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HIV-1, HCV and Alcohol in the CNS: Potential Interactions and Effects on Neuroinflammation

Peter S. Silverstein*, Santosh Kumar, and Anil Kumar

Division of Pharmacology and Toxicology, School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO 64108, USA

Abstract

Approximately 25% of the HIV-1 positive population is also infected with HCV. The effects of alcohol on HIV-1 or HCV infection have been a research topic of interest due to the high prevalence of alcohol use in these infected patient populations. Although it has long been known that HIV-1 infects the brain, it has only been a little more than a decade since HCV infection of the CNS has been characterized. Both viruses are capable of infecting and replicating in microglia and increasing the expression of proinflammatory cytokines and chemokines, including IL-6 and IL-8. Investigations focusing on the effects of HIV-1, HCV or alcohol on neuroinflammation have demonstrated that these agents are capable of acting through overlapping signaling pathways, including MAPK signaling molecules. In addition, HIV-1, HCV and alcohol have been demonstrated to increase permeability of the blood-brain barrier. Patients infected with either HIV-1 or HCV, or those who use alcohol, exhibit metabolic abnormalities in the CNS that result in altered levels of n-acetyl aspartate, choline and creatine in various regions of the brain. Treatment of HIV/HCV co-infection in alcohol users is complicated by drug-drug interactions, as well as the effects of alcohol on drug metabolism. The drug-drug interactions between the antiretrovirals and the antivirals, as well as the effects of alcohol on drug metabolism, complicate existing models of CNS penetration, making it difficult to assess the efficacy of treatment on CNS infection.

Keywords

AIDS; alcohol; CNS; HCV; HIV-1; neuroinflammation

INTRODUCTION

HIV-1 infection has been a persistent problem for over 3 decades. Despite major advances in antiretroviral therapy and efforts to stem the transmission of the virus, at the end of 2011 it was estimated that there were 34 million people in the world living with AIDS (UN AIDS

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*Address correspondence to this author at the Division of Pharmacology and Toxicology, School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO 64108, USA; Tel: (816) 235-1999; Fax: (816) 235-1776; Silversteinp@umkc.edu.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

report). Approximately 25% of the HIV+ population is also infected with HCV [1, 2]. Alcohol use has been associated with an increase in risky sexual behaviors in both HIV-infected and HIV-uninfected populations [3, 4], and higher rates of HCV infection have also been associated with alcohol use [5, 6]. Thus, alcohol use plays a role in the transmission of, and the pathology associated with, both HIV-1 and HCV. Although HIV-1 infection of the CNS has been studied for a number of years, it is only recently that HCV infection of the CNS has been a topic of investigation. Clearly, one of the major organ systems subject to the pathological effects of HIV-1, HCV and alcohol is the central nervous system (CNS).

Our goal is to provide some insight into potential interactions between HCV, HIV-1 and alcohol in the CNS. It is through overlapping mechanisms and pathways that alcohol, HCV, and HIV have the greatest potential for interaction. Compared with the CNS effects of HCV, the effects of alcohol, as well as the effects of HIV-1 on the CNS have been more extensively investigated and reviewed. Thus, for each pathway or mechanism of potential interaction between these three agents, we first provide a review of the CNS effects of HCV *via* the particular mechanism/pathway, followed by a brief review of the CNS effects of alcohol and HIV that are mediated through the same mechanism/pathway. Finally, we summarize the effects of alcohol on HIV-1 and HCV infection of the CNS and provide some insight as to future directions of research in this area.

HCV IN THE CNS

In one early study investigating the potential for HCV infection of the CNS, negative strand HCV RNA was detected in CNS specimens obtained at autopsy in 3 out of 6 patients. This study provided the first evidence that HCV could replicate in the CNS. Further, in two of the three patients whose CNS specimens were positive for the negative strand replicative intermediate, sequence analysis demonstrated that the genotype present in the CNS was distinct from that present in the serum [7]. In a subsequent publication from this group, HCV sequences from CSF were detected and sequences obtained from PBMC were compared with those obtained from CSF [8]. In this study, HCV RNA was detected in 8/13 CSF cell pellets, and two of the CSF cell pellets contained negative strand HCV RNA which indicated active HCV replication. In 4 patients, there were differences between the HCV genotypes isolated from the serum and PBMC, and in these instances the HCV identified in the CSF was more closely related to that identified in the PBMC. This study provided further evidence that HCV may enter the brain through trafficking of infected leukocytes.

In order to identify the regions of the brain and the types of cells infected with HCV in patients who were co-infected with HIV, Letendre *et al.* [9] utilized post-mortem CNS samples from both HCV seropositive and HCV seronegative patients. All HCV seropositive patients had HCV RNA in the brain. The frontal cortex, basal ganglia and white matter were positive for PCR amplification of RNA, but the cerebellum, brainstem, occipital cortex and thalamus were negative. Using a polyclonal antibody against NS5A, the same regions that were PCR positive were also immunoreactive. A monoclonal antibody directed against the HCV core protein demonstrated that this protein was also prominently expressed in the CNS of these patients. Further, approximately 80% of the HCV immunoreactive cells were

astrocytes and about 15% of the cells that were positive for NS5A were cells of the monocyte/macrophage lineage as shown by co-localization with the CD68 marker.

Although previous reports had demonstrated that cells in the CNS were infected by HCV, the identity of these cells in HIV negative patients had not been determined. In order to address this question HCV+ cells in the brains of autopsied HIV-patients were analyzed by laser capture microscopy and found to be CD68+ [10]. While it was also noted that the presence of HCV in CD68+ cells was more common in patients co-infected with HIV than in patients only infected with HCV, the small sample size necessitates that this conclusion be viewed cautiously. Furthermore, the presence of negative strand HCV RNA was only detected in HIV+ patients, indicating the presence of an actively replicating virus population. Astrocytes (GFAP+ cells) were also found to contain viral RNA but not negative strand RNA, indicating a lack of evidence for viral replication in these cells.

Although the sample size was small due to the report being a pilot study, one investigation focused on some of the clinical and neuropsychological effects of HCV in patients who were infected with HIV-1 as well as in a group of patients only infected with HCV [11]. Although 6/10 subjects co-infected with HIV also had HCV sequences in the brain, in the monoinfected HCV group virus was detected in the brain in only 1 out of 3 patients. Of the 7 co-infected patients for which pre-mortem CSF viral loads were available, HIV was detectable in the CSF of all patients in which HCV was detected in the brain (4/4), while in the patients in which HCV was not detected in the brain, HIV RNA was undetectable in all CSF samples (3/3). Neuropsychological assessments of HIV-infected patients also suggested a pattern in which patients infected with only HIV-1 scored higher than patients coinfecting with both viruses but in whom HCV was not detected in the brain. Both of these former groups scored higher than HIV-infected patients in whom HCV was detected in the brain. In summary, HIV presence in the CSF was correlated with the presence of HCV in the brain and the presence of HCV in the brain was predictive of neuropsychological impairment.

INFLAMMATION AND NEUROTOXICITY

HCV and Neuroinflammation

A subsequent study utilized laser capture microscopy combined with real-time PCR to determine the levels of cytokines and other inflammatory mediators in infected CD68+ cells [12]. In this case, all autopsied patients were negative for HIV infection and comparisons were made between patients who were HCV positive and those who were HCV negative. Compared to those uninfected with HCV, samples of CD68+ cells obtained from the CNS of HCV positive patients showed increased levels of IL-1 α , IL-1 β , TNF- α , IL-12 and IL-18, along with increases in the chemokines IL-8, IL-16 and IP-10. Thus, like HIV, HCV may cause neuroinflammation through increased cytokine production and the neuroinflammation may be sustained by the chemoattractant effects of increased chemokines. However, as noted by the authors, HCV infection of the CNS does not result in dementia as it does in HIV infection.

Specimens obtained post-mortem, as well as *in vitro* and *in vivo* models, were used to demonstrate the neurotoxic effects of HCV core protein [13]. The specimens obtained at

autopsy included brain regions from HIV+ patients who were not infected with HCV and an HIV-infected patient suffering from HAD and infected with HCV. In the HCV-infected patient, positive strand RNA was detected in the white matter, basal ganglia and cortex. However, negative strand HCV RNA was only detected in the white matter and basal ganglia but not in the cortex. These results are essentially in agreement with the results reported by Radkowski *et al.* [7] and Letendre *et al.* [9]. Human fetal astrocytes and human fetal microglia were also found to be permissive for HCV. The HCV core protein was capable of inducing inflammatory cytokines in both human fetal astrocytes and human fetal microglial cells. Furthermore, HCV core protein was found to induce neurotoxic products in human fetal microglial cells when supernatants from these cells that were exposed to HCV core protein [13] were used to treat neurons. However, treatment of human fetal astrocytes failed to induce secreted neurotoxic substances in these cells. In microglial cells infected with HIV-1, exposure to HCV core protein resulted in levels of IL-6, TNF- α , CXCL8 and CXCL10 that were significantly elevated above those levels observed in infected cells that were not exposed to HCV core protein [13].

To characterize the pathways involved in HCV core protein-related neurotoxicity, frontal cortex samples were obtained post-mortem from patients who were positive for HIV or HCV [14]. Also included in the samples were patients with HIV encephalitis (HIVE) who were HCV seronegative. Compared to either the control group or the HIVE group, the patients positive for HCV exhibited decreased levels of β -tubulin and increased levels of phosphorylated ERK and astrogliosis as determined by GFAP staining. Rat neuronal cultures exposed to HCV core protein exhibited similar characteristics, i.e. decreased levels of β -tubulin and increased levels of ERK activation. In addition, morphological effects of HCV core protein, specifically a time-dependent decrease in neurite length were also evident in the cultures treated with active core protein; in cultures treated with inactivated core protein no such differences were evident. In addition to ERK activation, treatment of neurons resulted in increased levels of activated STAT3. Use of the MEK inhibitor U0126 in conjunction with treatment by HCV core protein resulted in an abrogation of the ability of the core protein to activate ERK or STAT3. ERK activation, as well as the inhibitory effect of HCV core on neurite length, was abrogated through the use of siRNA targeted against MEK. TLR2 was also shown to be involved in HCV core-mediated activation of ERK. This was demonstrated by pre-treating neurons with antibody to TLR2 which abrogated activation of ERK by HCV core protein. In addition, knockdown of TLR2 using siRNA also abrogated ERK activation as well as the activation of IRAK by HCV core protein. Injection of HCV core into the hippocampus of wild-type mice was also shown to cause ERK activation, astrogliosis and a reduction of neuronal density. These effects were not observed in animals injected with heat-inactivated core protein. Taken together these results indicate that HCV core protein acts through TLR2 to activate ERK/STAT3. Further, treatment of neurons with HCV core protein results in sustained activation of ERK, a situation associated with neurotoxicity [15].

HIV and Neuroinflammation

Increased expression of inflammatory cytokines in the CNS mediated by HIV-1 infection is thought to be a major contributor to HIV-associated neurocognitive disorder (HAND). In an

initial report of HIV-associated neurotoxicity Gendelman and colleagues utilized a coculture system involving astroglia and monocytes that were either infected, or uninfected with HIV-1 [16]. When cultured with astroglia, the monocytes proved to be toxic to neurons. Subsequent studies produced evidence that resulted in the model that neurotoxicity is caused by either direct or indirect effects of HIV-1 infection in the CNS. Direct neurotoxicity may be caused by interaction of viral proteins (e.g. Tat, gp120) with neurons, while indirect toxicity is due to inflammation, oxidative stress and the secretion of soluble neurotoxins from HIV-infected microglia. While an extensive review of this area is beyond the scope of this article, there have been a number of excellent reviews on HIV neurotoxicity in recent years (reviewed in [17–20]).

As described above, one of the pathways that mediate neurotoxicity of the HCV core protein is signaling through the ERK pathway. The ERK1/2 mitogen-activated protein kinase pathway has been demonstrated to mediate some of the pro-inflammatory effects of HIV-1 Tat. Using primary human microglial cells, it was shown that treatment of the cells with Tat resulted in ERK activation and an increase in the expression of several chemokines [21]. Treatment with a specific ERK 1/2 inhibitor prior to Tat exposure substantially inhibited the induction of CCL2, CCL4 and CXCL10. The increased expression of these chemokines, especially CCL2, may serve to stimulate additional transmigration of monocytes across the blood-brain barrier, thereby exacerbating inflammation. Furthermore, a recent study using rat hippocampal slices indicated that, along with induction of MCP-1, induction of TNF- α by Tat was also mediated through the ERK1/2 pathway [22]. In addition to increasing MCP-1 in microglial cells, Tat has also been demonstrated to alter the expression of ZO-1 and claudin-5 in brain endothelial cells (see below). Thus, Tat stimulates the ERK1/2 pathway in both microglial cells and brain microvascular endothelial cells. By doing so, it increases the expression of a chemotactic protein, CCL2, which will tend to increase monocyte infiltration across the BBB, and it will also increase the permeability of the BBB through altering tight junction proteins.

Ethanol and Neuroinflammation

The effects of alcohol abuse on inflammation in the CNS have also been well studied. In a series of papers Guerri and colleagues have investigated the effects of alcohol on the CNS and the role of TLR4 in mediating these effects [23, 24]. This group has demonstrated that acute exposure of astrocytes to ethanol induces the phosphorylation of IRAK and the three members of the mitogen-activated protein kinase family, ERK1/2, p38, and JNK [23]. They also demonstrated that the activation of these pathways could be blocked by the addition of antibodies to either IL-1R1 or TLR4. In a subsequent investigation utilizing TLR4 KO mice, it was demonstrated that TLR4 was critical for alcohol-mediated activation of signaling pathways, including ERK1/2. The KO mice also did not show increased TNF- α , IL-1 β or IL-6 in response to ethanol treatment [24]. In a recent study from this group it was demonstrated that ethanol treatment results in increased expression of TLR2 and TLR4 in microglial cells. In addition, ethanol also induced colocalization of TLR2 and TLR4 in these cells as shown by immunostaining and co-immunoprecipitation assays [25]. Finally, ethanol treatment did not increase the phosphorylation of the MAPK signaling molecules p38, ERK1/2, and JNK in the astrocytes of either TLR2 $^{-/-}$ or TLR4 $^{-/-}$ mice, whereas the levels

of the phosphorylated forms of these molecules were significantly increased by treatment of WT astrocytes with ethanol.

In reviewing the literature it is clear that there are several mutual pathways that are affected by HCV, HIV and alcohol. The most striking examples of this are the ERK1/2 and TLR signaling pathways. To date, there are no reports of the effects of these three agents on the CNS as determined by either *in vitro* or *in vivo* models. However, one should also keep in mind that the initial findings regarding the effects of HCV on inflammation are recent and further investigations in this area are likely in the near future.

BLOOD-BRAIN BARRIER (BBB)

HCV and the BBB

In an effort to determine whether cognitive abnormalities associated with HCV infection are an indirect result of hepatic infection or due to HCV infection of the CNS, postmortem brain specimens were examined for expression of the known HCV receptors, CD81, SR-B1, claudin-1, occludin and LDL-R [26]. Brain microvascular endothelial cells expressed all these markers while astrocytes expressed CD81 and neurons expressed CD81, claudin-1 and occludin. Two human brain endothelial cell lines were shown to be susceptible to infection with HCV-dependent glycoprotein pseudoparticles, and infection by the pseudoparticles could be inhibited by the addition of antibodies to CD81, SR-B1 or claudin-1. The brain endothelial cell lines were also demonstrated to be permissive for chimeric HCV constructs which could also be inhibited by antibodies to the viral receptors as well as to protease and polymerase inhibitors. These chimeric viruses could replicate in the human endothelial cell lines as well as spread to other cells. However, replication levels were lower than in the Huh-7 cell line. Infection of the brain endothelial cell line with the chimeric virus also increased paracellular permeability in cell monolayers and increased the level of apoptosis as evidenced by an increase in TUNEL staining.

Ethanol and the BBB

Much of our understanding of the effects of ethanol on the BBB has come through the use of *in vitro* models of the BBB that utilize either bovine brain microvascular endothelial cells (BBMEC) or human brain microvascular endothelial cells (BMVEC). In a system that utilized BBMEC in conjunction with human monocytes Haorah *et al.* [27] demonstrated that ethanol caused an increase in phosphorylation of several proteins including occludin, claudin-5, myosin light chain kinase (MLCK), along with an increase in trafficking of monocytes across the endothelial cell barrier. There was also an overall decrease in the expression levels of claudin-5 and occludin. Interestingly, the effects of ethanol could be reversed by treatment with an inhibitor of MLCK or pre-treatment with an inhibitor of ethanol metabolism. This suggested that MLCK mediated the effects of ethanol on BBB permeability. Subsequently it was demonstrated that ROS was induced by ethanol treatment of BMVEC [28]. Treatment of cells with ROS donors resulted in increased phosphorylation of MLCK, suggesting that the increased permeability of the BBB was due to an increase in oxidative stress. Ethanol has also been shown to increase BBB permeability through increased expression of MMP-1, MMP-2 and MMP-9 and decreasing the expression of

TIMP-1 and TIMP-2 [29]. The effects of ethanol on these proteins could be reversed by treating the cells with either MMP inhibitors or inhibitors of ethanol metabolism.

HIV-1 and the BBB

The HIV-1 protein Tat has been demonstrated to have a deleterious effect on the BBB through its effects on the expression of two tight junction proteins, claudin-5 and ZO-1. In one investigation, Tat was injected into the hippocampus of mice and it was observed that ZO-1 expression was decreased and accompanied by an infiltration of inflammatory cells [30]. Interestingly, the effects of Tat on ZO-1 expression were significantly reduced when the mice were pre-treated with an i.p. injection of the ERK1/2 inhibitor U0126. Using a cell culture model consisting of primary brain microvascular endothelial cells isolated from rats, Tat was demonstrated to have a similar effect on claudin-5, another tight junction protein that is important for maintenance of BBB integrity [31]. As was the case with ZO-1, pre-treatment of the cells with U0126 significantly reduced the effect of Tat on claudin-5 expression. Taken together, these experiments suggest that Tat, which can be secreted from HIV-1 infected microglia, acts to reduce the integrity of the blood-brain barrier.

In the literature presented above it is clear that the BBB may play a significant role in mediating the effects of alcohol use in the context of HIV/HCV coinfection. All three agents increase the permeability of the BBB. When viewed in the context of some of the limited data from the clinical studies, it is quite possible that the initial CNS infection with HIV-1 increases the probability of subsequent infection with HCV. Clearly, there is substantial potential for the effects of one of these agents to be exacerbated by the actions of the other two. The effects of alcohol on the BBB are of paramount importance, as they may affect the entry of HCV and HIV-1 into the CNS.

METABOLIC ABNORMALITIES

HCV

Changes in brain metabolites have been used as markers of the CNS effects of many pathological conditions. In reviewing studies of the CNS effects of alcohol or infection with either HIV-1 or HCV, four metabolites are commonly used as indicators of CNS effects. N-acetyl-aspartate (NAA) is used as a marker of neuronal density and decrease is indicative of neuronal damage. Choline (Cho) is a marker for cellular turnover and is found in higher concentrations in white matter than gray matter. Myo-inositol (MI) is used as a marker for glial cells and higher levels indicate glial cell activation. Creatine (Cr) is a marker for cellular energy metabolites and was thought to be relatively stable, but has been subsequently shown to be variable under some conditions [32, 33].

Forton and colleagues [34] provided some of the first evidence for an impact of Hepatitis C virus on brain metabolites in a report in which they noted elevated Cho/Cr ratios in the white matter and basal ganglia of patients with HCV. These patients suffered from mild HCV infection with no evidence of cirrhosis or fibrosis. In a study that investigated the CNS metabolic differences in HCV-infected patients suffering from either mild or moderate fatigue it was found that an increased level of fatigue correlated with poorer performance on the psychometric tests [35]. Furthermore, patients with either mild or moderate fatigue had

significantly reduced NAA/Cr levels in their cerebral cortex compared with healthy controls, as determined by magnetic resonance spectroscopy (MRS). However, there were no significant differences between the groups in either the Cho/Cr or MI/Cr ratios of the four brain areas measured.

A group of HCV-infected patients from which individuals with numerous risk factors had been excluded, as well as an uninfected control group [36], were assessed for neurocognitive problems and metabolite abnormalities in the CNS. Excluded from this study were those patients with conditions that might confound the results such as a recent history of injection drug use, substance abuse disorder or depression. Compared with the uninfected control group, the HCV-infected patients exhibited significantly increased CHO and reduced NAA in the central white matter. These results are of particular significance, because this study reported the absolute concentrations of these metabolites rather than ratios normalized to creatine.

Bokemeyer *et al.* [37] utilized MRS to determine the levels of creatine, choline, myo-inositol and N-acetyl aspartyl glutamate + N-acetyl aspartate (NN) in patients infected with HCV, but who only exhibited mild liver disease. The patients were negative for HIV-1, as well as other disorders. Compared with uninfected controls, levels of choline and glutamate + glutamine were higher in white matter of the HCV-infected patients. In the basal ganglia, HCV-infected patients exhibited higher levels of choline, creatine, and NN than did the uninfected controls. These results suggest metabolite abnormalities that are usually attributed to activation of glial cells and are associated with neuroinflammation. The patients also exhibited significant differences from the control group in cognitive tests that measured such parameters as alertness and verbal learning ability. However, one of the more interesting results concerned the Fatigue Impact Score, which was negatively correlated with metabolite levels in the white matter and basal ganglia. The authors hypothesized that a mild neuroinflammatory response may lead to cerebral dysfunction, whereas a more pronounced inflammatory response also results in a setting that promotes neurogenesis and repair. N-acetyl aspartate, a component of NN, is a marker that is normally reduced in situations reflecting neuronal loss. Thus, the authors proposed that the increase in NN suggests the presence of compensatory neurogenesis in response to a more pronounced inflammatory response.

An *in vivo* study that utilized positron emission tomography (PET) and MRS demonstrated that microglial cells were activated in patients with mild chronic hepatitis C infection [38]. It is important to note that these patients were not on any antiviral therapy and were HIV negative. The binding potential of PK11195, an indicator of microglial activation, was significantly increased in the caudate nucleus of HCV patients as compared with uninfected controls. In addition, the ratios of myo-inositol/creatine and choline/creatine were also significantly elevated in basal ganglia of infected patients. Importantly, elevated levels of myo-inositol and choline have been associated with neuroinflammatory conditions, including those involving demyelination and neuroinflammation associated with HIV. A subsequent investigation of acute HCV infection in the context of coinfection with HIV-1 found an increased ratio of myo-inositol/creatine in the basal ganglia, but failed to detect an increase in microglial activation using the PK11195 ligand [39]. However, it should be noted

that the previous results describing increased levels of microglial activation were from patients with chronic HCV infection [38], while the patients in this study were acutely infected with a mean elapsed time of 21 weeks from a negative HCV RNA test [39].

The impact of HCV infection on brain metabolites has led to several investigations that have focused on the effects of viral clearance on CNS metabolic abnormalities. In a group of patients that included both sustained virological responders (SVR) and non-responders, no differences in cognitive measures were seen 6 months after discontinuation of antiviral therapy. Furthermore, no correlation could be seen between metabolite ratios and neurocognitive tests. However, as noted by the authors this initial study had the drawback of a small sample size [40]. As the authors also noted, the turnover of cerebral cells is slow, which suggests that the benefits of viral clearance may only be apparent after longer periods post-treatment.

A subsequent investigation focused on the effects of viral clearance on cerebral metabolites and neurocognitive tests following antiviral therapy with pegylated IFN and ribavirin [41]. In this case, the final time point was 12 weeks following discontinuation of antiviral therapy. At this time, in SVRs there was a decrease in the basal ganglia of both CHO/Cr and MI/Cr in comparison to the pre-treatment levels. These responses were not seen in non-responders. There was also a significant increase observed in some of the neurocognitive parameters measured in the SVRs. However, there was not a comparable change seen in the non-responders. As with the previous study [40], this study was also limited by a small sample size.

A recent study regarding HCV viral clearance did not determine metabolite concentrations in SVR, but did measure neurocognitive performance in these patients [42]. Although previous studies yielded conflicting results regarding viral clearance and neurocognitive improvement, this study had a larger sample size and the neurocognitive tests were performed at least 12 months after therapy ended. At the 12 month follow up several aspects of neurocognitive performance were significantly improved, including measures of vigilance, and working memory.

HIV

An investigation of correlations between neurometabolites and various clinical and neurocognitive parameters utilized ARV-naïve patients [43]. Compared to uninfected controls, HIV-infected patients exhibited higher levels of myo-inositol, creatine and choline in the frontal white matter. These elevated levels strongly correlated with CD4 counts. The elevated myo-inositol also correlated with viral loads, and all three neurometabolites correlated with a component of the Stroop neuropsychological test.

In an effort to correlate chemokine levels with cerebral metabolites, Letendre *et al.* [44] examined HIV-infected patients that were either neurologically unimpaired or neurologically impaired. The cerebral metabolites NAA, Cho, Cr and MI were measured in three brain regions using MRS, and the chemokine levels that included fractalkine, IP-10, IL-8, MCP-1, and MIP-1b were measured from CSF samples. Higher levels of IP-10 or MCP-1 were inversely correlated with neuronal metabolite scores ($\text{NAA/Cr}_{\text{WM}} + \text{NAA/}$

CrPC). Higher levels of IL-8 were correlated with an increased inflammatory pattern which consisted of MI/Cr and Cho/Cr ratios in different brain regions. Thus, the data supported the hypothesis that IP-10 and MCP-1 are primarily neurotoxic. Correlations between IP-10 and IL-8 and metabolite levels were differentially affected by ART. This suggests that ART may influence the relationship between chemokine levels and cerebral metabolites.

Clinical studies have shown that HIV-1 has detrimental effects on the CNS during acute infection as well as during the asymptomatic phase. In an investigation that examined changes in metabolism during the first year of infection, it was determined that HIV+ subjects exhibited altered levels of choline and glutamate-glutamine (Lentz *et al*, 2011). Upon enrollment in the study, when subjects were identified with acute viral infection, levels of choline and glutamate-glutamine were below those of control samples. Later samples showed an increase in these metabolic markers that were then equivalent to those of control.

A recent longitudinal study examined levels of neurometabolites in a group of patients who had been infected with HIV for a mean period of 11 years at the start of the study [45]. Over the course of the 2 year study, levels of NAA and Cho decreased in the frontal white matter and NAA, Cho and Cr decreased in the midfrontal cortex. As the patients were on CART, the authors proposed that the unexpected decline in choline may have been due to a stabilizing effect of the medications on neuroinflammation.

Animal models of HIV infection and neuroAIDS have yielded substantial information regarding the alteration of metabolites in the CNS during viral infection. The SIV/macaque model of HIV-1 was utilized to investigate the effects of viral infection on cerebral metabolites during acute infection [46]. Levels of both choline and myo-inositol were increased in most brain regions early after infection. This is suggestive of cerebral inflammation and activation of astrocytes and microglia. In the frontal cortex, levels of NAA were significantly decreased suggesting that neuronal injury was restricted to this region early in infection. During this early phase of infection, many of the metabolic changes observed returned to baseline levels after a brief period of perturbation.

In an effort to determine the role of altered creatine levels in neuroAIDS, macaques were infected with SIV and scanned using MRS at 4 weeks and at 8 weeks after infection [47]. Infected animals were sacrificed at 8 weeks p.i., which enabled immunohistochemical analysis of brain sections for markers of glial activation. As in the previous report from this group, choline levels increased early in infection and tended to return to baseline levels by 4 weeks p.i., but were elevated again by 8 weeks p.i. NAA levels in the white matter decreased over the course of 8 weeks, while creatine levels were significantly higher than baseline. Furthermore, changes in the NAA/Cr ratios were significant in the white matter, the frontal cortex, the parietal cortex and the basal ganglia. In the other brain regions examined, creatine levels were either significantly higher than baseline, or were trending towards higher levels. Importantly, in the frontal cortex and the parietal cortex, GFAP was significantly higher at 8 weeks. In the frontal cortex IBA staining was at higher levels at 8 weeks p.i. Thus, the increased levels of creatine correlated with increased levels of astrocyte and microglial activation.

Alcohol

As with studies focused on the CNS effects of HCV infection or HIV infection, proton magnetic resonance spectroscopy has been extensively used to investigate the effects of alcohol on metabolites in the brain. We will focus on the effects of alcohol on the levels of myo-inositol, choline, NAA, and creatine. The reader should keep in mind that other metabolites (e.g. GABA, glutamate, etc) will also be affected by alcohol use. However, since those metabolites have not been shown to be affected by HCV infection of the CNS, they will not be discussed in this review. However, we refer the reader to an extensive review on this topic [33].

MRS was used to evaluate metabolic changes in a group of recently detoxified alcoholics (RDA) and the results were compared with those from non-alcoholic controls [48]. In this study the RDA were abstinent for a median of 41.5 days. RDA had significantly increased levels of myo-inositol in the anterior cingulate gyrus (ACG), which is predominantly gray matter. No significant differences were noted in the anterior centrum semiovale (ACS), which is predominantly white matter. No significant differences were seen in either choline or NAA between groups in either the ACS or right thalamus. In another study the RDA group was abstinent for an average of 28 days at the time of imaging. The RDA exhibited a significant decrease of NAA in frontal white matter and a significant increase in MI in parietal white matter [49].

In a study that investigated metabolic changes in newly abstinent alcoholics, MRS was performed between 1–3 days after the cessation of drinking and at 36–39 days after cessation [50]. At the early time point the NAA/Cr ratio in the frontal lobes and cerebellum along with the Cho/Cr ratio in the cerebellum showed significant decreases in comparison to the control. However, at the second time point both ratios increased to levels not significantly different than control. A longitudinal study that determined metabolite concentrations rather than ratios found that in the cerebellar vermis the NAA concentration was significantly decreased 3–5 days after cessation of drinking as was the Cho concentration [51]. While the NAA concentration increased to normal values within 3 months the Cho concentration remained lower than in the control group. Taken together, these results indicate that the cerebellum is one of the primary targets for alcohol-induced metabolite alterations.

In a comparison between heavy drinkers (HD) and light drinkers (LD), NAA in the frontal white matter was significantly lower in the HD, whereas parietal gray matter MI and Cho were higher in the HD group [52]. This study also determined that among the HD, those who were binge drinkers had significantly higher levels of metabolites in their parietal gray matter than did the remaining HD. Furthermore, in neurocognitive assessments, the HD were impaired with respect to working memory, executive function and balance.

A longitudinal study that determined metabolite concentrations at the start of abstinence and 3 and 6 month time points after the cessation of drinking, found a significant decrease in Cho levels in frontal white matter, cerebellar cortex and cerebellar vermis at abstinence, but showed a substantial recovery in Cho at 3 months into abstinence [53]. This study did not detect a difference in NAA levels at the start of abstinence, but the authors noted that their

time point was a mean of 16.5 days, whereas previous studies detecting a decrease in NAA utilized time points between 1 and 5 days of abstinence. In a subsequent study that investigated Cho levels in actively drinking subjects, a positive correlation between Cho levels and alcohol consumption was observed [54]. This raised a question as to whether the decreased Cho levels were an early effect of abstinence.

In addition to neurocognitive effects, alterations in metabolite concentration have been shown to have some correlation to whether an individual will continue abstinence or resume drinking after treatment for alcohol use disorder. Lower concentrations of frontal white matter NAA, temporal gray matter NAA, or frontal gray matter Cho were associated with a greater probability of resumption of drinking behavior [55]. A subsequent study from this group showed that those who resumed drinking had decreased levels of NAA in all regions examined than did light drinkers [56].

It is clear that alcohol, HCV and HIV-1 all affect the levels of neurometabolites in the CNS. It is also clear that the effects of these agents on neurometabolites are dependent upon the timing of the MRS study relative to the time of infection and the alcohol consumption pattern. Some studies have been using absolute concentrations of metabolites rather than the metabolite/creatinine ratio because the levels of creatinine have been shown to be variable. However, this is far more demanding in terms of time and resources.

ANTIRETROVIRAL TOXICITY

HAART regimens generally contain at least 3 drugs from at least 2 of the following categories of antiretroviral agents: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) [57, 58]. NNRTIs and PIs are not only metabolized by CYP3A4, but these drugs also induce and inhibit CYP3A4, respectively, leading to drug-drug interactions (DDI) [59, 60]. CYP3A4-mediated DDI, especially when other medications are used in combination with HAART [59], are known to cause hepatotoxicity [61, 62]. DDI-mediated hepatotoxicity is generally caused by accumulation of NNRTIs and PIs or their metabolites, which are toxic at high doses, in addition to the generation of reactive oxygen species (ROS). These constituents cause toxicity through DNA damage, lipid peroxidation, and protein oxidation, and ultimately lead to mitochondrial damage, lipodystrophy syndrome, and steatohepatitis [62–64]. In addition to NNRTIs and PIs, NRTIs are also known to cause hepatotoxicity such as hepatic steatosis, lactic acidosis, and chronic hyperlactatemia [65]. These adverse effects of NRTIs are mediated by inhibition of human DNA polymerase γ resulting in mitochondrial dysfunction in the liver. Inhibition of DNA polymerase γ may lead to impaired oxidative metabolism causing an increased level of NADH that promotes conversion of pyruvate to lactate in mitochondria and results in mitochondrial dysfunction. Similarly, impaired oxidative metabolism may also lead to a decrease in fatty acid oxidation, resulting in fatty acid accumulation ultimately causing hepatic steatosis. Furthermore, co-infection with hepatitis significantly increases the risk of NNRTI- and PI-induced hepatotoxicity through HCV-induced impaired liver function [63, 66]. Therefore, an HIV-HCV international panel recommended that all HIV-HCV co-infected patients should be screened for viral hepatitis and liver function (i.e. level of transaminases) prior to commencing HAART [67].

In addition to hepatotoxicity, there is increasing evidence of neurotoxicity that can have adverse effects on CNS function. Similar to hepatotoxicity, neurotoxicity due to NRTIs has been shown to be a result of mitochondrial dysfunction caused by inhibition of DNA polymerase γ [68]. Upon analyzing HIV-1-infected patients, MRI studies revealed that the individuals who were on NRTI regimens containing didanosine and stavudine exhibited decreased N-acetylaspartate concentrations in frontal white matter suggesting a reduction in mitochondrial integrity [69]. In another study when 15 ARTs were tested *in vitro* using rat neuronal cells, NRTIs (abacavir, didanosine, and etravirine) and NNRTIs (nevirapine) caused neuronal lesions and cell death at the concentrations that are present in the CSF [70]. Furthermore, using primary rat forebrain cultures, direct effects of five NRTIs (2',3'-dideoxycytidine, 2',3'-dideoxyinosine, zidovudine, emtricitabine, and tenofovir), one NNRTI (efavirenz) and two PIs (ritonavir, atazanavir sulfate) on neuronal integrity were demonstrated [70]. In general, NRTIs and NNRTIs show significantly higher neurotoxicity than PIs and therefore monotherapy using boosted PIs has been considered as an option to eliminate HIV-1 infection from the CNS [71]. In fact, the most common PIs (lopinavir and darunavir) that are recently used to treat HIV-1 infection in the presence of ritonavir booster have been shown to be present in the CSF at the levels of IC₉₀ and IC₅₀ for HIV replication, respectively [72–74]. These findings further suggest the use of PIs for the treatment of HIV-1 infection in the brain. However, this approach may also lead to increased PI-mediated neurotoxicity.

The mechanism(s) by which NNRTIs and PIs may cause neurotoxicity is not known. However, based on the information from NNRT- and PI-mediated hepatotoxicity [59–62], we speculate that CYP3A4-mediated DDI may play an important role in NNRTI- and PI-mediated neurotoxicity. This hypothesis is strengthened by the fact that CYP3A4 is significantly expressed in the brain [75]. While total CYP content in the brain is <1% of that of liver [76], recent evidence demonstrates that CYP-mediated metabolism in the brain may contribute significantly to neurotoxicity [75]. This notion is based on the fact that the distribution of CYPs in the brain vary significantly among different brain regions, and that the levels of CYPs in specific brain regions is comparable to, or even higher than, levels in hepatocytes [75, 77, 78]. Furthermore, similar to liver CYP3A4, brain CYP3A4 can also be induced in hippocampal pyramidal neurons by many classical inducers such as oxycarbazepine, carbamazepine, and phenytoin, and this may lead to increased metabolism of CYP3A4 substrates [79, 80]. Therefore, owing to the fact that the level of CYP3A4 in specific brain regions is high, and CYP3A4 can be further induced by NNRTIs and inhibited by PIs, we speculate that CYP3A4-mediated DDI may play important role in NNRTIs- and PIs-mediated neurotoxicity.

Antiviral and Antiretroviral Drug Interactions and Neurotoxicity

Since HIV-1-HCV co-infections are very common, peginterferon and ribavirin are generally recommended for the treatment of HCV infection in HIV-1-infected patients [81]. However, the effectiveness of this HCV treatment is low, even at higher doses, because of their limited efficacy in patients infected with HCV genotype 1 [81–83]. For example, in a study with several hundred patients, increased ribavirin dosing (800 mg/day to 1200 mg/day) did not significantly increase sustained virological response in HCV (genotype 1) patients who were

co-infected with HIV-1 [83]. The introduction of PIs (telaprevir and boceprevir) in combination with peginterferon/ribavirin has resulted in a significant increase in sustained viral response rates in HIV-1-uninfected patients with HCV genotype 1 infection [84]. Peginterferon/ribavirin therapy is generally not associated with the occurrence (or worsening) of peripheral neuropathy in patients with HCV infection [85]. Although peginterferon, ribavirin, and antiviral PIs are not reported to cause neurotoxicity, the use of these agents in patients with HIV-1-HCV co-infections has been suggested to cause potential drug-drug interactions between antiretrovirals (NNRTIs and PIs) and antivirals (PIs) [81, 86]. This hypothesis is based on the fact that NNRTIs induce CYP3A4 and PIs inhibit CYP3A4 [59, 60], which are expected to alter CYP3A4-mediated metabolism of antiviral PIs in the brain, resulting in neurotoxicity. On the other hand, telaprevir and boceprevir are also inhibitors of CYP3A4, which may alter the metabolism of antiretroviral PIs and/or NNRTIs leading to increased neuronal toxicities [87, 88]. This is based on the observations in the liver, in which CYP3A4-mediated DDI with NNRTs and PIs causes hepatotoxicity [61, 62]. In fact, a study has revealed that the levels of boceprevir are decreased in patients taking efavirenz [62]. On the other hand, efavirenz compromises the levels of telaprevir and thus the use of efavirenz should be avoided in HCV patients [89]. Further, a recent review has reported a significant DDI between telaprevir and many antiretroviral drugs based upon a series of studies in healthy subjects [90]. Overall, these studies report DDI from low-dose ritonavir, ritonavir-boosted HIV PIs (atazanavir, darunavir, fosamprenavir and lopinavir), efavirenz, etravirine, rilpivirine, tenofovir disoproxil fumarate and raltegravir. Therefore, close monitoring and management has been suggested for the treatment of patients who are co-infected with HIV-1 and HCV [91]. Taken together, these results strongly suggest that concurrent treatment with antiretroviral agents and antiviral agents will lead to altered drug metabolism and neurotoxicity in the CNS.

Effect of Alcohol on Combined Antiviral/Antiretroviral Therapy

Chronic alcohol intake has been shown to increase the activity level of CYP2E1 up to 20-fold [92]. Alcohol-inducible CYP2E1 plays the major role in ethanol metabolism leading to generation of reactive oxygen species (ROS) and accumulation of acetaldehyde, both of which contribute to liver damage in chronic alcohol users [93–96]. Since CYP2E1 is also induced by alcohol in hippocampus, cerebellum, and brainstem leading to increased oxidative stress and increased permeability of BBB [97, 98] alcohol alone may cause neurotoxicity. Further, alcohol has been reported to induce CYP3A4 in hepatocytes [96, 99], which may contribute to the decreased effect of many drugs including NNRTIs and PIs in the liver. Indeed, alcohol has been shown to interact with NNRTIs/PIs leading to altered levels of NNRTIs/PIs plasma concentrations in human [100, 101]. Similarly, we have shown that alcohol induces CYP3A4 in extrahepatic monocytes/macrophages, which are one of the major targets of HIV-1 infection and a major viral reservoir [102]. Furthermore, using an *in vitro* system we have recently shown that alcohol at physiological concentration alters the binding characteristics (type I or type II) of eight antiretroviral PIs tested with recombinant CYP3A4 enzyme [103, 104]. Based on their altered binding characteristics, these PIs showed altered inhibitory characteristics with CYP3A4 [103]. Therefore, we hypothesized that alcohol may alter the metabolism of these PIs [105] in liver as well as in monocytes/

macrophages. Indeed, ethanol showed an altered metabolism of nelfinavir when we performed experiments in *in vitro* using recombinant CYP3A4 enzyme [104]. Based upon our results, we hypothesize that increased CYP3A4 induction and altered CYP3A4-PI interactions by alcohol may mediate alcohol-DDI with antiretroviral drugs, which in turn may cause cell toxicity [105]. Since CYP3A4 is also present in the brain, and is expressed at very high levels in specific regions [37, 75, 77], we further speculate that CYP3A4-mediated alcohol-DDI may cause toxicity in the brain [105]. Similarly, since antiviral PIs are also substrates and inhibitors of CYP3A4 [87, 88], alcohol may also alter the binding of antiviral PIs with CYP3A4 leading to altered PI metabolism and increased neurotoxicity. Thus, the simultaneous presence of antiretroviral and antiviral PIs in alcoholic individuals is expected to cause very complex drug-drug interactions that may further enhance neurotoxicity. Furthermore, since alcohol exposure has been shown to disrupt BBB [106], the CSF concentration of the antiviral and antiretroviral drugs is expected to increase that may ultimately lead to increased neurotoxicity. Therefore, it is important to study these interactions in detail so that the dose of these drugs is optimized in alcoholic individuals being treated for HIV-HCV coinfection.

FUTURE DIRECTIONS

In reviewing studies that have focused on the effects of either HIV-1 or HCV infection of the CNS, or those that have investigated the effects of alcohol on the CNS, it is clear that there are common mechanisms or pathways involved in mediating these impacts. Perhaps the most striking example of this is the involvement of ERK1/2 signaling in mediating the effects of Tat on both induction of inflammatory chemokines and the reduction of BBB integrity, as well as the neurotoxic effects of HCV core protein and some of the pro-inflammatory effects of ethanol on the CNS. However this raises many unanswered questions regarding the effects of alcohol use in those coinfecting with HCV and HIV-1. For example, the incidence of HCV infection of the CNS seems to be higher among those patients with detectable HIV-1 in the CSF. It is unknown whether this is due to the effects of HIV-1 on the BBB, or whether other mechanisms might be involved. Determination of this mechanism will hopefully identify a therapeutic target that could be used to limit the effects of HCV on the CNS.

Another question that arises concerns the potential neurotoxic effects of HCV. It is only in the past few years that HCV infection of the CNS has been examined, and only limited, but very compelling, information is available regarding HCV neurotoxicity. In addition to further investigations of the direct neurotoxicity of HCV proteins, there remain questions regarding potential secretion of neurotoxins from infected microglial cells as is seen in HIV infection. Using the discussion on HIV-associated neurocognitive disorders as a model, one might also investigate potential effects of HCV infection on molecules involved in neurotrophic support such as brain-derived neurotrophic factor.

The potential interactions between HIV, HCV and alcohol in the CNS are also an area that is not well studied. Studies on alcohol have demonstrated that alcohol increases the expression levels of TLR2 and TLR4 in microglial cells. Since HCV core protein signals through TLR2, this raises the question as to whether alcohol-mediated increases in TLR levels

sensitize cells to the effects of HCV core protein. This is just one mechanism by which alcohol could exacerbate the effects of HCV on the CNS. For example, it is known that HIV-1 Tat, HCV core protein and alcohol all signal through ERK1/2. The combined effects of these three agents on ERK1/2 signaling are virtually unknown.

It is also apparent that HIV-1, alcohol and HCV have effects on levels of neurometabolites in the CNS. Obviously, one of the first areas to be investigated will be that of clarifying the relationship between HCV clearance, neurometabolite abnormalities and neurocognitive effects. There are currently no published studies that examine all of these parameters in a large group of patients. There are also major questions to be answered as to the combined effects of all three agents on neurometabolite concentrations and determination of the correlation with neurocognitive and behavioral effects.

Now that the fact of HCV infection and replication in the CNS is firmly established, the importance of investigating interactions between HCV, HIV-1 and alcohol is clearly important. The prevalence of CNS infection with HIV and HCV needs to be monitored, and the effects of coinfection in the presence of substances of abuse such as alcohol, need to be determined in order to effectively treat this population.

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