HCV protein, core, is released and soluble in blood as a part of HCV morphogenesis [27] and serum levels of HCV core protein ranges from pg/ml to ng/ml [28]. HCV core protein is cytotoxic to hepatocytes through death-receptor mediated apoptosis [29,30]; conversely, other studies have demonstrated that HCV core protein has anti-apoptotic effects in hepatocytes [29]. Recombinant HCV core protein fused to β -galactosidase induces the production of inflammatory cytokines, e.g. interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in monocytes [31] as well as the chemokine CXCL8 in monocytes [31] and lung fibroblasts [32]. Additionally, HCV core protein has been implicated in suppressing differentiation, proliferation and function of T cells, dendritic cells and macrophages [33]. Herein, we investigated cell culture-derived HCV (HCV^{cc}) infection of astrocytes and microglia as well as the effects of HCV core protein on neurons, astrocytes and microglia in the presence or absence of HIV-1 Vpr protein. The present results demonstrated that HCV core protein caused immune activation of glial cells and was neurotoxic in an additive manner with the HIV-1 Vpr protein.

Materials and Methods

Reagents

Recombinant HCV core protein fused to β -galactosidase (Gal-core, genotype 1b) and β -galactosidase (Gal) were obtained from Virogen (Watertown, MA, USA). The full-length recombinant HIV-1 Vpr protein was prepared as previously described [15].

Standard Protocol Approvals, informed consents and ethic statements

The use of autopsied brain tissues and blood were approved under the protocol number 2291 by the University of Alberta Human Research Ethics Board (Biomedical) and written informed consents were signed before or at the collection time. Human fetal tissues were obtained from 15–19 week aborted fetuses with written consent approved under the protocol 1420 by the University of Alberta Human Research Ethics Board (Biomedical). All animals were housed and monitored on a regular schedule immediately following surgical implantation and behavioral tests according to the Center For Animal Care and Control Guidelines. This study was approved under the protocol number 452 by the University of Alberta Animal Care & Use Committee for Health Sciences.

Cell culture

Human fetal neurons were prepared and cultured in the presence of cytosine arabinoside [34] while human fetal astrocytes (HFAs) and human fetal microglia (HF μ ϕ) were grown in the absence of cytosine arabinoside. Microglia were collected from supernatants at 7 to 10 days after isolation while astrocytes were split and used from the fifth to the tenth passage [35].

Human tissue samples

Human white matter, cortex, basal ganglion and spleen were collected at autopsy and stored at -80°C . Non HIV-infected controls were comprised of other neurological diseases including Alzheimer’s disease, multiple sclerosis and stroke [16,36].

Hepatitis C viral preparation and infection

J6/JFH plasmid is a gift from Dr. Charles Rice (The Rockefeller University, New York) [19]. The HCV-J6/JFH infectious virus was prepared in Huh7.5 cells, as previously described [37]. HFAs and HF μ ϕ were infected with supernatant containing HCV^{cc} overnight before replacing with fresh media.

HIV-1 cultures

Human peripheral blood mononuclear cells (PBMCs) were purified from healthy subjects’ blood with Histopaque (Sigma, Oakville, Ontario, CA) [38]. Peripheral blood lymphocytes (PBLs) were isolated from PBMCs and maintained in RPMI 1640 medium with 15% FBS with PHA-P stimulation for 3 days, followed by hIL-2 stimulation. At day 3 post-isolation, PBLs were infected with a neurotropic HIV-1 strain (HIV-1 SF162) and supernatants were collected at day 7 and 10 post-infection.

Immunofluorescence

HFNs, HFAs and HF μ ϕ were plated on poly-L-ornithine (Sigma) coated coverslips or Lab-Tex chambered coverglass (Nunc, Rochester, NY). After 72 hr exposure to HCV^{cc}, HF μ ϕ and HFAs were fixed, permeabilized and immuno-labelled with mouse anti-HCV core (clone 11-B3, GeneTex, Irvine, CA) or mouse anti-HCV NS3 (ab65407, Abcam, Cambridge, MA) and rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1,

Wako Chemicals, Neuss, Germany) in HF $\mu\phi$ or rabbit anti-gial fibrillary acidic protein (GFAP, DAKO, Denmark) for HFAs. After 48 hr of exposure to either Gal or Gal-core proteins, HFNs were fixed, permeabilized and stained with antibodies to microtubule-associated protein-2 (MAP-2, Sigma) and LC3 (Novus Biological, Littleton, CO). Paraffin-embedded sections (6 μm) of mice brain tissue were deparaffinized and hydrated followed by antigen retrieval with boiled 0.01 M citrate buffer, pH 6.0, for 10 min. Brain sections were immunostained with anti-GFAP, anti-Iba-1 or anti-LC3 antibodies. After primary antibody incubation, cells and tissue sections were exposed to species-specific secondary antibodies labeled with Alexa Fluor 488 (Invitrogen, Eugene, OR) and/or Cy3 (Jackson ImmunoResearch, West Grove, PA) [15]. Images were collected using an inverted LSM510 Meta confocal microscope.

Real-time RT-PCR

HF $\mu\phi$ and HFAs in 6-well plates were exposed to either Gal or Gal-core proteins for 12 hr or as stated. RNA was extracted with TRIzol (Invitrogen) and purified by RNeasy mini columns (Qiagen, Mississauga, Ontario, Canada). First-strand complementary DNA (cDNA) was synthesized from 1 μg of total RNA mixed with random hexamer primers (Roche) and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's recommended protocols. Semi-quantitative real-time PCR was performed using Bio-Rad iQ SYBR green supermix (Bio-Rad, Hercules, CA) on Bio-Rad iQ5. All PCR primers are shown in Table S1. Data were normalized to GAPDH mRNA levels and expressed as relative fold increases compared with controls \pm SEM. For the positive-strand or negative-strand assays, antisense HCV-114R (5'-GAGGCTGCACGACACTCATACT-3') and sense primer HCV-20F (5'-CGACACTCCACCATGAATCACT-3') were used for cDNA synthesis [39]. Platinum[®] Quantitative PCR SuperMix-UDG and TaqMan probe HCV-P43 (5'-FAM-CCCTGTGAGGAAGTACTGTCTTCAC-GCAGA-T-AMRA3') were used for strand-specific real-time PCR.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants from Gal- or Gal-core treated HFAs were collected at 6, 12, 24 and 48 hr after treatments and amount of CXCL8 was measured using a human CXCL8 ELISA Ready-SET-Go (eBioscience, San Diego, CA).

Electrophysiological studies

Whole-cell patch-clamp recordings from HFNs were performed under voltage-clamp conditions [15].

Western blotting

6 hr after Gal or Gal-core exposure, HFNs were lysed in lysis buffer [20 mM Tris, 1% NP-40, 50 mM NaCl, Protease Inhibitor cocktail set III (1:1000, Calbiochem, La Jolla, CA)]. Crude protein lysates were separated by 16% SDS-PAGE and the membrane were blotted for LC3 and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized using HRP-conjugated secondary antibodies and quantitated using Quantity One[™] (Bio-Rad) imaging software.

In vitro cytotoxicity assay

HFNs or HFAs were plated at 5×10^4 and 2×10^4 cells in 96-well flat bottom plates and exposed to either Gal or Gal-core protein with and without Vpr. After 48 h of exposure, cells were fixed, permeabilized and stained with anti- β -tubulin antibodies (Sigma). Neuronal injury was quantified by β -III-tubulin immunoreactivity

using Odyssey[®] Imager (LI-COR, Lincoln, NE). Diminished β -III-tubulin immunoreactivity in neurons is an indicative of reduced cellular viability including neurite retraction and impaired survival [15].

Neurobehavioral studies

Vpr transgenic mice express Vpr under the control of the *c-fms* (M-CSF receptor) promoter, driving transgene expression chiefly in monocytoid cells [40]. *In vivo* neurological injury was assessed using the Ungerstedt model [41,42] in Vpr transgenic mice. Female animals (4 weeks, n = 6) were placed in a stereotaxic frame under Ketamine/Xylazine anesthesia. The coordinates of implantation were 3.5 mm posterior, 2.5 mm lateral and 3 mm deep relative to the bregma. 2 μl of Gal or Gal-core was stereotactically implanted into the right striatum of mice. Ipsiversive rotation number was counted over 10 min after intraperitoneal injection of amphetamine (1 mg/kg) on days 4, 7 and 14 following intra-striatal injection.

Neuronal count

After deparaffinization and hydration, mice brain sections were stained with 0.1% cresyl violet solution. Neurons were counted at 400 \times magnification in 4 separate non-overlapping fields. The number of neurons in the ipsilateral (right) side was normalized to those in the non-implanted contralateral (left) side [41].

Statistical analysis

Data were tested by one-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests or a two-tailed unpaired Student's *t* test. The level of significance was defined as $p < 0.05$.

Results

HCV RNA quantification in infected brain and protein detection in infected microglia and astrocytes

HCV-encoded proteins as well as positive and negative RNA strands have been reported in different brain regions and identified specifically in microglia/macrophages and astrocytes [9,10,18]. To verify HCV infection of the brain occurred, autopsied brain tissues were investigated in HIV/AIDS persons without HCV infection (n = 3) and an individual with HIV/AIDS and HCV infection at death. The latter individual was a 46-year old female patient with HIV-associated dementia (HIV Dementia Scale score = 6; cranial MRI: cerebral atrophy with increased diffuse white matter signal on T2-weighted images), CD4⁺ T cell level = 105 cells/ μl and detectable HIV and HCV viremia. HCV RNA positive- and negative-strand copy numbers were determined in brain samples from all patients by a strand-specific reverse transcriptase with subsequent PCR amplification and interpreted from a standard curve. Positive-strand HCV RNA was detectable in white matter, cortex and basal ganglia from the HIV/HCV co-infected patient at 10^2 viral copies/ μg RNA, which were 10-fold lower than viral copy numbers in the matched spleen (Figure 1A). In contrast, negative-strand RNA, an indication of viral replication, was found only in white matter and basal ganglia but not in cortex (Figure 1B), as previously reported [10]. HCV-encoded RNA was not detected in control groups and HIV/AIDS persons without HCV viremia. To determine if human glia were permissive to HCV infection, primary cultures of HFAs and HF $\mu\phi$ were exposed to supernatants from J6/JFH1 transfected Huh 7.5 cells containing infectious virus (10^3 FFU/ml, multiplicity of infection or m.o.i. = 1). At day 3 post-infection, HCV positive-strand RNA were detected in all HCV^{cc}-infected astrocytes at 10^2 to 10^3 viral copies/ μg RNA while negative strands of HCV RNA were evident in only 75% of infected astrocyte cultures (Figure 1C). Similarly, HCV positive- and

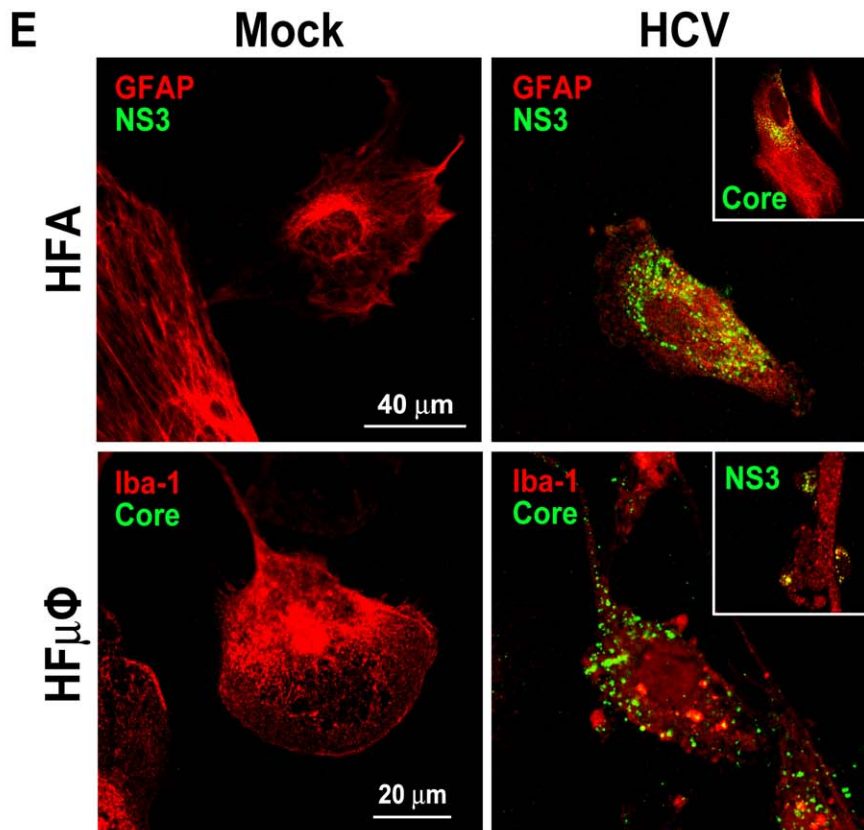
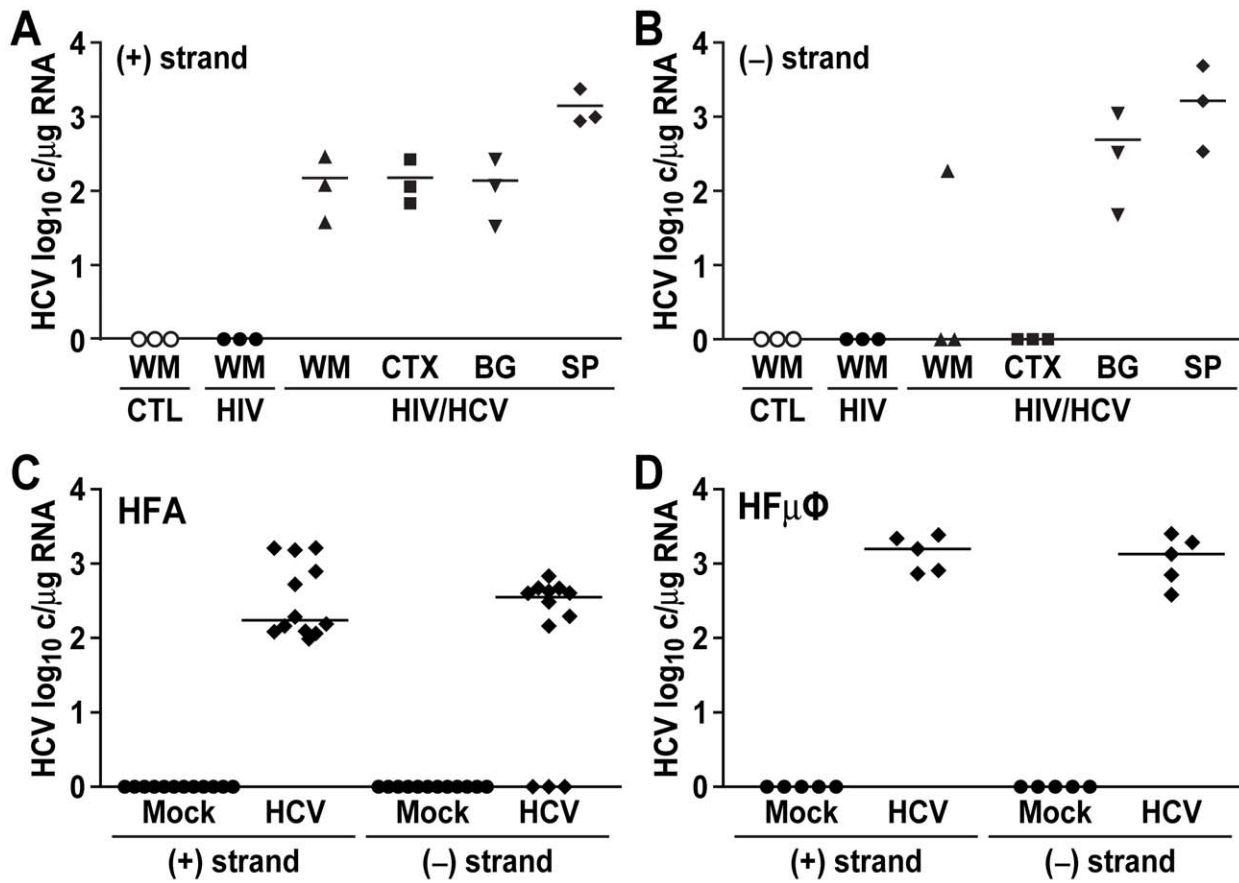


Figure 1. HCV RNA and proteins were detectable in different brain regions and infected glia cells. (A and B) Quantification of HCV-encoded positive- (A) and negative-strand (B) RNA of three brain regions from an HIV/HCV co-infected patient displayed different abundance of RNA in white matter (WM), cortex (CTX) and basal ganglion (BG) compared to spleen (SP). Negative-strand RNA of HCV was only detectable in basal ganglion, spleen and one sample from white matter. HCV RNA was not detected in white matter tissues from control (CTL) and HIV-1 mono-infected (HIV) individuals. (C and D) Primary cultures of human fetal astrocytes (HFA) and human fetal microglia (HF $\mu\phi$) were exposed to supernatant containing HCV^{cc}. The levels of HCV positive- and negative-strands were measured by real time RT-PCR with strand specific primers at day 3 post-infection (HFA, n = 12; HF $\mu\phi$, n = 5). (E) Immunofluorescence of HCV core and NS3 proteins in J6/JFH1-infected primary HFA and HF $\mu\phi$ showed co-labeling of HCV-encoded proteins (green) with GFAP-immunoreactive astrocytes (red) or Iba-1-immunoreactive microglia (red) in HCV-infected cells at day 3 post-infection but not in mock-infected cells. Insets showed core and NS3-immunopositive HFA and HF $\mu\phi$. (original magnification 630x) doi:10.1371/journal.pone.0012856.g001

negative strands were measurable at 10^3 viral copies/ μg RNA in HCV^{cc}-infected microglia (Figure 1D). Both HCV core and NS3 proteins were detectable in cytoplasm of GFAP (astrocytes) and Iba-1 (microglia) immunopositive cells compared to mock infection (Figure 1E and Figure S1). These studies highlighted the capacity of HCV to infect the select neural cells, particularly glial cells.

HCV core protein induces differential inflammatory responses in microglia and astrocytes

Exposure of HCV core protein to human blood monocytes activated the expression and release of pro-inflammatory cytokines [31]. To investigate the neuroinflammatory effects of HCV core on microglia/macrophages and astrocytes, HF $\mu\phi$ and HFAs were exposed to Gal-core or Gal (10 nM) for 12 hours and transcript levels of cytokines and chemokines were measured. HCV core induced expression of the pro-inflammatory cytokine *IL-1 β* by 80 fold in HF $\mu\phi$ (Figure 2A, $p < 0.05$) while there was only a trend of increased *IL-1 β* expression in HFA (Figure 2B). Likewise, the expression of *IL-6* and *TNF- α* transcripts was significantly higher in HCV core-exposed HF $\mu\phi$ (Figure 2C and 2E, $p < 0.05$) but not in HCV core-exposed HFAs (Figure 2D and 2F). Of interest, HCV core protein exposure did not activate the expression of interferon-alpha (*IFN α*) transcripts in both glial cell types (data not shown).

In addition to cytokine induction, HCV core protein has been reported to promote expression of chemokines, e.g. CXCL10, CXCL8 and CCL5, in monocytes and other cell types including hepatocytes and fibroblasts [31,32,43,44]. After HCV core exposure (12 hr), HF $\mu\phi$ expressed elevated levels of *CXCL10* and *CXCL8* transcripts (Figure 3A and 3C, $p < 0.05$). Similarly, transcript levels of *CXCL10* and *CXCL8* in HFAs were increased at 6 and 12 hr after HCV core protein application (Figure 3B and 3D, $p < 0.05$). There was no difference in transcript levels for *CCL5* and *CXCL12* in HFAs (data not shown). The supernatant levels of CXCL8 protein derived from exposed HFAs increased with time and reached a plateau by 24 hr post-exposure (Figure 3E, $p < 0.05$).

Direct or indirect exposure of HCV core protein results in neuronal injury

Given HCV core protein has been detected in white and gray matters [9], its cytotoxic effects on HFAs and HFNs were investigated. HCV core protein exposure was not toxic to astrocytes (Figure 4A) while the highest concentration of HCV core protein (100 nM) was cytotoxic to neurons, indicated by the loss of β -III-tubulin expression compared to controls (Figure 4B, $p < 0.05$). HCV core protein (100 nM) also reduced neuronal survival compared to controls as indicated by DAPI staining (data not shown). To determine if HCV core protein exerted its neurotoxic effects indirectly through induction of neurotoxic factors secreted by microglia or astrocytes, supernatants from HFAs or HF $\mu\phi$ exposed to Gal-core (10 nM) were collected at 24 and 48 hour post-exposure and then applied to HFNs. Supernatants from HCV core-exposed HFAs showed no neurotoxicity (Figure 4C) while supernatants from HCV core-exposed HF $\mu\phi$

caused neuronal injury (Figure 4D, $p < 0.05$). These results suggested that HCV core protein was toxic to neurons through both direct and indirect mechanisms.

HCV core protein alters neuronal membrane response and suppresses neuronal autophagy

To define the mechanisms that underlie the direct neurotoxic effects of HCV core protein, the actions of HCV core protein were examined on neuronal membrane activity. Under voltage-clamp conditions in purified HFNs, HCV Gal-core (100 nM) reduced whole-cell outward currents in the voltage range from -30 to $+30$ mV relative to Gal exposure. This effect was reversible after the wash-out period (Figure 5A), indicating that HCV core protein was directly active at the neuronal membrane. Recently, neuronal autophagy suppression has been recognized as a key mechanism in several neurodegenerative disorders [45] and participates in the neurovirulence of several viruses [16,46]. The role of autophagy in HCV core-mediated neurotoxicity was investigated by measuring the levels of light chain 3 (LC3), a marker of autophagosome formation. HFNs exposed to Gal protein displayed healthy MAP-2 immunopositive cells with long and complex neurites (Figure 5Bi); in contrast, HCV core-exposed neurons exhibited fewer MAP-2 immunopositive cells with shorter processes and atrophied soma (Figure 5Bii). Additionally, LC3 was highly expressed in Gal-exposed neurons (Figure 5Bi, inset). Western blot analysis of neuronal lysates showed a reduction of LC3-II expression in HCV core-exposed HFNs compared with control HFNs (Figure 5C, $p < 0.05$), indicating that HCV core protein inhibited LC3-I to LC3-II conversion. These data implied that HCV core protein could act at the neuronal membrane, contributing to neuronal death by modulating neuronal autophagy.

HIV-1 infection and Vpr protein potentiate the neuroimmune activation and neurotoxic effects of HCV core protein

Brain mononuclear cells in HIV-infected persons are known to produce several proinflammatory cytokines such as *IL-1 β* and *TNF- α* [14]. To determine if HCV core protein exacerbated cytokine and chemokine expression in HIV-1-infected microglia, HCV core protein was applied to HF $\mu\phi$ infected by HIV-1 SF162, a neurotropic HIV-1 strain. HIV-1 infection in microglia was confirmed by detecting HIV-1 *pol* expression (data not shown). As expected, HIV-1 infection induced expression of *IL-1 β* , *IL-6*, *TNF- α* and *CXCL8* (Figure 6A). However, HCV core exposure (12 hr) increased *IL-6*, *TNF- α* , *CXCL10* and *CXCL8* expression from 2 to 29 fold compared to Gal-exposed cells (Figure 6A, $p < 0.05$). These studies were extended by showing that concurrent exposure of HIV-1 Vpr with HCV core proteins caused higher *IL-6* expression than HCV core-treated HF $\mu\phi$ (Figure 6B) while there was no additive effect on the induction of other cytokines and chemokines (data not shown). In addition, soluble HIV-1 Vpr protein at a subneurotoxic concentration (50 nM) together with HCV core protein (10 and 100 nM) caused significantly increased HFN injury (Figure 6C) indicated by reduced β -III-tubulin

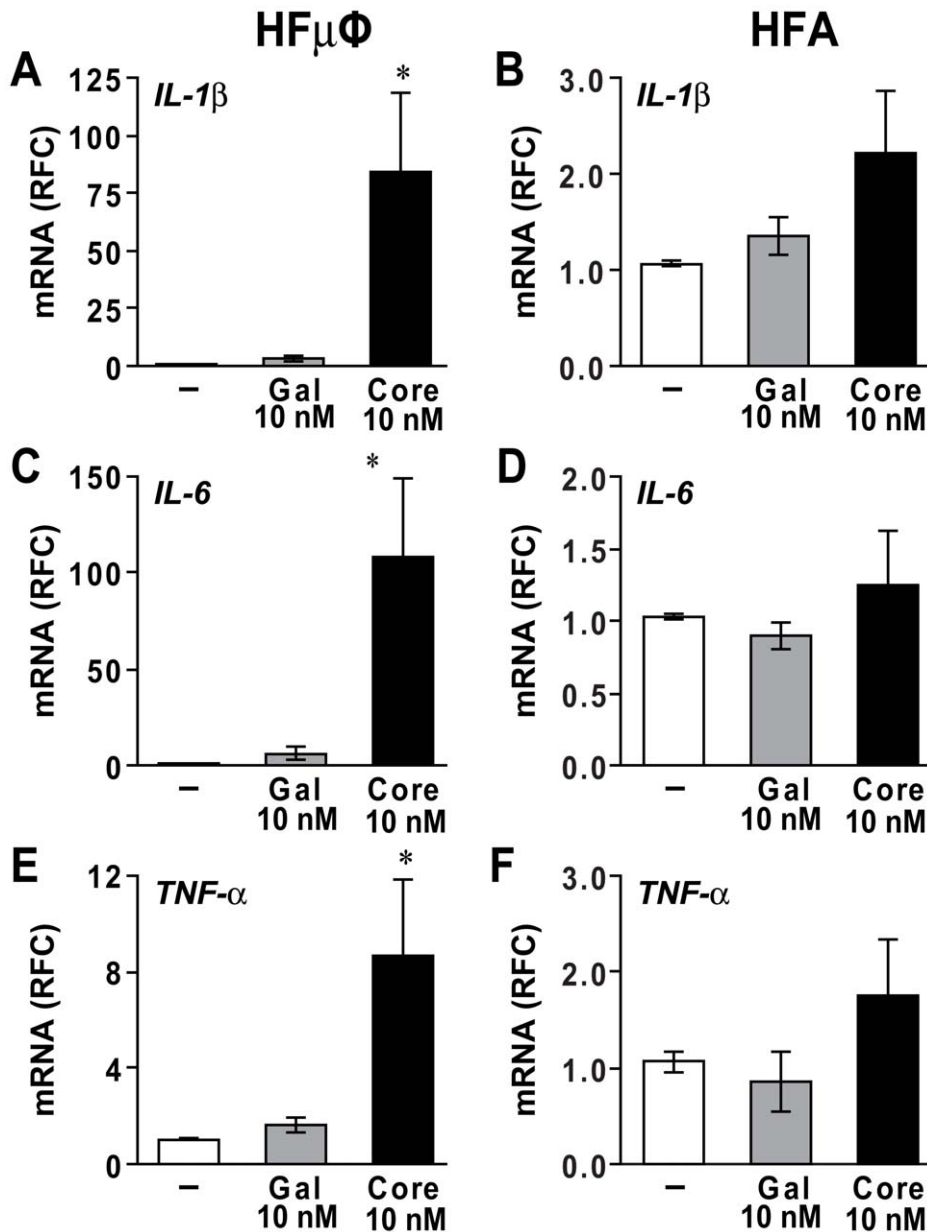


Figure 2. Recombinant HCV core protein activated microglia. Primary human fetal microglia (HF $\mu\Phi$) and astrocytes (HFA) were exposed to culture medium (mock, -), Gal (10 nM) or Gal-core (10 nM) for 12 hr. Gal-core-exposed HF $\mu\Phi$ displayed elevated mRNA expression for *IL-1 β* (A), *IL-6* (C) and *TNF- α* (E) while cytokine expression in Gal-exposed HF $\mu\Phi$ was similar to mock-exposed cells. In contrast, Gal-core-exposed HFAs showed similar transcript levels for *IL-1 β* (B), *IL-6* (D) and *TNF- α* (F) compared with mock-treated cells (n=4). mRNA expression was reported as relative fold change (RFC) compared to *GAPDH* expression. Data represent mean \pm SEM for five or more independent experiments (one-way ANOVA with Bonferroni *post hoc* tests, * $p < 0.05$ compared to mock-treated cells). doi:10.1371/journal.pone.0012856.g002

immunodetection, compared with HFNs exposed to either HIV-1 Vpr or HCV core proteins at the same concentrations ($p < 0.05$). These results indicated that concurrent exposure of HIV-1 and HCV encoded proteins potentiated the neuroimmune activation and neurotoxicity effects of HCV core protein.

HCV core protein causes neuropathological and neurobehavioral deficits in Vpr-transgenic mice

As the present *in vitro* studies exhibited additive neurotoxic effects of HCV core and HIV-1 Vpr proteins, Gal-core or Gal proteins were stereotactically implanted into the right striatum of HIV-1 Vpr-

transgenic mice. These transgenic animals were previously shown to exhibit features indicative of neurodegeneration [15,47]. Morphological analysis at day 14 post-implantation disclosed increased immunoreactivity of GFAP, an astrocyte marker, and Iba-1, a microglia/macrophage marker, in the right striatum of Vpr-transgenic mice receiving HCV core protein implantation (Figure 7Aiv and v). Additionally, HCV core-implanted animals displayed lower LC3 immunoreactivity (Figure 7Avi) and reduced numbers of cresyl violet-positive cells in the right striatum (Figure 7Bii). Mean ratios of ipsilateral to contralateral neuronal counts confirmed neuronal loss in the implanted striatum (Figure 7B). In conjunction with these latter neuropathological features, Vpr

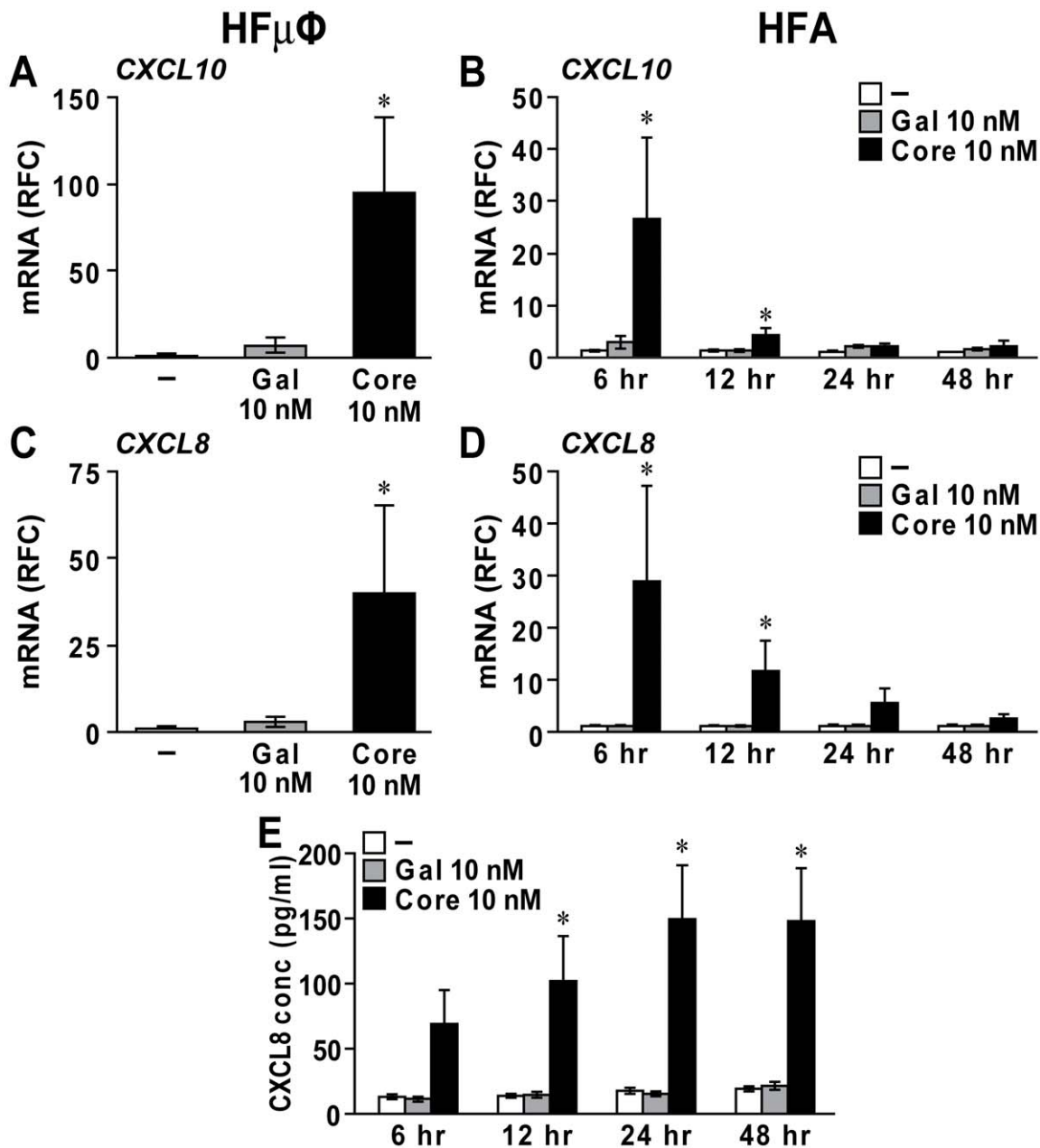


Figure 3. HCV core protein induced chemokine expression in microglia and astrocytes. Primary human fetal microglia (HFμΦ) (A, C) and astrocytes (HFA) (B, D) exposed to 10 nM Gal-core displayed elevated mRNA expression for *CXCL10* (A, B) and *CXCL8* (C, D). The increased *CXCL10* and *CXCL8* expression in Gal-core-treated HFA was highest at 6 hr post-exposure and extinguished at 24 hr post-exposure. (E) Elevated levels of *CXCL8* protein were detected in supernatants from Gal-core-treated HFAs after 12 hr exposure by ELISAs. Data represent mean ± SEM for three or more independent experiments (one-way ANOVA with Bonferroni *post hoc* tests, * $p < 0.05$ compared to mock-treated cells). doi:10.1371/journal.pone.0012856.g003

transgenic mice receiving HCV core implantation exhibited greater ipsiversive rotation frequency at days 7 and 14 post-implantation (Figure 7C, $p < 0.05$). Taken together, the *in vivo* neurotoxicity of HCV core protein was likely a consequence of autophagy suppression in neurons coupled with activation of proximate astrocytes and microglia leading to neuronal injury and loss.

Discussion

During the last decade, there is mounting evidence to suggest HCV is neuroinvasive and HIV/HCV co-infected patients display

higher rates of neuropsychological deficits. To delineate the underlying pathogenic mechanisms of HCV infection of the brain, we report for the first time that primary human astrocytes and microglia were permissive to HCV infection with cell culture-derived HCV particles. Moreover, this report demonstrates for the first time that HCV core protein activates human glia and contributes to neurotoxicity. Direct exposure of HCV core protein to primary human neurons suppressed the neuronal autophagy, leading to neurite retraction. The change in neuronal membrane potential after exposure to HCV core protein indicated that core was biologically active at the cell membrane and was able to

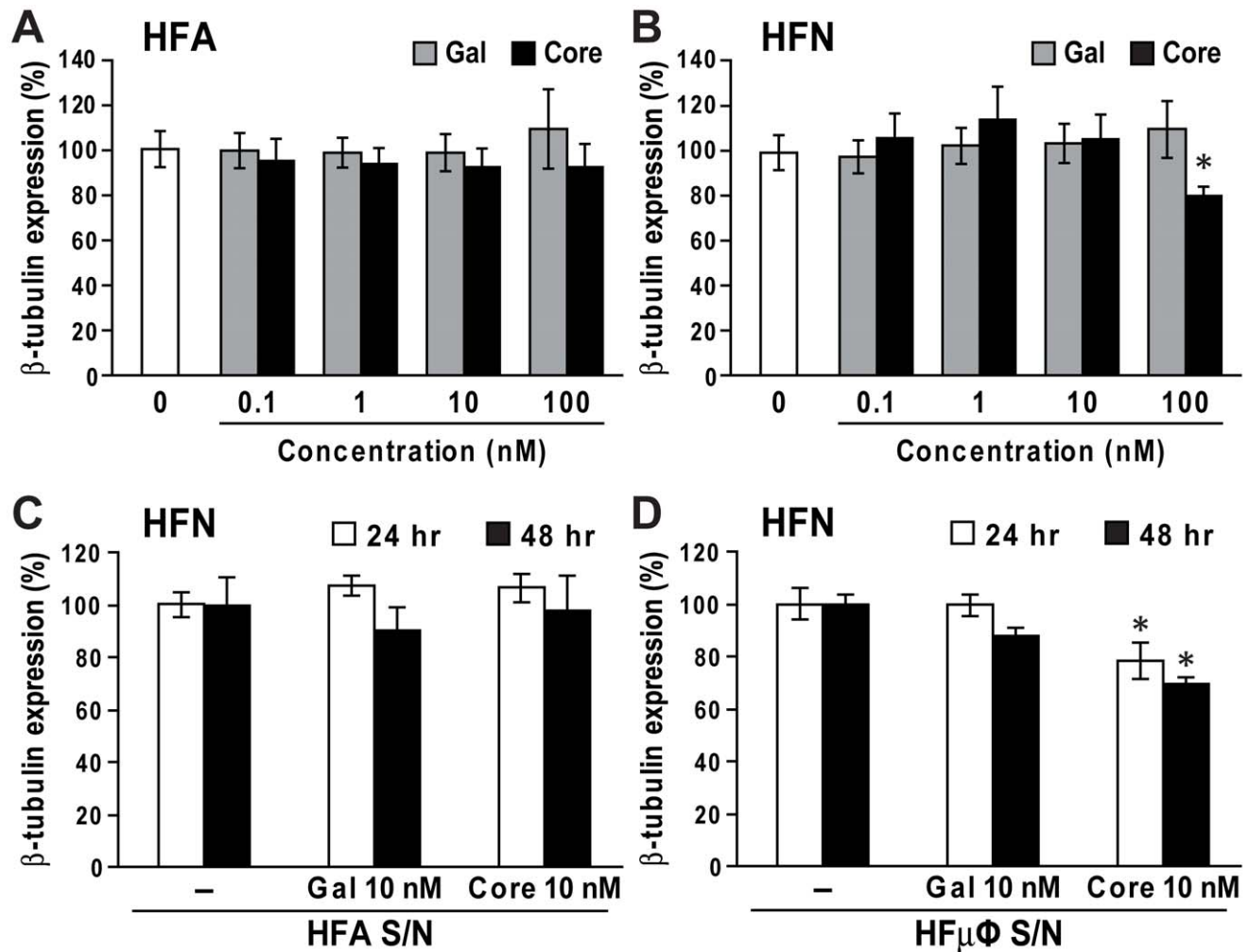


Figure 4. Neurotoxicity mediated by HCV core protein. Cell injury was determined by measuring β -III tubulin immunoreactivity at 48 hr post-exposure. (A) Gal or Gal-core exposure did not affect the viability of primary human fetal astrocytes (HFA). (B) Gal-core (100 nM) exposed to HFNs suppressed neuronal viability in terms of reduced β -tubulin immunoreactivity. (C) Supernatants from Gal- or Gal-core-treated astrocytes (HFA S/N) was not toxic to primary human fetal neurons (HFN, $n=3$). (D) Supernatants from Gal-core-exposed microglia (HF μ Φ S/N) collected at 24 and 48 hr post-exposure also caused a reduction in β -tubulin immunoreactivity of HFNs. Data represent mean \pm SEM for three or more independent experiments (one-way ANOVA with Bonferroni *post hoc* tests, * $p<0.05$ compared to mock-treated cells). doi:10.1371/journal.pone.0012856.g004

modulate ionic conductance in neurons. In addition to direct neurotoxicity, proinflammatory cytokines and other neurotoxins released from HCV core-activated microglia into supernatants were toxic to neurons. The *in vitro* and *in vivo* aberrant immune activation and neurotoxicity mediated by HCV core protein were amplified in the presence of HIV-1 Vpr protein. These findings support the concept that the presence of HCV-encoded proteins cause neuronal damage and perhaps neurocognitive impairment in individuals co-infected with HIV and HCV.

Although HCV tropism is principally recognized in human liver cells, extrahepatic replication has been reported in PBMC [48], myocardium [49,50] and brain cells [10,18] by detecting HCV negative-strand RNA. Herein, detection of positive- and negative-strand RNA in different brain regions was performed in a patient with HIV and HCV co-infection and was consistent with previous reports [10,18]. HCV^{cc} infects and replicates in Huh 7.5 hepatocytes. However, productive infection was not detected in other hepatic cell lines (e.g. HepG2) [20], non-hepatic cell lines (e.g. HeLa, 293T and U-937) [20] and PBMC (e.g. B and T

lymphocytes, monocytes and dendritic cells) [51], likely due to the lack of some entry receptors e.g. claudin-1 and their partner proteins [51] or host factors required for HCV infection e.g. liver specific microRNA miR-122 [52,53,54]. Herein, expression of HCV proteins, core and NS3, were immunodetected in the cytoplasm of primary human astrocytes and microglia infected with HCV^{cc}. Although the levels of infectivity in primary astrocytes and microglia were low (less than 1%), HCV positive- and negative-strand RNA were measurable in infected astrocytes, suggesting that HCV is capable of replication in astrocytes. Both microglia and astrocytes were previously identified as HCV-infected cells in autopsied brains [9,18] and were shown to express SR-BI, CD81, claudin-1 and occludin [22,23,24,25,26]. Although the expression levels of claudin-1 and occludin were 15 to 50 fold lower in primary human astrocytes and microglia compared with Huh 7.5 cells (Figure S2A), claudin-1 expression in glial cells was approximately 10 fold higher than in PBMC or 293T cells [51]. The higher expression of HCV entry receptors in primary astrocytes compared to microglia was also consistent with less

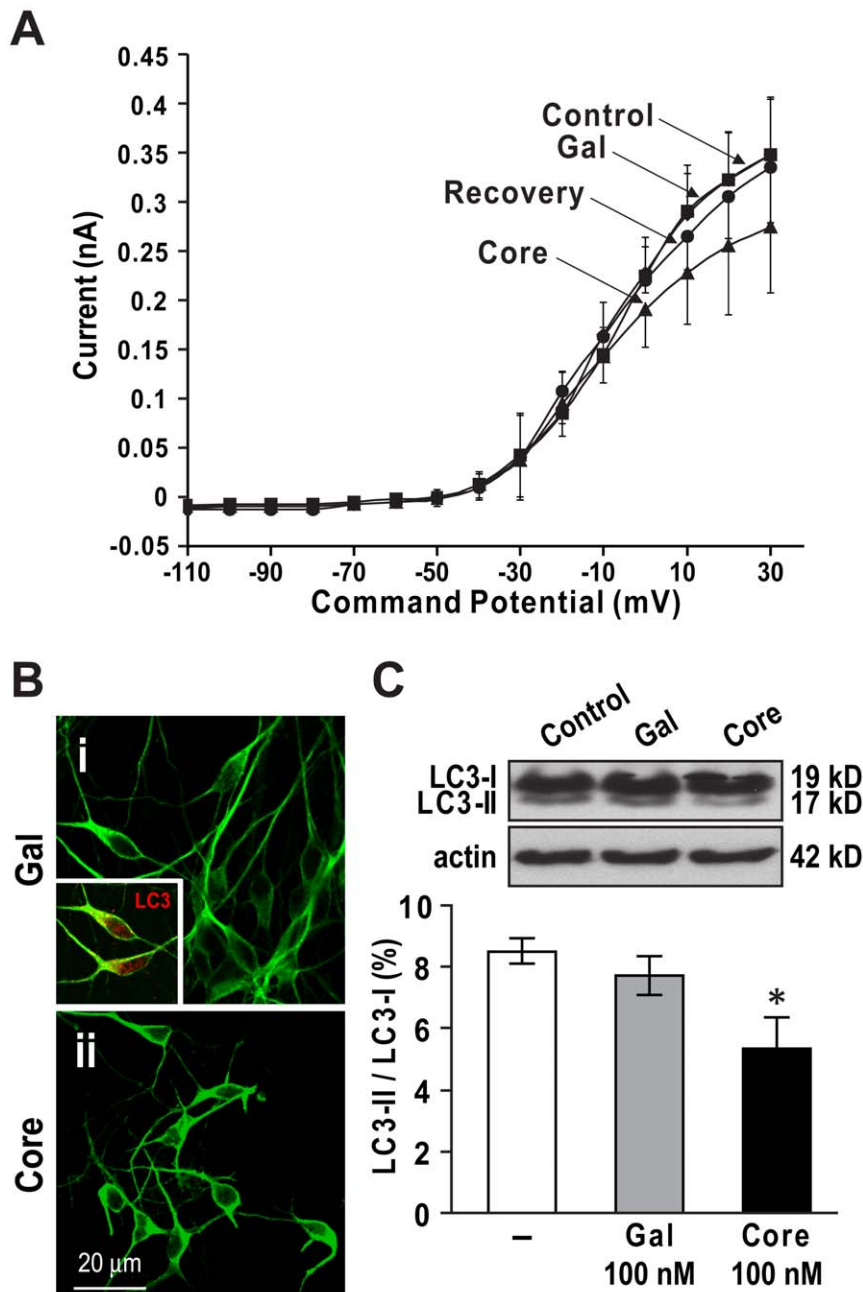


Figure 5. HCV core protein modulated neuronal membrane conductance. (A) Whole-cell currents recorded from human fetal neurons (HFNs) were reduced after exposure to Gal-core (100 nM, $n=4$) but not Gal (100 nM, $n=6$). (B) After Gal-core (100 nM) exposure, MAP-2-immunopositive HFNs (green) showed neurite retraction, fewer neurite processes, soma atrophy and loss of neurons (ii) compared to Gal (100 nM) exposure (i). The autophagy-related light chain 3 (LC3) molecule was widely expressed in unexposed neurons (red, i, inset) (original magnification 63 \times). (C) LC3 immunoreactivity of HFN lysates showed two isoforms of LC3: cytosolic LC3-I (top band) and its phosphatidylethanolamine-conjugated LC3-II (bottom band). 6 hr after exposure to 100 nM Gal-core, the band density ratio of LC3-II to LC3-I was reduced by 37.5% compared to mock-exposed cells. Data represent mean \pm SEM ($n=4$, one-way ANOVA with Bonferroni *post hoc* tests, * $p<0.05$). doi:10.1371/journal.pone.0012856.g005

frequently-detected HCV-immunopositive microglia after HCV^{cc} infection. HCV replication in infected or transfected hepatocytes was reported in the membranous web structure closely associated with rough endoplasmic reticulum [55]. In the present studies, localization of HCV proteins in astrocytes was similarly associated with structures resembling endoplasmic reticulum. Further studies to determine if HCV-infected microglia or astrocytes release infectious viruses will be of interest.

HCV^{cc} used herein belongs to the HCV genotype 2a [19] while most HCV strains in North America are genotypes 1a or 1b, which are associated with higher HCV RNA levels, poor responses to IFN- α treatment and lower mean CD4 count in HIV/HCV co-infected individuals [2]. The strain difference and other comorbidities such as intravenous drug use might influence the infection and production of HCV in human brain, as demonstrated for HIV infection [56].

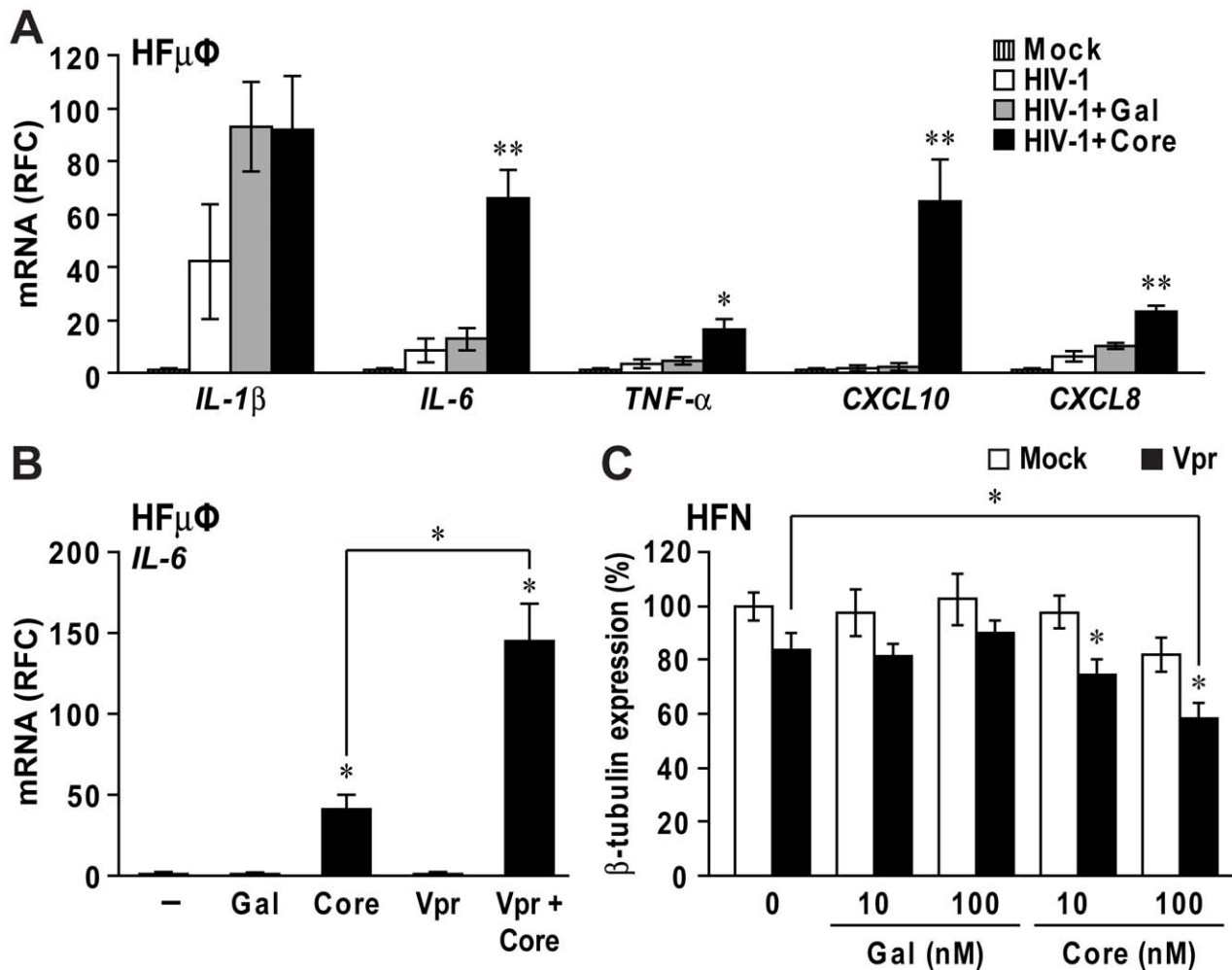


Figure 6. HCV core protein increased microglia activation and neurotoxicity induced by HIV-1 infection and Vpr protein. (A) Primary human fetal microglia (HFμΦ) were infected with HIV-1 SF162. At day 2 post-infection, HIV-1-infected HFμΦ were exposed to culture medium (HIV-1), Gal (10 nM, HIV-1+Gal) or Gal-core (10 nM, HIV-1+Core) for 12 hr. HIV-1 infection caused increased mRNA expression of *IL-1β*, *IL-6*, *TNF-α* and *CXCL8*. Exposure to Gal-core potentiated transcript expression of *IL-6*, *TNF-α*, *CXCL10* and *CXCL8* expression compared to HIV-1-infected microglia exposed to mock or Gal. (B) HFμΦ exposed to HIV-1 Vpr (50 nM) and Gal-core (10 nM) proteins displayed elevated *IL-6* transcript levels compared to Vpr- or Gal-core-treated cells. (C) Concomitant exposure of primary human neurons (HFN) to HIV-1 Vpr and Gal-core also resulted in reduced β-tubulin immunoreactivity compared to mock- or Vpr-exposed cells in a concentration dependent manner. Data represent mean±SEM for three or more independent experiments (one-way ANOVA with Bonferroni *post hoc* tests, * $p < 0.05$ and ** $p < 0.01$ compared to mock-treated cells). doi:10.1371/journal.pone.0012856.g006

HCV infection has been correlated with cognitive impairment with changes indicative of neuronal damage by magnetic resonance spectroscopy [8]. Herein, we demonstrated that the direct exposure of HCV core protein or exposure to supernatants from HCV core-exposed microglia were toxic to primary human neurons together with neurobehavioral deficits and neuronal loss in HCV core-implanted animals. Although the toxic concentration of HCV core for primary human neurons in our *in vitro* experiments was higher than the reported serum levels in HCV-infected patients, it was consistent with previous studies [31,32,57]. It is plausible that HCV core concentrations at the surface of infected and proximate (target) cells are higher than in serum; moreover, chronic and repeated HCV exposures might yield augmented neurotoxic effects. Remarkably, the concentration of HCV core required to activate microglia and astrocytes was one log less than the concentration needed for direct neurotoxicity.

HIV-1 Vpr is highly neurotoxic without substantial proinflammatory properties [58] and its expression in brain monocytic cells

contributes to synaptic injury and neurobehavioral abnormalities in transgenic mice [15]. At subtoxic concentrations, simultaneous exposure of HCV core and HIV-1 Vpr proteins to neurons exerted additive effects, resulting in increased *IL-6* expression in glial cells and neuronal injury. Likewise, implantation of HCV core into the striatum of HIV-1 Vpr transgenic mice resulted in marked glial activation coupled with neuronal loss. It is likely that Vpr and HCV core act through different mechanisms to yield cumulative neurotoxic effects as Vpr's putative receptor is the nuclear glucocorticoid receptor [59] while HCV core's receptor might be a cell membrane protein [31,32,57,60]. Additionally, it is conceivable that HCV core protein might also potentiate the neurotoxic or neuroinflammatory effects of other HIV-1 proteins e.g. Tat, Nef or gp120. This possibility might highlight the importance of interactions between HCV and HIV and warrants further investigation.

Our data showed that core protein from HCV genotype 1b triggered *in vitro* and *in vivo* activation of microglia and astrocytes,

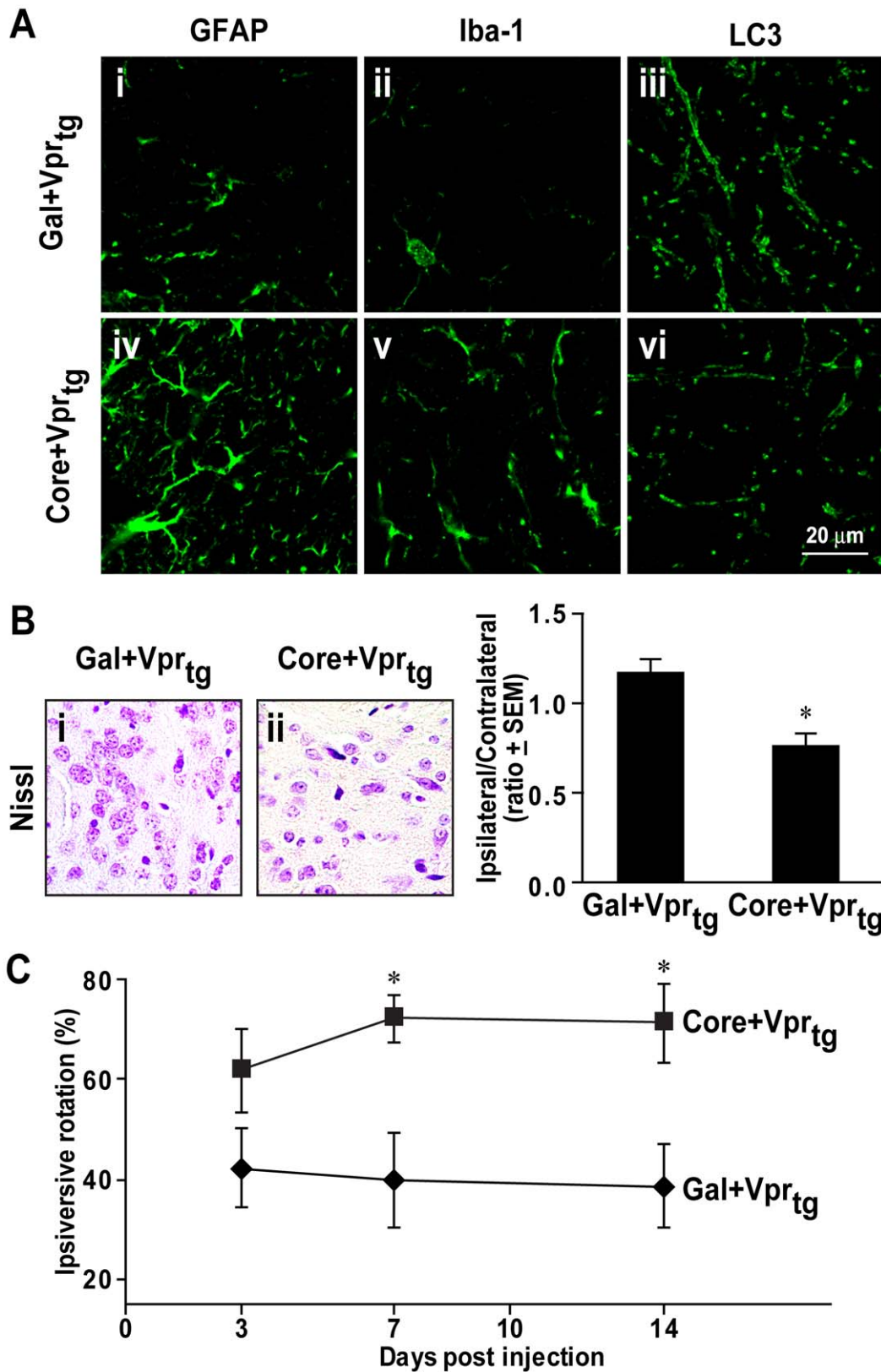


Figure 7. Implanted HCV core protein caused glial activation, neuronal loss and neurobehavioral deficits in Vpr-transgenic mice. (A) GFAP and Iba-1 immunoreactivities were minimally detected in ipsilateral brain hemispheres of animals implanted with Gal (i, ii) while higher numbers of GFAP and Iba-1 immunopositive cells were evident in Gal-core-implanted animals (iv, v). LC3 immunoreactivity was lower in Gal-core-implanted animals (vi) compared to Gal-implanted animals (iii) (original magnification 63 \times). (B) Nissl staining of ipsilateral basal ganglia displayed lower numbers of neurons in Vpr transgenic mice implanted with Gal-core (ii) compared with Gal (i) (original magnification 400 \times). Neurons were

counted in four different fields of four sections from ipsilateral and contralateral hemispheres. Gal-core-implanted Vpr transgenic mice displayed a lower ratio of ipsilateral to contralateral neuronal counts ($n=3$, two-tailed unpaired Student's t test, $*p<0.05$). (C) *In vivo* neurological damage in Gal-core-implanted Vpr transgenic mice was indicated by higher frequencies of ipsiversive rotations at days 7 and 14 post-implantation ($n=6$, repeated measured ANOVA with Bonferroni *post hoc* tests, $*p<0.05$ compared to Gal-implanted animals). Data are presented as the mean \pm SEM. doi:10.1371/journal.pone.0012856.g007

which is consistent with a recent magnetic resonance spectroscopy study that revealed an increased level of *myo*-inositol, a hallmark of gliosis, in frontal white matter of HCV-infected individuals [61]. Increased serum TNF- α and IL-1 β levels in HCV-infected individuals were correlated with severity of neuropsychiatric dysfunction [62]. *In vitro* HCV infection in primary human macrophages resulted in increased TNF- α and CXCL8 [63]. Increased expression of CXCL10 and CXCL8 in astrocytes was also reported in brains of individuals with HIV encephalitis [64]. A recent study demonstrated that HIV-infected macrophages induced CXCL8 production from astrocytes through IL-1 β and TNF- α [65]. The chemoattractant CXCL10 plays an important role in leukocyte infiltration into various tissues including CNS [66]. CXCL8 recruits not only neutrophils but also monocytes and lymphocytes [67]. It is plausible that the increased expression of CXCL10 and CXCL8 by HCV core-exposed microglia and astrocytes might enhance recruitment of T helper cells and monocytes in the brain, leading to deleterious effects. Of interest, we also observed that HCV core exposure to microglia induced the transcript levels of indoleamine 2,3-dioxygenase (IDO) (data not shown), which has been linked to neuronal injury [68,69]. Additionally, we also demonstrated that supernatants from HCV core-treated microglia were neurotoxic. In these experiments, the concentration of HCV core protein was subtoxic to neurons but was sufficient to induce the expression of neurotoxic factors. It is also possible that there was carry-over of HCV core protein in supernatants which might play a role in the overall neurotoxicity. However, these results indicate that exposure of HCV core protein to glial cells could lead to deleterious effects on neurons.

Several studies have reported that recombinant HCV core activated cell surface receptors e.g. Toll-like receptor 2 (TLR2) on monocytes [31] and dendritic cells [70] or the putative HCV core receptor, gC1qR, on T lymphocytes [57,60] and lung fibroblasts [32]. Microglia/macrophages are known to express both receptors [71,72] while astrocytes express gC1qR but not TLR2 [72,73] and there was a 30% increase in the gC1qR transcript level in HIV-infected brains (Figure S2B). Inhibition of either TLR2 or gC1qR by blocking antibodies or RNA silencing will be of interest. Nonetheless, HCV core might also be endocytosed by the microglia or astrocytes, and subsequently act through intracellular or nuclear receptors.

In summary, we reported primary human microglia and astrocytes were permissive to HCV infection and HCV-encoded protein, core, was neurotoxic but also activated pro-inflammatory responses in human microglia and astrocytes. The augmented glial activation and neurotoxicity mediated by HCV core protein in the

presence of HIV-1 Vpr protein or HIV-1 infection highlighted the additive effects of HCV- and HIV-encoded proteins in the pathogenesis of neurologic disease. Future studies are required to delineate the precise mechanisms by which HIV and HCV proteins interact and amplify neuropathogenesis, thereby permitting the development of potential management and therapeutic strategies.

Supporting Information

Table S1 Real-time RT PCR primer sequences for detecting human genes.

Found at: doi:10.1371/journal.pone.0012856.s001 (0.06 MB DOC)

Figure S1 Primary human astrocytes and microglia were permissive to infection by HCVcc. At day 3 post-infection, HCV core protein immunoreactivity (green) was colocalized with GFAP or Iba-1 (red) in HCVcc-infected primary human fetal astrocytes (HFA) and microglia (HF $\mu\Phi$) but not in mock-infected cells. (original magnification 630x)

Found at: doi:10.1371/journal.pone.0012856.s002 (4.39 MB TIF)

Figure S2 Expression of HCV entry receptors in glial cells and brains. (A) Transcript expression of HCV entry receptors were determined by real time RT-PCR. Primary human microglia and astrocytes expressed all known HCV entry receptors, albeit at the lower levels than Huh 7.5 hepatoma cell line. (B) Similar levels of HCV entry receptors were found in white matter tissues from control and individuals with HIV encephalitis (HIV) while the expression of the *gC1qR* transcript was elevated in HIV compared to control ($n=5$, $*p<0.05$). Data represent mean \pm SEM for three or more independent experiments.

Found at: doi:10.1371/journal.pone.0012856.s003 (0.81 MB TIF)

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Author Contributions

Conceived and designed the experiments: PV CR BA EAC JJ CP. Performed the experiments: PV FM LTL HN JJ. Analyzed the data: PV FM. Contributed reagents/materials/analysis tools: LTL CR EAC. Wrote the paper: PV CP.

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