Published in final edited form as:

Brain Res. 2009 June 12; 1275C: 81–86. doi:10.1016/j.brainres.2009.03.024.

HIV-1 Vpr deregulates calcium secretion in neural cells

Inna Rom¹, Satish L. Deshmane¹, Ruma Mukerjee^{1,‡}, Kamel Khalili¹, Shohreh Amini^{1,2}, and Bassel E Sawaya^{1,‡,¶}

¹Department of Neuroscience & Center for Neurovirology, Temple University School of Medicine, 1900 North 12th Street, Philadelphia, PA 19122

²Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA 19122

Abstract

The lack of productive infection of neurons by HIV-1 suggests that the neuronal damage seen in AIDS patients with cognitive disorders is caused indirectly via viral and cellular proteins with neurotoxic activity. Among HIV-1 proteins, Vpr has been shown to deregulate expression of various important cytokines and inflammatory proteins in infected and uninfected cells. However, the mechanisms underlying these changes remain unclear. Here, we demonstrate that neurons can take up Vpr that is released into the supernatant of HIV-infected microglia. We also found that administration of recombinant Vpr (rVpr) to human neurons resulted in a slow but sustained elevation of intracellular calcium $[Ca^{2+}]_i$. Interestingly, our data also show that $[Ca^{2+}]_i$ elevation by Vpr leads to ROS production and impairs glutamate signaling in neuronal cells. Vpr disturbs calcium homeostasis through downregulation of endogenous PMCA. Finally, we found that the permeability of the plasma membrane increases in neurons treated with Vpr. Therefore, we conclude that soluble Vpr is a major viral factor that causes a disturbance in neuronal communication leading to neuronal dysfunction. The outcome of these studies will advance the understanding of HIV-1 pathogenesis and will help in the development of new therapeutic approaches.

INTRODUCTION

Although the molecules involved in HIV-associated neurological disturbances have not been completely identified, many data indicate that HIV-infected macrophages or microglial cells produce neurotoxic factors such as viral proteins, excitotoxins and/or cytokines (González-Scarano and Martín-García, 2005). Viral proteins that are released from HIV-infected macrophages or microglial cells can be deleterious to the central nervous system (CNS). HIV-1 envelope glycoprotein 120 (gp120), transcriptional transactivator (Tat), and viral protein R (Vpr) have been shown to be toxic to neurons (Jones et al., 2007). However, Vpr is the only viral protein released from HIV-infected macrophages that can cause the retardation of neuronal growth and plasticity (Kitayama et al., 2008).

The HIV-1 accessory protein viral protein R (Vpr) is synthesized late in the HIV-1 life cycle, packaged into the virion, and is essential for HIV-1 replication in macrophages (Nitahara-

¶Corresponding Authors: Bassel E. Sawaya, PhD, Department of Neurology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, Phone: 215-707-5446, Fax: 215-707-5948, E-mail: sawaya@temple.edu.

‡Present Address: Laboratory of Molecular Virology, Department of Neurology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Kasahara et al., 2007). In addition, studies from many groups have demonstrated that Vpr mediates multiple functions, including nuclear import of the HIV-1 pre-integration complex (Jacquot et al., 2007), G₂ cell cycle arrest caused by the induction of the damage-specific DNA-binding protein 1 (DDB1) and the Cullin 4A (Cul4A) E3 ubiquitin ligase pathway (Le Rouzic et al., 2008), transactivation of both viral replication and host genes and induction of caspase-dependent cellular apoptosis (Siddiqui et al., 2008). Most of these Vpr functions have been confirmed in *in vitro* systems. However, the real effect of Vpr *in vivo*, especially in neurons, remains to be identified. In this regard, Vpr has been detected in a soluble form in CSF and sera of HIV-1-infected patients displaying neurological disorders (Kitayama et al., 2008). It is thought that Vpr is toxic to mature neurons. *In vitro* studies using cultured neurons derived from rat hippocampal, cortical, and striatal neurons or human neuronal cell lines and *in vivo* studies using Vpr transgenic mice have shown that Vpr can cause neuronal apoptosis (Jones et al., 2007). Further, it is also known that Vpr-induced apoptosis is mediated by its binding to the adenine nucleotide translocator (ANT) in the inner membrane of mitochondria (Sabbah et al., 2006). Mitochondria play important roles in the establishment of axonal polarity and the regulation of neurite outgrowth during neuronal development. The trafficking of mitochondria may be a necessary event in neuronal development (Maltecca et al., 2008). Recently, Vpr was shown to inhibit axonal outgrowth and disturb neuronal plasticity through induction of mitochondrial dysfunction (Kitayama et al., 2008).

Regarding neuronal calcium, transient elevations of cytosolic calcium concentration $[Ca^{2+}]_i$ serve as a second messenger signal that controls many neuronal functions from development to death (D'Antoni et al., 2008). Similar to other cells, signaling in neurons uses both calcium influx through plasmalemmal Ca^{2+} channels and Ca^{2+} release from internal stores (Verkhratsky A, 2002). Intracellular Ca^{2+} release channels are inositol 1,4,5-triphosphate (IP_3)-gated, referred to as IP_3 receptors (IP_3Rs) and Ca^{2+} -gated also known as ryanodine receptors (RyRs). RyRs are located only in the ER (Fill and Copello, 2002), while IP_3Rs are present in ER, Golgi apparatus (Giorgi et al., 2008) and caveolae. Recent evidence suggests that cADPR, a metabolite NAD, is the endogenous modulator of ryanodine receptors (Lee et al., 2001). IP_3Rs and RyRs are regulated by cytosolic Ca^{2+} . Low concentrations of Ca^{2+} potentiate Ca^{2+} release giving rise to Ca^{2+} -induced Ca^{2+} release (CICR) whereas higher concentrations are inhibitory (Bezprozvanny et al., 1991). This biphasic regulation likely underlies both the temporal and spatial complexity of Ca^{2+} signals in response to these messengers. Depletion of ER Ca^{2+} stores can activate Ca^{2+} entry through a store-operated Ca^{2+} entry (SOCE) or capacitative Ca^{2+} entry (CCE) mechanism (Putney JW Jr, 2007). Calcium has a critical role in the process of neuronal injury and perturbed calcium homeostasis can mediate cell death by apoptosis (Mattson and Chan, 2003). Both calcium influx and release from ER are considered apoptogenic (Giorgi et al., 2008). An apoptotic cascade involving interplay between mitochondria and ER through cytochrome c/ IP_3Rs interactions has been described (Boehning et al., 2004). Moreover, inhibition of cytochrome c/ IP_3R binding can block the apoptotic process (Boehning et al., 2005).

Therefore, given the uncertainty regarding the neuropathogenic properties of HIV-1 Vpr and moreover its *in vivo* effects, especially its effect on calcium, we performed studies where we demonstrated the involvement of Vpr in neuronal dysfunction. Our results will help in determining the pathway and participant elements that are employed by Vpr upon viral infection to induce CNS injury.

RESULTS

Detection of HIV-1 Vpr in neuronal cells

The role of the HIV-1 Vpr protein in the pathogenesis of AIDS-associated disorders of the brain is not well established. However, a few studies including our own have demonstrated the

ability of HIV-1-infected microglia, astrocytes and uninfected neurons to release and re-uptake the Vpr protein. Until this time, the exact amount of Vpr released and subject to uptake remains unclear. We addressed this question by Western blot analysis to show the presence and the amount of Vpr involved. First, we examined whether neurons have the capability to take up Vpr protein. The neuronal cell line, SH-SY5Y, was treated with 1, 10 and 100 pg/ml or 1, 10, and 100 ng/ml of Vpr. As a control, cells were also treated with supernatant collected from HIV-1-infected (JR-FL strain) or uninfected U937 cells. Briefly, 50 ng of p24 containing virus stock were added per 1×10^6 cells to U937 for 10 days as previously described (Sawaya et al., 2000). Supernatant was collected every alternate day and viral replication measured by p24 ELISA (data not shown). Supernatant was centrifuged to remove virus, after which 3 ml were added to SH-SY5Y. After 24h, cells were lysed; total extracts were collected and analyzed for Vpr by Western blot (Amini et al., 2005). Note that recombinant Vpr (rVpr) was prepared using a bacterially expressed GST-Vpr fusion protein as previously described (Amini et al., 2004). Vpr was then cleaved from GST and treated to remove endotoxins. The integrity and purity of the Vpr protein was analyzed by SDS-PAGE followed by Coomassie blue staining. Vpr was also tested for its functional ability using the HIV-1 promoter. Twenty-four hours after the treatment with rVpr or supernatant, cell extracts were analyzed by Western blot using anti-Vpr or -Grb-2 antibodies. As shown in Figure 1, Vpr was detected in cells treated with 1, 10 and 100 ng of rVpr (Vpr panel, lanes 1-3). Vpr was also detected in cells treated with supernatant prepared from HIV-infected cells (lane 8). To ascertain equal protein loading, anti-Grb2 antibody was used. These results confirmed the ability of HIV-infected cells to release Vpr and the ability of uninfected cells (a neuronal cell line in this case) to re-uptake the Vpr that was released.

Vpr induces mitochondrial and NADPH oxidase ROS production in microglia and neurons

Next, we examined the effect of Vpr on the growth and plasticity of neurons using cytosolic calcium as an indicator. Primary neurons were treated with 100 nM rVpr (~ 10 ng/ml) and the calcium concentration was measured as previously described (Brailoiu et al., 2006). Our results demonstrated the ability of Vpr to increase the calcium concentration inside the cells (data not shown).

Deregulation of $[Ca^{2+}]_i$ homeostasis has been reported to induce mitochondrial calcium overload (Dong et al., 2006). This process has major potential implications for cellular metabolism generating ROS and altering NADPH metabolism (Brookes et al., 2004). Indeed, in microglial cells, we found an elevation in ROS as well as an altered level of NADPH. Microglial cells were treated with 10 ng/ml of rVpr for 24h after which the cells were treated with the redox-sensitive probe Redox Sensor Red CC-1 and the mitochondria-specific dye MitoTracker green FM. As shown in Figure 2A, only Vpr-expressing cells exhibit bright yellow/orange fluorescence in mitochondria due to the co-localization of oxidized Red CC-1 and Mito Tracker green, whereas oxidation of Red CC-1 in cytoplasm shows red/orange fluorescence, indicative of increased ROS production in the mitochondria and cytosolic compartments (compare Vpr and mock panels). Figure 2B shows quantification of the ROS levels. In comparison with mock-treated cells, ROS production was approximately 11-fold higher in the cells treated with rVpr.

Similar results were obtained with neuronal cells even when the cells were treated with less Vpr (100 pg/ml of rVpr for 24h). As shown in Figure 2C, Vpr-expressing cells exhibit bright yellow/orange fluorescence in mitochondria when compared to the mock-treated cells. Interestingly, results point to the ability of Vpr to induce mitochondrial and NADPH oxidase ROS in microglia and neurons.

Vpr limits mitochondria transport in neuronal cells

The ability of Vpr to induce mitochondrial ROS, which can lead to neuronal injury, gave us a rationale to examine whether this injury affects the transport of mitochondria within axons. This transport requires motor proteins, which utilize ATP for their mobilization (Chang and Reynolds, 2006). Thus we measured levels of ATP in primary neurons mock-treated or treated with rVpr. Briefly, cellular ATP levels were measured using the ATP bioluminescence assay kit HSII as recommended by the manufacturer (Roche, Indianapolis, IN). As shown in Figure 3A, the concentration of ATP was significantly reduced in Vpr-treated cells ($1.46 \pm 0.44 \mu\text{M}$) when compared to the mock-treated cells ($4.32 \pm 0.71 \mu\text{M}$).

Vpr inhibits calcium release from the cell

The plasma membrane Ca^{2+} ATPase (PMCA) is a transport protein in the plasma membrane of cells that serves to export calcium out of the cell (Garcia and Strehler, 1999). PMCA is vital for regulating $[\text{Ca}^{2+}]_i$. There is a very large transmembrane electrochemical gradient of $[\text{Ca}^{2+}]_i$ driving the entry of Ca^{2+} ions into cells, yet it is very important for cells to maintain low concentrations of $[\text{Ca}^{2+}]_i$ for proper cell signaling; thus it is necessary for the cell to employ ion pumps to export Ca^{2+} ions. PMCA and the sodium calcium exchanger (NCX) are together the main regulators of $[\text{Ca}^{2+}]_i$ (Blaustein et al., 2002). Since it transports Ca^{2+} ions into the extracellular space, the PMCA is also an important regulator of the calcium concentration in the extracellular space. Hence, we examined the status of PMCA in Vpr-treated neurons. SH-SY5Y cells were treated 10 ng/ml of rVpr for 24 h. Twenty-five micrograms of extracts were analyzed by Western blot using anti-PMCA antibody. As shown in Figure 3B, treatment of the cells with rVpr led to decreased levels of endogenous PMCA (compare lane 1 to lanes 2). Anti-Grb2 was used as a control for protein loading.

Vpr promotes change in membrane permeability

The reduction in PMCA levels by rVpr gave us a rationale to examine the permeability of the plasma membrane of neuronal cells. SH-SY5Y cells were treated 10 ng/ml of rVpr for 24 h. Twenty-five micrograms of extracts were analyzed using a fluorescent dye loading assay which determines the plasma membrane permeability by allowing the measurement of protein leakage. Briefly, the leakage of calcein-AM (Molecular Probe, Eugene, OR), a fluorescent membrane integrity probe, from the cells was used to monitor the hyperpermeability state of the plasma membrane. Calcein-AM is membrane permeable and virtually non-fluorescent until intracellular esterase catalyzes the hydrolysis of the AM portion of the molecule resulting in the intensely fluorescent product, calcein, which fluoresces green (485 and 500 nm) (Menconi et al., 1997). Therefore, an increase in calcein flux across the membrane indicates hyperpermeability corresponding to damage of the plasma membrane. As shown in panel C, treatment of the cells with rVpr increased cellular leakage (lane 2).

DISCUSSION

Our data indicate that HIV-1 Vpr can be released from HIV-infected cells and taken up by uninfected neuronal cells. Once inside the cells, Vpr increases the intracellular calcium concentration leading to the activation of the reactive oxygen species-signaling pathway (ROS). This induction limited the transport of mitochondria within the cells. Further, although Vpr increase the permeability of the cytosolic membrane, we found that Vpr inhibits calcium release from the cells by affecting endogenous levels of PMCA. Thus it is possible that Vpr induces an array of biological events leading to neuronal deregulation through disturbing calcium homeostasis.

Vpr was found to induce an abnormality in mitochondrial transport at low concentrations through the suppression of ATP synthesis (Figure 3A). One of the major functions of

mitochondria is to generate intracellular ATP. Neuronal homeostasis is a highly dynamic process marked by an intense need for ATP, as ATP production is critical for the full functioning of cellular motor proteins such as kinesin and dynein (Chang and Reynolds, 2006) and also for the functioning of key metabolic pathways. As shown in Figure 3A, the reduction of the ATP concentration in rVpr-treated neuronal cells appeared to have a direct impact on the velocity of mitochondrial transport. Within the CNS, mitochondria are enriched in regions of high metabolic demand, especially in synapses. The necessity of mitochondrial transport in neuronal development and homeostasis is becoming clear. During neuronal cell differentiation from undifferentiated neuroblasts, cells acquire many neuronal processes. From these processes, axonal and dendritic branches form, and then synaptic connections assemble. These highly dynamic processes need large amounts of ATP to be produced at locations where it is needed. Therefore, the appropriate trafficking of mitochondria is a necessary task beginning in the earliest steps of neuronal development. Therefore, the suppression of ATP synthesis following Vpr treatment may induce the retardation of mitochondrial transport into and within neurites, which may lead to cell death. In this regard, Vpr is well known to induce apoptosis when a high concentration of Vpr protein is added to cell culture or is exogenously expressed in cells (Jacotot et al., 2000). Further, synthetic Vpr peptides are found to directly bind to ANT, a component of the mPTP located in the inner mitochondrial membrane. As a result, a decrease of mitochondria membrane potential and the release of cytochrome c are thought to induce the intrinsic apoptosis pathway. However, these functions require a high concentration of Vpr to induce the severe depression of $\Delta\psi_m$ in neuronal cells. Therefore, our future studies will aim to determine whether the amount of Vpr released from HIV-infected cells and taken up by neuronal cells is enough to cause all these neuronal disturbance or if it also requires the help of other toxins secreted along with Vpr from HIV-infected cells.

Based on these observations, we concluded that Vpr could be a major player in neuronal injury mainly observed in AIDS patients whom develop minor cognitive motor disorder (MCMD) (Kaul M, 2008) even in the HAART era.

MATERIALS AND METHODS

Cell Culture

The human microglial and neuroblastoma cell lines (SH-SY5Y) were maintained in DMEM + 10% FBS. The human microglial cell line was established after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen (Janabi et al., 1995). SH-SY5Y was chosen because of its ability to mirror pathways involved in neurodegenerative process associated with HIV-E (Everall et al., 2002; Sanders et al., 2000). Cells were plated at 1×10^5 /ml and cultured in DMEM. Confluent cells were replated at $1-5 \times 10^5$ cells/ml for different experiments and differentiated by treatment with 10 mM retinoic acid (Sigma, St. Louis, MO) for 7d with medium changes every two days. For all of the experiments, cells were serum starved for 6h in the presence of 10 mM Retinoic Acid prior to treatment with rVpr.

ROS measurement

The trafficking of 2,3,4,5,6-pentafluorodihydro-tetramethylfosamine (PF-H₂TMROS or Redox Sensor Red CC-1; Molecular Probes-Invitrogen, Carlsbad, CA) was used to detect reactive oxygen intermediates (Menconi et al., 1997). Redox Sensor Red CC-1 is oxidized in the presence of O₂⁻ and H₂O₂. Briefly, microglial cells mock, or treated with rVpr were loaded at 37°C for 20 min with 1 μ M of Redox Sensor Red CC-1 and a mitochondria-specific dye, MitoTracker green FM (50nM; Molecular Probes). Culture slides were washed with PBS and visualized with a Nikon fluorescence microscope (Nikon Eclipse E800) equipped with a triple-filter cube and charge-coupled device camera (Nikon DXM1200). Note that all ROS measurement experiments were performed at least three times.

Western blot

Microglia and SH-SY5Y were treated with rVpr at 37°C. Twenty-four hours post-infection, 30 µg of cell extracts were analyzed by Western blot using several antibodies as indicated with a 1/1000 dilution. Note that most of Western blot assays were performed at least twice as previously described (Saunders et al., 2005).

Plasma membrane permeability

SH-SY5Y cells were treated 10 ng/ml of rVpr for 24 h after which the cells were collected, washed and the cell extracts were prepared. Twenty-five micrograms of extracts were analyzed using a fluorescent dye loading assay which determines the plasma membrane permeability by allowing the measurement of protein leakage.

HIV-1 infection

The human U-937 monocytic cell line was maintained in RPMI + 10% FBS, 100 units/ml penicillin, 50 µg/ml streptomycin-G. Cells in log phase were infected with JR-FL strains of HIV-1 as follows. Fifty nanograms of p24-containing virus stock were added per 1×10^6 cells. Cells were incubated with virus stock in a small volume of serum-free medium for 2h at 37°C. The cells were then washed twice with PBS and fresh medium containing 2% of FBS was added (500,000 cells /ml). Supernatant was collected and added to SH-SY5Y cells for 6 hours after which Western blot analysis was performed. All infection experiments were performed three times.

Acknowledgments

The authors wish to thank past and present members of the Department of Neuroscience and Center for Neurovirology for their support, and sharing of reagents and ideas. We also thank Drs. Shongshan Fan and Eugene Brailoiu for technical support. This work was supported by grants awarded by NIH to BES, SA and KK.

References

1. Amini S, Mameli G, Del Valle L, Skowronska A, Reiss K, Gelman BB, White MK, Khalili K, Sawaya BE. p73 interacts with HIV-1 Tat in astrocytic cells and prevents its acetylation on Lys²⁸. *Mol Cell Biol* 2005;25:8126–8138. [PubMed: 16135803]
2. Amini S, Saunders M, Kelley K, Khalili K, Sawaya BE. Interplay between HIV-1 Vpr and Sp1 modulates p21WAF1 gene expression in human astrocytes. *J Biol Chem* 2004;279:46046–46056. [PubMed: 15302882]
3. Bezprozvanny I, Watras J, Ehrlich BE. Bell-shaped calcium-response curves of Ins(1,4,5)P₃- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature* 1991;351:751–754. [PubMed: 1648178]
4. Blaustein MP, Juhaszova M, Golovina VA, Church PJ, Stanley EF. Na/Ca exchanger and PMCA localization in neurons and astrocytes: functional implications. *Ann NY Acad Sci* 2002;976:356–366. [PubMed: 12502582]
5. Boehning D, Patterson RL, Snyder SH. Apoptosis and calcium: new roles for cytochrome c and inositol 1,4,5-trisphosphate. *Cell Cycle* 2004;3:252–254. [PubMed: 14726673]
6. Boehning D, van Rossum DB, Patterson RL, Snyder SH. A peptide inhibitor of cytochrome c/inositol 1,4,5-trisphosphate receptor binding blocks intrinsic and extrinsic cell death pathways. *Proc Natl Acad Sci U S A* 2005;102:1466–1471. [PubMed: 15665074]
7. Brailoiu E, Churamani D, Pandey V, Brailoiu GC, Tuluc F, Patel S, Dun NJ. Messenger-specific role for nicotinic acid adenine dinucleotide phosphate in neuronal differentiation. *J Biol Chem* 2006;281:15923–15928. [PubMed: 16595650]
8. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol* 2004;287:C817–C833. [PubMed: 15355853]

9. Chang DT, Reynolds IJ. Differences in mitochondrial movement and morphology in young and mature primary cortical neurons in culture. *Neuroscience* 2006;141:727–736. [PubMed: 16797853]
10. D'Antoni S, Berretta A, Bonaccorso CM, Bruno V, Aronica E, Nicoletti F, Catania MV. Metabotropic Glutamate Receptors in Glial Cells. *Neurochem Res.* 2008 Apr 26;
11. Dong Z, Saikumar P, Weinberg JM, Venkatachalam MA. Calcium in cell injury and death. *Annu Rev Pathol* 2006;1:405–434. [PubMed: 18039121]
12. Everall IP, Bell C, Mallory M, Langford D, Adame A, Rockenstein E, Masliah E. Lithium ameliorates HIV-gp120-mediated neurotoxicity. *Mol Cell Neurosci* 2002;21:493–501. [PubMed: 12498789]
13. Fill M, Copello JA. Ryanodine receptor calcium release channels. *Physiol Rev* 2002;82:893–922. [PubMed: 12270947]
14. Garcia ML, Strehler EE. Plasma membrane calcium ATPases as critical regulators of calcium homeostasis during neuronal cell function. *Front Biosci* 1999;4:D869–882. [PubMed: 10577388]
15. Giorgi C, Romagnoli A, Pinton P, Rizzuto R. Ca(2+) signaling, mitochondria and cell death. *Curr Mol Med* 2008;8:119–130. [PubMed: 18336292]
16. González-Scarano F, Martín-García J. The neuropathogenesis of AIDS. *Nat Rev Immunol* 2005;5:69–81. [PubMed: 15630430]
17. Jacotot E, Ravagnan L, Loeffler M, Ferri KF, Vieira HL, Zamzami N, Costantini P, Druillennec S, Hoebeke J, Briand JP, Irinopoulou T, Daugas E, Susin SA, Cointe D, Xie ZH, Reed JC, Roques BP, Kroemer G. The HIV-1 viral protein R induces apoptosis via a direct effect on the mitochondrial permeability transition pore. *J Exp Med* 2000;191:33–46. [PubMed: 10620603]
18. Jacquot G, Le Rouzic E, David A, Mazzolini J, Bouchet J, Bouaziz S, Niedergang F, Pancino G, Benichou S. Localization of HIV-1 Vpr to the nuclear envelope: impact on Vpr functions and virus replication in macrophages. *Retrovirology* 2007;4:84. [PubMed: 18039376]
19. Janabi N, Peudenier S, Héron B, Ng KH, Tardieu M. Establishment of human microglial cell lines after transfection of primary cultures of embryonic microglial cells with the SV40 large T antigen. *Neurosci Lett* 1995;195:105–108. [PubMed: 7478261]
20. Jones GJ, Barsby NL, Cohen EA, Holden J, Harris K, Dickie P, Jhamandas J, Power C. HIV-1 Vpr causes neuronal apoptosis and in vivo neurodegeneration. *J Neurosci* 2007;27:3703–3711. [PubMed: 17409234]
21. Kaul M. HIV's double strike at the brain: neuronal toxicity and compromised neurogenesis. *Front Biosci* 2008;13:2484–2494. [PubMed: 17981728]
22. Kitayama H, Miura Y, Ando Y, Hoshino S, Ishizaka Y, Koyanagi Y. Human immunodeficiency virus type 1 Vpr inhibits axonal outgrowth through induction of mitochondrial dysfunction. *J Virol* 2008;82:2528–2542. [PubMed: 18094160]
23. Le Rouzic E, Morel M, Ayinde D, Belaïdouni N, Letienne J, Transy C, Margottin-Goguet F. Assembly with the Cul4A-DDB1-DCAF1 ubiquitin ligase protects HIV-1 Vpr from proteasomal degradation. *J Biol Chem.* 2008 Jun 4;
24. Lee CJ, Kong H, Manzini MC, Albuquerque C, Chao MV, MacDermott AB. Kainate receptors expressed by a subpopulation of developing nociceptors rapidly switch from high to low Ca2+ permeability. *J Neurosci* 2001;21:4572–4581. [PubMed: 11425885]
25. Maltecca F, Aghaie A, Schroeder DG, Cassina L, Taylor BA, Phillips SJ, Malaguti M, Previtali S, Guénet JL, Quattrini A, Cox GA, Casari G. The mitochondrial protease AFG3L2 is essential for axonal development. *J Neurosci* 2008;28:2827–2836. [PubMed: 18337413]
26. Mattson MP, Chan SL. Calcium orchestrates apoptosis. *Nat Cell Biol* 2003;5:1041–1043. [PubMed: 14647298]
27. Menconi MJ, Salzman AL, Unno N, Ezzell RM, Casey DM, Brown DA, Tsuji Y, Fink MP. Acidosis induces hyperpermeability in Caco-2BBE cultured intestinal epithelial monolayers. *Am J Physiol* 1997;272:G1007–1021. [PubMed: 9176208]
28. Nitahara-Kasahara Y, Kamata M, Yamamoto T, Zhang X, Miyamoto Y, Muneta K, Iijima S, Yoneda Y, Tsunetsugu-Yokota Y, Aida Y. Novel nuclear import of Vpr promoted by importin alpha is crucial for human immunodeficiency virus type 1 replication in macrophages. *J Virol* 2007;81:5284–5293. [PubMed: 17344301]
29. Putney JW Jr. New molecular players in capacitative Ca2+ entry. *J Cell Sci* 2007;120:1959–1965. [PubMed: 17478524]

30. Sabbah EN, Druillennec S, Morellet N, Bouaziz S, Kroemer G, Roques BP. Interaction between the HIV-1 protein Vpr and the adenine nucleotide translocator. *Chem Biol Drug Des* 2006;67:145–154. [PubMed: 16492162]
31. Sanders VJ, Everall IP, Johnson RW, Masliah E. Fibroblast growth factor modulates HIV coreceptor CXCR4 expression by neural cells. *J Neurosci Res* 2000;59:671–679. [PubMed: 10686595]
32. Saunders M, Eldeen MB, Valle LD, Reiss K, Peruzzi F, Mameli G, Gelman BB, Khalili K, Amini S, Sawaya BE. p73 modulates HIV-1 Tat transcriptional and apoptotic activities in human astrocytes. *Apoptosis* 2005;10:1419–1431. [PubMed: 16235026]
33. Sawaya BE, Khalili K, Gordon J, Taube R, Amini S. Cooperative interaction between HIV-1 regulatory proteins Tat and Vpr modulates transcription of the viral genome. *J Biol Chem* 2000;275:35209–35214. [PubMed: 10931842]
34. Siddiqui K, Del Valle L, Morellet N, Cui J, Ghafouri M, Mukerjee R, Urbanska K, Fan S, Pattillo CB, Deshmane SL, Kiani MF, Ansari R, Khalili K, Roques BP, Reiss K, Bouaziz S, Amini S, Srinivasan A, Sawaya BE. Molecular mimicry in inducing DNA damage between HIV-1 Vpr and the anticancer agent, cisplatin. *Oncogene* 2008;27:32–43. [PubMed: 17653096]
35. Verkhatsky A. The endoplasmic reticulum and neuronal calcium signalling. *Cell Calcium* 2002;32:393–404. [PubMed: 12543098]

Figure 1. Uptake of Vpr by a neuronal cell line

SH-SY5Y cells were either untreated or treated with concentrations of Vpr as indicated or conditioned medium from HIV-infected U937 cells for 24h. Fifty micrograms of lysates prepared from untreated, rVpr-treated were analyzed by Western blot with α -Vpr or α -Grb-2 antibodies.

Figure 2. Induction of ROS production by Vpr at 24 hr

A and **C**. Microglia (**A**) and SH-SY5Y cells (**C**) were treated with rVpr for 24h. Cells were loaded with redox-sensitive dye Redox Sensor Red CC-1 (red panels) and the mitochondrial-specific dye MitoTracker green FM (green panels). Induction of ROS production and its localization in the mitochondria (yellow/orange and arrows) or in the cytoplasm (red/orange and arrows) is also shown. **B**. Total ROS fluorescence was averaged from individual cells and expressed as a percentage of Mito fluorescence. Measurements were made from 100 cells per field. The columns (means $\pm$ SE) represent the average of five fields. Statistically significant differences were seen between experimental values and controls: ($p < 0.05$, **, Student's *t* test). Experiments were carried out 2 times, with similar results.

Figure 3. Vpr reduces ATP production in neuronal cells

A. The cellular ATP concentration of neuronal cells treated with Vpr was measured. ($P < 0.01$ compared to the mock). **B.** rVpr decreases the levels of PMCA. SH-SY5Y cells were treated with rVpr for 24 h after which protein extracts were prepared. Twenty-five micrograms of extract were analyzed by Western blot with anti-PMCA, or -Grb2 antibodies. **C.** Induction of cellular leakage in Vpr-treated neurons. The permeability of the plasma membrane was measured in the presence of rVpr. Data shown here are from a single, representative experiment replicated three times.