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Critical implication of the (70–96) domain of human immunodeficiency virus type 1 Vpr protein in apoptosis of primary rat cortical and striatal neurons

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The human immunodeficiency virus (HIV)-1 regulatory protein Vpr has been detected in the serum of HIV-seropositive individuals and in the cerebrospinal fluid of acquired immunodeficiency syndrome (AIDS) patients suffering from neurological disorders. Therefore, Vpr could play a critical role in the neuronal apoptosis observed postmortem in the brain of patients, often connected to a severe AIDS-related disease termed HIV-associated dementia (HAD). This suggests that the Vpr neurotoxicity already observed *in vitro* on hippocampal neurons could also occur in other brain structures. In this study the authors have investigated the ability of synthetic Vpr to induce apoptosis in primary cultures of rat cortical and striatal neurons. Moreover, the authors have explored the Vpr minimal proapoptotic region using synthetic Vpr fragments and mutants of the protein. Treatments of both neuronal types with Vpr, its C-terminal domain, Vpr(52–96), or a shorter fragment, Vpr(70–96), led to dose- and time-dependent cell death as determined by flow cytometry after propidium iodide labeling, phase-contrast microscopy, and TUNEL labeling. Taken together, these results support an apoptosis-induced death of these neurons. The (71–82) Vpr peptide, previously shown toxic to isolated mitochondria, was inactive on neurons. Vpr-induced neuronal apoptosis was associated with activation of caspase-3 beginning 3 h after Vpr extracellular addition and peaking 3 h later. Moreover, an hyperproduction of reactive oxygen species was observed. In addition to hippocampal neurons, the extension of the apoptotic property of Vpr to cortical and striatal neurons could account for several signs observed in HAD and is thus consistent with a possible involvement of Vpr in this syndrome. *Journal of NeuroVirology* (2005) **11,** 489–502.

Keywords: apoptosis; HIV-1; neurons; Vpr

Introduction

The central nervous system (CNS) is affected during human immunodeficiency virus (HIV)-1 infection. Clinical manifestations of CNS infection ranged from moderate troubles,recorded in half of the infected subjects, to severe neurological disorders resulting in the so-called HIV-associated dementia (HAD), developed by 15% to 30% of HIV-1 infected individuals on late stages of the illness (MacArthur *et al*, 1993). Cognitive dysfunctions related to HAD include impaired memory, difficulty of concentration, and mental slowing. Motor problems caused by HAD are tremor, incoordination, and fine motor slowness. Behavioral symptoms include apathy, social withdrawal, irritability, and depression. The most specific morphological characteristics of HAD are formation of multinucleated giant cells (resulting from the fusion between infected macrophages or microglia),

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Figure 1 Primary sequence of Vpr. Vpr, from the HIV-1 LAI strain, as well as native or mutated fragments of the protein, were obtained by solid phase synthesis. Purity, checked by mass spectroscopy and HPLC, was ≥95%. Figure shows the primary Vpr sequence; the arginines residues at positions 73, 77, and 80, which were mutated to alanine, are indicated in bold.

myelin pallor, astrocytosis, and neuronal cell loss (Kolson *et al*, 1998; Rausch and Stover, 2001). HIV seems to cause neuronal cell depletion by inducing apoptosis as shown by neuropathological studies carried out *postmortem* on brains of AIDS patients (Adle-Biassette *et al*, 1997; Everall *et al*, 1991; Gray *et al*, 2000; Petito and Roberts, 1995). However, neurons, unlike macrophages and microglia, do not seem to be directly infected by the virus (or sparsely infected) and the cause of neuronal apoptosis during HIV infection remains unknown. It is speculated that it may result from the release of endogenous neurotoxic compounds from the host-immune cells present in brain (Xu *et al*, 2004). Another hypothesis is the implication of HIV-encoded neurotoxic soluble proteins either released from brain infected cells or able to cross the blood-brain barrier (reviewed in Van de Bovenkamp *et al*, 2002).

Among these proteins, a great interest was granted to the HIV-1 protein Vpr that was detected in the serum of seropositive individuals and in the cerebrospinal fluid of acquired immunodeficiency syndrome (AIDS) patients with neurological disorders (Levy *et al*, 1994). Vpr is a 96–amino acid protein encoded by HIV-1 and HIV-2 (Figure 1), whose solution structure has been solved by nuclear magnetic resonance (NMR) spectroscopy (Morellet *et al*, 2003). HIV-1 Vpr regulates viral transcription (Goh *et al*, 1998; Gummuluru and Emerman, 1999) by inhibiting cell cycle at the G2/M transition (He *et al*, 1995; Re *et al*, 1995). Vpr participates in the translocation to the nucleus of the preintegration complex (Heinzinger *et al*, 1994; Popov *et al*, 1998) and is therefore required for HIV replication in cells that do not undergo mitosis (Connor *et al*, 1995; Vodicka *et al*, 1998). Extracellular addition of Vpr triggers apoptosis of lymphocytes, monocytes, neuroblastomas, and hippocampal neurons (Arunagiri *et al*, 1997; Huang *et al*, 2000; Jacotot *et al*, 2000; Macreadie *et al*, 1996; Patel *et al*, 2000), suggesting thus that Vpr affects cellular pathways common to many cell types. Moreover, transfection by a *vpr*-expressing vector (Stewart *et al*, 1999) or viral delivery of Vpr (Muthumani *et al*, 2003; Patel *et al*, 2002; Stewart *et al*, 1999, 2000) leads also to apoptosis.Mapping studies done on lymphocytes or isolated mitochondria reveals that the cytotoxic domain of Vpr lies in the (70–96) fragment (Arunagiri *et al*, 1997; Jacotot *et al*, 2000; Macreadie *et al*, 1996). *In vivo*, transgenic mice overexpressing Vpr exhibit an enhanced apoptotic death of T lymphocytes (Yasuda *et al*, 2001) and intravenous (i.v.) injections of Vpr(52–96) in mice induce depletion of CD4 and CD8 cells (Lum *et al*, 2003). Activation of caspases during Vpr-induced apoptosis was suggested but not firmly confirmed (Muthumani *et al*, 2002a, 2002b; Patel *et al*, 2000, 2002; Stewart *et al*, 2000; Yasuda *et al*, 2001).

Vpr involvement in the HIV-associated dementia by triggering neuronal apoptosis is consistent with previous *in vitro* studies on Vpr neurotoxicity showing that Vpr was able to form ion channels in the cell membrane of cultured rat hippocampal neurons leading to cell death (Piller *et al*, 1999). However although apoptotic effects of Vpr are well recognized on hippocampal cells (Huang *et al*, 2000), they remains some contradictions regarding the region of the protein involved in this process and the extension of apoptosis to other brain cells. Indeed, the N-terminal part of Vpr was suggested to be proapoptotic by Piller and colleagues (Piller *et al*, 1999), whereas the C-terminal domain was reported to be essential by others (Arunagiri *et al*, 1997; Jacotot *et al*, 2000; Macreadie *et al*, 1996; Roumier *et al*, 2002). In addition, Vpr, although able to induce apoptosis on hippocampal neurons, did not seem to induce cortical neurons death (Huang *et al*, 2000). Hence, with the aim to determine the Vpr minimal fragment able to trigger *in vitro* neuronal apoptosis, Vpr and several Vpr native and mutated fragments were synthesized and added over primary cultures of rat cortical and striatal neurons. We demonstrate here that the neurotoxic domain of Vpr on both neuronal types is located in the (70–96) fragment, thus encompassing the ^{71}H(S/F)RIG^{82} mitochondriotoxic domain (Jacotot *et al*, 2000).However, the repeated motif H(S/F)RIG was not sufficient to induce neuronal death, and the C-terminal flexible domain of Vpr is also required. The Vpr induced death of cortical and striatal neurons occurred by an apoptotic process with the caspase-3 activated and reactive oxygen species (ROS) production increased. Taken together with the previously reported apoptosis of hippocampal neurons (Piller *et al*1999; Huang *et al*, 2000), the results of this study could account for several neurological disorders observed in the HIV-associated dementia syndrome.

Results

Vpr induces death of striatal and cortical neurons

Striatal neurons were extracted from rat embryos and seeded onto poly-D-lysine coated plates or chamber

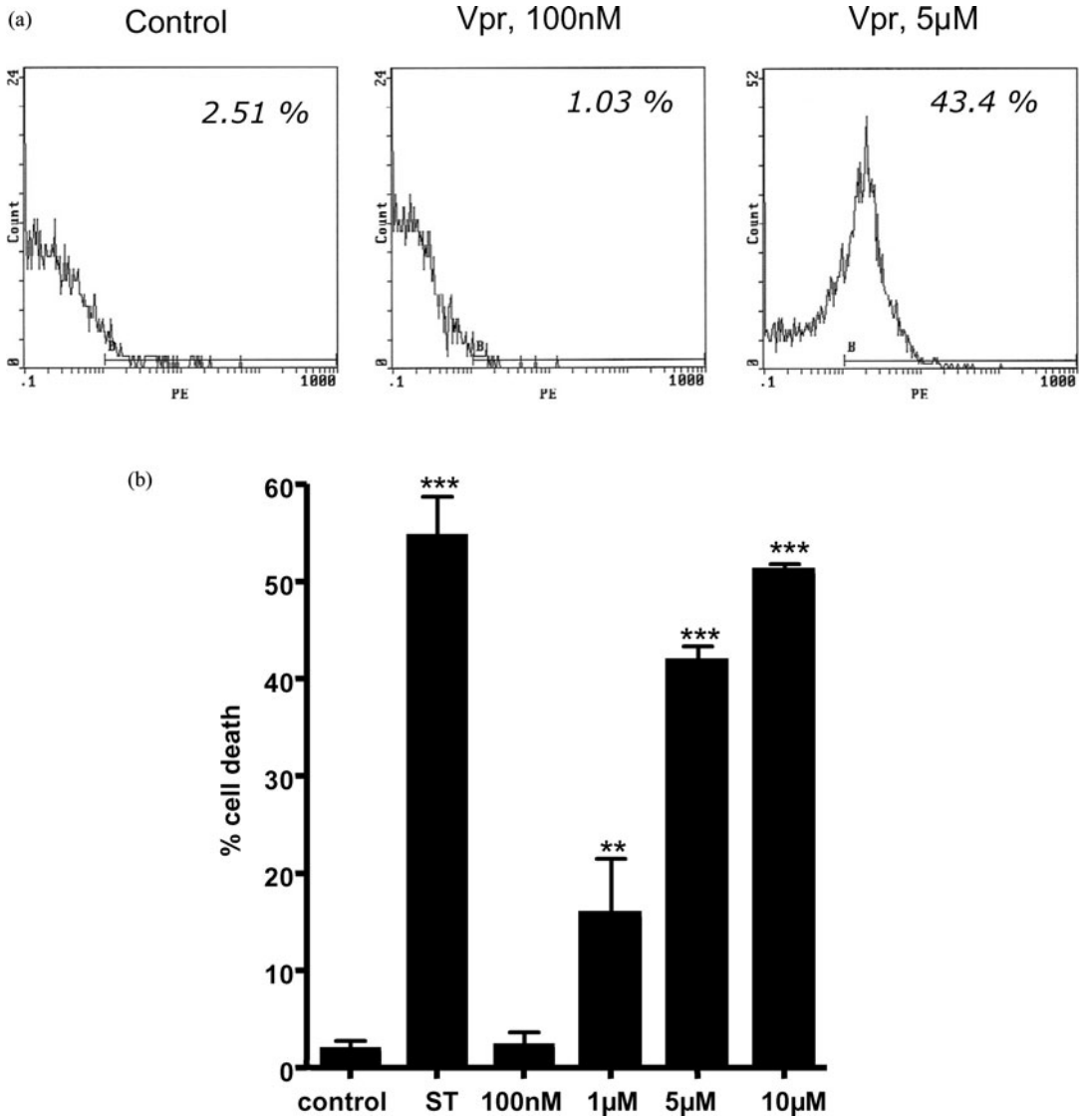

Figure 2 Overnight Vpr exposure of striatal neurons induces cell death. Striatal neurons were extracted from rat embryos. After 8 to 10 days in culture on poly-D-lysine-coated plates, they were subjected to overnight incubation with various concentrations of pure synthetic Vpr. Control corresponds to neurons treated with buffer alone and ST to neurons incubated with staurosporine, an apoptosis inducer, at 1 μM. Then the cells were collected and labeled with propidium iodide (PI; 1 μM) and analyzed by flow cytometry (minimum 10,000 events per experiment with at least three independent experiments). (**a**) Representative fluorescence signals of propidium iodide are shown here for two Vpr concentrations and buffer-treated cells (control). The percentages indicate the value of PI-positive cells. (**b**) Results expressed as means ± SEM, show the strong neurotoxicity of Vpr. $^{**}P < .01$, $^{***}P < .001$ compared to buffer-treated cells. ST: staurosporine, 1 μM.

slides. After 8 to 10 days of culture in a medium without serum to prevent proliferation of contaminating glial cells, neurons were subjected to overnight treatment with various concentrations of pure synthetic Vpr (primary sequence, Figure 1). Cell viability was visualized by observation of the cells under a light microscope and by flow cytometry after labeling with propidium iodide (PI). Examples of PI fluorescence signals are displayed (Figure 2a). Overnight exposure to micromolar concentrations of Vpr induced an increase in the number of PI-labeled striatal cells in a dose-dependent manner: at concentration below 1 μM, Vpr did not produce any cytotoxic effects, whereas at 10 μM, Vpr induced the death of more than half of the striatal neurons in culture (Figure 2b).

In order to assess the cell-type specificity of Vpr-induced neuronal death, experiments were performed also on primary rat cortical neurons. As observed with striatal neurons, extracellular addition of Vpr caused death of cortical neurons after overnight incubation as determined by microscopy and flow cytometry (Table 1). Moreover, no statistically significant difference was found between the levels of cell death in cortical and striatal cultures (data not

Table 1 Neurotoxic effects of Vpr and Vpr fragments over cortical and striatal neurons

Cells[a]	*Buffer*	*ST*[b]	*Vpr*	*(1–51)*	*(52–96)*	*(70–96)*	*(80–96)*	*(55–76)*	*(71–82)*
Cortical neurons	6.2 ± 1.5	$44.6 \pm 3.0^{***}$	$30.9 \pm 3.3^{***}$	4.6 ± 1.8	$47.9 \pm 3.4^{***}$	$34.1 \pm 4.3^{***}$	6.6 ± 2.4	8.7 ± 3.1	11.6 ± 3.2
Striatal neurons	1.9 ± 0.9	$52.8 \pm 4.1^{***}$	$41.8 \pm 1.6^{**}$	9.8 ± 9.0	$41.9 \pm 3.2^{***}$	$32.8 \pm 9.3^{*}$	10.8 ± 9.3	9.2 ± 2.5	11.8 ± 4.6

[a]Cortical and striatal neurons were incubated overnight with 5 μM Vpr or Vpr-derived fragments and then subjected to cell viability analysis by flow cytometry after propidium iodide labeling. Results corresponding to at least three independent experiments in triplicate indicate the percentage of cell death and are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$.
[b]ST: staurosporine. Staurosporine, an inducer of apoptosis, was used at 1 μM as a positive control in all experiments.

shown). Thus, it seems that unlike previously published results (Huang *et al*, 2000), Vpr does induce the death of cortical neurons in pure primary cultures.

The cytotoxic domain of Vpr on cortical and striatal neurons lies in the (70–96) fragment

In order to caracterize the neurotoxic domain of Vpr, several fragments and mutants of Vpr were synthesized and tested at 5 μM for their effects on neuron viability after overnight incubation. The percentage of dead cells was quantified by flow cytometry after propidium iodide labeling. As shown in Figure 3a and Table 1, the N-terminal fragment of Vpr, (1–51), affected nor the striatal viability neither the cortical one. On the other hand, the C-terminal domain of Vpr, (52–96), was neurotoxic (Figure 3a and Table 1). The use of fragments of Vpr(52–96) allowed us to demonstrate that the toxic domain of the protein was in the (70–96) sequence (Figure 3a and Table 1). The Vpr(71–82) peptide shown to be toxic to isolated mitochondria (Jacotot *et al*, 2000) displayed toxicity for both neuronal cell types that were not statistically different from the percentage of dead cells in the control experiments. Thus, among the tested Vpr peptides, the shortest neurotoxic one was Vpr(70–96). Figure 4 displays dose-response death rate of the cortical cells incubated overnight with increasing concentrations of Vpr(52–96) or Vpr(70–96). For each concentration, Vpr(70–96) is less potent that Vpr(52–96) to induce cell death.

Taking into account that the neurotoxic region of Vpr was in the (70–96) domain of the protein, we investigated the effects of point mutations of some of the arginine residues located in this fragment. Indeed, arginine residues in the C-terminal part of Vpr were shown to be critical for Vpr-induced apoptosis of various cell types (Di Marzio *et al*, 1995; Jacotot *et al*, 2000; Sawaya *et al*, 2000), but the effects resulting from mutations of these amino acids were not investigated in neurons. Therefore, three point-mutated Vpr(52–96) peptides were synthesized by replacement of one arginine in position 73, 77, or 80 by alanine, a neutral amino acid expected to be unable to modify the peptide conformation. Mutation of one of these residues led to a redution in neuronal death compared to wild-type Vpr(52–96), although the mutants still displayed significant differences in neurotoxicity with buffer-treated cells (Figure 3b). The more pronounced decrease of neurotoxicity is observed for the R73A mutant, with a dead neuronal population half-reduced as compared to Vpr(52–96)–treated cells.

Time course of Vpr-induced neuronal death

Time-course experiments of Vpr-induced neuronal death induced by Vpr were performed. Striatal and cortical neurons were incubated with Vpr or Vpr(52–96) for various periods and either examined through a phase-contrast microscope or PI-labeled and subjected to viability analysis by flow cytometry. A minimum of 6 h of Vpr incubation at 3 μM was required to cause neuronal death as detected by PI labeling (data not shown). Figure 5 displays representative photomicrographs of striatal neurons cultures incubated either with Vpr or Vpr(52–96) at 3 μM. The C-terminal domain of Vpr, Vpr(52–96), induced neuronal death faster than the entire protein, because 3 h were enough for Vpr(52–96) to induce morphological features characteristic of neuronal death, whereas a minimum of 6 h was required for entire Vpr protein, confirming the flow cytometry results. Neuronal death is more pronounced with the Vpr C-terminal domain than with the entire protein, although decrease of dendrites and cell number are also clear with Vpr.

Vpr-induced death of cortical and striatal neurons occurs by apoptosis

Experiments were then carried out to establish if the Vpr-induced neuronal death was due to an apoptotic or necrotic process. For this purpose, cortical and striatal cells were plated onto chamber slides, incubated with Vpr, Vpr(52–96), or Vpr(70–96), TUNEL-labeled, and examined by fluorescence microscopy. The TUNEL method labels chromatin strand breaks generated during the apoptotic process with fluorescent dUTP. The Vpr-treated cells displayed statistically significant increase of the TUNEL staining in comparison to buffer-treated cells (Figure 6 and Table 2). These data suggested that the mechanism of Vpr-induced death on cortical and striatal neurons was apoptotic. Moreover, the Vpr-treated neurons exhibited nuclear morphology changes with the DNA in discrete clusters, characteristic of the pyknotic nuclei produced during the apoptotic process (Figure 6b). Counting of TUNEL-labeled cells depicted in Table 2 is consistent with the results obtained by flow cytometry analysis after PI labeling (Table 1).

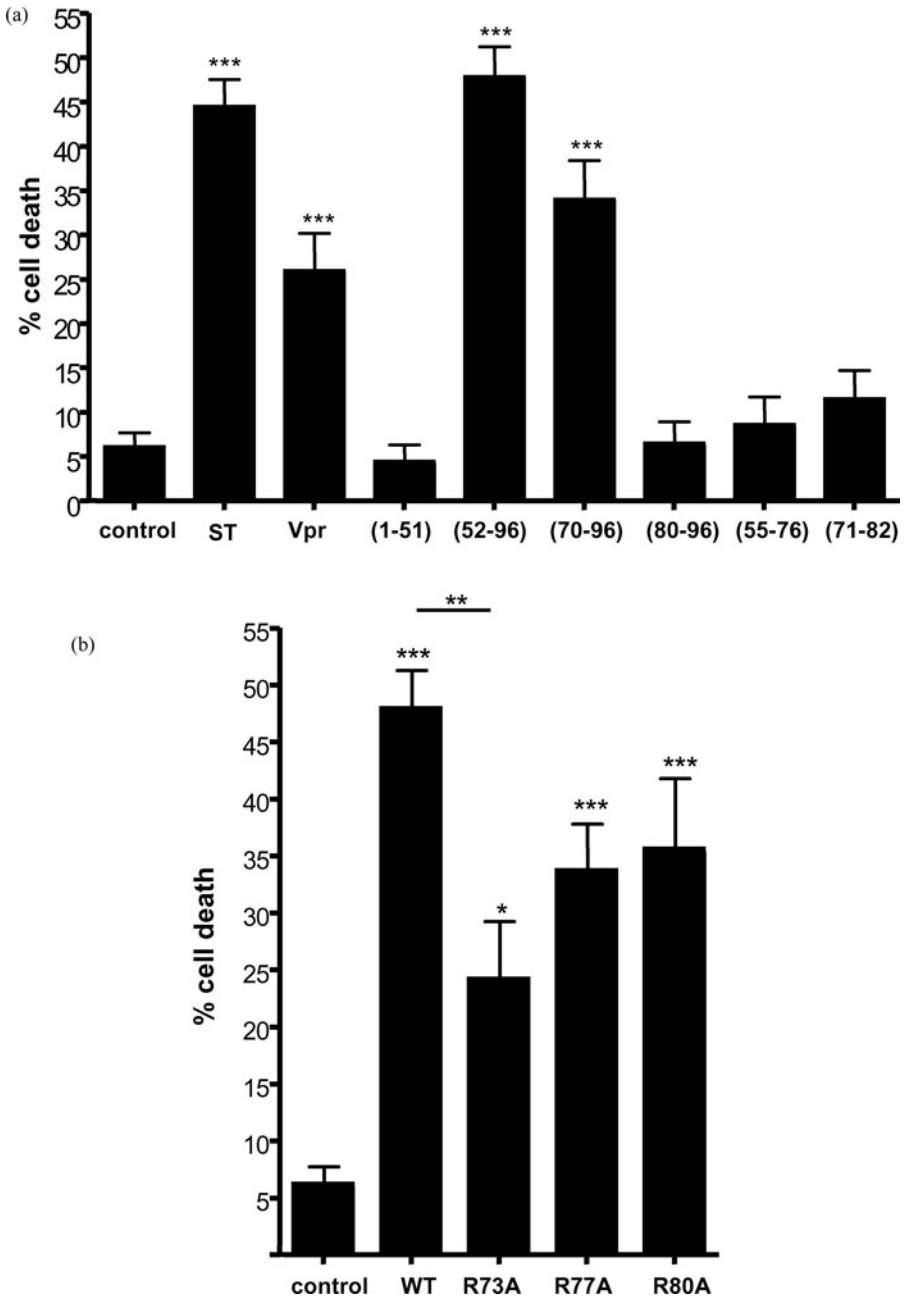

Figure 3 Neuronal death induced by Vpr and Vpr-derived peptides. Striatal and cortical cells were incubated overnight with overlapping Vpr peptides (**a**) or Vpr(52–96) point-mutated peptides (**b**), at 5 μM, followed by staining with propidium iodide (1 μM). Cell viability was measured by flow cytometry with at least 10,000 events and independent experiments were done at least three times. Histograms show the results obtained with cortical cells. $^{*}P < .05$; $^{**}P < .01$, $^{***}P < .001$ compared to buffer-treated neurons. In contrast to Vpr(52–96) and Vpr(70–96), the N-terminal region of Vpr, (1–51), was unable to induce neuronal death. The integrity of the C-terminal 70–96 sequence of Vpr is required to induce neuronal death (**a**). Note the decrease in the neurotoxicity of Vpr induced by mutation of one of the arginines 73, 77, or 80. This is particularly clear when Arg73 was replaced by Ala (**b**).

Implication of the caspase-3 in Vpr-induced apoptosis on primary neuronal culture

Activation of caspases, a family of cysteine-apartate–specific proteases, appears to play a pivotal role in apoptosis. The activity of the caspase-3, an executioner caspase, was measured using a fluorescence-specific caspase substrate in neuronal cells incubated with Vpr during various times. Activation of the caspase-3 started 3 h after Vpr treatment and increased gradually, reaching a peak after 6 h of incubation with a two- to threefold enhancement as compared to control (Figure 7a). No activation of the caspases-2, -8, and -9 was detected.

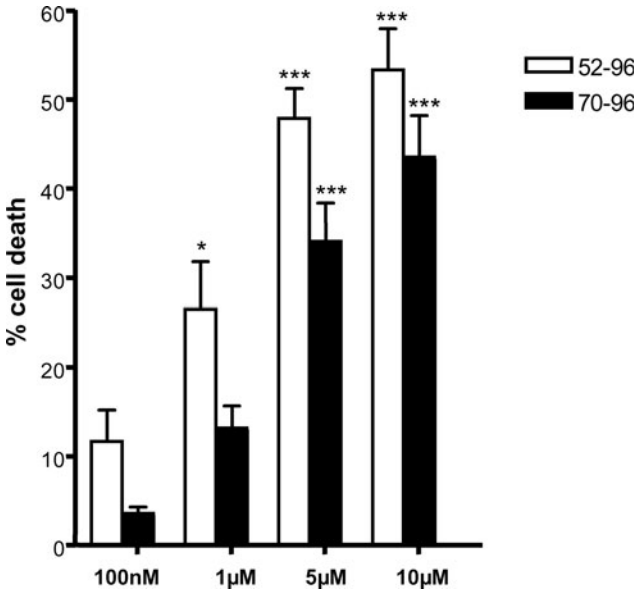

Figure 4 Vpr(52–96) and Vpr(70–96) induce neuronal death in a dose-dependent manner. Cortical cells were incubated overnight with various amounts of the Vpr fragments shown to be neurotoxic (see Figure 3a and Table 1), Vpr(52–96) or Vpr(70–96). Cells were then labeled with propidium iodide and cell death was quantified by flow cytometry. $^{*}P < .05$, $^{***}P < .001$.

Caspase-3 activation was also confirmed by immunoblot analysis (Figure 7b). Cells were treated with Vpr, Vpr(1–51), or Vpr(52–96) at 5 μM during 5 h; then lysed and equal amounts of proteins loaded on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Revelation with an anti-caspase-3 antibody demonstrated a decrease of the pro-caspase-3 in the cells treated with Vpr or its C-terminus fragment, whereas neurons incubated with the buffer or Vpr(1–51) displayed equivalent amounts of pro-caspase-3. A control provided by the manufacturer of the antibody (Jurkat cells treated with camptothecin, an apoptosis inducer) was also loaded.

Studies of mitochondrial membrane potential and production of reactive oxygen species during Vpr-induced apoptosis

We investigated the possible dissipation of the mitochondrial membrane potential during the Vpr-induced apoptosis by flow cytometry using the DIOC(6)3 fluorescent probe. In the case of cortical and striatal neurons, no reduction in the DIOC(6)3 fluorescence, indicating mitochondrial membrane depolarization, was observed after incubation with Vpr or Vpr fragments during different periods ranging from 1 h to overnight exposure (data not shown).

In many cases excessive production of ROS is believed to play a role in cell death including by apoptosis. Therefore, we used the hydroethidine (HE) probe to analyze the ROS production of the neuronal cells after incubation with Vpr. Flow cytometry analysis of the HE-labeled cells incubated with Vpr or Vpr(52–96) led to an increase of ROS concentration in both cell types (Figure 8).

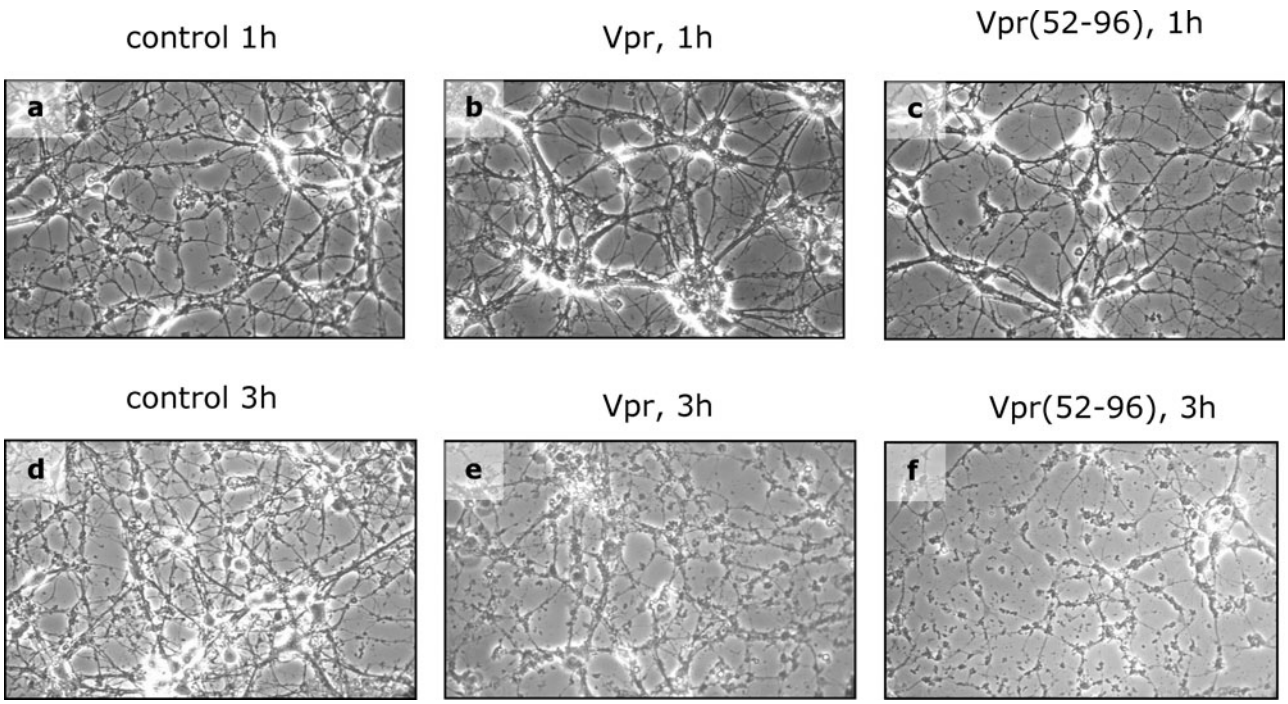

Figure 5 Time-dependant triggering of apoptosis by Vpr and its C-terminal domain. Striatal neurons were incubated during various periods with Vpr or Vpr(52–96), 3 μM. Phase-contrast microscopy reveal a faster neuronal death induced by Vpr(52–96) than by the entire protein (**e** and **f**).

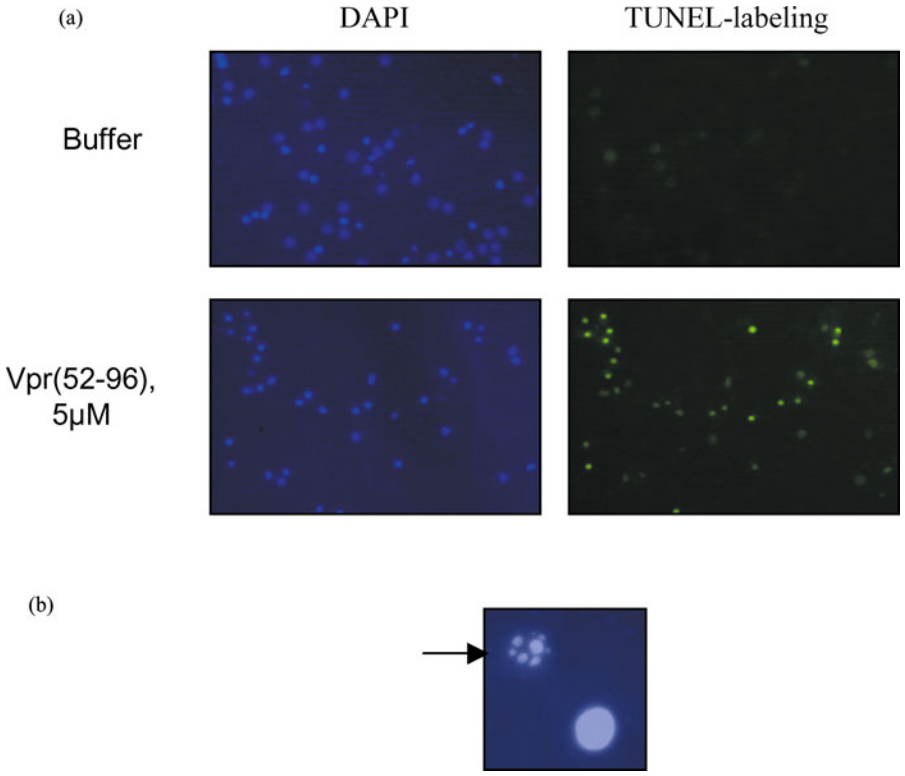

Figure 6 TUNEL labeling of neuronal cell incubated with Vpr. Neurons were incubated overnight with Vpr or Vpr-derived peptides. After fixation and permeabilization of the cells, TUNEL labeling was done. Counterstaining was done with DAPI. (**a**) TUNEL-labeling of cortical cells treated with Vpr(52–96). (**b**) Example of a pyknotic nucleus (*arrow*), indicating cell apoptosis. A normal nucleus is also displayed.

Discussion

The presence of the HIV-1 regulatory protein Vpr in an active form in the serum of seropositive individuals and the enhanced Vpr concentration in the cerebrospinal fluid of patients suffering from neuropathogenic clinical manifestations (Levy *et al*, 1994) suggest a possible role of this protein in the AIDS-associated neurodegenerative process. In addition, Vpr has been involved in the apoptosis of several cell lines including neurons (Huang *et al*, 2000; Patel *et al*, 2000, 2002; Piller *et al*, 1998). These data prompted us to study the neurotoxic properties of Vpr and the region of the protein responsible for these effects. With this aim, we analyzed the neurotoxicity of Vpr and of selected Vpr fragments to cortical and striatal neurons in primary culture. The choice of primary culture was justified by the fact that it is a well-adapted model for the evaluation of neurotoxicity without displaying some of the disadvantages of other available models. Indeed, cells inducible to differentiate in mature neurons (such as NT2 cells), could still contain in the culture some undifferentiated cells that release factors influencing the toxicity of exogenous compounds (Patel *et al*, 2002). Clonal cell lines such as neuroblastoma are convenient models but are immortalized cells whose sensitivity to toxic agent may differ from that of neurons in primary culture. This feature has been illustrated by the different toxicity pattern reported between transformed and primary neuronal cultures subjected to another HIV-1 encoded protein, Nef (Trillo-Pazos *et al*, 2000).

Synthetic Vpr with a sequence from the HIV-1_{NL4-3} strain was reported to be efficiently uptaked by cells,

Table 2 Neuronal viability assessed by microscopic experiments (TUNEL labeling)

	Buffer	*ST*[b]	*Vpr*	*(52–96)*	*(70–96)*
TUNEL+[a]	8.8 ± 2.9	61.2 ± 11.4**	45.7 ± 9.7**	56.3 ± 10.4 **	36.8 ± 10.9*

[a]After overnight exposure to Vpr or Vpr fragments, striatal cells were TUNEL labeled. Percentage of apoptotic cells (TUNEL-positive) was determined after examination under fluorescence microscope. A minimum of 500 cells were counted in each field and each experience repeated at least three times. Values indicated the percentage ±SEM of cell deaths. $^{*}P < .05$, $^{**}P < .01$.
[b]ST: staurosporine, 1 μM.

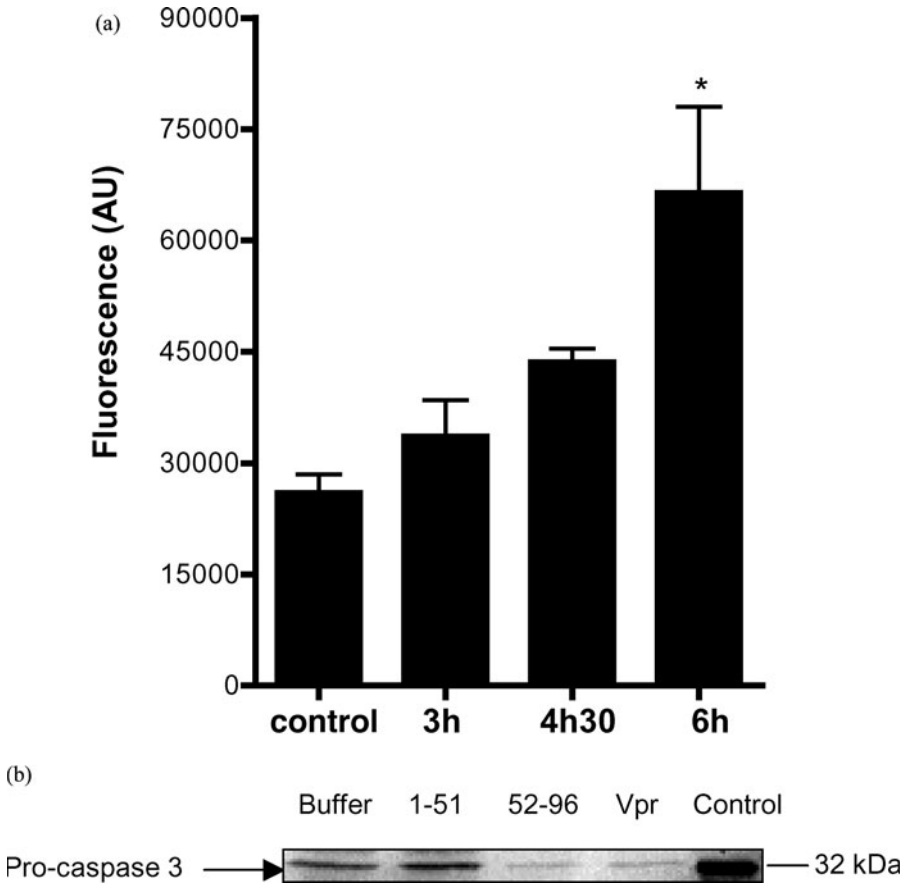

Figure 7 Caspase-3 activation after Vpr treatment of neuronal cells. (**a**) Five micromolar of Vpr was added to striatal neurons in primary culture for 1 h to overnight (see Materials and Methods for details). After collection of the cells, they were lysed and 25 μg of proteins were incubated with fluorescent-substrates specific from caspase-2, -3, -8, or -9 for 2 h at 37°C. The measured fluorescence, related to the substrate cleavage, corresponds to the activity of the corresponding caspase. (**a**) Time-dependent activation of the caspase-3. $^{*}P < .05$ compared to control (cells treated only with buffer). No activation of caspases-2, -8, and -9 was detected (not shown). (**b**) Striatal neurons were incubated with Vpr, Vpr(1–51) or Vpr(52–96), 5 μM, for 5 h. After lysis of the cells, equal amounts of proteins and control provided by the manufacturer (camptothecin treated Jurkat lysate) were loaded on a 14% SDS-PAGE. A semidry transfer to a nitrocellulose membrane was done and the membrane incubated with an anti-caspase-3 antibody. As compared to buffer, a decrease in pro-caspase-3 amounts (32 kDa) can be observed in the cells treated with Vpr or Vpr(52–96) but not with Vpr(1–51).

leading to cell cycle arrest in the G2 phase and found at least partly localized in the nucleus (Henklein *et al*, 2000). Similar experiments were done using C-terminal synthetic Vpr or Vpr fragments (Arunagiri *et al*, 1997; Henklein *et al*, 2000; Jacotot *et al*, 2000), either with a Vpr sequence from the NL4-3 or the LAI strains. The Vpr sequence used in our study, from the HIV-1 LAI strain, is almost identical to the Vpr sequence from the NL4-3 strain; it is thus very likely that our synthetic Vpr is also able to enter the cells (Kichler *et al*, 2000; Sherman *et al*, 2001). Moreover, as indicated in the introduction, extracellular addition of synthetic Vpr produce the same biological effects as Vpr expressed in cells (Yasuda *et al*, 2001).

Therefore, in an attempt to define the minimal Vpr apoptotic region, synthetic Vpr, Vpr fragments, and mutants were added in the medium of cortical and striatal neuronal cultures. Effects resulting from acute rather than chronic administration of the various peptides were analyzed. Direct neurotoxic effects of Vpr and of its (52–96) and (70–96) fragments, but not of the N-terminal region, (1–51), were observed on both neuronal cells types (Figures 2, 3a, 4, and Table 1). Moreover, the levels of cell-death induced by Vpr on cortical and striatal neurons are not statistically different. The higher potency of Vpr(52–96) versus Vpr to induce neuronal death (Figures 2 and 3a and Tables 1 and 2) may be due to a better accessibility of the critical (70–96) region in the Vpr(52–96) fragment, in agreement with NMR results (Morellet *et al*, 2003; Schuler *et al*, 1999). Comparison of the neurotoxicity of Vpr(52–96), Vpr(70–96) and Vpr(80–96) investigated by both flow cytometry after propidium iodide labeling (Table 1) and TUNEL experiments (Table 2) show that the (70–96) fragment of Vpr is an essential component of the neurotoxicity of the protein. The reduction (20% to 30%) observed in the measured neurotoxicity between the longer fragment Vpr(52–96) and Vpr(70–96) could be due

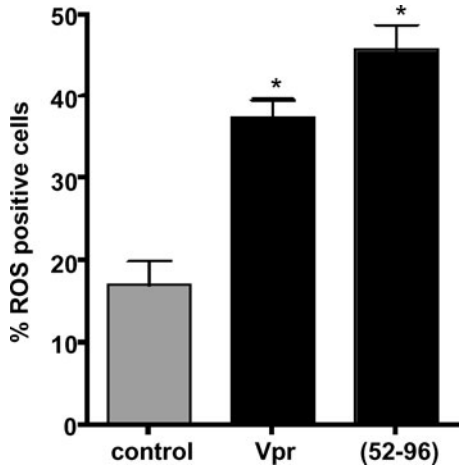

Figure 8 Production of reactive oxygen species (ROS) after incubation of primary neuronal cells cultures with Vpr. Neuronal cells were treated overnight with Vpr and Vpr(52–96), then collected by centrifugation. After labeling with hydroethidine, they were subjected to flow cytometry for quantification of the production of reactive oxygen species (ROS). Histograms presented in this figure shows increased production of ROS with Vpr and Vpr(52–96). $^{*}P < .05$ compared to control cells.

both to the participation of some amino acids of the longer sequence to toxic effects of Vpr and/or to a better conformation for neurotoxicity induction of the (70–96) fragment when it is included in Vpr(52–96). The dose-dependent neurotoxicity of Vpr was further characterized and shown to be an apoptotic process because the neurons were TUNEL-labeled (Figure 6a and Table 2) and involved a caspase pathway (Figure 7). Moreover, hyperproduction of ROS was detected (Figure 8). Apoptotic and necrotic effects of Vpr were previously observed on primary cortical astrocytes but not on cortical neurons (Huang *et al*, 2000). The apparent discrepancies with the present results could be explained by the use in the experiments of Huang and colleagues of mixed rat cortical cells that contained both neurons and astrocytes. In our conditions, the majority of cells (more than 80%) were neurons, as determined by cell type–specific staining (data not shown) and (Brewer, 1995). It can be noted that our results are consistent with the reported decrease of the neuron population in mixed cells culture: neurons were 26% of the cells in the control, but this value dropped to 10% following treatment with Vpr (Huang *et al*, 2000).

The (71–82) Vpr fragment was unable to reproduce the apoptotic effects observed with Vpr(70–96), thus confirming that Vpr(71–82) is mitochondriotoxic but cannot induce apoptosis over intact cells (Arunagiri *et al*, 1997; Jacotot *et al*, 2000; Macreadie *et al*, 1995). Our results demonstrate that one difference between the mitochondriotoxic and neurotoxic activities of Vpr lies in the importance of the arginines 73, 77, and 80, which cannot be mutated without an almost complete loss of action at the mitochondrial level (Jacotot *et al*, 2001), whereas such mutations led only to a slight decrease in apoptosis of neurons (Figure 3b).

Absence of mitochondriotoxicity for Vpr-treated neurons is supported by the lack of alteration of the mitochondrial membrane potential observed in this study, whereas Vpr and Vpr(52–96) induce this effect on isolated mitochondria (Jacotot *et al*, 2000) and Vpr on HeLa cells (Muthumani *et al*, 2002a). This is consistent with reported results showing that reduction of this membrane potential appeared dispensable for neuronal apoptosis (Budd *et al*, 2000; Krohn *et al*, 1999).

Two studies carried out on hippocampal primary culture have previously demonstrated the ability of Vpr to induce apoptosis *in vitro* (Huang *et al*, 2000; Piller *et al*, 1999). The work presented here demonstrates that Vpr displays also apoptotic properties over two other neuronal cell types: cortical and striatal neurons. These results and the neuronal apoptosis observed postmortem in the cortex and in the basal ganglia (where the striatum is located) of HIV-infected patients (Adle-Biassette *et al*, 1997; Asare *et al*, 1996; Gray *et al*, 2000; Shi *et al*, 1996) support our hypothesis that Vpr may be one of the causing agents of the neurodegenerative processes observed in AIDS patients. In addition to the acute Vpr neurotoxicity observed at relatively high Vpr concentrations, it has also been reported that chronic exposure to low dose (pM to nM range) of Vpr *in vitro* led to neuronal cells exhibiting signs of apoptosis (Patel *et al*, 2000). There is no clear indications in the litterature about Vpr concentration in patients but it could be in the range of 100 pg/ml, taking into account that the ratio Vpr/p24 is around 1.34 (Levy *et al*, 1994).

Of note, caspases activation was reported during HIV-induced neurodegeneration. Postmortem examination of cortical tissues showed specific active caspase-3 immunoreactivity in the neurons of patients with HIV-related neurological disorders; whereas in the cortex of AIDS individuals without such troubles or in seronegative subjects, no caspase-3 activity was detected (Garden *et al*, 2002). Moreover, neurons, macrophages, and microglia from pediatric patients with HIV-1 encephalitis overexpress caspase-3 (James *et al*, 1999). It is tempting to speculate that Vpr is one of the proteins that initiate caspase activation in the brain of seropositive individuals with neurological troubles and subsequent neuronal apoptosis, as we showed here caspase-3 activation after addition of Vpr on cortical and striatal neurons. No activation of one of the two initiator caspase-8 and -9 was detected in our experiments. Besides the fact that stimulation of caspase-3 by Vpr is demonstrated (Patel *et al*, 2002; Stewart *et al*, 2000), there is a debate about the activation of upstream caspases by the protein. Indeed, Vpr seems to activate the caspase -8 and -9 of NT2 cells (Patel *et al*, 2000, 2002) but only the caspase-9 in other tumor cell lines (Muthumani *et al*, 2002a, 2002b). It might be that according to the cell line, Vpr activates one way or another or acts directly over the executioner caspase-3 as this seems to be the case in our experimental model.

The HIV-1 produces viral proteins with redundant toxic activities on brain, eventually through stimulation of the caspase pathway in infected brain or in neuronal cells culture. Thus, after injection into rat brain, the gp120 protein activates the caspase-3 in the cortex (Acquas *et al*, 2004); Tat induces caspase activation and apoptosis of hippocampal neurons (Kruman *et al*, 1998); Nef is toxic to primary human neuronal cultures (Trillo-Pazos *et al*, 2000) and leads to caspase-3 activation (James *et al*, 2004). We can hypothesize that synergetic toxic effects might exist *in vivo* between these viral proteins as it was demonstrated after injections of Tat and gp120 in rat striatum (Bansal *et al*, 2000).

This study extents the neurotoxicity of Vpr observed on hippocampal cells to cortical and striatal neurons and demonstrates that this occurs via a caspase-3 involved apoptosis pathway. The direct neurotoxicity of Vpr over neurons located in three essential brain structures probably explains the cognitive dysfunction, the tremor and movement incoordination, in addition to behavioral disturbances associated with HAD. Moreover, we demonstrated that the neurotoxic domain of the protein requires the (70–96) domain and not only the (71–82) sequence, as shown by previous studies on Vpr toxicity (Arunagiri *et al*, 1997; Jacotot *et al*, 2000; Macreadie *et al*, 1996). Some recently published results established an original mechanism by which Vpr might play an important role in the HIV-associated dementia: the long-terminal repeats of the viral genome of patients suffering from HAD displays a majority of high-affinity binding sites for Vpr as compared to nondemented seropositive individuals (Burdo *et al*, 2004). Because Vpr is a weak viral transactivator (Cohen *et al*, 1990), these data indicate that Vpr might be responsible for the HAD emergence by increasing the viral load in the brain.

Antiretroviral therapies have led to significant life lengthening and improvements of the life conditions of AIDS patients. Nevertheless, penetration of most of the medications in the CNS remains poor (Price *et al*, 1999) and HAART is less effective for the neurological disorders caused by AIDS than for other AIDS-induced diseases (Dore *et al*, 1999). Therefore, it is fundamental to understand the mechanism which leads to neuronal apoptosis in order to treat and prevent the neurological abnormalities observed in most of AIDS patients.

Materials and methods

Primary neuronal culture

Cortical and striatal cell cultures were prepared from embryonic day 16 (E16) Sprague-Dawley rat fetuses (Charles River Laboratories). The fetuses were collected and dissected in cold phosphate-buffered saline (PBS)–0.6% glucose. Cortical and striatal neurons were maintained in culture medium (Neurobasal-B27 supplement, Gibco) with 1% penicillin-streptomycin (Gibco) until completion of the dissection. Dissociation of neuronal cells was done mechanically by trituration through the mouth of a Pasteur pipette, then the cells were allowed to settle for 15 min. This step was reproduced three to five times until no pellets were observed. After centrifugation (10 min, 600 rpm), cells were counted and seeded on poly-D-lysine (15 μg/ml; Pharmingen, BD Biosciences)-coated 6-or 12-well culture plates (Nunc) at respective density of 800×10^3 cells and 275×10^3 cells per well. For immunofluorescence microscopy experiments, cells were plated onto poly-D-lysine–coated LabTek in 4-wells chamber slides. Cells were maintained at 37°C in a 5% CO_2 humidified incubator. Cytosine-β-arabinoside (Sigma), 1 μM, was added to minimize non-neuronal cell growth. Medium was changed every 4 days. Cells were treated for experimentation after 8 to 10 days of culture. The use of a culture medium devoid of serum and the addition of an antiproliferation agent provide nearly pure neuronal cultures (Brewer, 1995).

Peptide synthesis and purification

Vpr from the HIV-1 LAI strain and Vpr-derived peptides or mutants were synthesized by solid-phase synthesis using the Fmoc strategy on Applied Biosystems models 431A and 433A peptide synthesizers as already described (Cornille *et al*, 1999; Morellet *et al*, 2003). All chemicals used for the synthesis were purchased from Applera. Briefly, 1 mmol of Fmoc-amino acids was coupled to 0.1 mmol of HMP (4-hydroxymethyl phenoxymethyl copolystyrene–1% divinylbenzene) resin using dicyclohexylcarbodiimide (DCC)/hydroxybenzotriazole (HOBt) in *N*-methyl-2-pyrrolidone (NMP). Deprotections were carried out with piperidine and followed by spectrophotometric monitoring. The peptide resins were treated for 3 h at room temperature with a trifluoroacetic acid/phenol/triisopropylsilane mixture in order to cleave the peptides from the resin and to remove the protecting groups from the amino acids side chains. Purification of the peptides was done by reverse-phase liquid chromatography with acetonitrile gradient. Peptides mass and purity were controlled by mass spectroscopy; all peptides used were more than 95% pure. Vpr and fragments were solubilized in H_2O/5 mM DTT (dithiothreitol; Sigma) (to avoid oxidation) at 1 mg/ml (stock solution) and diluted in appropriate buffer for the experiments.

Vpr treatment of cortical and striatal neurons

The ability of Vpr to cross cell membrane has been well demonstrated by several groups (Henklein *et al*, 2000; Kichler *et al*, 2000; Sherman *et al*, 2001; Jacotot *et al*, 2000). Moreover, previous studies showed that extracellular Vpr induce apoptosis on NT2 neurons (Patel *et al*, 2000) and hippocampal primary cultures (Piller *et al*, 1998). Therefore, entry of Vpr in the cortical and striatal neurons was not investigated in this study.

Due to the high propensity of Vpr to bind to medium culture proteins, incubation of the neuronal cells with Vpr was done in a isotonic buffer (13 mM Hepes, 2.4% glucose, 68 mM NaCl, 1.3 mM KCl, 4 mM Na_2HPO_4, 0.7 mM KH_2PO_4, pH 7.2) for 1 h, as already described (Arunagiri *et al*, 1997; Jacotot *et al*, 2000). Cell culture medium was then added in threefold excess and incubation of Vpr was pursued. Controls showed that incubation with the isotonic buffer did not induce by itself neuron death. As a positive control (included in all experiments), neurons were treated with 1 μM staurosporine, a nonselective protein kinase inhibitor well known to induce apoptosis.

Microscopy

Phase-contrast microscopy: Morphological assessment of neuronal death was done through a careful study of unfixed cortical and striatal neurons cultures by phase-contrast microscopy. Light images were captured using a Zeiss Axiovert camera with $\times$20 and $\times$32 objectives.

TUNEL assay: Fragmentation of DNA occurs during the late stages of apoptosis. The terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay is based on the specific incorporation at the generated 3′ free hydroxyl groups of fragmented DNA ends of fluorescent dUTP thanks to the terminal deoxynucleotidyltransferase (TdT).

TUNEL assays were performed using the ApoAlert DNA fragmentation assay kit (Clontech, BD Biosciences). After incubation of the cells with Vpr or its fragments or mutants (controls correspond to cells incubated with the buffer), cells were washed with PBS then fixed for 30 min at 4°C with 4% paraformaldehyde in PBS. After two washes, cells were permeabilized with 0.2% triton X-100 in PBS for 5 min at 4°C. Slides were then equilibrated in the supplied equilibration buffer for 10 min. After removal of the buffer, 50 to 70 μl of the reaction mixture were added per coverslip, following recommended manufacturer's instructions. Labeling reaction was done for 1 h at 37°C in a dark humidified chamber and stopped by immersing the slides in 2$\times$ SCC buffer for 15 min. Counterstaining was done using the DAPI-vectashield mounting medium (Vector laboratories). Cells were visualized by fluorescence microscopy using a Zeiss Axiovert camera.

Cell viability assessed by flow cytometry

Flow cytometry was used to screen the Vpr fragments and mutants toxic properties. After overnight incubation with Vpr, Vpr fragments, or mutants, cortical and striatal neurons were collected by centrifugation (10 min, 600 rpm) and rinsed with PBS. Pellets were resuspended in culture medium (Neurobasal-B27; Invitrogen) containing propidium iodide (PI; 1 μg/ml; Sigma) and incubated for 15 min at 37°C before flow cytometry analysis. Red fluorescence was detected through a 575 to 600-nm bandpass filter using a Coulter Epics XL cytometer. A minimum of 10,000 events was recorded for each condition. DiOC(6)3 was used to evaluate mitochondrial membrane potential of the cells and hydroethidine (HE) to assess the reactive oxygen species (ROS) production. Protocols of incubation of these two reagents was the same as the one described for PI except that 20 nM of DiOC(6)3 were incubated for 15 min and 1 μM of HE for 30 min. Green fluorescence of the DiOC(6)3 was detected through a 525 to 550-nm bandpass filter; for the fluorescence of HE, the same filter as the one used for the IP was selected.

Caspases assay

The BD ApoAlert caspase assay plates (BD Biosciences) was used to determine which caspase is activated during Vpr-induced apoptosis. After treatment with Vpr, cells were collected and centrifuged at 600 $\times$ *g* for 10 min and then lysed in the lysis buffer supplied by the manufacturer for 1 h at 4°C. Pellets collected after centrifugation (30 min, 4°C, 13000 rpm) were subjected to Bradford assay and 25 μg of cell lysates were incubated with each specific caspases fluorescent-substrates during 2 h at 37°C. Fluorescence was read with excitation at 340 to 380 nm (bandpass filter) and emission at 440 to 480 nm (bandpass filter) on a Cytofluor plate reader (Perseptive biosystem).

Determination by Western blot of the effects of Vpr, Vpr(1–51), and Vpr(52–96) on pro-caspase-3 cleavage in striatal neurons

Striatal neurons were incubated for 5 h with either isotonic buffer, Vpr, Vpr(1–51), or Vpr(52–96) at 5 μM. Cells were collected by centrifugation at 1000 rpm for 10 min then lysed for 1 h at 4°C. Supernatants after centrifugation at 10,000 rpm for 30 min were collected and a Bradford assay was done to determine the proteins concentration in each sample. Loading buffer was then added and 50 μg of proteins loaded for each sample on a 14% SDS-PAGE. The same concentration of proteins issued from a Camptothecin-treated Jurkat cells lysate provided by the manufacturer was added as control to the gel. Semidry transfer (BioRad apparatus) to a nitrocellulose membrane (Amersham) was done. After treatment for 2 h at room temperature with Superblock buffer (Pierce), the membrane was incubated overnight at 4°C with a rabbit anti-caspase 3 antibody (BD Biosciences) diluted at 1:1000 in Tris buffer–5% nonfat dry milk–0.4% Tween 20. Membrane was washed and incubated with ahorseradish peroxydase-conjugated anti-rabbit antibody (Amersham), 1:6000 in Tris buffer–5% nonfat dry milk–0.4% Tween 20 and revelation was done with the chemiluminescence method (ECL kit; Amersham).

Statistical analysis

Controls are cells treated with the incubation buffer. Data are expressed as mean $\pm$ SEM and evaluated

for statistical significance with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. The criteria of significance were set as $^{*}P < .05$, $^{**}P < 0.01$, and $^{***}P < .001$.

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