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HIV-1 gp120 and Drugs of Abuse: Interactions in the Central Nervous System

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Abstract

HIV-1 infection is a global public health problem with more than 34 million people living with HIV infection. Although great strides have been made in treating this epidemic with therapeutic agents, the increase in patient life span has been coincident with an increase in the prevalence of HIV-associated neurocognitive disorders (HAND). HAND is thought to result from the neurotoxic effects of viral proteins that are shed from HIV-infected microglial cells. One of the primary neurotoxins responsible for this effect is the HIV-1 glycoprotein gp120. Exposure of neurons to gp120 has been demonstrated to cause apoptosis in neurons, as well as numerous indirect effects such as an increase in inflammatory cytokines, an increase in oxidative stress, and an increase in permeability of the blood-brain barrier. In many patients, the use of drugs of abuse (DOA) exacerbates the neurotoxic effects of gp120. Cocaine, methamphetamine and morphine are three DOAs that are commonly used by those infected with HIV-1. All three of these DOAs have been demonstrated to increase oxidative stress in the CNS as well as to increase permeability of the blood-brain barrier. Numerous model systems have demonstrated that these DOAs have the capability of exacerbating the neurotoxic effects of gp120. This review will summarize the neurotoxic effects of gp120, the deleterious effects of cocaine, methamphetamine and morphine on the CNS, and the combined effects of gp120 in the context of these drugs.

Keywords

CNS; cocaine; gp120; HIV; methamphetamine; morphine; central nervous system; HAND; drug of abuse; ARV

INTRODUCTION

HIV is a public health problem of immense proportions. In 2009 there were over 34 million people living with HIV infection [1]. HIV enters the central nervous system (CNS) within the first few days after infection; the primary mechanism of entry is postulated to be the “Trojan Horse” mechanism. In this scenario, infected monocytes from the periphery are extravasated through the blood-brain barrier (BBB)[2]. HIV neurotoxicity is thought to be primarily an indirect effect of HIV infection. Some of the evidence for this is that neurons are refractory to infection by HIV-1, while the virus in the CNS replicates in microglial cells, and to a lesser extent, astrocytes [2, 3]. The neurotoxic effects of HIV infection of the CNS are manifested as a set of abnormalities known as HIV-associated neurocognitive disorders (HAND). Although the most severe forms of HAND, e.g. HIV-associated dementia (HAD), have declined in incidence since the advent of antiretroviral therapy (ART), the prevalence of HAND is increasing and is thought to be due to the longer survival times of AIDS patients on HAART [4, 5]. Much of the neurotoxicity associated with CNS infection by HIV has been attributed to the effects of viral proteins such as gp120 that can be shed from infected microglial cells [6–8]. The viral envelope protein, gp120, has been the focus of numerous studies because of its major impact on viral pathogenesis. gp120 not only mediates cell entry but it also determines much of the pathology of the infection. It has long been known that gp120 determines the cellular tropism (i.e. macrophage-tropic, T-cell tropic, or dual tropic) of the virus, but gp120 is also neurotoxic and its properties in this regard are dependent not only on cellular tropism but also clade [9].

A major factor that impacts the neurotoxicity of gp120 is the use of drugs of abuse (DOA). The use of DOA increases the likelihood of acquiring HIV infection. For example, methamphetamine (MA) use has been associated with behavioral parameters such as increased numbers of sexual partners and decreased use of condoms [10]. A recent study suggests an increase in the use of MA among patients newly diagnosed with HIV [11]. MA use is high among men who have sex with men (MSM) [12] and in MSM the incidence of HIV is greatly increased in MSM who use amphetamines as compared with those who do not use this drug [13]. In addition to MA, cocaine and opioids are drugs that are often injected, and injection drug users (IDU) represent a significant proportion of the HIV-infected population. In a study of drug users conducted over 25 years in New York City HIV seroprevalance peaked at just over 50% in IDU, with approximately equal rates of seroprevalance among cocaine and opioid users. This peak was followed by a decline after the initiation of a needle exchange program [14]. However, the same study also showed a trend of increasing seroprevalance in non-injecting drug users. Similar levels of HIV seroprevalance in IDUs and non-IDUs was also documented in another study that included North America and Brazil, where the use of crack cocaine is a major problem [15]. Furthermore, HIV risk behaviors appear to be similar in both IDU and non-IDU opioid users. In addition, drug use may also increase HIV susceptibility through a biological route by modifying immune parameters [16]. In those already infected with the virus, illicit drug use may exacerbate neuroinflammation and accelerate disease progression especially in the CNS [17].

In this review we will focus on the neurotoxic effects of gp120, along with the effects of drugs of abuse, and how these two neurological insults interact to affect the neuropathogenesis of HIV. The drugs of abuse on which we will focus are cocaine, methamphetamine, and morphine.

THE EFFECTS OF GP120 ON THE CNS

gp120 and Chemokine Receptors

Early work that demonstrated the neurotoxic effects of gp120 came from *in vitro* studies that focused on the effects of this protein on neuronal cultures [6, 18, 19]. At the time of these investigations it was known that neurons did not become infected with HIV, but it had been hypothesized that gp120 shed from infected microglial cells might be responsible for the toxicity observed. These investigations examined parameters such as cell death induced by purified gp120 [18], as well as the roles of calcium channel and NMDA antagonists on gp120-mediated neurotoxicity [6, 19]. Although these results demonstrated that gp120 was responsible for neurotoxicity there was also evidence that astrocytes and microglia were necessary for maximal effect [20, 21]. Meucci and Miller [22] showed that gp120-mediated neuronal toxicity was partially dependent upon the existence of glial feeder cells. They also demonstrated that the primary mechanism for cell death was apoptosis and that necrosis was observed only after prolonged exposure to gp120. Subsequent work from this group demonstrated the presence of a number of chemokine receptors on neurons, including those for CCR5, CXCR4, and CX₃CR1 (fractalkine) [23]. Stimulation of these receptors could modulate neuronal survival [23, 24]. Later work from Meucci's group [7, 25] demonstrated that gp120-mediated apoptosis in neuroblastoma cells was dependent upon CXCR4 signaling rather than gp120-mediated internalization of CXCR4. Kaul and Lipton demonstrated that in a mixed cell culture system, gp120 could induce apoptosis, and this effect could be abrogated by the β chemokines RANTES and MIP1 β [7]. Furthermore, the neurotoxicity of gp120 could be abrogated by inhibition of the p38 MAPK signaling pathway or by the addition of a peptide-based inhibitor of macrophage activation. Subsequent work from Kaul *et al.* [26] demonstrated the presence of both CCR5 and CXCR4 in neurons. Thus, exposure of mixed cerebrocortical cultures to gp120 derived from X4 or R5 virus resulted in neuronal death except in the case of cultures derived from animals deleted for both co-receptors. Interestingly, animals deleted for CCR5 exhibited greater neurotoxicity when exposed to dual-tropic gp120 than did wild type or CXCR4 deleted cultures. This suggested a neuroprotective effect could be mediated by CCR5, and this effect was demonstrated using the CCR5 ligands RANTES and MIP1 β . Furthermore, specific inhibition of p38 MAPK also resulted in a decrease in neurotoxicity, while the neuroprotective effects mediated by CCR5 were dependent upon the Akt pathway. In a subsequent paper from Marcus Kaul's group [27], it was shown that exposure of mixed cerebrocortical cultures to gp120 resulted in activation of the p38 and JNK pathways, albeit with different kinetics. In addition, it was demonstrated that exposure of THP-1 monocytes to gp120 resulted in the secretion of neurotoxic substances into the culture medium. However, generation of the neurotoxins were abrogated either by chemical inhibition of the p38MAPK pathway in the THP-1 cells, or by transfecting the THP-1 cells with siRNA targeted against p38. Meucci *et al.* [24] demonstrated the functional expression of the

fractalkine receptor, CX₃CR1 on neurons. Soluble fractalkine was shown to be able to inhibit neuronal apoptosis mediated by gp120, and that this effect was mediated through the Akt pathway.

Taken together these results demonstrate a direct neurotoxic effect of gp120 on neurons that is dependent upon CXCR4 and CCR5 signaling and is mediated through the activation of p38MAPK. Although signaling through CXCR4 or CCR5 can also be neuroprotective in some instances, this effect appears to be mediated through activation of the Akt pathway.

gp120-Mediated Apoptosis and BDNF

Differences in pathogenic potential between T-cell-tropic and macrophage-tropic viruses were identified by Zheng *et al.* [28]. This group found that HIV virions could produce apoptosis in astrocytes and MDM as well as in neurons. The effects on neuronal apoptosis of the virions from different viral strains varied with the most profound effects being seen with the T- tropic strains, intermediate effects with the dual tropic strain, and the lowest level of apoptosis was seen with the M-tropic strains. When signal transduction pathways known to be affected by gp120 were examined, the strains exhibited similar effects with M-tropic strains exhibiting the least impact and the greatest impact being observed with the T-tropic strains. This work was confirmed and extended by a later study that demonstrated that neuronal apoptosis induced by M-tropic or T-tropic strains could be abrogated through use of CCR5 or CXCR4 inhibitors [29].

Neuronal apoptosis induced by gp120 was shown to be dependent upon activation of caspase-3. Although both gp120 and Tat induced apoptosis in mouse striatal neurons, the timing and mechanisms involved were shown to be different. While apoptosis induced by gp120 occurred at 48 h after exposure, Tat-induced apoptosis occurred after 72 h of exposure. Furthermore, while gp120 induced higher levels of caspase-3 activation, Tat was shown to also induce endonuclease G which is a caspase-independent mediator of apoptosis [30]. In a subsequent paper from this group it was demonstrated that gp120 activated both the p38 MAPK and JNK pathways. However, while inhibition of the activation of p38 MAPK reduced both caspase-3 activation and neuronal death, inhibition of the JNK pathway was effective at blocking neither caspase activation nor increased neuronal death [31].

Bachis *et al.* [32] investigated the effects of M-tropic and T-tropic strains of HIV by injection of rats in the striatum with the M-tropic strain gp120Bal. The results in terms of extent of neuronal apoptosis, activation of microglia and induction of BDNF were significantly different than had been seen in previous experiments the group had done with the T-tropic strain gp120IIIB [33]. The damage observed with Bal affected only areas which are proximal to the injection site, whereas gp120IIIB affected areas which are distal to the site of injection, such as the substantia nigra (SN) and the somatosensory cortex. Injection with gp120Bal also caused an increase in IL-1b and an increase in BDNF in the striatum and SN. The difference in BDNF release induced by gp120IIIB and gp120Bal is especially striking. Injection of IIIB reduced BDNF release by approximately 40% while injection of gp120Bal increased BDNF levels to almost 200% of control. This is quite intriguing as BDNF has been demonstrated to be involved in regulating neuronal plasticity and survival [34]. Bachis *et al.* [35] had demonstrated that in cerebellar granule cells gp120IIIB is

internalized and this treatment resulted in an increase in apoptosis and an increase in caspase-3 expression. Treatment of neurons with BDNF decreased apoptosis caused by either gp120IIIB or SDF-1. BDNF also reduced the internalization of gp120. A subsequent report from this group showed that striatal injection of mice with gp120IIIB caused an increase in apoptosis and a decrease in BDNF levels in the striatum [36]. Interestingly, the effects of gp120 injection in mice heterozygous for BDNF (BDNF^{+/-}) were more pronounced than in wild type mice, suggesting that decreased levels of BDNF were involved in mediating the increased effects noted in these mice. Injection of gp120IIIB was also shown to decrease the levels of GDNF, another neurotrophic factor, in the substantia nigra [37]. Although the previous report had shown that exposure of neurons (or CGCs) to BDNF prior to treatment with gp120 prevented apoptosis, Nosheny *et al.* [38] demonstrated this effect using gp120-injected rats. In this case, the rats were first injected with an adenovirus vector encoding BDNF (rAAV-BDNF). Two weeks following this first injection, the rats were then injected with gp120 in the striatum. The rats injected with the BDNF-encoded in the adenovirus showed lower levels of caspase-3 expression, along with lower levels of apoptosis and tissue damage. Interestingly, in the rats treated with the rAAV-BDNF construct, expression levels of CXCR4 were also substantially reduced, whereas in the BDNF^{+/-} mice the expression levels of CXCR4 were substantially increased. This observation supports the role of the co-receptor in mediating the neurotoxic effects of gp120. Furthermore, these reports have identified BDNF, and possibly GDNF, as potential therapeutic targets for the amelioration of the effects of gp120-mediated neurotoxicity.

gp120 and Oxidative Stress

The induction of oxidative stress by gp120 is another pathway that has been demonstrated to cause neuronal damage. Viviani *et al.* [39] utilized a co-culture system to study the effects of gp120 on hippocampal neurons and mixed glial cells. Treatment with gp120 caused an increase in neuronal death and an increase in neuronal levels of intracellular calcium, but it failed to induce higher levels of reactive oxygen species (ROS) in these cells. However, higher levels of ROS and IL-1 β were observed in the glial cells. Higher levels of ROS, as well as decreased GSH/GSSG ratios were observed in primary rat astrocytes treated with gp120 from an M-tropic HIV strain (HIV-1_{96ZM651}) [40]. These investigators also found increased expression and activity of MRP-1, an efflux transporter that is involved in the efflux of GSH as well as various therapeutic agents. Treatment of primary mixed glial/neuronal cultures with gp120_{LAV} has also been shown to increase levels of ROS production, apoptosis, lipid peroxidation and a loss of dopaminergic neurons [41]. Exposure of rat brain endothelial cells to gp120 resulted in an increase in indicators of oxidative stress including decreased GSH/GSSG ratios, as well as reduced levels of glutathione peroxidase and glutathione reductase [42]. This suggests that the BBB is also negatively affected by the oxidative stress induced by gp120. Injection of gp120 into the caudate-putamen of rats has been shown to induce neurotoxic effects in brain regions that extend several millimeters from the site of injection, as well as regions more proximal to the injection site. Injection with HIV-1 Bal gp120 caused neuronal loss, loss of tyrosine hydroxylase-positive cells in the substantia nigra as well as loss of dopamine transporter-positive cells in the caudate-putamen. Delivery of antioxidant enzymes by a recombinant SV40 vector, either superoxide dismutase (SOD) or glutathione peroxidase (GPx1), prior to the gp120 treatment resulted in

substantial abrogation of the toxic effects of the HIV-1 protein [43]. Subsequent studies using cultured neurons demonstrated that dopaminergic neurons were more sensitive to gp120-induced apoptosis but that transduction with a viral vector encoding either SOD or GPx1 reduced the effects of the gp120 through inhibition of ROS [44]. Additional evidence that oxidative stress is involved in mediating the neurotoxic effects of gp120 comes from the observation that treatment of primary cultures of human astrocytes with gp120 resulted in increased expression of several genes involved in antioxidant response including Nrf2, heme oxygenase 1 and NAD(P)H dehydrogenase quinone 1 [45]. The induction of increased levels of these genes could be abrogated by pretreatment of the cells with N-acetyl cysteine and catalase prior to treatment with gp120.

gp120 and Inflammatory Cytokines

In addition to various other mechanisms of neurotoxicity attributed to gp120, the increased expression of pro-inflammatory cytokines has been of interest. As mentioned above, Viviani *et al.* [39] detected increased levels of IL-1 β in primary glial cells treated with gp120. In primary rat astrocytes treated with gp120, increased levels of IL-1 β , TNF- α , and IL-6 were observed [46]. In studies from our laboratory, gp120 has been determined to cause increased expression of pro-inflammatory cytokines including IL-6, IL-8 and CCL5 in both the SVGA astrocyte cell line, as well as in primary human fetal astrocytes (Fig. 1) [47–49]. The role of gp120 in induction of proinflammatory cytokines was determined by treating astrocytes with gp120 added extracellularly as well as by transfecting cells with a plasmid encoding gp120. The expression levels of these cytokines increased in a time-dependent manner in response to gp120 at the levels of both mRNA as well as in proteins secreted in the supernatants. Different strains of gp120 showed a differential response in terms of IL-6 expression in astrocytes, depending upon whether they were M-tropic or T-tropic [49]. The increase in the pro-inflammatory cytokines was gp120- specific, since siRNA against gp120 abolished the expression of cytokines induced by transfection of a plasmid encoding gp120. The role of the nuclear factor-kappa B (NF- κ B) pathway was also determined because there are NF- κ B binding sites in the promoters of IL-6, IL-8 and CCL5 [50, 51]. It was determined that gp120 increased the activation of I κ B and increased translocation of p50 from cytoplasm to the nucleus [47,48,49]. The involvement of the NF- κ B pathway was confirmed using SC514 and BAY11–7082, specific inhibitors for this pathway. Additional confirmation of the role of gp120 was obtained through the use of siRNA for RelA (p65) and NF- κ B1 (p50) [49].

The different clades of HIV-1 have been associated with different rates of disease progression as well as different neuropathogenic effects [52]. For example, although this study involved small sample sizes, more rapid disease progression and a higher incidence of dementia was associated with clade D as compared to those patients infected with clade A [53]. The differential effects of Tat from clade B and clade C have been studied in terms of the induction of inflammatory cytokines and chemokines [54, 55], as well as the induction of the serotonin transporter, 5-HTT, and indoleamine 2,3-dioxygenase [56]. These reports suggest that Tat from clade B induces higher levels of proinflammatory cytokines and lower levels of anti-inflammatory cytokines, as well as more severe alterations in terms of tryptophan and serotonin synthesis and transport, than does Tat from clade C. Although these reports have focused on the effects of Tat from different clades, a recent report

demonstrated differential pathogenic effects of gp120s derived from clade B and clade C viruses [57]. This report showed that gp120 from clade B reduced the levels of the NMDA receptor and intracellular glutamine, while increasing the intracellular levels of glutamate. In comparison, gp120 derived from clade C virus had no effect on these parameters. In addition, gp120 from clade B also induced higher levels of COX-2 and the thromboxane A2 receptor, strongly suggesting an increase in the neurotoxin arachidonic acid. Thus, the differential effects of HIV-1 clades on neuropathogenesis that have been observed with regards to Tat parallel those effects that have been observed with gp120.

The Effects of gp120 on the BBB

Alterations in permeability of the blood-brain barrier (BBB) have also been shown to be caused by gp120. Using both M-tropic and T-tropic strains, Kanmogne and colleagues have shown that exposure of HBMEC to gp120 reduces the expression levels of the tight junction proteins ZO-1, ZO-2 and occludin. Furthermore, permeability of the HBMEC monolayers to FITC-dextran was also increased by exposure to the viral protein [58]. A subsequent study by this group determined that gp120 activates STAT1 signaling in HBMEC that is also associated with increased expression levels of IL-6 and IL-8, as well as increased migration of monocytes in an *in vitro* model of the BBB [59, 60]. The decrease in ZO-1 along with increased BBB permeability in HBMEC in response to gp120 have been reported by others [61, 62]. Injection of gp120 into the caudate putamen of rats resulted in increased expression of matrix metalloproteinases (MMP) 2 and 9. Injected rats also displayed reduced levels of claudin-5 and laminin. Oxidative stress induced by gp120 was implicated as one of the mechanisms responsible for injury to BBB function, as delivery of antioxidant enzymes to the caudate putamen prior to injection with gp120 ameliorated the effects of the viral protein [63].

Taken together, the studies presented above demonstrate that gp120 is not only directly toxic to neurons, but it is able to exert indirect effects through induction of inflammatory cytokines, the induction of oxidative stress, and a reduction in factors important for neuronal survival and plasticity. In addition, gp120 increases BBB permeability which may contribute to an increased level of CNS infection with HIV. A number of signaling pathways, including p38MAPK, NF- κ B and STAT1 have been shown to be involved in mediating these neurotoxic effects, while induction of Akt signaling, as well as the induction of various neurotrophic factors, can ameliorate these effects.

THE EFFECTS OF COCAINE ON THE CNS

Early work on transcription factors involved in CNS responses to cocaine administration determined that NF- κ B expression was induced in the brains of cocaine-treated mice. Using an mRNA microarray followed by western blot analysis, it was determined that chronic exposure, but not acute exposure, of mice to cocaine resulted in increased expression of NF- κ B [64]. Subsequent work showed chromatin modifications indicative of higher levels of activation of three NF- κ B genes, p105/p50, p65/RelA, and I κ B β [65]. An NF- κ B-LacZ reporter was used to show greater NF- κ B dependent transcriptional activity in the nucleus accumbens, as well. Morphological changes in response to cocaine (i.e. induction of dendritic spines), was also found to be dependent upon the NF- κ B pathway. By employing

an elegant approach that utilized rats that express a *cfos-LacZ* transgene in activated neurons, followed by FACS purification of the activated neurons and microarray and PCR analyses of these neurons, several genes associated with cocaine activation of neurons were identified. The genes whose expression levels were increased include *fosB*, *arc* and *nr4a3*. In contrast, the expression level of *Map2k6*, which activates p38 was decreased [66]. The expression level of a phosphatase that inactivates p38, *Mkp1*, was increased. Although, this might suggest that p38 signaling is inhibited in cocaine-activated neurons, Yao *et al.* [67] as described below, has determined that p38 is activated in rat neurons exposed to cocaine and gp120 (see below).

Many of the effects of cocaine have been shown to be mediated by the σ -1 receptor (reviewed in 68). This receptor is a non-opioid receptor that resides on the interface between the endoplasmic reticulum and the mitochondrion. This receptor is activated by a number of agents, including cocaine and methamphetamine [68, 69]. This receptor has been found to activate several ion channels, including those for calcium, potassium and sodium. In addition, signaling through the σ -1 receptor regulates apoptosis [68]. The σ -1 receptor physically interacts with the dopamine receptor D1 and modulates the function of the dopamine receptor in mouse brain [70]. Recently, stimulation of the σ -1 receptor in rats has been demonstrated to activate the Akt pathway and affect phosphorylation of the eNOS protein [71].

The Effects of Cocaine on the BBB

Using BV-2 mouse microglia, Yao *et al.* [72] showed that cocaine induces MCP-1 expression at the mRNA and protein levels. The findings were reproducible in primary rat microglia. The use of the σ -1R antagonist, BD1047 demonstrated that the effect was mediated by the σ -1R; similar results were seen when the BV-2 cells were transfected with σ -1R siRNA. ROS generated by microglia in response to cocaine, as well as the Src tyrosine kinase and the PI3/Akt pathways were also necessary for maximal induction of MCP-1. Other signaling work showed that the ERK1/2 and JNK pathways, but not p38, were involved in mediating MCP-1 induction by cocaine. Furthermore, it was shown that these pathways, as well as Src kinase and σ -1R were upstream of NF- κ B, and that the activities of these signaling molecules was necessary for binding of p65 to the MCP-1 promoter. Yao *et al.* [72] went on to further demonstrate that the MCP-1 induction by cocaine was responsible for increased monocyte migration across the BBB. Through this pathway, cocaine may increase the numbers of HIV-infected cells in the CNS, as well as increase neuroinflammation. Similarly, subsequent work from Yao *et al.* [73] demonstrated that cocaine, through σ -1R, as well as the MAP kinase, Src tyrosine kinase, PI3/Akt, and NF- κ B pathways, induced higher levels of ALCAM, an adhesion molecule that plays a key role in transmigration of leukocytes across the BBB in several neurological disorders. Using HBMECs it was demonstrated that cocaine, acting through the σ -1 receptor, was responsible for induction of ALCAM expression in these cells, as well as increasing monocyte adhesion to the endothelial cells and stimulating transmigration of monocytes across a monolayer of HBMEC's. This group also determined that exposure of HBMECs to cocaine resulted in the activation of PDGF- β R as well as a physical interaction between this receptor and the sigma-1 receptor. Furthermore, the use of antagonists for either sigma-1 receptor or PDGF-

β R resulted in the abrogation of the cocaine-mediated activation of the Akt and MAPK pathways. Subsequent work from this group demonstrated that in HBMEC exposure to cocaine upregulated PDGF-B and PDGF-BB and that this was mediated by the Egr-1 transcription factor [74]. Consistent with their previous results, the sigma-1 receptor was important for the induction of PDGF-BB. In turn, the upregulation of PDGF-BB was shown to mediate a decrease in zona occludens 1 (ZO-1), a tight junction protein important for maintaining the integrity of the BBB. Furthermore, this group also confirmed both the cocaine-mediated induction of PDGF-BB and the involvement of PDGF-BB in maintaining the integrity of the BBB [74]. Recently, Notch 1 has been demonstrated by this group to be a critical mediator of cocaine-induced PDGF-BB upregulation [75].

In addition to the changes in regulatory and signal transduction pathways, Fiala *et al.* [17, 76] have shown that cocaine induces morphological changes in HBMECs through cytoplasmic remodeling and the disruption of endothelial cell junctions. These changes, termed “ruffling” could be visualized by scanning electron microscopy, and were accompanied by a reduction in TEER values in endothelial cell monolayers that had been exposed to cocaine.

Taken together, the results described above demonstrate that cocaine may impact HIV CNS infection by at least two different, but related mechanisms: 1- an increase in permeability of the BBB as evidenced by a decrease in tight junction proteins (e.g. ZO-1) and a decrease in TEER values and 2- an increase in transmigration of monocytes across the BBB, possible in response to an increase in cytokines in microglial cells (e.g. MCP-1). Together, these mechanisms may result in an increased influx of HIV-infected monocytes into the CNS which will eventually lead to an increased viral load in the CNS and exacerbation of the neurocognitive effects of HIV infection.

THE EFFECTS OF COCAINE AND HIV-1/GP120 ON THE CNS

The interest in the effects of cocaine on HIV infection of the CNS was initially triggered by several factors, including the prevalence of cocaine use among HIV-infected patients and the fact that the CNS is a target for both HIV and cocaine. This led to some initial reports that focused on the effects of cocaine on viral replication in macrophages as well as in animal models of HIV infection. In one early report, it was found that cocaine enhanced replication of HIV in PBMC, and that the enhancement was dependent on TGF- β [77]. Interest in the effects of cocaine on HIV pathogenesis was also stimulated by the demonstration that cocaine could stimulate HIV replication in PBMC. As measured by an increase in p24 antigen, cocaine exposure resulted in increases in HIV replication ranging between 128–280% [78]. A case report from Nath *et al.* [79], described the onset of accelerated dementia in an HIV-infected patient who used cocaine and/or methamphetamine on a regular basis. Of note, dementia progressed rapidly even after the patient discontinued drug use. The patient displayed generalized cerebral atrophy that was prominent in both the frontal and the temporal regions of the brain. Interestingly, Gekker *et al.* [80] demonstrated that cocaine could stimulate HIV replication in microglial cells. However, exposure of the cocaine-treated microglial cells to κ -opioid receptor agonists resulted in suppression of cocaine-induced enhancement of HIV replication. This was shown to be mediated by the abrogation

of the cocaine-mediated activation of ERK1/2. ERK1/2 activation had been shown to be responsible for the upregulation of CCR5, and the k-opioid ligand also suppressed this. A subsequent report demonstrated that exposure to cocaine significantly increased HIV replication in human astrocytes [81]. This group also reported that proteomic analysis revealed that the expression levels of a number of proteins, some of which may affect HIV replication, were altered in these cells when exposed to cocaine. Accompanying this was an increased level of the phosphorylated form of ERK2.

The Effects of Cocaine and gp120 on Apoptosis and Cell Viability

The observations of increased viral replication in the presence of cocaine led to explorations of the potential interactions between various virotoxins, including gp120, and cocaine. Bagetta *et al.* [82] examined apoptosis in the brains of rats that had been injected with either gp120 or gp120 + cocaine. Of note, the dose of cocaine that was utilized was a lower dose that did not produce increased apoptosis when administered alone. Injection of gp120 into the lateral cerebral ventricle caused an increase in apoptosis in the neocortex of treated rats that was further increased by the administration of cocaine. Levels of apoptosis were significantly reduced by pretreatment with inhibitors of either NMDA receptors or iNOS. Immunohistochemical staining confirmed the increase in iNOS associated with cocaine treatment. In addition to their work on the effects of cocaine on the BBB, Buch and colleagues have also investigated the mechanisms responsible for the combined toxicities of gp120 and cocaine on cells of the CNS. Using rat primary neurons, Yao *et al.* [67] determined that treatment of these cells with either cocaine or gp120 resulted in decreased cell viability, an increased level of apoptotic cells, and increased expression of caspase-3, Bax, and reactive oxygen species. Treatment with both cocaine and gp120 resulted in increases of all of these effects compared to cells treated with either agent alone. Examination of the signal transduction pathways and transcription factors that mediate these effects revealed that all three branches of the MAPK pathway were activated by cocaine and gp120, and that the effects of both agents in combination were generally greater than the effects seen with either agent alone. In addition to the MAPK signaling pathway, the NF- κ B pathway was also activated by these agents. Finally, chemical inhibition of the MAPK or NF- κ B pathways resulted in increased cell viability.

Using primary rat astrocytes, Yang *et al.* [83] determined that exposure to cocaine resulted in dose-dependent loss of cell viability when cells were exposed to the drug in combination with a constant dose of gp120. The loss in viability produced by the cocaine with the gp120 was significantly greater than the loss in viability observed with either agent alone. As with the loss of cell viability, gp120 administered with cocaine also produced greater levels of caspase-3 activation and ROS production than was seen with either agent administered alone. In addition, mitochondrial dysfunction as determined by a decrease in mitochondrial membrane potential was also increased in the presence of both gp120 and cocaine. In terms of signaling pathways, Yang *et al.* [83] examined the MAPK pathways (i.e. p38, JNK and ERK), and found that the phosphorylated forms of all three proteins were increased in the presence of both agents to a greater extent than seen with either agent alone. Increased translocation of the p65 subunit of NF- κ B to the nucleus was also observed in astrocytes treated with both cocaine and gp120. Treatment with chemical inhibitors of ERK, p38, or

JNK activation, in the presence of both gp120 and cocaine, resulted in an increase in cell viability.

Thus, cocaine and gp120 activate overlapping signaling pathways that mediate neurotoxicity in microglia, neurons and astrocytes. In addition to overlapping signaling pathways such as p38MAPK family and NF- κ B, some of the effects of gp120 such as increased permeability of the BBB are induced independently by cocaine. This opens up numerous possibilities for this DOA to exacerbate the neurotoxic effects of gp120.

THE EFFECTS OF METHAMPHETAMINE ON THE CNS

Methamphetamine and the Dopaminergic System

The basis for the neurochemical effects of methamphetamine (MA) is thought to be the chemical similarity between the drug and the neurotransmitter dopamine. Early investigations of methamphetamine toxicity utilized approaches that determined the effects of MA exposure on dopamine synthesis, metabolism, transport and disposition. In one of the first reports regarding MA-toxicity, Gibb and Kogan [84] determined that MA-treatment inhibited the activity of tyrosine hydroxylase (TH), a key enzyme involved in the dopamine biosynthetic pathway, in the neostriatum of rats. Subsequent work determined that the inhibitory effects of MA were even more pronounced with regard to the activity of tryptophan hydroxylase (TPH), an enzyme responsible for serotonin synthesis [85, 86]. As with TH, the effects of MA on TPH could be abrogated by α -methyl-*r*-tyrosine and the protection conferred by this agent could be reversed by L-DOPA. These early experiments demonstrated that the effects of MA impact both the dopaminergic and the serotonergic systems.

The discovery of the effects of MA on the dopaminergic system led to investigations regarding the mechanism of these effects and one of the first avenues explored was the effect of MA on dopamine transporters (DAT). Using a rat model, Wagner *et al.* [87] demonstrated a depletion of dopamine levels in the striatum as well as a loss of sites for dopamine uptake following administration of multiple doses of MA. Subsequently, Schmidt and Gibb [88] demonstrated that treatment of animals with amfonelic acid, an inhibitor of DAT, abrogated the inhibitory effects of MA on TH activity. The effects of amfonelic acid on DAT, as well as the effects of other DAT inhibitors, were confirmed by other investigators [89]. The use of mice in which the gene for the DAT was deleted provided additional confirmation of the role of the dopaminergic system, and specifically the DAT, in mediating the effects of MA. Mice that were homozygous for the DAT deletion and that were treated with MA did not exhibit the depletion of dopamine levels in the striatum that was seen with wild type animals. Mice that were heterozygous for the deletion exhibited levels of striatal DA that were intermediate between wild type and homozygous KO animals [90]. Levels of oxidative stress and MA-induced astrogliosis also positively correlated with the levels of MA observed in the wild type and mutant animals.

In addition to DAT, the vesicular monoamine transporter 2 (VMAT-2) also mediates DA disposition (reviewed in 91). VMAT-2 packages dopamine and other monoamine transmitters into synaptic vesicles. As with DAT, knockout mice have been a valuable tool

for the study of the effects of MA on VMAT-2. Mice that were heterozygous for the VMAT-2 deletion exhibited higher levels of neurotoxicity and aberrant distribution of DA compared to that observed in wild type mice [92]. Subsequent work demonstrated that MA treatment altered the interaction between a VMAT-2 ligand and the transporter [93]. A single dose of MA also caused a decrease in vesicular DA uptake [94], while repeated administration of MA resulted in a decreased level of expression of VMAT-2 in the vesicular fraction [95]. Treatment with the dopamine transport inhibitor abrogated the effects of MA on VMAT-2. Eyerman and Yamamoto [96] also demonstrated the function of VMAT-2 on dopamine disposition through the use of lobeline, a compound that abrogates some of the behavioral effects of MA. Although lobeline did not alter the increase in striatal dopamine caused by MA, it did abrogate the MA-mediated decrease in immunoreactivity of VMAT-2.

The effect of MA on the dopaminergic system is not only mediated by transporters involved in the disposition of dopamine, but it is also mediated by dopamine receptors. This line of investigation has been facilitated by the availability of antagonists for the D1 and D2 receptors. The D1 antagonist SCH23390 and the D2 antagonist sulpiride have both been shown to abrogate the effects of MA on striatal dopamine levels [97]. Administration of SCH23390 has also been shown to protect against MA-induced neurotoxicity [98]. Both the D1 antagonist SCH23390 and a D2 antagonist, raclopride, reduced the levels of astrogliosis, depletion of DAT levels, and cell death [99]. SCH23390 was also shown to inhibit the induction of apoptosis that was MA-induced, as determined by caspase-8 cleavage and TUNEL staining [100].

Methamphetamine and Oxidative Stress in the CNS

One of the major effects of MA on the CNS is the induction of oxidative stress. Administration of MA, in either repeated acute doses or chronic administration of a lower dose of MA, induced higher levels of SOD activity and lipid peroxidation in the brains of rats [101]. Repeated doses of MA have also been shown to alter levels of enzymes involved in the response to oxidative stress, including glutathione peroxidase and catalase, while increasing lipid peroxidation in the striatum of mice [102]. Administration of MA to mice deleted in the VMAT-2 gene demonstrated higher levels than their wild-type counterparts of ROS and cysteinyl-DA, a protein modification associated with an increase in levels of oxidized DA [103]. Previous results had demonstrated an increased level of protein cysteinyl-DA using a rat model of MA neurotoxicity [104]. Similarly, administration of MA was found to result in an increase in levels quinoproteins and NAD(P)H quinine oxidoreductase-1 in mice [105]. Elevated levels of protein carbonyls, as well as other markers of oxidative stress, have been observed in mice treated with repeated doses of MA [106]. Administration of repeated injections of MA to rats was found to reduce the level of parkin, while increasing the level of parkin modified by a by-product of lipid peroxidation. Both of these effects could be reproduced by incubating synaptosomes from control animals in H₂O₂. MA-treatment also induced a significant reduction in the activity of the 26S proteasome that could be abrogated by pretreatment with vitamin E [107]. Using an *in vitro* model of the BBB, MA treatment of human brain microvascular endothelial cells was shown to increase the phosphorylation of the p47 subunit of NAD(P)H oxidase along with

increased NAD(P)H oxidase activity, increased generation of ROS, decreased levels of occludin and increased transmigration of monocytes through a monolayer of the brain microvascular cells [108]. The decrease in occludin expression induced by MA could be abrogated by an inhibitor of ERK1/2. Other groups have identified signaling pathways activated by MA. In human SH-SY5Y cells treatment with MA was demonstrated to increase the phosphorylation of JNK and ERK. However, phosphorylation of p38 MAPK was not increased by MA [109]. Another study utilized mice that were injected subcutaneously and then sacrificed between 6 and 48 h later. Striatal homogenates revealed increased phosphorylation of JNK and ERK [110].

Taken together it is clear that MA induces changes in DA disposition that result in neurotoxicity. Some of these effects are mediated by oxidative stress that is the result of altered levels of expression and activity by the DAT and other components of the dopaminergic system.

THE EFFECTS OF GP120 AND METHAMPHETAMINE ON THE CNS

The effects of MA and gp120 on several aspects of BBB integrity were investigated by Mahajan *et al.* [111]. Exposure of a BBB model comprised of human BMEC cocultured with human astrocytes to MA was shown to exhibit decreased transendothelial electrical resistance (TEER). Decreases in TEER were shown to be dose dependent, and TEER was further decreased by treatment with gp120 and MA. Expression levels of tight junction proteins were examined and treatment with a low dose of MA (10 nM) produced no significant changes in ZO-1, JAM-2, Claudin-3, Claudin-5, or occludin. However, treatment with the low dose of MA in conjunction with gp120 did produce significant changes in all of these proteins. Interestingly, while expression of all of the other genes encoding tight junction proteins decreased, expression of occludin increased. Of particular importance is that this study also demonstrated that the changes in the BBB were associated with an increase in transmigration of PBMC across the endothelial cell layer. Transmigration increased in a dose-dependent manner in response to MA, and transmigration was further increased by the addition of gp120.

The use of mice that are transgenic for gp120 has also helped reveal the effects of gp120 on MA-induced behaviors [112]. Mice that are transgenic for gp120, along with their non-transgenic littermates, were subjected to a series of 4 MA injections over a period of 2 months. After MA treatment, mice were assessed for various behaviors including locomotion and stereotypic (i.e. repetitive) behaviors. Although MA treatment resulted in several types of behavioral changes, the gp120 transgenic mice were significantly different from their non-transgenic littermates in only stereotypic behaviors. This brief report was of interest not only because it demonstrates an interaction between gp120 and MA at a behavioral level, but also because the behavioral effect strongly suggests an interaction between MA and gp120 at the same site that is targeted by both MA and HIV infection. This further validates the relevance of the gp 120 transgenic mice in studies of HIV neuropathogenesis.

THE EFFECTS OF MA IN CONJUNCTION WITH TAT AND GP120 ON THE CNS

Although there is a paucity of data on the effects of gp120 and MA on the CNS, there are a few studies that have been performed which have included gp120 in combination with Tat and MA. Using primary cultures of human fetal neurons, Turchan *et al.* [113], demonstrated that gp120 in combination with Tat served to exacerbate neuronal cell toxicity induced by MA. A gradation of MA doses demonstrated that MA exerted this effect in combination with the viral proteins in a dose-dependent manner. Furthermore, a neuroprotective effect was observed when the combination of MA and viral proteins was administered in conjunction with 17 β -estradiol.

Both gp120 and MA are known to cause oxidative stress in the CNS when administered separately [40, 114]. Banerjee *et al.* [115] showed that intrastriatal MA injection in mice resulted in synergistic interactions between MA and a mixture of Tat and gp120 that were mediated through oxidative stress. Mice treated with both MA and the HIV proteins showed an increased level of various measures of oxidative stress in the brain, including levels of malonyl dialdehyde (MDA), lipid peroxidation and protein carbonylation. Increased levels of antioxidant enzymes such as GSH and GPx were also increased in the presence of MA and a mixture of Tat and gp120.

As was the case with cocaine it can be seen that MA and gp120 induce effects that are mediated by overlapping pathways. With regard to MA and gp120, this is especially relevant in terms of oxidative stress because the NF- κ B pathway is involved in mediating the response to both gp120 and oxidative stress.

MORPHINE AND THE CNS

Pathways of Morphine Activity

Morphine is the primary product resulting from the metabolism of heroin. Although there are 3 different classes of opioid receptors, μ , δ , and κ morphine appears to exert its greatest effects through its interactions with the μ -opioid receptor. While acute stimulation of the μ -opioid receptor results in a decrease in cAMP levels, chronic stimulation of the μ -opioid receptor causes an increase in adenylyl cyclase activity that results in increased intracellular levels of cAMP. The high cAMP levels stimulate PKA activity which then result in higher levels, and higher DNA-binding activities, in CREB transcription factors (reviewed in [116]). Opioid receptors have also been shown to regulate other signaling pathways including phospholipase C- PKC pathways, the MAPK pathways, as well as Ca⁺⁺-mediated signaling (reviewed in [117]).

The NF- κ B transcription factor has been shown to be involved in regulating a number of cytokines as well as other genes in cells of the immune system. In addition, NF- κ B has also been shown to play a role in opioid signaling. In one of the first demonstrations that morphine can modulate NF- κ B activation, Roy *et al.* [118] exposed mouse peritoneal macrophages to either a low dose (50 nM), or a high dose of morphine (50 μ M), in combination with LPS. The low dose of morphine and LPS resulted in increased production

of IL-6 and TNF- α compared to cytokine levels produced by administration of LPS alone. However, the high dose of morphine followed by exposure to LPS resulted in decreased production of IL-6 and TNF- α compared to levels of the cytokines produced by LPS alone. The increase in cytokine levels was paralleled by increased binding activity of the NF- κ B p50-p65 heterodimer. Although the increased cytokine levels induced by a low dose of morphine could be reversed by treatment with an opioid receptor antagonist, the antagonist could not reverse the inhibitory effect of the higher morphine dose.

The role of the NF- κ B pathway in the induction of TNF- α by morphine was also explored in astrocytes and microglia by Sawaya *et al.* [119]. It was demonstrated that morphine induced TNF- α expression in primary astrocytes to a level approximately 5-fold higher than in untreated cells. The induction of TNF- α by morphine could be abrogated by the opioid antagonist, naloxone, as well as by the specific μ -opioid antagonist, β -funaltrexamine hydrochloride. Because this induction showed that TNF- α mRNA and protein were increased, these investigators utilized deletion analysis of the TNF- α promoter to identify the region of the promoter responsible for mediating induction by morphine. An NF- κ B binding site was present in the region responsible for morphine-mediated induction, and further analysis demonstrated that siRNA against p65 abrogated induction of the promoter by morphine. Notably, p65 translocation to the nucleus was also greatly increased by morphine treatment of the cells. Taken together with the results from Roy *et al.* [118], these papers demonstrate that morphine can potentiate the induction of TNF- α in response to LPS, and morphine alone can also induce TNF- α expression in microglia and astrocytes.

Morphine, Chemokines, and Chemokine Receptors

CXCR4 is a chemokine receptor that has been well studied as one of the co-receptors for HIV-1 infection, but it is also a receptor for SDF-1 that is involved in mediating pro-survival signaling in neurons [120]. The effects of signaling through the m-opioid receptor on CXCR4 signaling have been investigated by Olimpia Meucci and colleagues [121, 122]. Although acute treatment of rat cortical neurons with DAMGO, a μ -opioid agonist, resulted in increased phosphorylation of Akt and ERK1/2, long term exposure (24 h) to DAMGO resulted in inhibition of CXCL12-induced phosphorylation of both Akt and ERK [121]. The inhibitory effect of DAMGO on Akt phosphorylation induced by CXCL12 could be abrogated by treatment with the opioid antagonist naloxone. Analysis by flow cytometry revealed that DAMGO did not have an effect on CXCR4 levels expressed on the cell surface. While CXCL12 exerted a significant neuroprotective effect against NMDA-induced cell death, this protective effect was abrogated by 24 h treatment with DAMGO but restored when cells were treated with DAMGO in the presence of a μ -opioid specific antagonist (CTAP). These results were confirmed and extended by Sengupta *et al.* [122] who demonstrated that CXCL12 could induce phosphorylation of CXCR4 and that either morphine or DAMGO could inhibit this phosphorylation. Addition of an opioid antagonist along with either DAMGO or morphine restored the ability of CXCL12 to induce phosphorylation of CXCR4. Of note is that acute DAMGO or morphine treatments did not affect CXCR4 phosphorylation. Treatment of neurons with CXCL12 was also shown to result in the internalization of the receptor, and this effect was inhibited by long-term treatments with DAMGO. This raised the question of the mechanism by which DAMGO

inhibited these neuroprotective effects of CXCL12. Long-term treatment with DAMGO was shown to increase the expression of ferritin heavy chain (FHC), a protein previously known to be a regulator of CXCR4 [123]. In neurons transfected with siRNA targeted against FHC, DAMGO treatment had no effect on CXCL12 induction of Akt and ERK phosphorylation. Similar results were obtained using an *in vivo* transgenic mouse model in which FHC expression had been inhibited through the use of shRNA. Taken together this body of work has elucidated the mechanism by which morphine can inhibit neuroprotection, and thus make neurons more susceptible to damage through various mechanisms, including oxidative stress.

Although morphine was not shown to alter CXCR4 expression on neurons, morphine and DAMGO have been shown to increase expression of CCR5 and CXCR4 on lymphoblasts and monocytes [124]. Induction of the chemokine receptors was shown to be dose-dependent, and the maximal effect of two- to seven-fold increase was seen after 72 h of treatment. Treatment of PBMC with DAMGO also resulted in a two-fold increase in HIV infection as evaluated by p24 assays and PCR amplification of the HIV LTR. In a subsequent study from this group it was shown that DAMGO acted through TGF- β to increase CXCR4 expression. However, the increase in CCR5 expression did not appear to be mediated through the increase in TGF- β induced by DAMGO [125]. Using both the astrocytoma cell line U87, as well as normal human astrocytes, morphine has been demonstrated to upregulate the expression of CCR5 [126, 127]. The use of antagonists demonstrated that the increased expression of CCR5 was mediated through the μ -opioid receptor [126] and observed increases in the phosphorylation of p38 MAPK suggested that this signaling pathway may be involved in this effect of morphine [127].

Morphine and the BBB

The increase in HIV co-receptor and HIV replication induced by morphine has an even greater potential impact in terms of the CNS when viewed in the context of data that indicate that morphine also increases permeability of the BBB [128,129]. Using primary human brain microvascular endothelial cells, Mahajan *et al.* [128] demonstrated that treatment with morphine decreased ZO-1 and occludin expression while increasing expression of JAM-2, another component of the BBB. Morphine treatment also resulted in a significant dose-dependent reduction in TEER values and an increase in transmigration of PBMC across an *in vitro* model of the BBB. Significantly, morphine also caused an increase in transmigration of HIV-infected PBMC when compared with transmigration levels of non-treated HIV-infected PBMC. In a subsequent study, Wen *et al.* [129] demonstrated that morphine increased PDGF-B expression through activation of the p38 MAPK, ERK1/2 and JNK pathways, and the increase in PDGF-B was involved in mediating the decrease in ZO-1 expression. Furthermore, this group also demonstrated that treatment with morphine increased the permeability of their *in vitro* model of the BBB.

Morphine has also been demonstrated to induce MCP-1 in astrocytes. Using primary cultures of mouse astrocytes, El-Hage *et al.* [130] showed that conditioned media obtained from morphine-treated astrocytes increased the chemotactic response in microglial cells. It was further demonstrated that these effects could be abrogated by blocking the activity of

MCP-1 with an antibody to the chemokine. It is also of note that there are several research papers on the effects of morphine in conjunction with Tat e.g., [131]. Although the results reported little or no effect of morphine, treatment with morphine plus Tat often resulted in an increased response when compared to the results observed with Tat alone.

The Effects of Morphine in Combination with gp120

Interest in the combined effects of HIV proteins and morphine has been heightened by several studies that demonstrate that morphine exacerbates parameters associated with viral infection in various macaque models of AIDS. In those models which have shown a positive correlation between opiate treatment and more rapid disease progression, the presence of higher viral loads in the CSF has been a fairly consistent finding [132–134]. This strongly suggests that the effects of morphine seen in the studies described above have relevance to primate models of HIV/AIDS.

The Effects of Morphine and gp120 on Apoptosis and Expression of Cell Surface Receptors

There are relatively few studies on the combined effects of gp120 and morphine in the CNS. One of the first studies to examine gp120 neurotoxicity in the context of morphine abuse investigated the effects of morphine and gp120 on apoptosis in mixed neuronal cultures derived from human fetal brain tissue [135]. In these cultures, which were a mix of neurons (70–80%), astrocytes (15–25%), and microglia (3–7%), treatment with morphine produced a slight increase in the level of apoptosis, treatment with gp120 resulted in a level of apoptosis slightly more than morphine, and treatment with both morphine and gp120 resulted in a significantly increased level of apoptosis that was reported to be the result of synergy between the two agents. The effects of morphine could be blocked by treatment with naloxone, suggesting that the effect of morphine was mediated through the μ -opioid receptor. Induction of apoptosis could be blocked by SB203580, which inhibits the p38 MAPK pathway. Furthermore, TPK, a peptide inhibitor of macrophage activation reduced the level of synergy between gp120 and morphine. This suggests that some of the apoptotic effects were contributed by macrophages. However, the effects of gp120 and morphine are not restricted to neurons or microglia, as demonstrated by an investigation that utilized the human astrocytoma cell line U373 [136]. In this cell line treatment with morphine suppressed MIP-1 β expression and secretion and increased CCR3 expression at the mRNA and protein levels. Morphine treatment also increased the expression of CCR5, as did gp120. Treatment with both morphine and gp120 further increased CCR5 expression at levels more indicative of a synergistic rather than an additive interaction between the two agents. Morphine also increased expression of the β -opioid receptor, and treatment with morphine and gp120 resulted in a slight increase over that seen with morphine alone (2.7 vs 3.1 fold). As was seen in the case of morphine-induced apoptosis [135], naloxone could abrogate the effects of morphine on CCR5 and MIP-1 β expression. Intracellular levels of calcium were also increased by treatment with either morphine or gp120, and calcium levels were higher in cells treated with both agents when compared to those cells treated with either gp120 or morphine. It has recently been demonstrated that the level of neurotoxicity of the interactions between morphine and gp120 are dependent upon the strain of the virus from which the gp120 was derived. Although all the strains of gp120 tested were neurotoxic,

morphine exacerbated the neurotoxicity of some strains but had no effect upon the neurotoxicity exhibited by other strains [137].

In order to address the effects of morphine and HIV proteins on immune responses in the CNS, El- Hage *et al.* [138] used primary mouse astrocytes to determine changes in TLR expression in response to different combinations of morphine, Tat, and gp120. Exposure of astrocytes to gp120 resulted in increased TLR2 expression. However, increased expression of TLR2 was largely abrogated by concurrent treatment with gp120 and morphine. In contrast, TLR9 expression was decreased by exposure to gp120, and the gp120-induced decrease was exacerbated by exposure to morphine. Flow cytometric analysis demonstrated that while the changes in expression level of TLR2 tended to reflect changes in the proportion of astrocytes expressing that TLR, significant changes in the proportion of astrocytes expressing TLR9 were not induced by gp120, morphine, or a combination of the two agents. However, in untreated cells, the proportion of astrocytes that expressed TLR2 (39%) was approximately twice the proportion that expressed TLR9 (21%). Both TLR2 and TLR9 were functional in these cells as shown by the induction of nitric oxide by LTA stimulation of TLR2 and MCP-1 induction by CpG stimulation of TLR9. Thus, morphine and gp120 have the capability to modulate the host innate immune response to other pathogens.

The Effects of Morphine and gp120 on Synaptic Repair

Sphingomyelin is a signaling lipid that is involved in synaptic activity and is also thought to play a role in synaptic plasticity. Banderu *et al.* [139] used mice that were transgenic for gp120, along with their non-transgenic counterparts, to examine the effects of morphine withdrawal. Baseline levels of morphine-induced oxidative stress were the same in the transgenic mice as in the non-transgenic mice. However, during withdrawal, oxidative stress parameters (e.g. GSH/GSSG, SOD levels, antioxidant capacity) did not recover in transgenic mice to the same extent they did in the non-transgenics. In mice carrying the gp120 transgene and that were administered placebo, the levels of sphingomyelin were decreased and ceramide levels were increased. In chronic morphine treatment, no difference was observed between the transgenics and non-transgenics in terms of ceramide and sphingomyelin levels. During morphine withdrawal, levels of sphingomyelin either normalized or decreased in the transgenics compared to the non-transgenics, and all levels of ceramides were increased. In chronic morphine treatments, there was no difference between two different groups of mice in terms of decrease in levels of PSD95. However, during withdrawal from morphine PSD95 levels in the gp120 transgenics did not recover to pre-treatment levels of PSD95, as did the non-transgenics. Taken together, these results suggest that morphine withdrawal in mice carrying the gp120 transgene results in altered sphingomyelin metabolism and synaptic repair. This further suggests that gp120 may reduce synaptic plasticity in the context of morphine.

Although morphine is one of the primary metabolites of heroin it is also a commonly used treatment for pain. The effects of the chemokines CCL5 and CXCL12 on the antinociceptive effects of morphine were examined by Chen *et al.* [140]. Using a rat model, this group determined that CCL5 or CXCL12 blocked the antinociceptive effects of morphine. The

antinociceptive effects of DAMGO, a synthetic opioid peptide were also blocked, but not as completely as the effects of morphine. Injection of gp120 into the rat brain also has been demonstrated to block the antinociceptive effects of morphine [141]. Treatment with AMD3100, a CXCR4 antagonist, substantially abrogated gp120-inhibition of morphine antinociception. Results from *in vitro* experiments also demonstrated that gp120 inhibited hyperpolarization of neurons by morphine.

Taken together the results described above suggest many pathways at which gp120 and morphine pathways may intersect. As was the case with MA and cocaine, the cellular responses to both morphine and gp120 involve the NF- κ B pathway. In addition, both morphine and gp120 are involved in regulating the expression levels of the HIV coreceptors CXCR4 and CCR5. Thus, there are many opportunities for interplay between the cellular responses to gp120 and morphine.

SUMMARY AND FUTURE DIRECTIONS

The reports described above clearly show the involvement of overlapping signal transduction pathways that are activated by gp120 and drugs of abuse. However, a review of the literature also demonstrates that much remains to be done. Many of the studies that have been reported have utilized either a mix of Tat and gp120 or they have utilized supernatants from infected cells. Certainly, those approaches are valid and they have been useful in determining the biological effects of HIV infection of the CNS. It is also important, especially in terms of the development of therapeutic agents for HAND, to understand the various roles of the individual viral proteins of HIV. The development of a mouse transgenic for HIV gp120 will certainly facilitate such studies.

The translation of basic science into research that has clinical relevance will also include studies that focus on the neurotoxicity of HIV proteins in conjunction with a single drug of abuse as well as different combinations of drugs of abuse. Patients engaging in drug abuse often utilize multiple drugs at once. Thus, the clinically relevant model may include cocaine in conjunction with alcohol or MA and cocaine. The pathways that would be activated in polydrug abuse may result in synergy and exacerbation of some pathways leading to CNS damage, while other overlapping pathways may serve to inhibit one another and ameliorate damage to the CNS. As can be seen in the accompanying table, there are several pathways that are affected by more than one drug of abuse and/or gp120. Since multiple drugs of abuse are often utilized at the same time, this provides multiple opportunities for drug-drug interactions (Table 1). For example, both cocaine and morphine have been demonstrated to affect the BBB by inducing PDGF [73, 129]. Drug addiction is often comprised of periods of high dosage of the DOA followed by abrupt cessation of the exposure. As has been seen with the case of morphine and gp120, the biological response is mediated not only by the pathway activated immediately after administration of a drug of abuse, but the effects of withdrawal can also have adverse affects upon the CNS. Although at least one such study has been performed with gp120 and morphine, additional work with gp120 and other drugs of abuse is needed.

In summary, the identification and characterization of interactions between drugs of abuse and gp120 has been a productive area of research. However, there are many questions that remain to be answered. The availability of new tools, as well as the development of new model systems, will facilitate rapid progress in this area.

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Fig. (1). Confocal images demonstrating gp120-mediated IL-6 expression in SVGA astrocytes
 SVGA astrocytes were grown on cover slips and transfected with a plasmid encoding gp120. The media was supplemented with golgi-plus during the transfection and the cells were fixed 6 hours post-transfection. The cells were washed, permeabilized, and blocked in PBST containing 1% BSA for 30 minutes and co-stained for GFAP (red) and IL-6 (green). The cells were then incubated with anti-mouse conjugated with Alexa Fluor 555 (for GFAP) and anti-rabbit conjugated with Alexa Fluor 488 antibodies for IL-6 following which cover slips were mounted using Vectashield mounting medium with DAPI. Individual images for different fluorophores were captured using confocal microscopy and merged using EZ C1

confocal microscope software. The non-transfected control showed basal levels of IL-6 and GFAP (**A-C**). Mock-transfected astrocytes exhibited a marginal increase in IL-6 expression as compared to the control (**D-F**), while gp120-transfected astrocytes demonstrated higher levels of accumulation of IL-6 in the processes of astrocytes that colocalized with GFAP (**G-I**). (Magnification - 60X in **A-I**).

Fig. (2). Confocal images demonstrating gp120-mediated IL-8 expression in SVGA astrocytes SVGA astrocytes were grown on cover slips and transfected with a plasmid encoding gp120. The media was supplemented with golgi-plus during the transfection and the cells were fixed 6 hours post-transfection. The cells were washed, permeabilized, and blocked in PBST containing 1% BSA for 30 minutes and co-stained for GFAP (red) and IL-8 (green). The cells were then incubated with anti-mouse conjugated with Alexa Fluor 555 (for GFAP) and anti-rabbit conjugated with Alexa Fluor 488 antibodies for IL-8 following which cover slips were mounted using Vectashield mounting medium with DAPI. Individual images for different fluorophores were captured using confocal microscopy and merged using EZ C1 confocal microscope software. The non-transfected control showed basal levels of IL-8 and

GFAP (**A-C**). Mock-transfected astrocytes exhibited a marginal increase in IL-8 expression as compared to the control (**D-F**), while gp120-transfected astrocytes demonstrated higher levels of accumulation of IL-8 in the processes of astrocytes that colocalized with GFAP (**G-I**). (Magnification - 60X in **A-I**)

Table 1

Mutual Molecular Targets for Different Drugs of Abuse and/or gp120

Target		Effector	References
MAPK	P38	gp120	[7, 26, 27, 31]
		cocaine	[60, 61, 68]
		morphine	[121]
	JNK	gp120	[27, 31, 61, 77]
		cocaine	[61, 67, 68, 77]
		methamphetamine	[103, 104]
		morphine	[121]
	ERK	gp120	[61, 77]
		cocaine	[61, 67, 68, 74, 77]
		methamphetamine	[102–104]
		morphine	[113, 121]
NF- κ B		gp120	[47–49]
		cocaine	[58, 59, 61, 67, 68]
		morphine	[110, 111]
Tight Junction Proteins		gp120	[5, 53–57]
		cocaine	[67–69]
		methamphetamine	[103]
		morphine	[120, 121]
Markers of Oxidative Stress		gp120	[39–45, 78]
		cocaine	[61, 67, 77]
		methamphetamine	[96–102]
Akt		cocaine	[67, 68]
		morphine	[122]
TGF- β		cocaine	[71]
		morphine	[117]