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Neurotoxic effects of the HCV core protein are mediated by sustained activation of ERK via TLR2 signaling

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Abstract Hepatitis C virus (HCV) infection is a serious problem among those co-infected with human immunodeficiency virus; however, its impact in the central nervous system (CNS) remains unclear. This study aimed to investigate the mechanisms underlying HCV core protein-mediated neurodegeneration. Analysis of human HCV seropositive cases demonstrated widespread damage to neuronal dendritic processes and sustained activation of extracellular signal-related kinase (ERK); analogous pathologies were observed in wild type injected with HCV core protein into the hippocampus. In vitro analysis in neuronal cells exposed to HCV core demonstrated retraction of the neuronal processes in an ERK/Signal Transducer and Activator of Transcription 3 (STAT3)-dependent manner dependent on toll-like receptor 2 (TLR2) signaling activation. These results indicate that HCV core protein neurotoxicity may be mediated by the sustained activation of ERK/STAT3 via TLR2-IRAK1 signaling pathway. These pathways provide novel targets for development of neuroprotective treatments for HCV involvement of the CNS.

Keywords HCV· TLR2 · Neurodegeneration · ERK

Introduction

Approximately 200 million people worldwide are infected with hepatitis C virus (HCV; Yang et al. 2008); it is a major cause of sustained liver disease and has recently been shown to cause cognitive impairment (Forton et al. 2004b;

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Laskus et al. 2005; Ryan et al. 2004). HCV is highly prevalent among patients with human immunodeficiency virus (HIV) and a history of drug abuse (Letendre et al. 2005). In the USA alone, approximately 30% of the people infected with HIV/AIDS are co-infected with HCV (Blackard and Sherman 2008). HCV, a member of the Flavivirdae family, has a positive single-strand RNA genome that is roughly 9.5 kb. The genome encodes a polyprotein of approximately 3,000 amino acids. At least 11 polypeptides are produced from the polyprotein; these include three structural proteins (core, E1, and E2), six non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and two others, F and p7, that result from a core frame-shift and protein processing, respectively (Chevaliez and Pawlotsky 2006; Polyak et al. 2006).

Considerable attention has been devoted to the effects of HCV in the liver, and studies have investigated the cellular receptors utilized by HCV in hepatocytes and fibroblasts for signaling or virus entry. These receptors include CD81, scavenger receptor class B type I, heparin proteoglycans, toll-like receptor 2 (TLR2), and claudin-1 (Bartosch and Cosset 2006; Cocquerel et al. 2006; Evans et al. 2007; Sato et al. 2007).

Eleven human TLRs have been identified and described; they play a role in innate cell immunity and are responsible for pathogen detection. TLR activation in the brain has been shown to cause neural inflammation and may have a role in neurodegeneration (Crack and Bray 2007). Activation of TLR2 initiates a complex signaling cascade that includes the interleukin-1 receptor-associated kinase (IRAK) family. After activation, TLR2 binds myeloid differentiation factor 88 (MyD88) and associates with IRAK1 and tumor necrosis factor receptor-associated factor 6 (TRAF6). IRAK1 then forms a cytosolic complex with TRAF6. TGFβ activated kinase 1 (TAK1), and Tak binding protein 1 (Table 1), an activator of TAK1, then associate with the IRAK1:TRAF6 complex, which eventually activates both NFK-B and extracellular signal-related kinase (ERK) pathways (Akira and Takeda 2004; Dolganiuc et al. 2004; Gottipati et al. 2008).

The HCV core protein, a structural polypeptide derived from HCV, is involved in formation of the viral capsid but also acts as an RNA binding protein (Chang et al. 2007; McLauchlan 2000; Ray and Ray 2001) and can interact with cellular components such as the ER and mitochondrial membranes (Polyak et al. 2006). HCV RNA and core protein have been found in the cerebral spinal fluid and the brain (Laskus et al. 2002; Letendre et al. 2007; Maggi et al. 1999; Radkowski et al. 2002; Vargas et al. 2002) of HCV seropositive and HIV co-infected patients, particularly in those with neurocognitive impairment (Laskus et al. 2005; Letendre et al. 2007). However, the mechanisms underlying HCV neurotoxicity and the receptors involved in the central nervous system (CNS) pathology remain unclear. In this context, the objective of this study was to investigate the signaling pathways involved in HCV core neurotoxicity. We demonstrate that HCV core hyperactivation of ERK and Signal Transducer and Activator of Transcription 3 (STAT3) via TLR2 signaling results in neurodegenerative pathology both in vitro and in vivo.

Materials and methods

Subjects and postmortem examination

A total of nine HIV seropositive (HCV seronegative), nine HIVE (HCV seronegative), and three HCV seropositive (HIV seronegative) cases were included in the present study (Table 1).

Subjects had a thorough neuromedical assessment including antemortem laboratory data. HIV encephalitis was defined based on the presence of HIV+ microglial nodules, astrogliosis, and myelin pallor. Brain samples were obtained from the California NeuroAIDS Tissue Network based on clinical and postmortem data from their enrollment in a longitudinal study at the University of California, San Diego HIV Neurobehavioral Research Center.

HCV core intra-hippocampal injections into mice

One microgram per milliliter (2 nm) of HCV core protein (Virogen, Watertown, MA, USA), vehicle control, or heat-inactivated protein was injected into the hippocampus of 3-month-old wild type (n=6 per group). Following NIH guidelines for the humane treatment of animals, under anesthesia, mice were sacrificed, and brains were removed 1 week post-injection. The brains were cut into 40-μm vibratome sections and stored at −20°C until processed for immunocytochemical analysis.

All experiments were approved by the animal subjects committee at the University of California, San Diego (UCSD) and were performed according to National Institutes of Health (NIH) recommendations for animal use.

Immunoblotting and immunocytochemistry analysis of neuronal cultures exposed to HCV core

B103 rat neuronal cultures were used in all in vitro studies. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA, USA) media supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, USA) and 1% penicillin/streptomysin (Invitrogen, Carlsbad, CA, USA). Cells were grown in serum-free DMEM for a minimum of 6 h before being exposed to the HCV core protein. The cultures were

Table 1 Characteristics and demographics of cases examined in this study

N	HIV+ HCV− 9	HIVE HCV− 9	HIV− HCV+ 3
Age, mean±SD, years	45.67±5.29	41.38±10.23	49±4.24
Sex, male/female	7/2	8/1	3/0
History of IDU, yes/no	2/9	4/9	0/3
Brain weight (g)	1,347±135	1,233±177	1,403±110
Liver weight (g)	2,054±607	1,867±713	2,475±318

exposed to 1 μg/mL of HCV core protein from 30 min to 24 h. Control experiments were conducted with NS3 and NS5. For immunoblotting, cells were lysed in 50 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 0.4% sodium cacodylate [pH 7.4] with protease/phosphatase inhibitors (EMD Calbiochem, San Diego, CA, USA) and collected. Cultures grown for immunocytochemistry were fixed in 4% paraformaldehyde for 20 min and remained in 1% paraformaldehyde at 4°C until immunocytochemical analysis. Additional complimentary studies using the HCV core protein, inactivated HCV core protein, and NS3 control protein were conducted in neuronal cells derived from Neuronal Precursor Cells (NPCs) as previously described (Crews et al. 2011).

For immunoblot analysis, 20 μg of protein from the collected samples, as determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), were placed on a 4–12% Bis–Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA, USA) and then transferred onto a 0.45-μm polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 3% bovine serum albumin and probed with antibodies against pERK, ERK, or β-tubulin. All antibodies were used at a 1:1,000 dilution. After primary antibody incubation overnight, blots were incubated with an anti-mouse or anti-rabbit IgG conjugated with a horseradish peroxidase antibody (1:5,000; American Qualex, San Clemente, CA, USA). Western Lightening Chemiluminescence Reagent (PerkinElmer, Waltham, MA, USA) followed with analysis on the Versadoc gel imaging system (BioRad, Hercules, CA, USA). For all 24-h core protein exposure in vitro experiments, a CytoTox Non-Radioactive Cytotoxicity Assay (Promega, Madison WI, USA) was performed according to manufacturer's instructions.

For immunocytochemical analysis, fixed coverslips were incubated overnight with antibodies against β-tubulin, CD81, or TLR2. This was followed by incubation with biotinylated anti-mouse or anti-rabbit IgG (1:100; Vector Laboratories, Burlingame, CA, USA) and Avidin D-HRP (1:200; Vector Laboratories, Burlingame, CA, USA) and reacted with diaminobenzidine tetrahydrochloride with 0.001% hydrogen peroxide.

For double-immunocytochemical analysis, fixed cells were incubated with an antibody against phospho-ERK (1:100) detected with the Tyramide Signal Amplification-Direct (Red) system (1:100; NEN Life Sciences, Waltham, MA, USA) and a mouse MAb against β-tubulin (1:250; Millipore, Temecula, CA, USA) detected with fluorescein isothiocyanate-conjugated secondary antibodies (1:75; Vector Laboratories, Burlingame, CA, USA). Cells were imaged with a Zeiss ×63 (numerical aperture, 1.4) objective on an Axiovert 35 microscope (Zeiss, Thornwood, NY, USA) that has a MRC1024 laser scanning confocal microscope system (BioRad, Hercules, CA, USA) attached.

To observe the cytoskeletal effects of the core protein over time, neuronal cultures were exposed to HCV core protein for 1, 12, and 24 h. Following exposure, cultures were collected for western blot analysis for β-tubulin immunoreactivity. Cultures exposed to core for 12 and 24 h were also fixed in 4% paraformaldehyde and processed for immunocytochemistry with an antibody against β-tubulin.

Co-immunoprecipitation of TLR2 pathway activation complexes

For co-immunoprecipitation studies, neuronal cultures were either untreated or exposed to HCV core protein for 30 min to induce TLR2 activation. After 30 min, the cells were lysed with a cell-lysing buffer (50 mM Tris–HCl pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.4% sodium cacodylate) with appropriate phosphatase/protease inhibitors (EMD Calbiochem, San Diego, CA, USA) and collected. Approximately 500 μg of protein was used in each immunoprecipitation (IP) reaction. Lysates were pre-cleared by constant mixing at 4°C for 1 h with 1.0 μg of the appropriate normal IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) species of the IP antibody and 30 μL of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After pre-clearing, 2 μg of immunoprecipitating antibody was added to the mixture and kept at 4°C overnight with constant mixing. An appropriate IgG control sample was included. The beads were centrifuged, and the supernatant was removed. The beads were then washed (2×) in a NET-BSA buffer (50 mM Tris/HCl, 150 mM NaCl, 0.1% Nonidet P-40, 1.0 mM EDTA, 0.25% BSA, 0.02% sodium azide, pH 7.5) with a

10-min incubation of mixing at 4°C between each wash. The agarose beads were then placed into a final wash buffer (10 mM Tris/HCl, 0.1% Nonidet P-40 [pH 7.5]). After removal of the wash, an elution buffer of 50 mM Tris pH 6.7 was added. Following the addition of NuPage reducing agent (Invitrogen, Carlsbad, CA, USA) and NuPage sample reducing agent (Invitrogen, Carlsbad, CA, USA), the beads were heated at 100°C for 5 min to allow the release of the attached proteins. The elution was then placed on an SDS-PAGE gel, and a western blot, as described above, was performed.

Receptor identification and neutralization assays

To verify the presence of HCV receptors CD81 and TLR2 in the B103 neuronal cells, cultures were probed with antibodies against CD81 and TLR2. Following receptor presence confirmation, cells were incubated for 30 min with antibodies against CD81 and TLR2 and Heparinase I (Sigma, St. Louis, MO, USA) to neutralize the receptors and then exposed to HCV core protein for 30 min. The cells were analyzed for CD81 and TLR2 protein levels and for pERK immunoreactivity in response to HCV core exposure.

Si-RNA

Si-RNA experiments against TLR2 and MEK were performed on the B103 cultures. In separate experiments, 30 nM of siRNA against MEK or TLR2 (Applied Biosystems, Austin, TX, USA) was applied to the cells using the NeoFX Transfection Agent (Applied Biosystems, Austin, TX, USA). Twenty-four hours following transfection, cells were exposed to HCV core protein (1 μg/ml) and were harvested 48 h later for immunoblot analysis.

Real-time quantitative PCR (qRT-PCR) analysis of TLR2 levels upon HCV core exposure

In order to examine the effects of HCV core protein on TLR2 mRNA levels, qRT-PCR was performed on neuronal cells exposed to increasing concentrations of HCV core. Cellular RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). Total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qRT-PCR analysis of TLR2 and control GAPDH was conducted using IQ SYBR green (Bio-Rad, Hercules, CA, USA) on the iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primer pair for TLR2 was forward primer 5′-TGTCAGTGGCCAGAAAAGATG-3′ and reverse primer 5′-GCAGAAGCGCTGGGGAATGGV-3′ (Shingu et al. 2003). QuantiTect Primer Assays (Qiagen, Valencia, CA, USA) were used for GAPDH control primers. PCR was carried out for 35 cycles with a denaturing temperature of 94°C for 10 s, annealing for 1 min at 57°C, and extension at 72°C for 1 min. Amplification was concluded with a final incubation of 10 min at 72°C. Data was analyzed using the comparative Ct method.

Immunohistochemical analysis of human and mouse tissue samples

Vibratome tissue sections (7 μm) from paraformaldehyde-fixed tissue blocks of the midfrontal cortex from the human cases or 40 μM mouse sections were incubated overnight with antibodies against β-tubulin (1:250, Millipore, Temecula, CA, USA), NeuN (1:1,000, Millipore, Temecula, CA, USA), MAP2 (1:100, Millipore, Temecula, CA, USA), and Glial Fibrillary Acidic Protein (GFAP) (1:500, Millipore, Temecula, CA, USA). This was followed by incubation with species-appropriate biotinylated secondary antibodies (1:100; Vector Laboratories, Burlingame, CA, USA) and Avidin D-HRP (1:200; Vector Laboratories, Burlingame, CA, USA) and reacted with diaminobenzidine tetrahydrochloride with 0.001% hydrogen peroxide.

HCV immunoreactivity in the human samples was verified using an antibody against HCV core protein as previously described (Letendre et al. 2007). For studies of cell counts, sections labeled with the antibody against NeuN were analyzed with the dissector method using the StereoInvestigator System (MBF technologies). Levels of GFAP and pERK immunoreactivity were determined with digitized bright field images analyzed with the Image Quant 1.43 program (NIH) and expressed as optical density (arbitrary units).

Statistical analysis

All experiments were conducted blind-coded and in triplicate. Values are expressed as means±SEM. Statistical significance was determined by one-way ANOVA with Dunnet, Tukey–Krammer, or Fisher post hoc tests as appropriate; significance was deemed at $p<0.05$.

Results

Patterns of neurodegeneration in the neocortex of HCV-infected patients and in neuronal cultures exposed to HCV core

Immunohistochemical analysis of dendritic morphology with an antibody against the neuronal cytoskeletal protein β-tubulin demonstrated damage to the neuronal dendritic processes with reduced complexity and vacuolization in the frontal cortex of HCV seropositive patients with verified

HCV core protein immunoreactivity in the CNS compared to HIV-positive HCV seronegative controls (Fig. 1a, c, d). HCV seronegative patients with HIV encephalitis (HIVE) displayed comparable neuronal pathology to the HCV seropositive patients (Fig. 1b, d). Consistent with previous reports (Letendre et al. 2007; Wilkinson et al. 2009), in comparison to control of HIVE patients, HCV seropositive patients display greater levels of HCV immunoreactivity in astroglial and some microglial cells (Fig. 1e–h). Neuronal damage in the HCV and HIVE was accompanied by widespread astrogliosis in comparison to controls (Fig. 1i–l). Consistent with the immunohistochemistry, immunoblot analysis demonstrated reduced levels of β-tubulin in the HIVE and HCV cases compared to controls (Fig. 1q, r). Moreover, immunoblot analysis confirms the presence of HCV core protein in samples from HCV patients but not in control or HIVE patients (Fig. 1q).

Previous studies have suggested that MAP kinase activation may mediate the toxic effects of the HCV core in hepatocytes; therefore, we examined the levels of ERK phosphorylation across our groups. Immunohistochemical analysis demonstrated significantly increased levels of phospho-ERK in the HIV-negative HCV seropositive cases, but not in patients with HIV alone or HIVE in comparison to controls (Fig. 1i–l). Similarly, by immunoblot analysis, ERK phosphorylation was significantly increased in the HIV-negative HCV seropositive cases, but not in cases with HIVor HIVE in comparison to controls (Fig. 1m, o). Levels of total ERK remained unchanged among the groups (Fig. 1m, o).

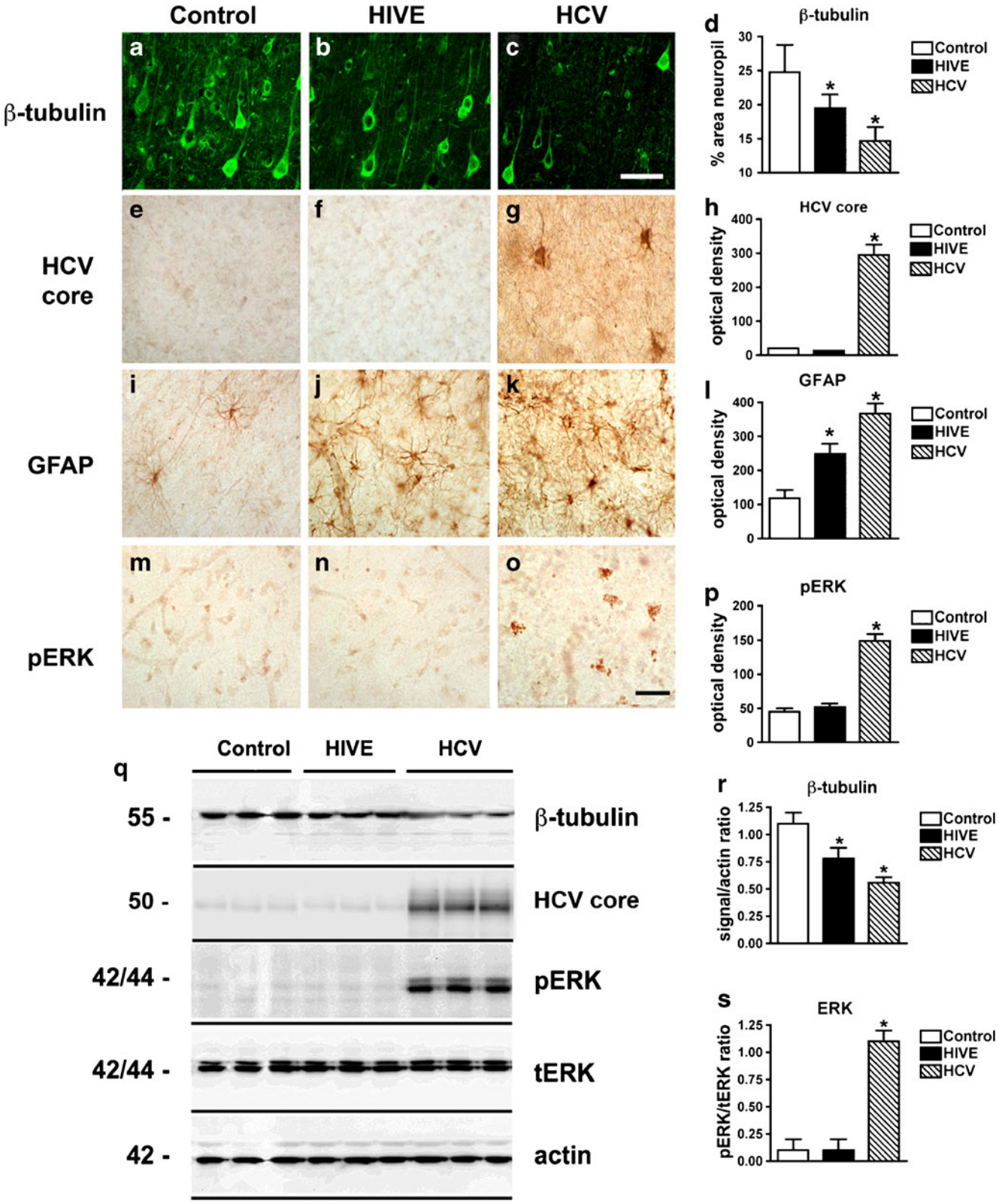
To investigate the mechanisms through which the HCV core promotes neuronal damage, a neuronal cell line (B103) was treated with the HCV core protein at time intervals for up to 24 h. Analysis by phase contrast microscopy and image analysis throughout the time course showed that the HCV core protein exposed cultures had damage to the integrity of the cell and a significant shortening of neuronal processes versus controls (Fig. 2a–e). Immunocytochemical analysis showed that after 12 h of exposure to HCV core protein, neurite length shortened (Fig. 2a–e), and overall β-tubulin immunoreactivity was reduced (Fig. 2f–j). The reduction in neurite length was not related to HCV protein-associated cell death, as cytotoxicity assays demonstrated comparable cell viability in the control and HCV-treated groups (data not shown). Consistent with the immunocytochemical analysis, the immunoblot studies showed a 60% reduction in total β-tubulin immunoreactivity after 24 h of exposure to HCV core protein (Fig. 2k, l). No significant effects on neurite length or β-tubulin immunoreactivity were detected when the neuronal cell cultures were treated with inactivated HCV core (Fig. 2d, e, i, j). Consistent with the immunohistochemical results observed in the human HCV cases, immunoblot analysis of cells treated with HCV core protein demonstrated enhanced levels of pERK immunoreactivity in comparison to vehicle-treated control cells (Fig. 2k). ERK activation (ratio of pERK to tERK) increased following 1 h of HCV core treatment (Fig. 2k, m) and, in contrast to the often transient activation of ERK, the HCV core-treated B103 cells continued to display a sustained high level of ERK activation up till 24 h of treatment (Fig. 2k, m). Treatment of the B103s cells using NS3 or NS5 as controls had no effect upon neurite length or β-tubulin immunoreactivity (data not shown). A complementary set of experiments was conducted in neuronal cells derived from NPCs and, consistent with the studies in the B103 cells, demonstrated an increase in pERK immunoreactivity 24 h following treatment with HCV core protein, in comparison to vehicle-treated NPCs (Supplementary Fig. 2b, e, m). The increase in pERK was specific to treatment with HCV core, as it was not observed in cells treated with inactive HCV core or the control HCV NS3 protein (Supplementary Fig. 2h, k, m). Furthermore, HCV core-related decreases in β-tubulin immunoreactivity in the NPCs were also not observed when the cells were treated with inactive HCV core or the control HCV NS3 protein (Supplementary Fig. 2a, d, g, j).

Prolonged activation of the ERK signaling pathway mediates the neurotoxic effects of the HCV core protein in a neuronal cell line

The involvement of the ERK signaling pathway in the neurotoxic effects mediated by HCV core was further investigated by immunoblot analysis. After 30 min treatment with HCV core, ERK phosphorylation was increased 1.5-fold over control levels, while the levels of total ERK remained unchanged (Fig. 3a, b). Similarly high levels of ERK phosphorylation persisted in neuronal cells treated with HCV core proteins at 24 h (data not shown). Levels of phosphorylated and total Akt, glycogen synthase kinase 3b (GSK3b), and cyclin-dependent kinase-5 (CDK5) were not modified by HCV core treatment either at 30 min or 24 h (data not shown).

To verify the specificity of the effects of HCV core on ERK phosphorylation, a known MEK inhibitor, UO126, was used. In neuronal cells treated with HCV core, pretreatment with U0126 completely blocked the phosphorylation of ERK (Fig. 3a, b), while inhibitors of other signaling pathways had no effects (data not shown).

To determine which transcription factors were activated upon HCV-mediated activation of ERK, immunoblots were performed for E-26 like protein 1 (ELK), janus kinase (JAK), and STAT3. This study showed that after 30 min of HCV core treatment, levels of phospho-STAT3 were elevated compared to controls, while levels of total STAT3 were unchanged (Fig. 3a–c). Levels of ELK and JAK were not affected by HCV (data not shown).



To further confirm by non-pharmacological approach that HCV core activates ERK, siRNA experiments were performed. Pre-treatment of the neuronal cell line with MEK siRNA blocked the effects of the HCV core on ERK phosphorylation, while levels of total ERK remained unchanged (Fig. 3d, e). While neuronal cells treated with HCV core alone displayed a 30–40% reduction in neurite outgrowth and β-tubulin expression by immunoblot, pre-

◀ **Fig. 1** Immunocytochemical and immunoblot analysis of frontal cortex of HCV and HIV cases. **a**–**c** Stained with an antibody against β-tubulin, analyzed in **d**. **e**–**g** Stained with an antibody against HCV core, analyzed in **h**. **i**–**k** Stained with an antibody against GFAP, analyzed in **l**. **m**–**o** Stained with an antibody against pERK, analyzed in **p**. *Scale bar*=20 μm. Immunoblot analysis of frontal cortex samples (**q**) with antibodies against β-tubulin (analyzed in **r**) pERK and tERK (analyzed in **s**). *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *HIV* human immunodeficiency virus, *HIVE* human immunodeficiency virus-encephalitis, *HCV* hepatitis C virus, *GFAP* glial fibrillary acidic protein, *pERK* phospho-extracellular signal-related kinase, *tERK* total extracellular signal-related kinase

treatment for 24 h with MEK siRNA significantly reduced the toxic effects of HCV core (Fig. 3f). Pre-treatment with an inactive compound or with an unrelated siRNA (siControl) had no protective effects on neuronal cells treated with HCV core (Fig. 3d–f).

Consistent with immunoblot, immunocytochemical analysis showed that even 24 h after exposure, levels of phospho-ERK immunoreactivity were increased in neuronal cultures exposed to HCV core compared to vehicle-treated cells (Fig. 4a–f). No significant effects on ERK phosphorylation were detected when the neuronal cell cultures were treated with inactivated HCV core or when neuronal cells treated with HCV core were pre-incubated with U0126 (Fig. 4g–m).

HCV core dysregulates neuronal signaling via the toll-like receptor 2

To investigate the possible receptors involved in ERK activation, analysis of CD81 and TLR2 expression was performed by immunochemical and qRT-PCR methods. CD81 and TLR2, among others, have been shown to be

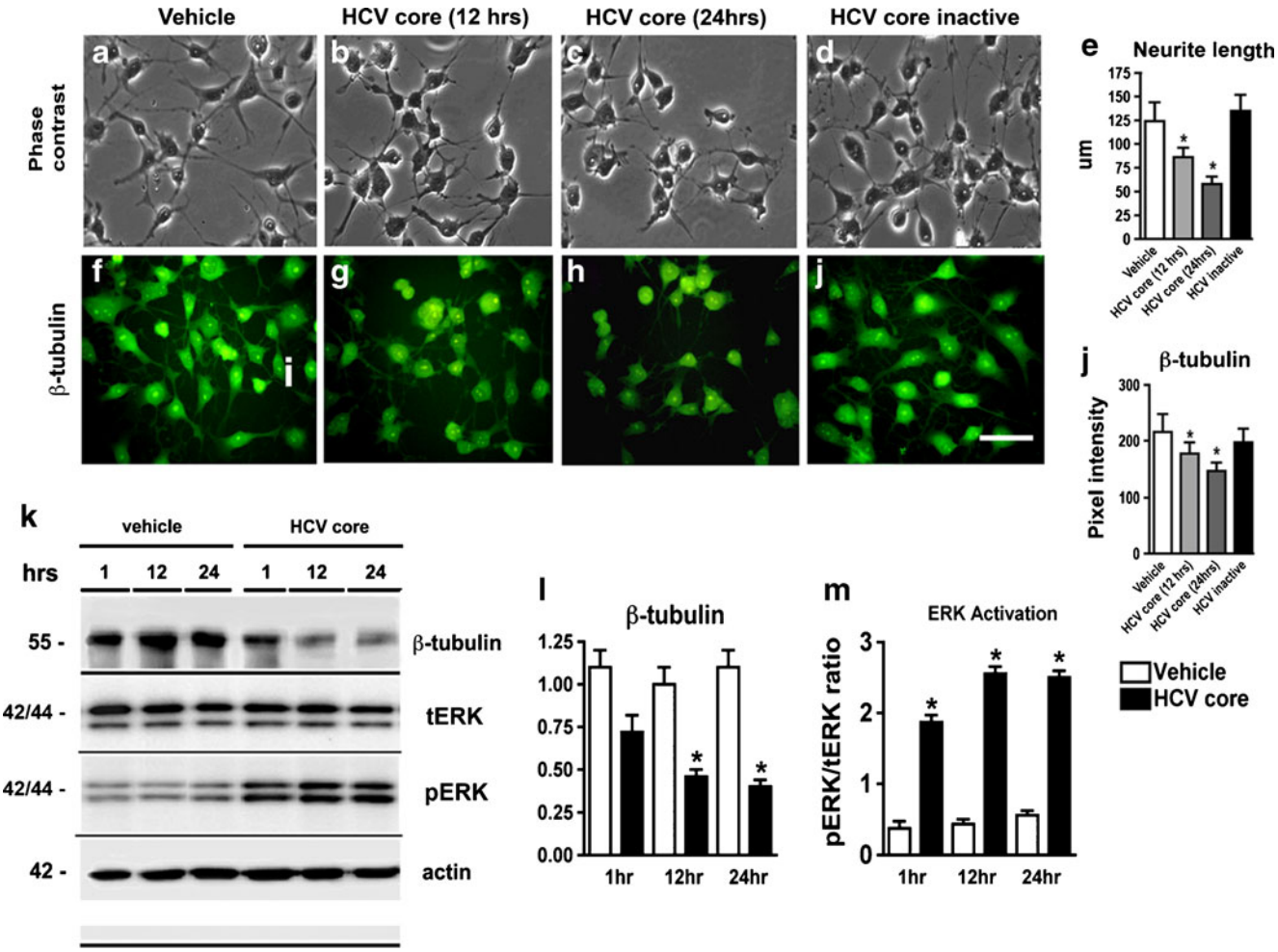


Fig. 2 Time course study of HCV core protein toxicity in neuronal cultures. Phase contrast images of neuronal cultures alone (**a**), HCV core treated at 12 (**b**), 24 h (**c**), and an inactive HCV core-treated sample (**d**). Quantitative analysis of neurite length over time was performed (**e**). Immunocytochemical analysis of β-tubulin on neuronal cultures alone (**f**), with HCV core over time (**g**, **h**), and with an inactive HCV core (**i**), analyzed in (**j**). *Scale bar*=25 μm. Immunoblot analysis (**k**) of β-tubulin, pERK, and tERK expression levels in neuronal cultures exposed to HCV core over time; β-tubulin analyzed in **l**; and ERK activation (pERK/tERK) analyzed in **m**. *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *HCV* hepatitis C virus, *pERK* phospho-extracellular signal-related kinase, *tERK* total extracellular signal-related kinase

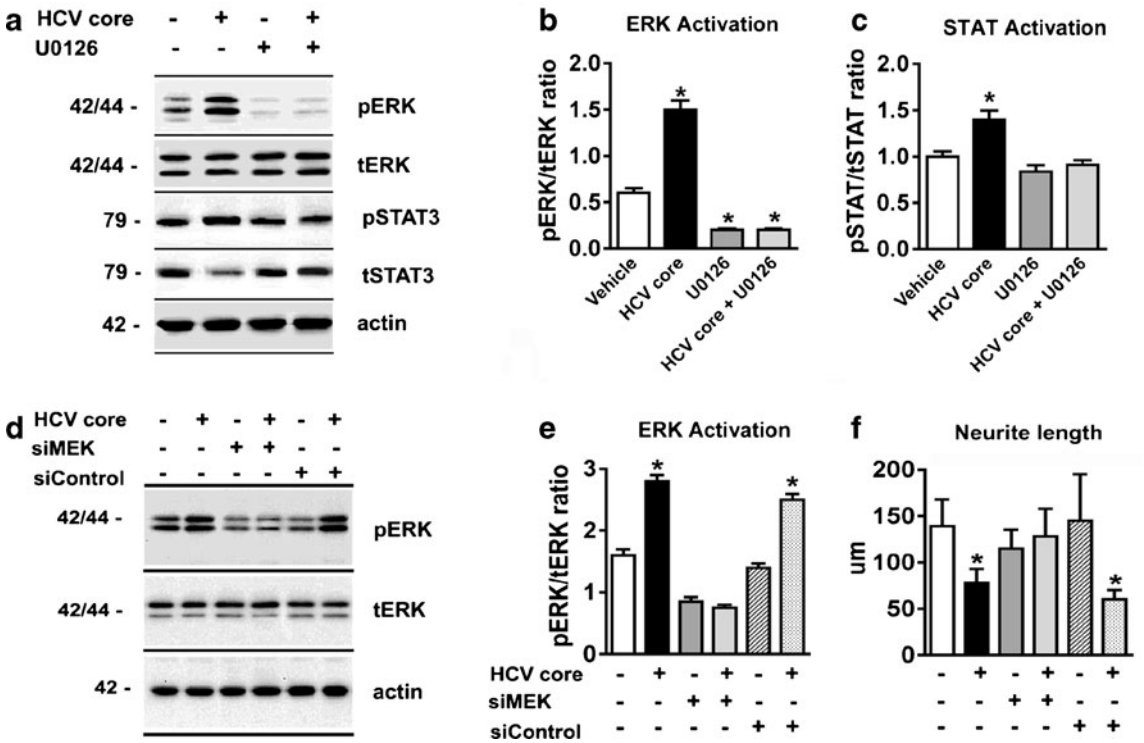


Fig. 3 Effects of HCV core on the ERK signaling pathway. Immunoblot analysis (**a**) of pERK, tERK, pSTAT, and tSTAT in HCV core-treated cultures compared to cultures treated with vehicle and MEK inhibitor UO126. Activation levels of ERK and STAT were determined by comparing ratios of pERK/tERK (**b**) and pSTAT/tSTAT (**c**), respectively. Immunoblot analysis (**d**) of levels of pERK and tERK in cultures treated with and without HCV core and siMEK. Activation levels of ERK were determined by comparing ratios of pERK/tERK (**e**). Neuronal neurite length (**f**) was also measured in these samples. *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *pERK* phospho-extracellular signal-related kinase, *tERK* total extracellular signal-related kinase, *pSTAT* phospho-Signal Transducer and Activator of Transcription, *tSTAT* total Signal Transducer and Activator of Transcription

important HCV receptors (Bartosch and Cosset 2006; Cocquerel et al. 2006; Sato et al. 2007). The presence of both CD81 and TLR2 protein were confirmed in the B103 neuronal cell line by immunoblot and immunohistochemistry (Fig. 5a, d, h). Treatment with HCV core protein for 24 h resulted in an increase of TLR2 immunoreactivity (Fig. 5b, g). No significant effects were seen on neuronal cultures treated with inactive HCV core (Fig. 5c, g). By immunoblot analysis, exposure of the neuronal cell line with the HCV core protein for 30 min resulted in a dose-dependent increase in TLR2 levels of by immunoblot compared to controls (Fig. 5h, i). No significant effects were observed on the levels of CD81 (Fig. 5e–g). Consistent with these observations, analysis by qRT-PCR showed a three- to seven-fold increase mRNA expression of TLR2 in neuronal cells treated with HCV core compared to controls (Fig. 5j).

Receptor neutralization assays were performed to investigate whether TLR2 mediates the effect of HCV core. Previously, we have shown that heparin, a known HCV receptor that is present on neurons, is able to bind to the HCV core protein (Letendre et al. 2007). Therefore, B103 neuronal cells were treated with antibodies against CD81 or TLR2 or heparinase I followed by exposure to HCV core protein. Immunoblot analysis confirmed that pre-treatment with the TLR2 antibody was able to prevent the phosphorylation of ERK in the presence of the HCV core protein (Fig. 5k, l). In contrast, pretreatment with an antibody against CD81 or heparinase I did not block the effects of HCV core or ERK phosphorylation (Fig. 5k, l).

To confirm by an independent method whether TLR2 mediated the effects of HCV core on ERK signaling pathway, RNA silencing experiments were performed. TLR2 levels were lowered by 75% when exposed to siRNA against TLR2 compared to a control siRNA (Fig. 5m, n). Consistent with the antibody neutralization studies, pretreatment with the TLR2 siRNA blocked the phosphorylation of ERK in neuronal cells challenged with the HCV core protein (Fig. 5m, n). In contrast, pretreatment with a control siRNA did not block the effects of HCV core or ERK phosphorylation (Fig. 5m, n).

HCV core protein interactions with TLR2 activate the IRAK signaling complex

To investigate if HCV core signaling alterations via TLR2 might involve IRAK, immunoblot and co-immunoprecipitations

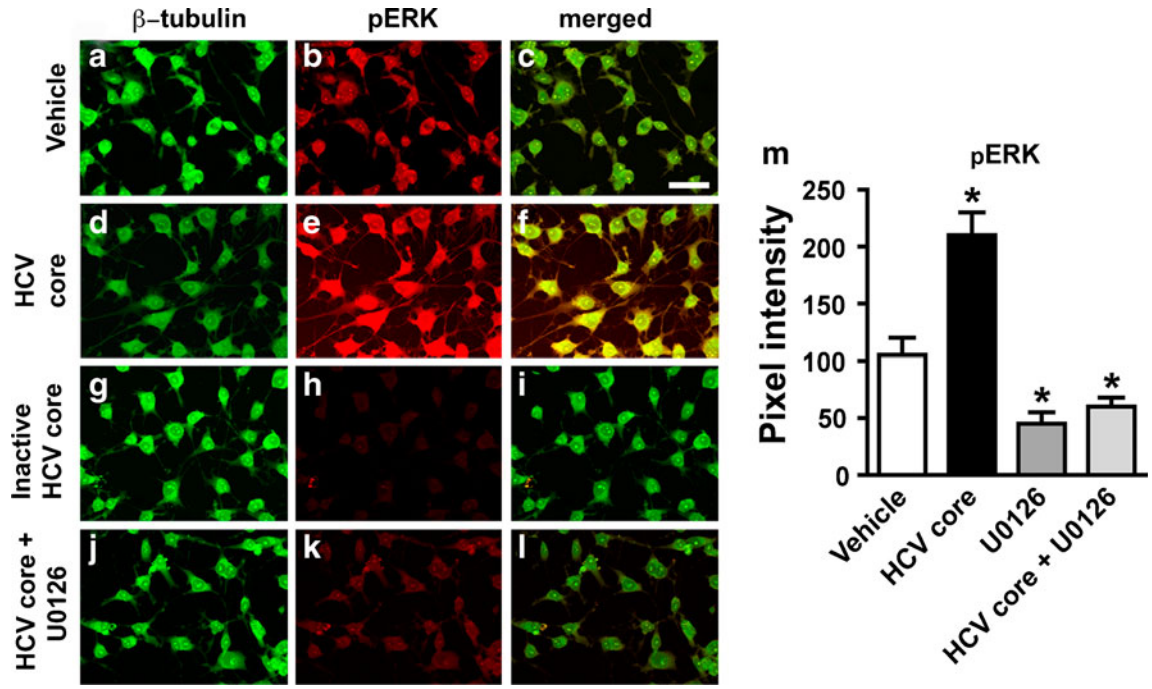


Fig. 4 Effects of HCV core on neuronal cell structure. Double immunocytochemical analysis of β-tubulin and pERK on untreated cultures (**a**–**c**), cultures treated with HCV core (**d**–**f**), inactive HCV core (**g**–**i**), and MEK inhibitor UO126 (**j**–**l**), levels of pERK analyzed in **m**. *Scale bar*=30 μm. *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *HCV* hepatitis C virus, *pERK* phospho-extracellular signal-related kinase, *MEK* mitogen-activated extracellular signal-related kinase kinase

studies were performed. We found that challenging the B103 neuronal cells with HCV core resulted in increased IRAK phosphorylation (Fig. 5o, p). The effects of HCV core on IRAK phosphorylation were blocked by reducing TLR2 levels with a siRNA (Fig. 5o, p). The levels of total IRAK1 and other signaling molecules in this pathway such as TRAF6 and Table 1 were not changed (Fig. 5o, p). Immunoprecipitation studies indicated that HCV core activated TLR2 by recruiting MyD88 (Table 1) and TRAF6 (Supplementary Fig. 1). Taken together, these studies suggest that binding of the HCV core to TLR2 might activate IRAK1 via recruitment of adaptors molecules TRAF6 (Table 1) and MyD88; the ERK signaling pathway is known to be downstream of IRAK1 and TRAF6 in this pathway.

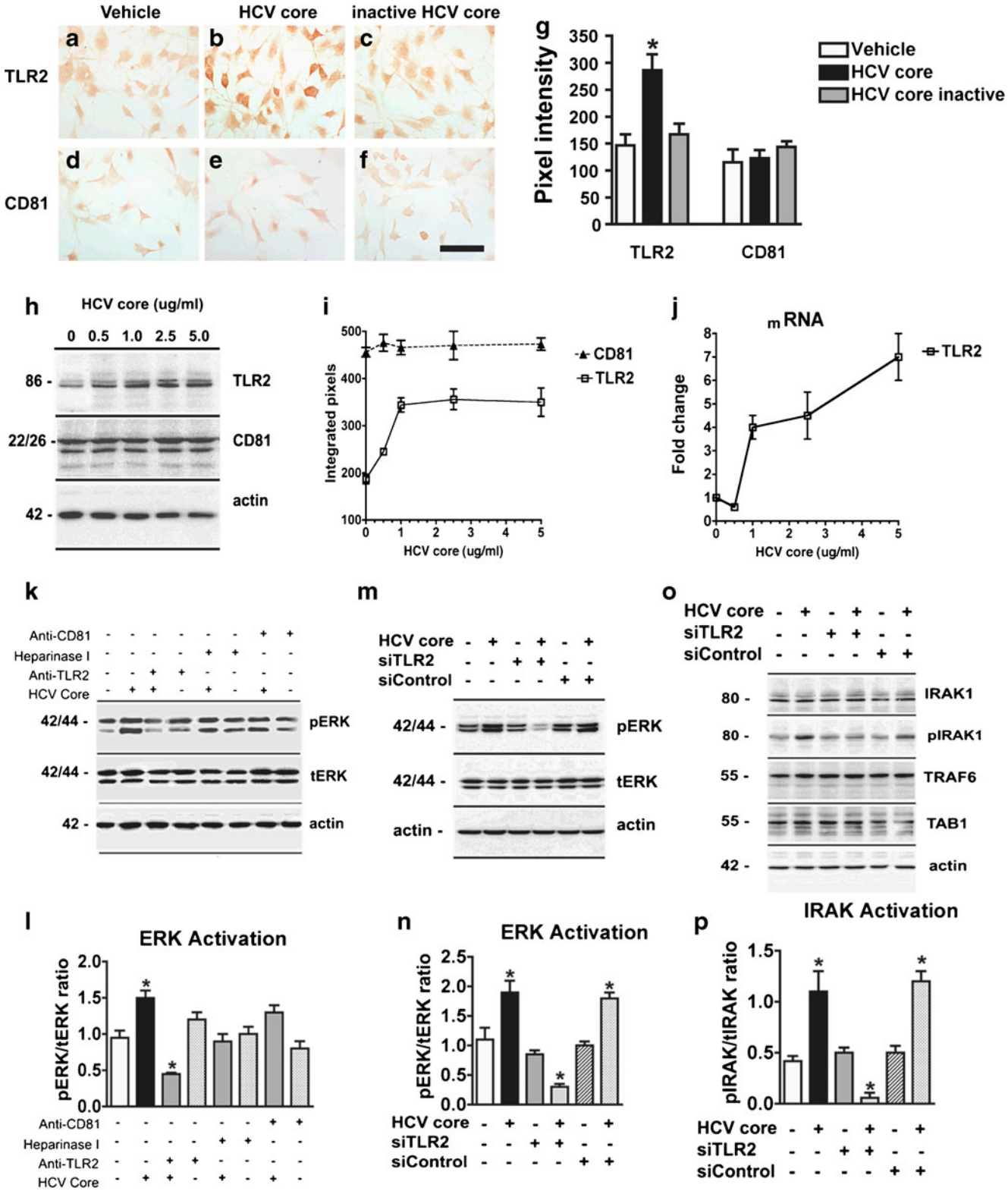
HCV core injection into the hippocampus of mice promotes neurodegeneration and ERK activation

To investigate the effects of the HCV core protein in vivo, the protein was injected into the hippocampus of non-transgenic mice and compared to vehicle control or the heat-inactivated HCV core protein (Fig. 6). One week post-injection, mice were sacrificed, and levels of HCV core protein were analyzed by immunohistochemistry. Extensive core protein immunoreactivity in the cortex and hippocampus of mice injected with HCV core was observed compared to the vehicle controls (Fig. 6a, b). Levels of core protein immunoreactivity were reduced in mice that had been injected with the heat-inactivated core protein (Fig. 6c).

Injection of HCV core resulted in reduced neuronal density in the hippocampus compared to vehicle control (Fig. 6d, e, g); this neuronal loss was not observed in the heat-inactivated control (Fig. 6f, g). Accompanying the neuronal damage, there was intense astrogliosis in the HCV core-injected mice compared to the vehicle control (Fig. 6h, i, k). No astrogliosis was observed in the heat-inactivated control (Fig. 6j, k). Activation of pERK was more prominent in the HCV core-injected mice (Fig. 6m, o) compared to vehicle (Fig. 6l, o) and heat-inactive (Fig. 6n, o) controls.

Discussion

The results from the present study support the hypothesis that the HCV core protein promotes neurotoxicity via the sustained activation of ERK through TLR2 signaling. Similar effects on ERK signaling have been shown in hepatocyte and fibroblast cell lines exposed to HCV core (Erhardt et al. 2002; Hayashi et al. 2000). Under physiological conditions, ERK signaling has been related to cell proliferation and neuronal survival (Wang et al. 2009). In contrast to the transient ERK activation often observed in connection with neurotrophic and survival signals, sus-



tained activation of ERK has been recently associated with non-apoptotic cell death in models of neurodegeneration (Subramaniam and Unsicker 2006). In agreement with these studies, we found that increased phosphorylation of ERK in the brains of patients with HCV infection as well as in mice that received intra-hippocampal HCV core injections was associated with neurodegenerative alterations including extensive dendritic damage accompanied by

◀ **Fig. 5** HCV core effect involves TLR2 and IRAK activation of ERK. Immunocytochemical analysis of TLR2 (**a**–**c**) and CD81 (**d**–**f**) in cultures exposed to HCV core and its inactive form, analyzed in **g**. *Scale bar*=40 μm. Immunoblot analysis (**h**) of TLR2 and CD81 levels at different doses (microgram per milliliter) of HCV core, analyzed in **i**. TLR2 immunoreactivity in HCV core-treated cultures (**j**). A dose-dependent increase of TLR2 was found in cultures exposed to HCV core (**h**, **i**) and is consistent with qRT-PCR data (**j**) demonstrating this dose-dependent three- to seven-fold change. Immunoblot analysis (**k**) of pERK and tERK levels in B103 cells following receptor neutralization assays with antibodies against TLR2, CD81, and heparinase I. Activation level of ERK was determined by comparing ratios of pERK/tERK (**l**). Receptor neutralization assays were confirmed by siRNA against TLR2. **m**, **o** ERK and IRAK activation analyzed in **n** and **p**, respectively. *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *HCV* hepatitis C virus, *TLR2* toll-like receptor 2, *IRAK* interleukin-1-receptor-associated kinase, *pERK* phospho-extracellular signal-related kinase, *tERK* total extracellular signal-related kinase, *siRNA* silencing RNA, *qRT-PCR* quantitative real-time polymerase chain reaction

astrogliosis. Moreover, supporting a role for sustained activation of ERK in neurodegeneration, HCV core toxicity was blocked by ERK inhibitors or by silencing the MEK-ERK signaling pathway in neuronal cultures.

Toll-like receptor pathways have been shown previously to activate the ERK signaling pathway (Gottipati et al. 2008). Here, the HCV core protein led to ERK activation through TLR2 signaling. Consistent with previous studies (Nagyoszi et al. 2010; Okun et al. 2010), we observe TLR2 expression in our neuronal cell line. It has been shown that the HCV core protein can activate the TLR2 receptor (Dolganiuc et al. 2004). In agreement with this effect, the present study demonstrated that the effects of HCV core on ERK activation and neurotoxicity can be blocked by neutralizing TLR2 with antibodies or siRNA. Moreover, upon exposure to HCV core, TLR2 expression levels increased in a dose-dependent manner. Therefore, TLR2 recruitment and activation plays a central role in modulating the HCV core cellular signaling that leads to neuronal damage.

Stimulation of TLR signaling pathways by HCV proteins initiate from the cytoplasmic TIR domain of TLRs (Akira and Sato 2003; Dolganiuc et al. 2004; Kirschning and Schumann 2002). The adapter molecule MyD88 associates with the TIR domain, and upon activation, MyD88 recruits IRAK (Dolganiuc et al. 2004; Kopp and

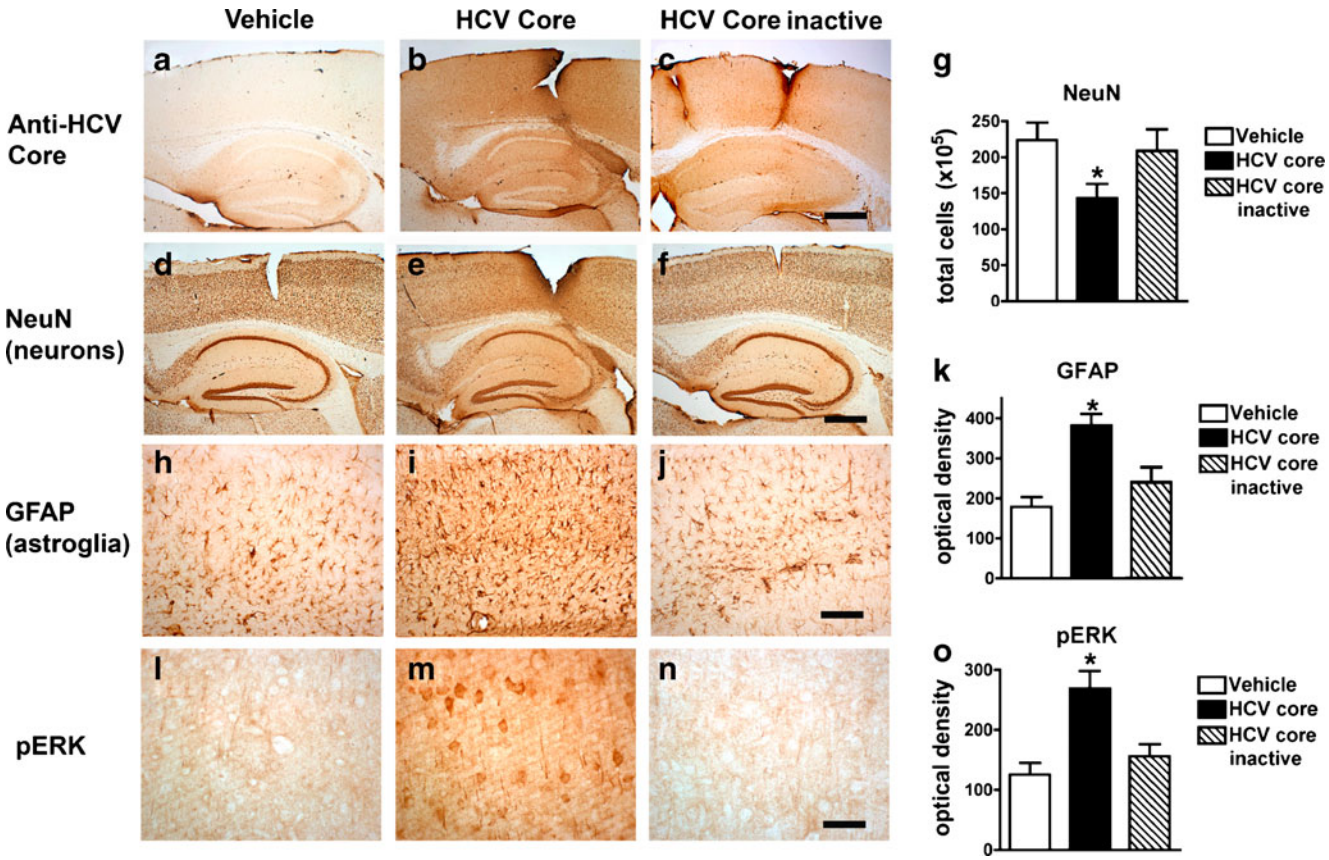


Fig. 6 In vivo effects of HCV core. Immunohistochemical analysis of hippocampus sections of non-transgenic mice injected with vehicle, HCV core or inactive HCV core for expression of HCV core protein (**a**–**c**), NeuN (**d**–**g**, analyzed in **g**), GFAP (**h**–**j**, analyzed in **k**), and pERK (**l**–**n**, analyzed in **o**). *Scale bar*=25 μM. *Asterisk* indicates $p<0.05$ one-way ANOVA with post hoc Fisher. *HCV* hepatitis C virus, *GFAP* glial fibrillary acidic protein, *pERK* phospho-extracellular signal-related kinase

Medzhitov 2003; Takeda and Akira 2004). IRAK is activated by phosphorylation which in turn associates with TRAF6, leading to the activation of distinct signaling pathways, including components of the MAPK pathway such as ERK and JNK (Akira and Takeda 2004; Gottipati et al. 2008). Consistent with this pathway, our data in neuronal systems demonstrate that via TLR2, HCV core activates IRAK1, which is accompanied by recruitment of MyD88 and TRAF6. Downstream activation involved sustained phosphorylation of ERK resulting which may have played a role in the HCV core-induced TLR2-mediated signaling activation of STAT3; however, more studies are necessary to fully understand the interactions between STAT3 activation and ERK signaling in relation to HCV core and TLR2.

TLR ligands induce transcription factors such as interferon regulatory factors and STATs (Hu et al. 2007). For example, the activation of the STAT3 via TLR and IRAK1 in macrophages plays an important role in immune regulation responses (Huang et al. 2004). This pathway has also been shown to be involved in sustained stimulation with HCV core in NIH-3T3 cells leading to cellular transformation (Yoshida et al. 2002). HCV proteins can act on the STAT signaling via direct and indirect mechanisms. In our neuronal cell system, the effects were mediated through IRAK1 and sustained ERK activation. In other studies, HCV core effects on STAT3 may depend on decreasing the dephosphorylation of this transcription factor (Yoshida et al. 2002). In addition, other HCV proteins such as NS5a have been shown to activate STAT3 via JAK1 in hepatoma cells (Sarcar et al. 2004). In our neuronal cell system, HCV core neurotoxicity manifested as reduced neurite outgrowth and cytoskeletal retraction. This is of interest as STAT3 activation has also been shown to influence tubulin expression and neuronal cytoskeleton organization (Ng et al. 2006; Yeh et al. 2008).

In recent years, increasing interest in the role of HCV trafficking to the CNS (Laskus et al. 2005; Letendre et al. 2007) and cognitive impairment (Acharya and Pacheco 2008; Hilsabeck et al. 2005; Letendre et al. 2005) has grown, in part because of the magnitude of the HCV epidemic but also because the frequent occurrence of HCV and HIV co-infection (Hilsabeck et al. 2005; Letendre et al. 2005).

Observations of the impact of HCV on the CNS have emerged over the past decade and have recently been reviewed (Forton et al. 2004b; Weissenborn et al. 2009). Many studies have identified that HCV disease is associated with cognitive and mood disorders and that these effects can be distinct from those that occur with liver disease alone (Letendre et al. 2005). The published findings include evidence that viral genotypes derived from brain tissue and blood-derived mononuclear cells diverge from those in other tissues (Forton et al. 2004a; Laskus et al. 2005), that viral proteins, such as core protein, are present in glial cells (Letendre et al. 2007), and that HCV disease is associated with neuroimaging abnormalities (Forton et al. 2001) and neurocognitive impairment (Morgello et al. 2005; Richardson et al. 2005). Acute HCV infection has also been increasingly recognized (Fierer et al. 2008) and has been reported to be associated with encephalitis (Fujita et al. 1999; Seifert et al. 2008) or myelitis (Annunziata et al. 2005). However, more remains to be investigated about how HCV injures the brain. Moreover, HCV is a common pathogen in patients with intravenous drug use displaying neuronal degeneration (Gonzalez and Cherner 2008; Martin-Thormeyer and Paul 2009), further highlighting the CNS-related roles of HCV.

Our neuropathological studies in the brains of patients with HCV, as well as in a murine model that received intrahippocampal injections of HCV core, support the notion that this HCV protein activates ERK and promotes cytoskeletal pathology with dendritic shortening. Thus, the HCV core might compromise neuronal integrity by damaging axons and dendrites in the absence of overt neuronal loss. The core protein alone has been shown to have pathogenic effects without the presence of the whole virus (Kang et al. 2009; McLauchlan 2000).

In conclusion, the present study illustrates that there is a direct association between the activation of TLR2 signaling by HCV core and the activation of ERK. Without affecting cell survival, the core protein had an effect on the overall structure and integrity of the neurons. This may play a role in the overall viral pathogenesis of the HCV itself and may play an important role in the HCV neuropathogenesis clinically manifesting as cognitive and behavioral alterations. The results of this study suggest that neurotoxicity is the result of the sustained activation of ERK/STAT3 by the HCV core protein and is mediated by the TLR2-IRAK1 signaling pathway. Overall, this study identifies novel targets that can be utilized to develop neuroprotective treatments for HCV infection and provides new insights into the understanding of HCVand HIV co-infection.

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