Published in final edited form as:

J Neurovirol. 2016 August ; 22(4): 403–415. doi:10.1007/s13365-016-0436-5.

Defining the roles for Vpr in HIV-1-associated neuropathogenesis

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Abstract

It is increasingly evident that the human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr) has a unique role in neuropathogenesis. Its ability to induce G₂/M arrest coupled with its capacity to increase viral gene transcription gives it a unique role in sustaining viral replication and aiding in the establishment and maintenance of a systemic infection. The requirement of Vpr for HIV-1 infection and replication in cells of monocytic origin (a key lineage of cells involved in HIV-1 neuroinvasion) suggests an important role in establishing and sustaining infection in the central nervous system (CNS). Contributions of Vpr to neuropathogenesis can be expanded further through (i) naturally occurring HIV-1 sequence variation that results in functionally divergent Vpr variants; (ii) the dual activities of Vpr as an intracellular protein delivered and expressed during HIV-1 infection and as an extracellular protein that can act on neighboring, uninfected cells; (iii) cell type-dependent consequences of Vpr expression and exposure, including cell cycle arrest, metabolic dysregulation, and cytotoxicity; and (iv) the effects of Vpr on exosome-based intercellular communication in the CNS. Revealing the effects of this pleiotropic viral protein is an essential part of a greater understanding of HIV-1-associated pathogenesis and potential approaches to treating and preventing disease caused by HIV-1 infection.

Keywords

HIV-1; Vpr; neuropathogenesis; brain; exosome

Introduction

Human immunodeficiency virus type 1 (HIV-1) viral protein R (Vpr), which is a 96 amino acid accessory protein incorporated into the viral capsid, acts as an intracellular and extracellular protein. It has been associated with increased susceptibility to HIV-1 infection, activation of latent integrated virus, and aiding in the functions of other accessory proteins

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Conflict of interests: The authors declare that they have no conflicts of interests.

such as Vpu, Tat, and Nef. This accessory protein is essential for infection of cells of the monocyte-macrophage lineage, terminally differentiated cells, and quiescent cells. However, it is dispensable in activated CD4⁺ T-lymphocyte infection and replication. Extracellular Vpr has varied cytotoxic effects dependent on concentration and cell type. Although Vpr has been found as an extracellular protein in the cerebrospinal fluid (CSF) from HIV-1-infected patients, there is still little known of its role in neurodegenerative disease during the course of infection (Jones *et al*, 2007). The mechanism of Vpr release into the extracellular space is still unclear, but it is possible that exosomes could be playing a role in Vpr trafficking in the periphery and neuronal environment (Desplats *et al*, 2013; Nitahara-Kasahara *et al*, 2007; Sampey *et al*, 2014).

Prior to the introduction of highly active antiretroviral therapy (HAART), the development of neurological impairment was usually an early indicator of disease progression and a possible harbinger of the development of HIV-1 associated dementia (HAD) and subsequent death (Langford *et al*, 2006; Tozzi *et al*, 2005). With the advent of more effective combination therapies, the severity of HIV-1-associated neuropathogenesis in HIV-1-infected patients has declined, but impairment is still evident in the aging HIV-1-positive population (Rao *et al*, 2014). Impairment in the face of effective antiviral therapy may be the result of several underlying mechanisms. Postmortem studies of patients on varied regimens of HAART and HAART-naïve groups showed increased HIV-1 RNA in the brain in the naïve group and several treated patients, suggesting that efficacious treatment and associated reductions in virus replication do not necessarily curtail the expression of neurotoxic viral proteins. Persistent viral replication in the brain made possible by inadequate penetration of anti-retroviral drugs into the central nervous system (CNS) may also result in neuropsychological impairment (Kramer-Hammerle *et al*, 2005; Langford *et al*, 2006). Some in vitro studies also point to the neurotoxic effects of antiviral therapy, suggesting global neuronal damage as a result of treatment.

Diagnosis of neurological impairment currently relies on numerous tests, including MRI imaging and CT scanning. In addition, clinical assessment tools, such as the Total Hopkins Modified Dementia Scoring system and others, test patient neuropsychological abilities, including mental flexibility, concentration, speed of mental processing, memory, visual-spatial function, motor function, and critical thinking (Kuslansky *et al*, 2004). Neurological impairment associated with HIV-1 infection, however, can be over-diagnosed or misdiagnosed, and also confounded by co-factors such as co-infection and substance abuse (Sacktor *et al*, 2001). These confounding factors have been a challenge in clinical psychiatric evaluations, leading to the need for better diagnostic tools used to identify neurological impairment.

Neuropathogenesis associated with HIV-1 infection is clearly multi-factorial, involving a complex web of interactions between infected and uninfected cells, soluble factors associated with inflammation in the brain, and neurotoxic extracellular viral proteins. It is increasingly evident that Vpr participates in mechanisms that contribute to HIV-1-associated CNS disease. Furthermore, structure-function studies of Vpr support the hypothesis that variation in Vpr sequence during the course of disease leads to alterations in the contributions of Vpr to neuropathogenesis (and immunopathogenesis). Insights into the

pathogenic roles of Vpr could provide a greater understanding of HIV-1-associated disease, provide new opportunities for therapy, and also point to potential biomarkers of disease progression involving Vpr.

This review offers an overview of the roles played by Vpr in HIV-1-associated disease, with an emphasis on understanding the contribution of Vpr to the genesis and severity of HIV-1-associated neuropathogenesis in the brain.

Vpr structure

Vpr is a 96-amino acid, 14-kDa intracellular and extracellular protein with three α -helical secondary structures (Fig. 1). The helices form at positions 17-33 (α_1), 38-50 (α_2), and 53-78 (α_3). These three helices are connected by two turns that span positions 34-37 and 51-52, allowing for both flexibility and steric stability of the protein's tertiary conformation. Non-structural N- and C-terminal domains flank this helix-turn-helix-turn-helix moiety. Of the three helices, α_1 and α_3 have amphipathic faces with hydrophobic amino acids that support leucine zipper-like interactions, while the opposite hydrophilic faces have unknown roles. Positions 74-78 in α_3 form a less strictly defined α -helix conformation (Schuler *et al*, 1999; Wecker *et al*, 2002; Wecker and Roques, 1999). Some functions of Vpr are attributed to the C-terminus of the protein, which includes a hydrophilic patch containing six arginine residues spanning positions 73-96. Previous studies demonstrated that mutation of these residues resulted in reduced nuclear localization and G₂/M arrest, but incomplete attenuation of the protein's cytotoxicity (Bolton and Lenardo, 2007).

The N-terminus of the protein is known to facilitate self-association, which aids in Vpr incorporation into the capsid via interactions with p6 derived from the viral structural protein pr55Gag (Bachand *et al*, 1999). Virion packaging has been connected to the three helices in Vpr, which contain leucine and isoleucine residues on the α_1 and α_3 hydrophobic faces (Schuler *et al*, 1999). The N-terminus is suggested to associate with the adenosine-nucleotide translocator (ANT). Additionally, Vpr binding has been mapped to a specific WxxF motif in ANT (Wecker *et al*, 2002; Wecker and Roques, 1999). This WxxF motif is also found in other proteins known to interact with Vpr, including transcription factor IIB (TFIIB) and uracil-DNA glycosylase (UNG2). However, not all proteins known to interact with Vpr exhibit this motif (Guenzel *et al*, 2014).

It is worth noting that variation in Vpr amino acid sequence has been associated with changes in disease severity/progression in HIV-1-infected patients. In three cases involving long-term non-progressors, slower disease progression, as gauged by CD4⁺ T-lymphocyte counts, was associated with the appearance of changes in specific amino acids within Vpr (Lum *et al*, 2003; Mologni *et al*, 2006; Somasundaran *et al*, 2002; Wang *et al*, 1996). In support of this connection between Vpr sequence variation and pathogenesis, in vitro studies using patient-derived Vpr variants demonstrated changes in Vpr cytotoxicity, G₂/M arrest, and HIV-1 replication (p24) consistent with a lack of progression in the source patients.

Roles of Vpr in viral infection and replication

Vpr is an intracellular and extracellular protein with functions that impact viral pathogenesis and sustained infection. The effects of Vpr include: induction of cell cycle arrest at G₂/M; boosting viral replicative capacity; aiding in the nuclear localization of the preintegration complex (PIC); aiding in the fidelity of reverse transcription; and facilitating infection in terminally differentiated and quiescent cells (Fig. 2). Vpr expression also results in cytotoxicity in several cell types, as well as alterations in metabolic processes and signaling pathways. The effects of Vpr depend on the cell type; varied effects have been shown in neurons, brain microvascular endothelial cells (BMVECs), oligodendrocytes, lymphocytes, astrocytes, and cells of the monocytic lineage, including monocytes, macrophages, and microglial cells (Barrero *et al*, 2013; Emerman, 1996; Kramer-Hammerle *et al*, 2005; Torres and Noel, 2014). The effects of Vpr in monocytes and terminally differentiated macrophages are particularly pertinent to HIV-1-associated neuropathogenesis, since macrophages and microglia serve as major viral reservoirs in the CNS (Barrero *et al*, 2013; Re and Luban, 1997).

As an accessory protein involved in the early steps of viral infection, Vpr is incorporated into the viral particle through interactions with the carboxy-terminal p6 protein after cleavage of this region from the Gag-encoded Pr55Gag precursor. Quantitative studies have established the ratio of Vpr/Pr55Gag at 1:7, which puts estimates of packaged Vpr at 275 molecules per virus particle. Once in the virion, Vpr forms tight associations with the viral RNA (Bachand *et al*, 1999). This association boosts the fidelity of the reverse transcriptase complex. In the absence of Vpr, mutations introduced into nascent DNA during reverse transcription increase by approximately 4-fold relative to reverse transcription in the presence of Vpr (Goh *et al*, 1998). While the effect of Vpr on reverse transcriptase fidelity is evident, the mechanism through which Vpr accomplishes this result is unknown.

After reverse transcription, the viral cDNA is transported to the nucleus in the preintegration complex (PIC), which is composed of several viral and host-derived proteins, including Vpr. Vpr uses its N-terminus for nuclear localization via the non-classical transport system by binding to importin- α . This interaction is essential for replication in macrophages, but not in monocytes due to the low expression of mature importin- α isoforms (Nitahara-Kasahara *et al*, 2007). Although Vpr is a karyophilic protein, studies of the karyophilic pathway demonstrated that, unlike the HIV-1 matrix protein, Vpr is not dependent on this pathway (Gallay *et al*, 1996). Vpr is also able to exit the nucleus using a nuclear export signal that interacts with exportin-1 to allow for nucleocytoplasmic shuttling. This activity was also noted to be required for replication in tissue macrophages from tonsils and spleen (Sherman *et al*, 2003). Subcellular localization of Vpr is also dependent on the cellular phenotype (Ferrucci *et al*, 2011a). Mutations in Vpr nuclear localization sequences resulted in reduced Vpr nuclear localization, but did not affect Vpr-induced apoptosis or cytotoxicity relative to wild type Vpr (Bolton and Lenardo, 2007). These results suggest that nuclear localization is not a requirement for Vpr-associated cytotoxicity.

Vpr also aids in replication by triggering G₂/M arrest, the duration of which can range from hours to days. The arrest of the cell cycle in G₂/M promotes LTR-directed viral transcription

and viral replicative capacity (Goh *et al*, 1998). Vpr stimulates gene expression in a glucocorticoid-dependent manner by activating the LTR through interactions with components of the glucocorticoid-induced transcription initiation complex (Kino *et al*, 1999; Kino *et al*, 2002), including the GR receptor, the TFIIB/TFIID complex, and TFIIF. It has been demonstrated that p300/CBP and SRC-1 participate in this complex to successfully induce transcription. Vpr G₂/M arrest is linked to increased p24 protein production and higher HIV-1 RNA, suggesting these two mechanisms may be mechanistically linked (Goh *et al*, 1998). However, other studies have suggested transcriptional activity and G₂ arrest can be enhanced differentially depending on the involvement of unknown host factors. While there are conflicting in vitro studies regarding the connection between G₂ arrest and cytotoxicity (Bolton and Lenardo, 2007), the known mechanisms underlying both will be discussed later in this review.

In addition to G₂/M arrest, multiple changes in metabolism are attributed to Vpr expression. These include alterations in glycolysis, gluconeogenesis, the pentose phosphate pathway, the tricarboxylic acid (TCA) cycle, and mitochondrial function, as well as increases in hypoxia-inducing factors due to greater levels of reactive oxygen species (ROS) in the cell. These changes are reminiscent of a metabolic state seen in cancer cells called the Warburg effect.

Clinical consequences of Vpr sequence variation

Connections between Vpr variation and pathogenesis stem from case studies that have demonstrated mutations in Vpr (including insertions and deletions) associated with long-term non-progressors. One of the first cases showing heritability of disease progression was a mother-child pair. In this case, sequencing of quasispecies from blood and plasma-derived HIV-1 Vpr clones revealed insertions/deletions and length polymorphisms clustered at the C-terminus between amino acid positions 83 and 89. The mother and child both exhibited stable CD4⁺ T-lymphocyte counts over 13 years and were characterized as long-term non-progressors. The results of this study suggest that Vpr mutations contributed to alterations in the set-point viral load (SPVL) in this donor/recipient pair (Wang *et al*, 1996). However, recent cohort studies suggest that host factors play roles in driving the selection of Vpr variants associated with long-term non-progression (Cali *et al*, 2005). However, the specific viral and host factors that drive Vpr selection are still largely unknown and need to be examined further.

Because of its ability to induce G₂/M arrest in infected cells, Vpr may have a role in determining the SPVL by changing the replicative capacity of the virus. Patient SPVL is the relative average viral load maintained during the course of infection with or without treatment. The SPVL is determined by the interaction of multiple host and viral factors (Fraser *et al*, 2014). Under the pressure of the host immune response, the virus must maintain a level of infectivity and replicative capacity to propagate during the initial infection and subsequent dissemination to other regions of the body. It is worth noting that multiple clinical investigations have shown strong correlations between SPVL in donors and recipients that correspond to viral genetic sequences (Kino *et al*, 1999; Lum *et al*, 2003; Mologni *et al*, 2006). These studies indicate the heritability of viral phenotype and point to

roles for variations in Vpr (and other elements of HIV-1) in determining the course of infection.

The only known mutation corresponding to attenuation in Vpr-mediated cytotoxicity is the Q3R polymorphism within the N-terminus (Fig. 1). The patient from whom this Vpr variant was isolated had a unique clinical presentation characterized by the absence of CD4⁺ T-lymphocyte depletion combined with a relatively high viral load (Somasundaran *et al*, 2002). In vitro studies demonstrated that the presence of the Q3R polymorphism in Vpr abrogated Vpr-mediated cytotoxicity relative to the parental LAI-derived Vpr.

Additionally, Vpr variation was highlighted during a screen for viral gene polymorphisms in HIV-1-infected patients between 10 long-term non-progressors (LTNP) and 15 patients with progressive disease. In this study, a R77Q variant in Vpr (Fig. 1) was associated with long-term non-progression (Lum *et al*, 2003; Mologni *et al*, 2006). In the context of a VSV-G pseudotyped virus, the R77Q Vpr variant had reduced cytotoxicity in the Jurkat T-lymphocyte cell line and in a mouse model of viral infection. The reduced in vitro and in vivo toxicity of this Vpr variant was consistent with its appearance in LNTN patients.

The appearance of Vpr variants and associated changes in pathogenesis do not, however, signal a permanent change in virus genotype and phenotype, especially when the virus is transmitted. In a study involving a non-progressor who carried a mutation at position S90R in the nonstructural C-terminus of Vpr, the virus was followed in two recipients who exhibited normal disease progression. Sequence analyses of the recipients' quasispecies demonstrated that the S90R mutation had reverted to the wild type sequence (Cali *et al*, 2005). Although position 90 in Vpr has been suggested to be a phosphorylation site, its function is unknown (Guenzel *et al*, 2014; Schuler *et al*, 1999). The loss and subsequent restoration of this putative phosphorylation site suggests functional changes that affect clinical outcomes. This study points to the influence of host factors and pressures on viral genotype and phenotype. Furthermore, this study again implies a connection between Vpr sequence variation and disease progression.

Roles for Vpr in HIV-1-associated neuropathogenesis

HIV-1 establishes residence in the CNS early in the infection and, once established, causes a host of changes in cells of the CNS, including neurons, oligodendrocytes, brain microvascular endothelial cells (BMVECs), astrocytes, and microglial cells (Barrero *et al*, 2013; Jones *et al*, 2007; Kramer-Hammerle *et al*, 2005; Liu *et al*, 2002; Re and Luban, 1997). These changes over the course of the infection lead to microgliosis, astrogliosis, and neuronal death, as seen in post-mortem sections of patients diagnosed with HAD or HIV-Associated Neurocognitive Disorders (HAND), and patients with HIV encephalitis (HIVE) (Desplats *et al*, 2013; Torres and Noel, 2014).

There are several proposed avenues of HIV-1 infiltration into the CNS, yet these mechanisms of infiltration are not fully understood. Studies of BMVECs have suggested the virion is able to penetrate the CNS after a loss of integrity of the blood-brain barrier (BBB) (Liu *et al*, 2002). Of the proposed models, one of the more favored is the Trojan Horse

hypothesis, in which HIV-1 is trafficked across the barrier from the peripheral blood in infiltrating activated CD4⁺ lymphocytes and CD163⁺/CD16⁺ monocytes, which differentiate into microglial cells in the CNS (Fischer-Smith *et al*, 2008). Consistent with the Trojan Horse model, histological staining of post-mortem sections of the frontal cortex and basal ganglia from HIV-1-infected brains showed the presence of Vpr co-localized to perivascular and parenchymal monocytoïd cells. Similar results were obtained in experiments involving transgenic mice expressing Vpr (Desplats *et al*, 2013; Jones *et al*, 2007). The results from these experiments make it apparent that Vpr is positioned to affect cells in and proximal to the BBB.

Once HIV-1 has gained a foothold in the CNS, numerous cell types are impacted by HIV-1 propagation in the brain and, specifically, the potential neuropathogenic effects of Vpr (Fig. 3). Cell types involved in the complex interplay of cells and soluble factors that lead to neuropathogenesis include neurons, astrocytes, oligodendrocytes, and cells of monocytic lineage (microglial cells and perivascular macrophages). Of these cells, little is known regarding oligodendrocytes and the effects of Vpr on cell viability and function (Desplats *et al*, 2013; Tozzi *et al*, 2005).

Vpr has been shown to affect neurons in both in vitro and in vivo experiments. In vitro experiments have shown that neurons have reduced outward whole-cell current in a voltage range of -30 to +3 mV in a Vpr dose-dependent manner. The electrochemical instability was attributed to a potassium channel current delay, which disrupts the calcium/potassium ionic balance in neurons. In other in vitro studies, exposure of an N-terminal His-tagged pNL4-3 Vpr recombinant protein to neurons lead to a reduction in phosphorylated Akt and caspase 3, as well as an increase in p53, ultimately leading to cytochrome C release and apoptosis in neurons (Jones *et al*, 2007). Neurons exposed in vitro to supernatants from infected astrocytes showed dose-dependent decreases in IL-1 β transcripts and increases in IL-6 production (Guha *et al*, 2012; Jones *et al*, 2007). In studies involving a Vpr-expressing transgenic mouse, a pNL4-3-derived Vpr expressed from a c-fms promoter specific for macrophage colony stimulating factor receptor resulted in the accumulation of Vpr in perivascular cells, as shown in postmortem brain sections. These results are consistent with post-mortem analyses of HIV-1-infected brain sections that showed a loss of pallor indicative of glial cell dysfunction or neuronal death.

Of the supporting glial cells in the CNS, astrocytes are the most abundant cell type, providing crucial metabolites and signaling for neuronal development, support, and survival. They are not readily infected in HIV-1-positive patients, as observed in histological staining. Astrocytes are one of the more resistant cell types to Vpr-induced death, withstanding very high concentrations of soluble Vpr (>100 nM). In contrast, most neurons die after exposure to concentrations <10 nM in vitro. Neuronal susceptibility to Vpr may be due to the higher concentration of mitochondria in neurons, leading to more apoptotic signals in neurons. In vitro studies have demonstrated that cell lines of astrocytic (and neuronal) origin exhibit changes in metabolism, including increases in ROS and lactate production, when exposed to extracellular Vpr (Ferrucci *et al*, 2012).

Vpr can also impact the roles that astrocytes play in modifying the extracellular environment of the brain. Studies conducted on astrocytes expressing Vpr have demonstrated up-regulation of metabolites and chemokines such as RANTES/CCL5, MCP-1, TGF- β , TNF- α , IL-8, IP-10, NO, and reactive oxygen species, all of which have detrimental effects on neurons in this altered microenvironment (Gangwani *et al*, 2013). This effect may be amplified by the high levels of viral protein expression (without parallel virus production) characteristic of HIV-1-infected astrocytes, and the effects of increased Vpr expression on their release of extracellular mediators.

Release of neurotoxic factors induced by Vpr expressed in astrocytes may also be dependent on interactions with neighboring cells, including microglia. In co-cultures of astrocytes and macrophages (Fiala *et al*, 1996), levels of Nef in HIV-1-infected astrocytes were greatly increased relative to astrocytes in monoculture, suggesting that Vpr may also be expressed at greater levels and may induce greater release of neurotoxic factors as a consequence. In contrast, it has been shown that astrocytes can have a neuroprotective effect during HIV-1 encephalitis by attenuating the overproduction of eicosanoids, platelet activating factor, and TNF- α produced from activated, HIV-1-infected macrophages (Desplats *et al*, 2013). It is therefore apparent that the ability of HIV-1-infected astrocytes to play neurotoxic or neuroprotective roles in the brain depend on the milieu of the CNS and the stage of HIV-1-associated neuropathogenesis. Identifying the exact role of Vpr in this network will require more studies of Vpr as an extracellular protein and as a viral protein expressed in infected cells (Minagar *et al*, 2002).

The role of Vpr in infection of cells of the monocyte-macrophage lineage is important since these cells are the primary reservoirs of HIV-1 infection in the CNS. Vpr has reduced cytotoxic effects in monocytic cells, while in vitro studies show it is essential for circulating CD163⁺/CD16⁺ monocyte infection (Barrero *et al*, 2013). During macrophage and microglial expression of and exposure to Vpr, the cell survives for 20+ days in in vitro studies, suggesting a similar life span in vivo (Kramer-Hammerle *et al*, 2005; Re and Luban, 1997). This extended life span suggests the potential for long-term effects of Vpr expressed by these long-lived cells. Monocytes exhibit some in vivo resistance to Vpr cytotoxicity at relatively high concentrations. In this study, Vpr induced an up-regulation of IL-1 β , while reducing IL-6 detected in post-mortem sections of patients with HAND (Desplats *et al*, 2013). In behavioral studies focused on the neurological effects of Vpr, transgenic mice expressing Vpr in cells of monocyte-macrophage lineage exhibited abnormalities in performance, hyper-excitability, and motor functions when compared to control mice (Jones *et al*, 2007).

In the brain, activated, HIV-1-infected macrophages are a source of viral neurotoxins (Tat, gp120, and Vpr), cytokines and chemokines (e.g., TNF- α), and other soluble mediators that act as neurotoxins [arachidonic acid, platelet activating factors (PAF), nitric oxide (NO), and quinolinic acid] (Minagar *et al*, 2002). The release of these factors, which can profoundly affect the microenvironment of the CNS and the ensuing neuropathogenesis, can be affected by Vpr expression. In experiments involving macrophages, Vpr expression increased the release of IL-1 β , IL-8, and TNF- α , which resulted in increased neuronal death (Guha *et al*, 2012). In HIV-1-infected microglia, Vpr, along with HIV-1 Nef, induced

RANTES/CCL5 expression (Si *et al*, 2002). Vpr expression in monocytes and macrophages also resulted in the increased expression of IL-8 (Roux *et al*, 2000). However, reductions in β -chemokines MIP-1 α , MIP-1 β , and RANTES/CCL5 as a result of Vpr expression suggest Vpr-associated mechanisms that increase HIV-1 replication while making a lesser contribution to the pro-inflammatory environment in the CNS (Muthumani *et al*, 2000). Reduced secretion of IL-12 from monocyte-derived dendritic cells (Tcherepanova *et al*, 2009) suggests a similar effect in HIV-1-infected macrophages and microglial cells in the brain. Vpr in brain-resident macrophages and microglia may also impact innate immune responses in these cells, as indicated by Vpr-induced STAT1 phosphorylation, expression of interferon-stimulated genes (ISGs), and differential expression of genes involved in innate immune responses (Zahoor *et al*, 2014). All of these observations suggest that Vpr expression in HIV-1-infected macrophages and microglia can have a profound impact on inflammation and neurotoxicity in the brain.

In addition to its effects as an intracellular protein expressed during infection, Vpr has also been shown to function as an extracellular protein. Extracellular Vpr has been found in the plasma and CSF samples collected from HIV-1-infected patients (Ferrucci *et al*, 2011b) at concentrations that increase with disease progression (Levy *et al*, 1994; Levy *et al*, 1995). In vitro experiments involving astrocytes demonstrated that the presence of extracellular Vpr resulted in reductions in ATP and glutathione, with concomitant increases in cellular reactive oxygen species (Ferrucci *et al*, 2012). Our studies involving primary human fetal astrocytes also revealed significant changes in the release of IL-6, IL-8, MCP-1, and MIF in response to the introduction of extracellular Vpr (Ferrucci *et al*, 2013), as well as demonstrable changes in cellular gene expression patterns (Ferrucci *et al*, 2013). These Vpr-dependent changes in astrocytes were also accompanied by increased activation of caspase-3 and -7 (Ferrucci *et al*, 2013). Extracellular Vpr can also have an indirect effect, as shown by experiments in which conditioned media from astrocytes exposed to extracellular Vpr caused increased neuronal apoptosis (Ferrucci *et al*, 2013). Extracellular Vpr has also been shown to potentiate glucocorticoid-induced suppression of IL-12 expression in monocytic cells (Mirani *et al*, 2002), suggesting that extracellular Vpr may also be involved in suppressing immune responses facilitated by cells of this lineage.

Considering the effects of intracellular Vpr on cells of the CNS, it is clear that Vpr acts as a neurotoxin and can lead to metabolic dysfunction in astrocytes and microglia, which ultimately alter the microenvironment of neuronal function and survival. However, Vpr in the extracellular microenvironment is also a neuronal toxin, which is linked to its ability to trigger mitochondrial instability and apoptosis (Fig. 3).

G₂/M arrest and cytotoxicity

During HIV-1 infection, G₂/M arrest occurs within hours of virus entry into the cell. In vitro studies involving Vpr exogenous expression as well as exposure to extracellular protein demonstrated that Wee1 and cyclin-dependent kinases (CDC25C) are hypophosphorylated, leading to Wee1 activation and CDC25C inactivation. This has the effect of stalling CDC2 activation by dephosphorylation by CDC25C, which inhibits CyclinB-CDC2 activation. Although the cell cycle proteins are present, they are not in an active form due to their

phosphorylation states (Emerman, 1996). Studies of Vpr-mediated cell cycle arrest have pointed to a protein phosphatase as a possible target of Vpr-mediated degradation by the 26S proteasome.

Vpr is known to associate with proteins involved in an E3 ligase complex known as CUL4A-DDB1 E3 ligase. Affinity studies have shown direct interactions between Vpr and Cullin 4A of the Cul4-associated factor, which is referred to as host Vpr binding protein-VPRBP/DCAF-1. This protein forms a complex with damage-specific DNA binding protein 1 (DDB1) to form the CUL4A-DDB1 E3 ligase complex (Gerard *et al*, 2014). This interaction is believed to alter the E3 ligase substrate specificity such that it targets a protein involved in cell cycle progression. Activation of this pathway is also suggested to have a role in the degradation of an unknown protein involved in the DNA damage response that triggers apoptosis (Bolton and Lenardo, 2007; Guenzel *et al*, 2014).

A link between G₂ arrest and apoptosis is also supported by Vpr interactions with the uracil-DNA glycosylase (UNG), which can be alternatively spliced at the N-terminus to form two isoforms UNG1 and UNG2, which are sorted to the mitochondria and nucleus respectively. The site of Vpr interaction has been demonstrated to be in the commonly shared C-terminus of the UNG proteins. After entering the nucleus via its nuclear localization signal, Vpr has been shown to associate with UNG2 (Mansky *et al*, 2000). The isoform UNG2 is also thought to aid in reverse transcription fidelity, since its presence in the reverse transcription complex has been demonstrated (Klarmann *et al*, 2003).

Structure-function studies focused on Vpr-mediated cell cycle arrest have pointed to specific residues and domains within Vpr. In vitro experiments demonstrated that a R88A mutation attenuates G₂ arrest (Bolton and Lenardo, 2007). Similar substitutions at nearby positions (R80A and R87A) that also result in attenuated G₂ arrest suggest that the C-terminal domain is associated with G₂ arrest and cytotoxicity (Re and Luban, 1997). Furthermore, mutagenesis studies of Vpr have linked these activities to a hydrophilic portion of C-terminus, which includes six arginine residues between positions 73-96.

With respect to Vpr-associated cytotoxicity, Vpr cytotoxicity has been linked to interactions with permeability transition pore complex (PTPC) and adenine nucleotide translocator (ANT), which lead to mitochondrial instability, increased calcium concentrations in the cytoplasm, and ultimately apoptosis (Orrenius *et al*, 2003). Prior studies hint at roles for amino acids 27-51 at the N-terminus and residues 71-82 at the C-terminus in interactions with ANT as possible mechanisms for disrupting ATP/ADP transport and leading to reduction in mitochondrial transmembrane potential. The Q3R mutation, which is outside this region in the nonstructural N-terminus, has been shown to attenuate cytotoxicity (Jacotot *et al*, 2001; Somasundaran *et al*, 2002). Additionally, Vpr interacts with the mitochondrial PTPC (Jacotot *et al*, 2001; Jacotot *et al*, 2000). This interaction leads to mitochondrial instability and causes the release of cytochrome C, which interacts with the apoptotic peptidase activating factor 1 (Apaf-1). As a result, Apaf-1 forms a complex with caspase-9 and initiates apoptosis (Ghiotto *et al*, 2010).

The connection between G₂/M arrest and cytotoxicity is still in dispute. Mutations of amino acids in the hydrophilic portion of the Vpr C-terminus reduce nuclear localization and G₂/M arrest without concomitant attenuation of cytotoxicity (Bolton and Lenardo, 2007; Guenzel *et al*, 2014), suggesting that cell cycle arrest and cytotoxicity are not necessarily linked. Other studies, however, have demonstrated a correlation between G₂ arrest and cytotoxicity (Andersen *et al*, 2006; Goh *et al*, 1998). Since Vpr cytotoxicity appears to be cell type-dependent, disparities in these studies might be attributed to differences in target cells and experimental conditions.

The metabolic effects of Vpr

Due to their greater resistance to the cytotoxic effects of Vpr and their roles in sustaining neuronal function, astrocytes and microglia may contribute to chronic neuropathogenesis through adverse, Vpr-dependent changes in cellular metabolism. The metabolic pathways affected in microglia and astrocytes by extracellular and intracellular Vpr are the pentose phosphate pathway (PPP), glycolysis, induced HIF-1 α signaling (due to ROS accumulation), and up-regulated ceramide synthesis, which is mainly attributed to Tat (Barrero *et al*, 2013; Borjabad *et al*, 2010; Ferrucci *et al*, 2012; Haughey *et al*, 2004; Trajkovic *et al*, 2008). In the presence of Vpr, the reducing equivalent NADP⁺/NADPH produced in the PPP is diverted to anabolism instead of regenerating glutathione. This leads to ROS accumulation in the cell. To compensate for this loss, the cell uses N-acetyl-cysteine (NAC) to rebalance the reducing equivalent levels in astrocytes (Ferrucci *et al*, 2012). In macrophages, Vpr causes the downregulation of glutathione S-transferase isoform kappa-1 and the upregulation of the pi isoform, which is not associated with ROS neutralization, but rather with anabolic processes in the cell (Harris *et al*, 1998). The glycolytic enzymes upregulated in astrocytes and microglia are glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase involved in NADPH production and subsequent anabolic processes.

There also appear to be parallels between the metabolic consequences of Vpr expression and cancer biology. One established aspect of cancer cell metabolism is a switch from the active enzyme PKM1 to the slower enzyme PKM2. This switch pushes metabolites of glycolysis into the alternative anabolic pathways for amino acid, lipid, and nucleic acid synthesis. This characteristic phenotypic switch is also accompanied by higher lactate production (Israelsen *et al*, 2013). It has also been shown that this isoform switch and lactate production occurs in macrophages and astrocytes subsequent to Vpr expression. The same study also noted macrophage up-regulation of hexokinase and down-regulation of GAPDH (Barrero *et al*, 2013; Torres and Noel, 2014). The shunting of glycolysis and Vpr-associated mitochondrial instability all leads to a drop in substrate availability for the citric acid cycle due to PKM2 up-regulation and down-regulation of enzymes of the TCA cycle. The loss in reduction equivalents from the PPP and glycolysis leads to a build-up of ROS in the cell. This build-up contributes to the activation of HIF-1.

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor activated by several products of cellular metabolism. Activation of this transcription factor leads to multiple cell type-specific alterations in gene transcription. The genes altered in HIF-1 activation have roles in vasomotor control, angiogenesis, iron metabolism, cell proliferation/cell cycle control, cell

death, energy metabolism, and exosome release (Sharp and Bernaudin, 2004). The two isoforms of HIF-1 – HIF-1 α and HIF-1 β – form hetero- and homo-dimers. HIF-1 β is expressed constitutively in all cells and does not respond to changes in oxygen availability. In contrast, HIF-1 α is made constitutively in hypoxic cells, but not in cells under normoxic conditions. In the brain, HIF-1 α is induced in neurons, astrocytes, and ependymal cells. It is expressed in microglia and oligodendrocytes, but little is known regarding HIF-1 regulation in these cell types. Studies of HIF-1 α -deficient mice show a reduction of neuronal cells and impairment in spatial memory (Tomita *et al*, 2003).

The exact connection between Vpr and HIF-1 remains to be fully defined. In studies of HIF-1 α in microglial cells, adenovirus-delivered Vpr resulted in a dose-dependent increase of HIF-1 α . This is consistent with the demonstration that extracellular Vpr-induced increases in ROS in astrocytes and neurons (Ferrucci *et al*, 2012). Vpr expression seems to induce a positive feedback loop through the generation of ROS and activation of HIF-1 α , which has been shown to upregulate GAPDH, pyruvate kinase (PKM), hexokinase (HK), lactate dehydrogenase A, and exosome release (Cohen *et al*, 2015) (Fig. 4).

Possible roles for Vpr in exosome biology

As previously discussed above, some of the metabolic changes attributed to expressed or extracellular Vpr occur in pathways connected to the release of exosomes. Exosomes released by cells in the peripheral blood and CNS upon HIV-1 infection (and particularly upon Vpr expression) could have important roles in the development and progression of HIV-1-associated immunopathogenesis and neuropathogenesis. Roles for exosomes during HIV-1 infection would be consistent with the roles that exosomes play in immune and neuro-glial cell communication (Fruhbeis *et al*, 2012). Recent studies have identified viral proteins and RNA in exosomes that alter target cell properties, promote virus infection, and downregulate molecules involved in local immune responses, such as MHC-like receptors (Arenaccio *et al*, 2014; Lenassi *et al*, 2010; Sampey *et al*, 2014). Studies of exosomes in the context of the immune system suggest that they play dynamic roles in altering the immune microenvironment. In light of the growing roles of these extracellular bodies in pathogenesis, this section will focus on the potential relationships between Vpr and exosomes.

Exosomes are distinguished from other vesicular secretions by their characteristic diameter (30-100 nm), density, protein components, and crescent-like morphology. Exosomes are derived from multivesicular bodies (MVBs) and intraluminal vesicles (IVLs). IVL budding and sorting is ESCRT (endosomal sorting complex required for transport)-dependent and -independent. ESCRT-dependent transport is associated with Tsg101, CD63, tetraspanin, and Alix, which serve as markers for this pathway (Bobrie *et al*, 2011; Simons and Raposo, 2009). In contrast, the ESCRT-independent pathway sorts vesicles through ceramide-mediated budding from the late endosomal vesicle (Trajkovic *et al*, 2008). It has also been observed that exosomes can originate directly from the plasma membrane. Exosomal vesicles are composed of higher levels (two-fold on average) of sphingolipid ceramide relative to the parental cell due to the raft-based microdomain-dependent method of budding.

HIV-1 infection and, in particular, Vpr expression appear to have functional connections to exosome biology (Fig. 4). In experiments involving HIV-1-infected lymphocytes, HIV-1 infection has been shown to affect exosome release and the exosome-associated proteins Tsg101 and Alix (Li *et al*, 2012). Of the metabolic changes associated with Vpr, ROS accumulation leading to HIF-1 α activation, changes in ceramide synthesis, and increases in Ca²⁺ due to mitochondrial instability are all known triggers of exosomal release (Bobrie and Thery, 2013; King *et al*, 2012; Simons and Raposo, 2009). In many in vitro studies, these mechanisms are exploited to induce exosomal release. Proteomic analyses of exosomes upregulated during HIV-1 infection demonstrated changes in proteins associated with multiple biological processes. One proteomic study showed Vpr-dependent increases in prostaglandin E synthase 3 (PTGES3) in exosomes (Li *et al*, 2012; Zhang *et al*, 2006). This enzyme is secreted by microglia after engulfment of apoptotic bodies through a COX-1-dependent pathway. This observation is consistent with quantitative studies of lipid production in HIV-1-infected lymphocytes that showed an increase in sphingomyelin production along with several lipid-based neurotransmitter precursors (Zhang *et al*, 2006). The increase in sphingomyelin synthesis is associated with the activity of Tat according to literature (Haughey *et al*, 2004). However, Vpr is associated with glucocorticoid receptor (GR) activation to aid in transcription of anti-inflammatory genes and interacts with the HIV-1 LTR (Kino *et al*, 1999; Kino *et al*, 2002).

Vpr modulation of exosome release in the CNS may make a considerable contribution to HIV-1-associated neuropathogenesis. Cells in the CNS have been shown to use exosomes as vehicles of intercellular communication. Exosomes released from oligodendrocytes have been shown to carry ROS-neutralizing peroxiredoxins, glycolytic enzymes including GAPDH, and pyruvate kinase (Kramer-Albers *et al*, 2007). Microglia secrete exosomes containing monocarboxylate transporter 1 (MCT1), which aids in supplying energy to mitochondria in neuronal axons (Poticchio *et al*, 2005). Astrocytes secrete exosomes containing heat shock protein 70 (Hsp70), which is known to associate with the mitochondrial transport complex (Fruhbeis *et al*, 2012). It is also known that exosomes in the CNS support neuronal function and survival (Fruhbeis *et al*, 2012; Fruhbeis *et al*, 2013).

Vpr could contribute to neuropathogenesis through disruptions in exosome communication within the CNS not only within the local microenvironment proximal to HIV-1-infected cells, but also throughout the CNS as exosomes are distributed in the CSF. Due to their particulate nature, exosomes may form gradients in CNS tissues and possibly within the draining circulatory system of the CNS. Exosomes that originate in the cerebral tissues would drain through the aqueduct of Sylvius into the CSF in the subarachnoid space and come in contact with the neurons traveling from the spinal cord via the anterior and posterior rootlets (Street *et al*, 2012). These neurons supply synaptic signals that travel from the brain through C1-T12 out to the brachial plexus and thoracic plexus. The flow of CSF from the brain would create a gradient of exosomes that is highest at C1 and dissipates down the column according to computational fluid dynamic models (Haughton and Mardal, 2014). A gradual increase of exosomes carrying viral proteins and other payloads during the course of infection would have a direct impact on cervical ganglion and ganglion of the thoracic plexus (C1 through T12), leading to impairment of upper body motor functions and problems in the thoracic region. The effects of disrupted exosome communication by HIV-1

infection would lead to neurological clinical manifestations observed during the course of HIV-1 disease progression (Cohen *et al*, 2015; Sampey *et al*, 2014). Exosome-based communication demonstrated between the brain and the peripheral circulation also suggest the possibility that exosome contributions to neuropathogenesis and immunopathogenesis may be linked (Xin *et al*, 2014).

The effects attributed to Vpr may also be due, in part, to Vpr protein packaged into exosomes. As a precedent for this speculation, the HIV-1 accessory protein Nef was found in exosomal bodies secreted by the Sup-T1 and Jurkat lymphocyte cell lines, and by primary T-lymphocytes. In vitro, these exosomes induced apoptosis in bystander CD4⁺ T-lymphocytes (Lenassi *et al*, 2010). Nef-containing exosomes from HIV-1-infected cells activate uninfected quiescent CD4⁺ T-lymphocytes and facilitate HIV-1 replication through a Nef- and ADAM17-dependent mechanism (Arenaccio *et al*, 2014). Nef has also been shown to interfere with natural killer T-lymphocyte (NKT) activation by interrupting intracellular trafficking of the MHC class I-like cell surface molecule CD1d, which is involved in lipid antigen presentation in dendritic cells (Chen *et al*, 2006; Lenassi *et al*, 2010; Li and Xu, 2008). Because Nef is packaged into exosomes, it is possible that the presence of CD1d on uninfected cells could be reduced by the uptake of Nef-containing exosomes. In this way, exosome release during HIV-1 infection could contribute to the dramatic decline of NKT cells in the first year of infection (Li and Xu, 2008). While these results suggest the possibility of detecting Vpr in exosomes, studies to definitively demonstrate its presence in exosomes released from infected cells, as well as the activities of Vpr-containing exosomes communicated to uninfected cells, have not yet been reported.

Conclusions

The HIV-1 accessory protein Vpr is a pleiotropic protein that has important roles in virus replication and effects on host cells that facilitate sustained viral infection and contribute to pathogenesis. The effects of Vpr, which have been attributed to different domains and amino acids within this relatively small protein, include changes in cell cycle progression, dysregulation of cellular metabolism and signaling, and losses in cell viability. The contribution of Vpr to HIV-1-associated pathogenesis is evident in demonstrated associations between the clinical presentation of virus-associated disease and the presence of specific Vpr amino acid sequence variants.

Because of the particularly important part that Vpr plays in facilitating HIV-1 infection of cells of monocytic lineage, this protein is also well positioned to participate in neuropathogenesis associated with viral invasion of the nervous system. As in cells of the peripheral immune system, Vpr expression in HIV-1-infected cells in the CNS, including microglial cells, perivascular macrophages, and astrocytes, has detrimental effects on cellular function and viability. The participation of Vpr in neuropathogenesis (and likely immunopathogenesis) is made more complex by effects mediated not only by intracellular Vpr, but also by Vpr molecules that are secreted into the extracellular space. This has the effect of extending the influence of Vpr to uninfected bystander cells.

More recent studies also point to an intersection between HIV-1 infection and the expanding field of exosome biology. Exosomes are increasingly acknowledged as vehicles of intercellular communication among cells of the immune and nervous systems. There is increasing evidence that exosomes are involved in HIV-1 infection and pathogenesis. Furthermore, specific changes in cellular metabolism and signaling associated with Vpr expression suggests that Vpr plays a role in modulating exosome release. This, again, would appear to be a mechanism that extends the influence of Vpr to nearby cells that are uninfected. This aspect of Vpr remains to be investigated.

It is increasingly apparent that Vpr is an integral part of HIV-1-associated disease in the immune and central nervous systems. A more complete understanding of Vpr functions and their contributions to pathogenesis will contribute to a more comprehensive picture of HIV-1-associated disease and potentially reveal novel therapeutic approaches that take advantage of this knowledge.

Acknowledgments

This work was partially supported by P30 MH092177 Comprehensive NeuroAIDS Center (CNAC, Program Director: Kamel Khalili, Brian Wigdahl, PI of the Drexel subcontract). These studies were also supported by the Public Health Service, National Institutes of Health, through grants from the National Institute of Neurological Disorders and Stroke (NS32092 and NS46263), the National Institute of Drug Abuse (DA19807; Dr. Brian Wigdahl, Principal Investigator), and the National Institute of Mental Health under the Ruth L. Kirschstein National Research Service Award (5T32MH079785; Jay Rappaport, PI, Brian Wigdahl, PI of the Drexel subcontract). The contents of the paper are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. Dr. Michael Nonnemacher was supported in part by the Public Health Service, National Institutes of Health, through a grant from the National Institute of Neurological Disorders and Stroke (NS089435) and faculty development funds provided by the Department of Microbiology and Immunology and the Institute for Molecular Medicine and Infectious Disease. Dr. Fred Krebs was supported by a developmental grant awarded by CNAC.

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Fig. 1. Vpr primary sequence and structural features

(A) Primary sequence of HIV-1 Vpr. Residues that make up the three helices and the arginine-rich C-terminus are indicated. (B) Vpr ribbon diagram. Major structural features are annotated, as are residues (Q3 and R77) featured prominently in clinical studies. Adapted from other sources (Biasini *et al*, 2014; Omasits *et al*, 2014).

Fig. 2. Vpr has roles throughout the HIV-1 replication cycle

Vpr is packaged into the virion and can be found associated with viral RNA inside the capsid during binding and entry. It aids in reverse transcription within the pre-integration complex (PIC), as well as PIC localization to the nucleus. After integration, Vpr interacts in a glucocorticoid receptor (GR)-dependent manner to facilitate viral transcription. Vpr also aids in assembly and is recruited to the capsid by cleavage of Gag55Pr/P6 prior to budding and release of the virion.

Fig. 3. Sources and effects of intracellular and extracellular Vpr in the HIV-1-infected CNS
Vpr is introduced into the central nervous system (CNS) in HIV-1-infected macrophages, infected T lymphocytes, and cell free virions that cross the blood-brain barrier (BBB) (dashed lines). Infected microglial cells, perivascular macrophages, and astrocytes within the CNS are directly affected by intracellular Vpr expression. These cells presumably also release extracellular Vpr into the cerebrospinal fluid (CSF) (dotted lines). Neurons, oligodendrocytes, and brain microvascular endothelial cells (BMVEC) can be indirectly affected by extracellular Vpr released from infected cells and virions (solid lines). Indirect effects include aberrant cytokine release and alterations in cellular metabolism.

Fig. 4. Vpr-dependent effects putatively contribute to increased exosome release

Vpr causes alterations in glycolysis, including up-regulation of pyruvate kinase isozyme M2 (PKM2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Mitochondrial instability contributes to accumulation of reactive oxygen species (ROS) and increases in cytoplasmic Ca²⁺. The up-regulation of ROS combined with a down-regulation of ROS neutralizing enzymes leads to activation of HIF-1 α . These combined, Vpr-dependent effects are speculated to stimulate exosome release.