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## HIV-1 infection and alcohol abuse: Neurocognitive impairment, mechanisms of neurodegeneration and therapeutic interventions

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### Abstract

Clinical studies indicate that alcohol dependence has an additive effect on cognitive deficits associated with HIV-1 infection. Findings in humans and animal models suggest that alcohol, similar to HIV-1, induces inflammatory processes in the brain leading to neurodegeneration. The causes of HIV-1-associated neurotoxicity are comparable to those mediating alcohol-induced neuronal injury. This review aims to present the mechanisms of the combined effects of HIV-1 and alcohol abuse in the brain and to discuss neuroprotective therapies. Oxidative stress, overproduction of pro-inflammatory factors, impairment of blood brain barrier and glutamate associated neurotoxicity appear to play important roles in alcohol driven neurodegeneration. Diminution of neuroinflammation constitutes a logical approach for prevention of HIV-1 and alcohol mediated neurodegeneration. Agonists of cannabinoid receptor 2 (CB<sub>2</sub>) possess potent anti-inflammatory and neuroprotective properties. We address multifaceted beneficial effects of CB<sub>2</sub> activation in the setting of HIV-1 brain infection and alcohol abuse.

### Keywords

HIV-1; alcohol abuse; neuroinflammation; cannabinoid receptor 2; glial cells; blood brain barrier

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The clinical and neuropsychological complex of HIV-1 associated dementia (HAD) is usually described as a frontal-subcortical pattern of impairment. The following cognitive areas demonstrate deterioration: fine motor coordination and speed, sustained attention,

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processing speed, executive function, learning efficiency and working memory (Grant, Heaton et al. 1995). Three forms of HAD have been described, including (1) frank HAD, characterized by severe impairment in two or more cognitive areas with marked impact on everyday function; (2) mild neurocognitive disorder with impairment in two or more cognitive functions that produces modest interference with everyday functioning (equivalent to minor cognitive motor disorder, MCMD); and (3) asymptomatic neurocognitive impairment, documented by impairment in two or more ability areas without apparent effect on everyday functioning.

One quarter of adults and 50% of children used to develop significant neurocognitive complications as a consequence of infection at a period of immunosuppression or AIDS before the introduction of anti-retroviral therapy (ART) (Navia, Jordan et al. 1986). While subclinical metabolic and structural abnormalities are detectable in neurologically asymptomatic subjects, overt HAD usually develops as a late complication and is accompanied by immunosuppression (Koralnik, Beaumanoir et al. 1990).

Introduction of ART considerably changed the clinical evolution of HIV-1 infection (Palella, Delaney et al. 1998). ART diminished the incidence and prevalence of major opportunistic infections and resulted in improvement in survival rates. 10-15% of patients are now over 50 years of age and, with continued advances in treatment, many will reach normal life expectancy (Navia and Rostasy 2005). Markedly improved survival rates brought to light other factors not previously considered to any great extent, including the effects of aging, chronic infection and the neurotoxic effects of ART itself. Indeed, nucleoside reverse transcriptase inhibitors (NRTIs) suppress HIV-1 replication, but they are associated with mitochondrial toxicity (Schweinsburg, Taylor et al. 2005). Reduction of the neuronal marker, N-acetylaspartate, in frontal lobe white and gray matter was found in individuals taking NRTIs, didanosine and/or stavudine, as a result of depleted brain mitochondria and/or alterations in cellular respiration (Schweinsburg, Taylor et al. 2005).

## **Combined effects of alcohol and HIV infection on neurocognitive performance**

According to the 2001-2 National Epidemiologic Survey on Alcohol and Related Conditions (NESARC) survey, more than 20% of men, age 18 to 29, met the criteria for a diagnosable alcohol use disorder (AUD): 9.3% Alcohol Abuse and 13% Alcohol Dependence. More than 50% of HIV-infected clinic patients reported heavy alcohol use (Samet, Phillips et al. 2004; Conigliaro, Justice et al. 2006). Alcohol abuse is associated with increased immune suppression (Wang and Watson 1995; Wang, Douglas et al. 2002). While some investigators reported a limited effect of alcohol exposure on the acute plasma viral load in an animal model for HIV infection, macaques infected with simian immunodeficiency virus (SIV), other studies showed opposite results - greater SIV viral load at various times post-infection (Bagby, Zhang et al. 2006; Poonia, Nelson et al. 2006). Bagby and colleagues (Bagby, Zhang et al. 2006) demonstrated a significantly more rapid progression to end-stage disease in SIV-infected monkeys. Underlying causes can be associated with diminished circulating memory CD4+ T cells and increased levels of monocytes expressing the viral co-receptor, CCR5, found in macaques exposed to alcohol (Marcondes, Watry et al. 2008). Another aspect of alcohol exposure is accelerated AIDS wasting that has been suggested as a mechanism by which alcohol might result in more rapid HIV disease progression (Molina, Lang et al. 2008). Most importantly, alcohol administration in SIV-infected monkeys produced greater behavioral deficits than either alcohol or SIV alone (Winsauer, Moerschbaecher et al. 2002).

With the widespread use of ART, the incidence of cognitive and motor deficits related to HIV infection has declined (Sacktor, McDermott et al. 2002); however, specific neuropsychological deficits persist (Sacktor, McDermott et al. 2002; Cysique, Maruff et al. 2004). These changes may be exacerbated by other conditions, like alcohol use disorders, that affect neural systems. ART, including NRTIs and protease inhibitors (PIs), are metabolized by the human cytochrome P450 system. It is known that alcohol can affect the metabolism of ART by two different mechanisms, enzymatic induction (Lieber and DeCarli 1970), associated with chronic alcohol use, and enzymatic inhibition, due to competition of ethanol for various cytochrome P450 isozymes, associated with acute ethanol use. Since the P450 pathway metabolizes multiple drugs, chronic alcohol users may be affected by altered drug concentrations in plasma.

Despite a number of reports documenting cognitive deficits associated with HIV infection and chronic alcoholism separately, very few studies have addressed the combined effects of these diseases. Farinpour and colleagues (Farinpour, Martin et al. 2000) reported impaired verbal working memory, and Martin et al. (Martin, Pitrak et al. 2004) found enhanced cognitive impulsivity in studies of individuals with HIV infection and drug use disorders, including alcoholism, compared with seronegative drug users. Synergistic negative effects of HIV infection and concurrent heavy alcohol consumption were reported on motor and visual spatial tasks (Rothlind, Greenfield et al. 2005). Examination of HIV-infected individuals with past alcohol use disorders (Green, Saveanu et al. 2004) provided evidence for additive and interactive effects of previous alcohol abuse and HIV infection on verbal reasoning, auditory processing, and reaction time tasks. Using a novel test (Match-to-Sample Stroop Task), Schulte and colleagues (Schulte, Mueller-Oehring et al. 2005) demonstrated that HIV-infected individuals with alcoholism had normally reduced reaction times when a valid match cue introduced a Stroop stimulus, but were disproportionately slow when the cue was invalid, indicating impairment in attention disengagement.

Significantly impaired memory and executive functions were reported both in HIV infection and chronic alcoholism (Fein, Torres et al. 2006; Pitel, Beaunieux et al. 2007; Pitel, Witkowski et al. 2007). Pitel and colleagues (Pitel, Beaunieux et al. 2007; Pitel, Witkowski et al. 2007) examined both memory and executive function abilities in abstinent alcoholics and demonstrated that executive function impairments did not account for the majority of variance in memory scores, concluding the memory impairment to be “genuine” rather than solely the result of executive function deficits.

## Mechanisms of HIV-1 and alcohol associated neurotoxicity

The causes of HIV-1-associated neurotoxicity include excitotoxic effects of glutamate, secretory products of chronically activated glial cells and oxidative stress (Boven, Gomes et al. 1999; Zhao, Lopez et al. 2004; Ellis, Langford et al. 2007), similar culprits to ones mediating alcohol-induced neuronal injury (Blanco, Pascual et al. 2004; Blanco and Guerri 2007). Dysfunction of the blood brain barrier (BBB; a common feature of HIV-1 neurodegeneration) (Persidsky, Ramirez et al. 2006) was recently documented in the setting of inflammation and chronic alcohol exposure in animal studies (Singh, Jiang et al. 2007). Taken together, alcohol abuse and HIV-1 infection of the central nervous system (CNS) could result in combined toxic effects leading to neuronal injury and cognitive dysfunction. Indeed, neuroimaging studies demonstrated more significant white matter abnormalities in HIV-1 positive alcohol abusers (especially with advanced infection) as compared to non-infected alcoholics (Pfefferbaum, Rosenbloom et al. 2007). While mechanisms of such injury are currently unknown, oxidative damage and glutamate imbalance contribute to neurodegeneration in both conditions (Zhao, Lopez et al. 2004; Melendez, Hicks et al. 2005). Interestingly, clinical studies support the efficacy of glutamate-blocking approaches for

treatment of alcohol withdrawal symptoms (Krupitsky, Rudenko et al. 2007) and dependence in humans (Ma, Ait-Daoud et al. 2006) promoting the idea that glutamatergic activation contributes to alcohol abuse. These observations establish a link between dysfunction of glutamate transporters and increased alcohol intake. Chronic neuroinflammation (like HIV-1 CNS infection) could cause glutamate transporter dysfunction and promote an alcohol-craving phenotype and alcoholism.

Recent findings in humans and animal models suggest that alcohol induces inflammatory processes in the brain leading to neurodegeneration (Potula, Haorah et al. 2006; He and Crews 2008; Qin, He et al. 2008). Furthermore, neuroinflammation appears to promote alcohol addiction in animals making this finding very relevant for HIV-1 brain infection associated with chronic inflammation (Blednov, Bergeson et al. 2005). A linear relation between the number of drinks consumed and higher relative risk for stroke in a human cohort indicates dysfunction of the BBB (Bazzano, Gu et al. 2007). We have demonstrated alcohol-induced BBB impairment (Haorah, Heilman et al. 2005; Haorah, Knipe et al. 2005; Haorah, Knipe et al. 2007) and delineated molecular mechanisms of this formerly unrecognized phenomenon (Haorah, Ramirez et al. 2007; Haorah, Schall et al. 2008) that further promotes the neurodegeneration mediated by ethanol metabolism in human neurons (Haorah, Ramirez et al. 2008) and in human astrocytes (Floreani, Rump et al. 2010). Furthermore, we showed alcohol exposure increased neuroinflammation (Potula, Haorah et al. 2006) in an animal model of HIV-1 encephalitis (HIVE), a pathologic correlate of HAD. Therefore, therapeutic strategies aiming at reduction of neuroinflammation is a logical approach to ameliorate, or reverse, CNS injury in the setting of alcohol abuse and HIV-1 CNS infection. Agonists of cannabinoid receptor 2 (CB<sub>2</sub>) possess potent anti-inflammatory properties as demonstrated in animal models and *in vitro* systems and are devoid of the psychoactive effects of CB<sub>1</sub> stimulators. CB<sub>2</sub> activation affords neuroprotection in animal models of stroke, multiple sclerosis and Alzheimer's disease (Benito, Tolon et al. 2008; Mestre, Docagne et al. 2009; Zhang, Adler et al. 2009; Zhang, Martin et al. 2009).

## Alcohol abuse and HIV-1 infection

There is an important connection between alcohol abuse and progression of HIV-1 infection and its contribution to HAD. Higher plasma LPS levels, together with increased monocyte activation markers and pro-inflammatory molecules (CD14, CCL2, and IL-6) in plasma, were associated with HAD or MCMD (Ancuta, Kamat et al. 2008). LPS levels predicted HAD, independent of viral load and CD4 counts. These findings support the idea that increased circulating LPS, a consequence of microbial translocation due to increased permeability of the gut barrier, contributes to monocyte activation in chronic HIV-1 infection and AIDS (Brenchley, Price et al. 2006) and provides evidence that circulating LPS may be linked to development of HAD via increased trafficking of activated monocytes into the brain. This study also indicated that both virologic and immune control in the periphery are linked to key events involved in HIV-1 neuropathogenesis. Validity of these observations was underscored by the finding that immediate episodic memory was impaired in individuals co-morbid with HIV-1 infection and alcoholism (Fama, Rosenbloom et al. 2009) and imaging studies showing faster brain atrophy in HIV-1 infected alcoholics as compared to uninfected heavy drinkers (Schulte, Muller-Oehring et al. 2008; Sullivan and Zahr 2008).

These data have certain correlates with animal studies of HIV-1 infection. SIV infected macaques exposed to alcohol showed increased viral load (Kumar, Perez-Casanova et al. 2005; Bagby, Zhang et al. 2006), faster progression of infection, increased levels of pro-inflammatory factors in the internal organs (Molina, Lang et al. 2008) and impaired immune responses (Marcondes, Watry et al. 2008). Using the mouse model for HIVE [non-obese

diabetic (NOD)/SCID mice reconstituted with human peripheral blood lymphocytes (PBL)] that reproduces important features of encephalitis, acquired anti-viral immune responses and neuronal injury], we showed that alcohol feeding resulted in increased viremia, diminished anti-viral immune responses associated with signs of oxidative stress resulting in increased levels of HIV-1 infected macrophages in the brain. Furthermore, we found increased microglial activation in an animal model for HIVE that was further enhanced by alcohol exposure (Potula, Haorah et al. 2006). Despite widely acknowledged inhibitory effects of ethanol *in vivo* on leukocyte migration in alcohol-treated animals, alcohol did not affect the amount of T cell brain infiltration in the HIVE model. Rather, the immunosuppressive effects of alcohol were mediated by impaired immune responses secondary to oxidative stress and dysfunction of immunoproteasomes playing important role in antigen presentation.

While combined adverse effects of HIV-1 and alcohol abuse in CNS are complex and multifaceted, recent studies suggest that both may have similar targets (glutamate excitotoxicity, pro-inflammatory responses, BBB injury).

### **Excitatory amino acid transporter 2 (EAAT-2) decreased expression and function correlate with astroglial activation**

Glutamate, being the main excitatory neurotransmitter (Fonnum 1984), is released from glutamatergic neuronal vesicles through a calcium-dependent mechanism [for review, see (Fillenz 1995)]. Glutamate is present in the micromolar range in the synaptic cleft under physiologic conditions (Fillenz 1995). Sustained glutamate receptor hyper-stimulation induces neuronal death (Rothman 1985) via calcium and sodium deregulation (so-called excitotoxicity). Extracellular glutamate concentration is controlled by EAATs removing extracellular glutamate (Gegelashvili and Schousboe 1997). There are five cloned EAAT subtypes (Tanaka 1994), and EAAT-1 and EAAT-2 are primarily detected in astrocytes (Rothstein, Martin et al. 1994). The astrocytic transporters EAAT-1 and -2 are essential for protection against excitotoxicity as was shown in EAAT gene knockout experiments in mice (Rothstein, Dykes-Hoberg et al. 1996). Excitotoxicity is seen in many acute and chronic neurological conditions, including HIV-1 CNS infection (Kaul, Zheng et al. 2005; Gras, Porcheray et al. 2006). Among other mechanisms, HIV-1-associated neurotoxicity involves glutamate-related excitotoxicity and oxidative stress, resulting in neuronal injury and apoptosis (Kaul, Zheng et al. 2005). *In vitro* experiments demonstrated that EAAT expression and function in astrocytes were diminished by HIV-1, presumably due to the effects of inflammatory mediators and viral proteins (Fine, Angel et al. 1996; Patton, Zhou et al. 2000; Rozyczka, Figiel et al. 2004). TNF $\alpha$  decreased astrocyte clearance of glutamate (Fine, Angel et al. 1996) and decreased EAAT-2 expression in these cells (Sitcheran, Gupta et al. 2005). The inhibitory effect of TNF- $\alpha$  may depend on the recently identified astrocyte-expressed gene-2, which affects EAAT-2 (Kang, Su et al. 2005). In addition, oxidative stress [present during HIV-1 brain infection (Turchan, Pocernich et al. 2003)] also results in decreased EAAT-2 expression and function (Trotti, Rizzini et al. 1997). Since oxidative stress plays an important role in alcohol-mediated neurotoxicity, EAAT dysfunction seen in HIV-1 CNS infection could be further exacerbated by alcohol abuse leading to increased levels of extracellular glutamate.

### **Glutamate and alcohol effects in the CNS**

While glutamate imbalance plays an important role in alcohol-associated brain effects, available data in the literature are somewhat conflicting. Following chronic exposure to ethanol (at high doses of 100 mM), EAAT-2 expression was increased in organotypic cortical slice brain cultures (Zink, Schmitt et al. 2004), did not change in brain tissues of animals exposed to ethanol (Melendez, Hicks et al. 2005), or was reduced (both density and

function) in alcohol-preferring rats (Schreiber and Freund 2000) or *in vitro* systems (Kim, Do et al. 2005). In cortical astroglial cell cultures exposed to ethanol, uptake of radioactively labeled glutamate was increased, and this was interpreted as a maladaptive process (Zink, Schmitt et al. 2004). Concentrations of extracellular glutamate were increased in animals exposed to ethanol for 4-8 days, suggesting deficits in glutamate transport (Melendez, Hicks et al. 2005). Increased gene expression for EAAT-1 was shown in the brains (frontal cortex) of alcoholics, while no results were reported for EAAT-2 (Flatscher-Bader, van der Brug et al. 2005). In contrast to discrepant experimental results, a number of clinical studies showed efficacy of anti-glutamatergic approaches for treating alcohol withdrawal (Krupitsky, Rudenko et al. 2007) and dependence (Ma, Ait-Daoud et al. 2006). Increased glutamate levels in animals with defective glutamate transporters enhanced their alcohol consumption (Spanagel, Pendyala et al. 2005) pointing to an association between dysfunction of glutamate transporters and enhanced alcohol intake. Therefore, chronic neuroinflammation (like HIV-1 CNS infection) could cause glutamate transporter dysfunction promoting alcohol-craving phenotype and alcoholism. How the combination of chronic inflammatory processes (like HIVE) and alcohol abuse affects glutamate transport and the resulting neurotoxicity is currently unknown.

Dysfunction of glutamate transport seen in neuroinflammation and alcohol abuse requires new therapeutic approaches. Tawfik and colleagues (Tawfik, Lacroix-Fralish et al. 2006) demonstrated a link between astrocyte activation and dysfunction of the glutamate uptake system. Propentofylline (PPF), a methylxanthine derivative, induces an astrocyte phenotypic switch from activated to differentiated phenotype (ramified, process-bearing form). These changes are accompanied by induction of transcription/translation of the glutamate transporters (EAAT-2), glutamate uptake and diminution of LPS-induced chemokine release from cultured astrocytes. *In vivo*, PPF was able to decrease astrocytic and microglial activation due to nerve injury-induced and attenuated expression of interleukin IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (Sweitzer, Schubert et al. 2001; Raghavendra, Tanga et al. 2003). Cytokines were shown to inhibit astrocytic glutamate uptake in a dose-dependent manner (Hu, Sheng et al. 2000). Utilization of the antioxidant, acetyl-L-carnitine (ALC), prevented ethanol-induced brain toxicity and lipid hydroperoxide levels, indicating important role for ALC in the prevention of alcohol-induced cellular damage (Calabrese, Scapagnini et al. 2001; Calabrese, Scapagnini et al. 2002). Furthermore, ALC treatment significantly reduced the signs of alcohol withdrawal syndrome and the level of ethanol intake in alcohol-preferring rats (Mangano, Clementi et al. 2000). Therefore, PPF and antioxidants could be potential treatments and/or intervening strategies to restore the alcohol-induced brain damage.

## Inflammation as the cause of alcohol-induced neurodegeneration

Alcohol abuse related neuronal injury and dysfunction are associated with increases in oxidative stress in the brain that coincide with the induction of pro-inflammatory cytokines and oxidative enzymes. Although neuroprotective effects of ethanol were observed in specific experimental settings (Janis, Hoane et al. 1998), previous studies clearly indicated that long-term alcohol abuse produced profound functional or morphological changes in the CNS regardless of nutritional status. Neuropathologic examination of brain tissue from chronic alcoholics suggested that alcohol abuse results in neuronal degeneration, ranging from minor dendritic structural change and synaptic changes to neuronal cell death in the CNS (Harper 1998). Multiple lines of evidence suggest that chronic and excessive ethanol consumption may enhance oxidative injury of neurons and result in cell death. By mechanisms not well understood, ethanol induces activity of CYP2E1 (a major alcohol-metabolizing enzyme), enhances ROS generation, changes the cytokine signaling pathways [resulting in up-regulation of inducible nitric oxide (iNOS) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>)], and increases production of prostanoids through the PLA<sub>2</sub>/cyclooxygenase (COX) pathways

[for review (Sun and Sun 2001)]. Knapp and Crews (Knapp and Crews 1999) showed that COX-2 immunoreactivity was progressively increased in rat brains during chronic alcohol administration. Expression of CYP2E1 (metabolizing ethanol to ROS and acetaldehyde) was detected in neuronal cell bodies and astroglia. Interestingly, CYP2E1 staining of blood vessels was prominent in the white matter, and immunoreactive astrocytes were seen to have end-feet on the microvessels (Hansson, Tindberg et al. 1990). Ethanol could increase inflammation caused by other factors (e.g., HIV-1 CNS infection). Davis and Syapin demonstrated that exposure of an astrocytic cell line to ethanol (0.5-6 hr at 50 mM) enhanced iNOS activity induced by a cytokine cocktail (IL-1 $\beta$ , TNF $\alpha$  and IFN $\gamma$ ) via nuclear factor kappaB (NF- $\kappa$ B) activation (Davis and Syapin 2004; Davis and Syapin 2004). Interestingly, chronic ethanol exposure resulted in an increased number of microglia *in vivo* before any ethanol-induced brain atrophy was detected (Riikonen, Jaatinen et al. 2002).

*In vivo* experimental data indicate that chronic ethanol administration followed by a secondary pro-inflammatory stimulus (LPS) resulted in sustained up-regulation of cytokines (TNF $\alpha$ , CCL2, IL-1b) in the brain for a long period of time (Qin, He et al. 2008). Activation of brain microglia and increased brain expression of COX-2 and gp91phox NOX subunit mRNA were found in the ethanol-pretreated LPS group. These results paralleled findings in the brains of chronic alcoholics where microglial activation coincided with enhanced CCL2 production (He and Crews 2008). We also found diffuse microglial activation affecting gray and white matter in the brain tissues of patients with a history of alcohol abuse as compared to control brain tissues (without alcohol exposure) (Fig. 1 A,C,E).

It was shown before that chronic alcohol consumption in humans is associated with increases in serum proinflammatory cytokines (McClain, Barve et al. 1999; Kiefer, Jahn et al. 2002). Monocytes isolated from the blood of alcoholics produce greater amounts of TNF $\alpha$  spontaneously and in response to endotoxin (McClain, Song et al. 2004). What causes such proinflammatory changes in glial cells is currently unknown.

Recent studies demonstrate that chronic ethanol exposure activates iNOS/COX (Blanco, Pascual et al. 2004) and increases the levels of IL-1 $\beta$  and TNF $\alpha$  in both the rat brain and in cultured astrocytes, activating signaling pathways that are usually associated with inflammation (MAPKs, NF- $\kappa$ B, AP-1). Such events coincided with an increase in cell death (Valles, Blanco et al. 2004) via RhoA activation (Minambres, Guasch et al. 2006). These data suggest that ethanol can activate glial cells by triggering the production of toxic compounds, such as ROS or NO (Valles, Blanco et al. 2004), cytokines and glutamate contributing to ethanol-induced brain damage. Our data indicate that EtOH exposure results in increased cytochrome P450-2E1 activity, generation of ROS and secretion of prostaglandin E2 (PGE2) in primary human astrocytes (Floreani, Rump et al. 2010). Secretion of PGE2 paralleled induction of PLA<sub>2</sub> and COX-2. Immunoprecipitation and Western blot analyses suggest that the tyrosine phosphorylation of TLR4-Src kinase complex at the cell membrane triggered Src kinase signaling and mediated activation of cPLA<sub>2</sub> and COX-2. Inhibition of ethanol metabolism, Src kinase activity or the TLR4 blockade prevented PLA<sub>2</sub>/COX-2 activation, and diminished PGE2 production, suggesting that interactive phosphorylation of TLR4-Src kinase regulates proinflammatory responses in astrocytes. Relevance of these observations was confirmed *in vivo*. Chronic ethanol feeding up-regulated the levels of CD11b (microglial marker) and glial fibrillary acidic protein (astrocyte marker), and iNOS, COX-2, and cytokine levels (IL-1 $\beta$ , TNF $\alpha$ , IL-6) in the cerebral cortex of wild-type mice, and TLR4 deficiency protects against ethanol-induced glial activation and induction of inflammatory mediators (Alfonso-Loeches, Pascual-Lucas et al. 2010).

We also demonstrated that metabolism of ethanol in primary human neurons by alcohol dehydrogenase (ADH) and cytochrome P450-2E1 generated ROS. In addition, ethanol metabolites further augment ROS/NO levels via induction of NADPH/xanthine oxidase (NOX/XOX) and nitric oxide synthase (NOS) in human neurons (Haorah, Ramirez et al. 2008). Marked increase in lipid peroxidation and decrease in neuronal-specific marker paralleled ROS generation.

In addition to neurons and astroglia, alcohol causes profound changes in brain endothelium. Ethanol caused dysfunction in brain endothelial cells (diminishing barrier tightness, increasing permeability due to phosphorylation of tight junction (TJ) and cytoskeletal proteins, thereby enhancing leukocyte migration across the BBB (Haorah, Heilman et al. 2005; Haorah, Heilman et al. 2005; Haorah, Knipe et al. 2007; Haorah, Ramirez et al. 2007; Haorah, Ramirez et al. 2008; Haorah, Schall et al. 2008). We delineated molecular mechanisms of BBB disruption by alcohol in pathophysiologically relevant concentrations (25-50 mM). Such mechanisms include alcohol metabolism in brain microvascular endothelial cells, BMVEC (via CYP2E1) causing oxidative stress, leading to Ca<sup>2+</sup> release via stimulation of inositol 1,4,5-triphosphate receptor, activation of myosin light chain kinase (MLCK), phosphorylation of MLC and TJ proteins (Haorah, Heilman et al. 2005; Haorah, Knipe et al. 2005; Haorah, Knipe et al. 2007). These changes decreased the structural integrity of BMVEC monolayers, increased BBB permeability *in vitro* and *in vivo* and enhanced monocyte migration across the BBB, events potentially enhancing neurotoxicity associated with HIV-1 CNS infection. Chronic exposure to ethanol (24-72 h) resulted in activation of MMP-2 and -9 via activation of protein tyrosine kinase, degradation of basement membrane components, increased barrier “leakiness” and monocyte migration across human brain endothelial monolayers (Haorah, Schall et al. 2008). We found that all functional alterations and signal transduction events caused by ethanol exposure can be reproduced by application of acetaldehyde (Ach) or exogenous donors of ROS (Haorah, Knipe et al. 2005; Haorah, Ramirez et al. 2007). Overall, a significant amount of data points to pro-inflammatory effects of chronic alcohol exposure in the brain that can cause neurodegeneration and further promote alcohol addiction (Blednov, Bergeson et al. 2005).

## **CB<sub>2</sub> receptor expression in immune cells and anti-inflammatory effects of cannabinoids**

There are two well-characterized CB receptors with very different physiological properties. The psychoactive effects of cannabinoids are associated with the CB<sub>1</sub> receptor; the CB<sub>2</sub> receptor mainly mediates anti-inflammatory and immunosuppressive actions (Miller and Stella 2008). Identification of CB<sub>1</sub> and CB<sub>2</sub> resulted in the recognition of endogenous cannabinoids (endocannabinoids) (Pacher, Batkai et al. 2006). The therapeutic limitations of CB<sub>1</sub> agonists are related to the short window of their beneficial actions and psychoactive effects at effective doses. CB<sub>2</sub> agonists are devoid of psychoactive activities. Since neuroinflammation plays a significant role in essentially all neurodegenerative processes, CB<sub>2</sub> stimulation became an attractive target for development of neuroprotective therapies.

CB<sub>2</sub> is expressed in different types of leukocytes mediating cannabinoid anti-inflammatory effects and immunomodulation (McKallip, Lombard et al. 2002). CB<sub>2</sub> stimulation affects B and T cell differentiation and the balance of pro-inflammatory to anti-inflammatory cytokines (Ziring, Wei et al. 2006). CB<sub>2</sub> activation in macrophages diminishes the release of pro-inflammatory factors (NO, IL-12p40, TNF $\alpha$ ) (Chuchawankul, Shima et al. 2004). Stimulation of CB<sub>2</sub> prevented ROS generation and secretion of TNF $\alpha$  and CCL2 in macrophages (Han, Lim et al. 2009). Activation of the CB<sub>2</sub> receptor by the selective agonist, JWH-133, in macrophages increased IL-10 production, counteracting pro-inflammatory genes (Correa, Docagne et al. 2009). Anti-inflammatory effects of CB<sub>2</sub> activation were

found in different animal models including colitis (Storr, Keenan et al. 2009), acute hind paw inflammation (Berdyshev, Boichot et al. 1998), acute lung injury (Conti, Costa et al. 2002) and sepsis (Tschop, Kasten et al. 2009) to name a few.

## CB<sub>2</sub> CNS expression and its mediated anti-neuroinflammatory responses

CB<sub>2</sub> expression has been detected in microglia. Carlisle et al. (Carlisle, Marciano-Cabral et al. 2002) found CB<sub>1</sub> and CB<sub>2</sub> mRNA in brain tissue and in primary rat microglia cultures. Cannabinoids were able to diminish LPS-induced mRNA expression for IL-1 $\beta$ , TNF $\alpha$  and IL-6 (Puffenbarger, Boothe et al. 2000) and to inhibit TNF $\alpha$  release in LPS-activated rat microglia cultures (Facchinetti, Del Giudice et al. 2003). Microglia activated with  $\beta$ -amyloid showed a significant increase in TNF $\alpha$  release blocked by CB<sub>2</sub> agonists (Ramirez, Blazquez et al. 2005). CB<sub>2</sub> expression in microglia is up-regulated during immune activation. The combination of IFN $\gamma$  and granulocyte mononuclear colony stimulating factor induces high levels of microglial CB<sub>2</sub> expression (Maresz, Carrier et al. 2005). Expression of the activation marker, CD40, up-regulated by IFN $\gamma$  in microglia, was decreased by a selective CB<sub>2</sub> agonist (JWH-015) and by non-selective cannabinoids (Ehrhart, Obregon et al. 2005). These data indicate that during inflammation more molecular targets are present that may dampen exaggerated immune responses (Cabral and Griffin-Thomas 2009).

Neuroprotective effects of CB<sub>2</sub> agonists are associated with suppression of microglia activation in animals (Klegeris, Bissonnette et al. 2003; Eljaschewitsch, Witting et al. 2006) via inhibiting the release of neurotoxic factors and by decreasing neuronal cell damage in cell or tissue culture models. CB agonists provided neuroprotection in *in-vitro* oxygen/glucose deprivation, a model for ischemic injury by acting on glutamatergic excitotoxicity, TNF $\alpha$  release, and iNOS expression (Fernandez-Lopez, Martinez-Orgado et al. 2006). *In vitro* and *in vivo* studies have shown that cannabinoids can act on glia enhancing the release of the anti-inflammatory factors, IL-4 and IL-10 (Molina-Holgado, Molina-Holgado et al. 1998). CB agonists inhibited LPS-induced ROS via NADPH oxidase (NOX) activation, p38 MAPK activation and increased TNF $\alpha$  production in microglial cells (El-Remessy, Tang et al. 2008). CB<sub>2</sub> signaling interfered with the enhanced expression of iNOS and CCR2 induced by IFN $\gamma$  in mouse microglial cells (Racz, Nadal et al. 2008).

Increased expression of CB<sub>2</sub> under neuroinflammatory conditions was found in human brain tissues (Benito, Tolon et al. 2008). Prominent CB<sub>2</sub> up-regulation was reported in CNS affected by MS, amyotrophic lateral sclerosis (ALS), Down syndrome and Alzheimer's disease (AD) (Benito, Kim et al. 2005; Yiangou, Facer et al. 2006; Benito, Romero et al. 2007; Benito, Tolon et al. 2008). Enhanced CB<sub>2</sub> expression was detected on microglia, perivascular macrophages and T cells in SIV encephalitis (Benito, Kim et al. 2005). We have investigated the presence of CB<sub>2</sub> in human brains from patients with HIV-1 infection affected by HIVE (n=8), no encephalitis (n= 4) and controls (n=3). We found increased expression of CB<sub>2</sub> on microglia/perivascular macrophages and brain microvascular endothelial cells in HIVE (Fig. 2E,H,K), brain endothelium in HIV<sup>+</sup> cases (data not shown) and very little in non-infected control human brains (Fig. 2B). Of note, no CB<sub>2</sub> expression was found in astrocytes in HIVE. As mentioned above, we found an increased amount of microglial cells with signs of activation (cell size and shape) in the frontal cortex (grey and white matter) in alcoholic brains (n=7) (Fig. 1A,C) as compared to control brains (n=6) (Fig. 1E). Importantly, microglial activation paralleled enhanced expression of CB<sub>2</sub> in brain microvascular endothelial cells (Fig. 1B,D) when compared to the control group (Fig. 1F).

Anti-inflammatory and neuroprotective effects of cannabinoids were confirmed in animal models for MS, AD, stroke and ALS. The synthetic cannabinoid, WIN55,212-2, prevented microglial activation, cognitive impairment, and loss of neurons caused by  $\beta$ -amyloid in rats

(Ramirez, Blazquez et al. 2005). Selective CB<sub>2</sub> agonists slowed disease progression in an ALS model (Kim, Moore et al. 2006). Amelioration of EAE was achieved by the synthetic cannabinoid that was accompanied by a reduction of axonal damage and diminished excitotoxicity (Docagne, Muneton et al. 2007). Using a viral model of MS, Mestre and colleagues observed that the cannabinoid agonist WIN55,212-2 administered at the time of virus infection suppressed ICAM-1 and VCAM-1 in brain endothelium, reduced perivascular CD4<sup>+</sup> T lymphocyte infiltrates and microglial responses (Mestre, Docagne et al. 2009).

In a stroke animal model, CB<sub>2</sub> activation with highly selective agonists (O-3853, O-1966) reduced infarct size by 30% in mice 24 h after induction of focal ischemia and reperfusion (Zhang, Martin et al. 2007), diminishing leukocyte rolling and adhesion. CB<sub>2</sub> agonists diminished adhesion of neutrophils and their recruitment into brain tissue in an ischemia model (Murikinati, Juttler et al. 2010). This effect on neutrophils was responsible for the neuroprotection mediated by CB<sub>2</sub> agonists since the agonist was no longer protective when neutrophils were depleted. In summary, cannabinoids can be neuroprotective via their immunomodulatory properties, which have been mainly attributed to CB<sub>2</sub> receptors.

## CB receptors and immune cell migration

Cannabinoids have been reported to inhibit chemokine-induced chemotaxis of various cell types including neutrophils, lymphocytes, macrophages, monocytes and microglia (Miller and Stella 2008). CB<sub>2</sub> appears to play a major role in leukocyte migration. Cannabinoids could reduce inflammation by interfering with the action of other chemoattractants (Montecucco, Burger et al. 2008). Human monocytes treated with the synthetic CB<sub>2</sub> agonist JWH-015 showed diminished migration in response to the chemokines CCL2 and CCL3 via down-regulation of their receptors and inhibition of IFN $\gamma$ -induced ICAM-1 expression. Leukocyte migration mediated by RhoA activation was inhibited by CB<sub>2</sub> agonists providing an additional mechanism for anti-inflammatory effects of CB<sub>2</sub> stimulation (Kurihara, Tohyama et al. 2006).

In addition to putative effects on leukocytes, anti-inflammatory properties of CB agonists may be related to their actions on the endothelium. CB<sub>2</sub> has been found in brain endothelium (Golech, McCarron et al. 2004; Lu, Avraham et al. 2008) and endothelial cells from other organs (Rajesh, Mukhopadhyay et al. 2007). Lu and colleagues (Lu, Avraham et al. 2008) demonstrated that HIV-1 gp120 caused secretion of substance P and dysfunction of brain endothelial cells (Ca<sup>2+</sup> influx, decreased monolayer tightness, diminution in TJ protein expression) that was prevented by a non-selective CB<sub>1</sub>/CB<sub>2</sub> or CB<sub>1</sub> agonist. These compounds diminished monocyte migration across endothelial monolayers pretreated with gp120. The molecular mechanism underlying the beneficial effects of CB agonists was not investigated. Synthetic CB<sub>2</sub> agonists (JWH-133, HU-308) reduced TNF $\alpha$ -induced activation of human coronary artery endothelial cells *in vitro* (Rajesh, Mukhopadhyay et al. 2007). CB<sub>2</sub> stimulation attenuated TNF $\alpha$ -induced NF- $\kappa$ B and RhoA activation, and decreased expression of ICAM-1 and VCAM-1 and CCL2 release. Pre-treatment of endothelial cells with CB<sub>2</sub> agonists diminished transendothelial migration and adhesion of a THP-1 monocytic cell line.

In summary, since CB<sub>2</sub> agonists modulate immune cell migration, they represent a promising pharmacological approach for development of anti-inflammatory therapeutics for HIV CNS infection and alcohol-induced neuroinflammation. It has been reported that chronic  $\Delta(9)$ -tetrahydrocannabinol administration decreased early mortality from SIV infection, and this was associated with attenuation of plasma and CSF viral load and retention of body mass (Molina, Winsauer et al. 2010).

Signaling events associated with inflammatory responses are complex in endothelial cells and monocytes, and few studies have addressed potential mechanisms of anti-inflammatory CB<sub>2</sub> stimulation. Gertsch et al. investigated intracellular signaling pathways triggered in monocytes by LPS-triggered TNF $\alpha$  and IL-1 $\beta$  production that were suppressed by CB<sub>2</sub> agonists. LPS treatment of human monocytes led to a rapid phosphorylation of p38 and JNK1/2 (Gertsch, Leonti et al. 2008), and a CB<sub>2</sub> agonist reduced Erk1/2 and JNK1/2 activation (phosphorylation) (Gertsch, Leonti et al. 2008). CB<sub>2</sub> agonists prevented neuronal injury during neuroinflammation via up-regulation of mitogen-activated protein kinase phosphatase-1 that resulted in Erk1/2 inhibition (Eljaschewitsch, Witting et al. 2006). These findings have relevance to the anti-inflammatory effects of CB<sub>2</sub> stimulation in BMVEC playing an important role in BBB dysfunction associated with HIV-1 infection (Ramirez, Fan et al. 2010) that are mediated by JNK1/2 in brain endothelium.

In summary, due to their multifaceted beneficial effects on inflammation, immune cell migration, and resulting neuroprotection, CB<sub>2</sub> agonists present a novel therapeutic opportunity for the treatment of HIV-1 brain infection and alcohol abuse.

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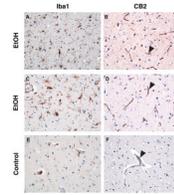
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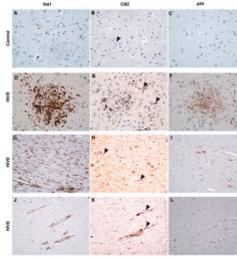
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**Figure 1.**

Up-regulation of CB<sub>2</sub> expression in microvessels in brain tissue of alcoholics. Frontal cortex brain tissue specimens were obtained from chronic alcoholics (n=8) or aged matched controls without a history of alcohol abuse (n=6). Serial paraffin sections (5 μm thick, A-B, C-D, E-F) were cut and stained for Iba1 (monocyte-macrophage marker) and CB<sub>2</sub>.

Microglial activation was found in the brain tissues of chronic alcoholics (Iba1 staining, A, C) and was accompanied by strong expression of CB<sub>2</sub> in microvascular endothelial cells (arrowhead, B, D). Occasional perivascular macrophages and microglia without signs of activation (E) were found in control brain tissues with light CB<sub>2</sub> staining in microvessels (F, arrowhead). Primary Abs were detected by Vectastain Elite Kit with DAB as a substrate. Stained sections were observed by light microscopy, and digital images were acquired by a cooled CCD camera. Original magnification: panels A-F × 200.

**Figure 2.**

Up-regulation of CB<sub>2</sub> expression in microvessels and macrophages/microglia in HIVE. Frontal cortex brain tissue specimens were obtained from 8 cases of HIV-1 encephalitis (HIVE) of different severity (moderate to severe (Persidsky, Ghorpade et al. 1999)) along with seronegative age-matched controls (n=4). Serial paraffin sections (5 μm thick, A-C, D-F, G-I, J-L) were cut and stained for Iba1 (macrophage marker), CB<sub>2</sub> and HIV-1 p24. (A-C) Brain tissue from a seronegative control demonstrates the usual component of non-activated microglia (A), minimal CB<sub>2</sub> on brain endothelium (arrowhead, B) and no p24 staining (C). (D-F) Cells of the microglial nodule are positive for Iba1 (D), CB<sub>2</sub> (E) and p24 (F). Microvascular endothelial cells show CB<sub>2</sub> staining (arrowhead, E). (G-I) Diffuse activation of Iba1+ microglia (G) is accompanied by CB<sub>2</sub> expression in these cells (H) and brain endothelium (arrowhead, H). (I) Some microglia cells are p24-positive. (J-L) Perivascular macrophages (J) express CB<sub>2</sub> (arrowhead, K) and part is HIV-1 p24<sup>+</sup> (L). Primary Abs were detected by Vectastain Elite Kit with DAB as a substrate. Digital images were acquired by a cooled CCD camera. Original magnification: panels A-F × 200; panels G-L × 100.