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# Neurotoxic effects of feline immunodeficiency virus, FIV–PPR

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**FIV is a lentivirus of domestic cats that causes neurologic disorders which are remarkably similar to those found in HIV-1 infected people. Using feline neuron cultures, we investigated the potential of both FIV virus and FIV-Env protein to cause neuronal damage through the excitotoxicity mechanism. The neuron swelling and lactate dehydrogenase (LDH) release assays were used as measures of cellular damage. The effects of FIV Env protein on glutamate receptor mediated increases in intracellular calcium were also examined. We found that FIV virus and FIV-Env protein significantly increased LDH release from the neuron cultures. Additionally, an increase in neuron size was detected in the cultures exposed to the virus, while swelling did not occur with exposure to either saline, denatured virus, or FIV-Env by itself. However, when both 20 $\mu$M glutamate and the FIV–PPR Env protein were added to the culture, a significant increase in neuron cell size was observed. The NMDA calcium signals were similar in general form between the control and FIV–PPR Env exposed cultures. However, the FIV–PPR Env protein treated cultures resulted in significant enhancement of the NMDA induced calcium signal. Our results indicate that FIV Env protein (either within the virion or baculovirus expressed) induced neurotoxicity as measured by neuron swelling and LDH release assays and that exposure of feline neurons to FIV Env protein alters the handling of intracellular calcium. These findings help to validate the FIV/cat system as a potential animal model for evaluating therapeutic approaches that target the excitotoxicity mechanisms of lentivirus induced CNS disease.**

**Keywords:** FIV; neurologic disease; HIV; lentivirus; calcium; NMDA

## Introduction

FIV and HIV-1 are lentiviruses that cause similar disease syndromes in their respective hosts (Elder and Phillips, 1993; Henriksen *et al*, 1995). Both of these viruses are associated with a broad spectrum of clinical syndromes, including a neurological form of the disease (Elder and Phillips, 1993; Henriksen *et al*, 1995; Phillips *et al*, 1994, 1996; Podell *et al*, 1993). FIV and HIV-1 produce similar neurologic defects in their respective hosts, including: anisocoria, alterations in pupillary reflexes, delayed auditory and visual evoked potentials, decreased nerve conduction velocities and abnormal sleep architectures (Jabbari *et al*, 1993; Norman *et al*, 1992; Phillips *et al*, 1994, 1996; Prospero-Garcia *et al*, 1994a). Histological examination of brains from FIV infected cats or HIV-1 infected people have shown that neuron dropout and neuron damage are common sequelae to infection (An *et al*, 1996; Gray *et al*, 1991; Meeker *et al*, 1997). Yet, there is a lack of compelling evidence that either virus infects neurons.

In the case of HIV-1 infection, there is increasing evidence that HIV-1 mediates its effects on neurons through indirect means rather than direct infection of the neurons (Dewhurst *et al*, 1996; Gendelman *et al*, 1994; Lipton *et al*, 1994). Multiple mechanisms are likely to be involved in HIV-1 induced neuropathology. However, recent studies in model systems implicate excitotoxicity as a potentially critical element in HIV-1-induced neuronal damage. Excitotoxicity refers to the ability of the excitatory neurotransmitter, glutamate or ligands that act at glutamate receptors, to damage neurons when the neurons are excessively stimulated with these agents (Choi, 1992). An increase in intracellular calcium, due to calcium influx through the NMDA subtype of glutamate receptors, appears to play a pivotal role in the excitotoxic process. In

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rodent systems (Barks *et al*, 1995; Diop *et al*, 1994; Dreyer *et al*, 1990; Lipton *et al*, 1991; Lo *et al*, 1992; Muller *et al*, 1992; Perovic *et al*, 1994; Savio and Levi, 1993; Sindou *et al*, 1994; Toggas *et al*, 1996; Toggas *et al*, 1994; Ushijima *et al*, 1993) and, more recently, in a human cell culture system (Lannuzel *et al*, 1995; Truckenmiller *et al*, 1993; Yeung *et al*, 1995), it has been demonstrated that the HIV-1 Env protein (gp120 and/or gp160) induces neuronal death through a pathway involving voltage-gated calcium channels and/or the NMDA subtype of glutamate receptors. These results are consistent with the involvement of excitotoxicity and calcium overload in HIV-induced neuropathology.

The similarity of the disease process and neuropathology in FIV-infected cats and HIV-1 infection in people suggests that the cellular and molecular mechanisms mediating the disease process are similar in FIV and HIV-1 infection. The FIV model has proven to be a valuable model for studying the neurological form of the disease. However, information is limited on mechanisms underlying the neuropathological changes induced by FIV. Such information is basic to the further development of the FIV/cat system as a model for potential therapeutic approaches which target neuropathogenic process.

To address this issue, we investigated the potential for FIV virions and FIV-Env protein to damage CNS neurons through the excitotoxicity mechanism by utilizing a culture model system of embryonic feline neurons. We also assessed the effects of FIV Env protein on calcium homeostasis and glutamate receptor mediated increases in intracellular calcium in the cultured feline neurons. Our results are consistent with the interpretation that FIV Env protein (either within the virion or baculovirus expressed) induced neurotoxicity as measured by neuronal swelling and lactate dehydrogenase (LDH) release assays and that exposure of feline neurons to baculovirus purified FIV Env protein alters intracellular calcium handling.

## Results

Feline embryonic cortical neurons survived well in culture and showed similar maturation changes in culture as described for embryonic rodent neurons (Diop *et al*, 1994; Lipton *et al*, 1991; Lo *et al*, 1992; Muller *et al*, 1992; Perovic *et al*, 1994; Savio and Levi, 1993; Sindou *et al*, 1994). At plating, the neurons exhibited a rounded morphology, and by day-one *in vitro* (DIV), bipolar migrating neurons were evident. By week-one *in vitro*, a dense interconnecting neuronal network was established that remained relatively stable for the remainder of the culture period. Immunohistochemical staining for microtubular associated protein-2 (MAP2), which is localized to neurons, showed that the main cell type in the cortical cultures was neurons (Figure 1). Immunostaining for glial fibrillary acidic protein (GFAP), which is localized to astrocytes, showed that scattered astrocytes were also present in the cultures, and uptake of fluorescently-tagged DiI-acetylated low density lipoprotein, a marker for microglial cells, revealed a relatively small population of microglial cells (data not shown). Viable neurons remained within these cultures for approximately 30 days, during which time the cultures were used for experiments. For most studies, no age-dependent effects were observed and results were combined.

In the first series of experiments, we assessed the potential for FIV–PPR to cause neurotoxicity in the cortical cultures. FIV–PPR is an infectious molecular clone that is neuropathogenic and has been completely sequenced and characterized at the molecular level (Elder and Phillips, 1993; Elder *et al*, 1993; Mancuso *et al*, 1994; Phillips *et al*, 1990, 1992; Prospero-Garcia *et al*, 1994a,b; Wagaman *et al*, 1993). Two assays were used to assess neurotoxicity: measurement of neuronal swelling and measurement of LDH levels in the culture supernatant.

Increase in neuronal size upon exposure to an agent is an early sign of neurotoxic properties of the agent (Choi, 1992). To determine if FIV–PPR virions exhibited this property, the neuron enriched cultures were exposed for 15 min to a vehicle (modified physiological saline, see methods) or an FIV–PPR preparation containing 67 600 RT counts which had been denatured (100°C for 20 min), heat inactivated (56°C for 1 h) or left untreated. The results, depicted in Figure 2, show that a 15-min-incubation in saline alone resulted in no detectable neuronal swelling. Similarly, when the virus

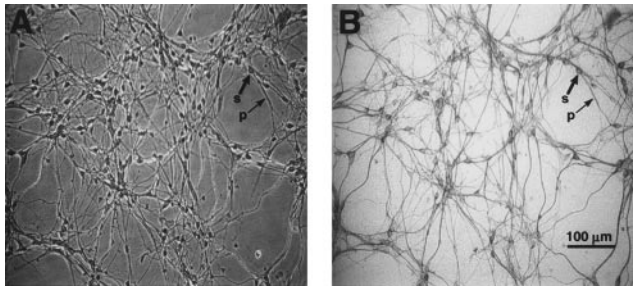


**Figure 1** Cultured cat cortical neurons. Digitized phase contrast (A) and bright field (B) images of cat cortical neurons (15 days *in vitro*) immunostained with an antibody to MAP-2. This antibody immunostains neuronal cell bodies and processes. The phase contrast image shows all cells in culture, whereas the bright field image shows the immunostaining. The majority of cells in the cultures were immunostained with the antibody indicating that they are neurons. The somata of one neuron is indicated by an 's' and a process indicated by a 'p'. The cultures consisted of a complex network of neurons and interconnected neuronal processes.

preparation had been denatured (100°C for 20 min), no obvious neuronal swelling was found. However, neuronal swelling was readily detected in the neuronal cultures exposed to untreated virus or virus that had been rendered non-infectious (56°C for 60 min) (Figure 2). Figure 3A graphically depicts the time course and dose dependency of neuronal swelling. The degree of neuron swelling increased with time after addition of the virus to the culture and was dependent upon the amount of virus added to the culture, as 1/5 dilution (67 000 RT counts) had a greater effect on cell size than the same virus preparation diluted to 1/20 (16 900 RT counts).

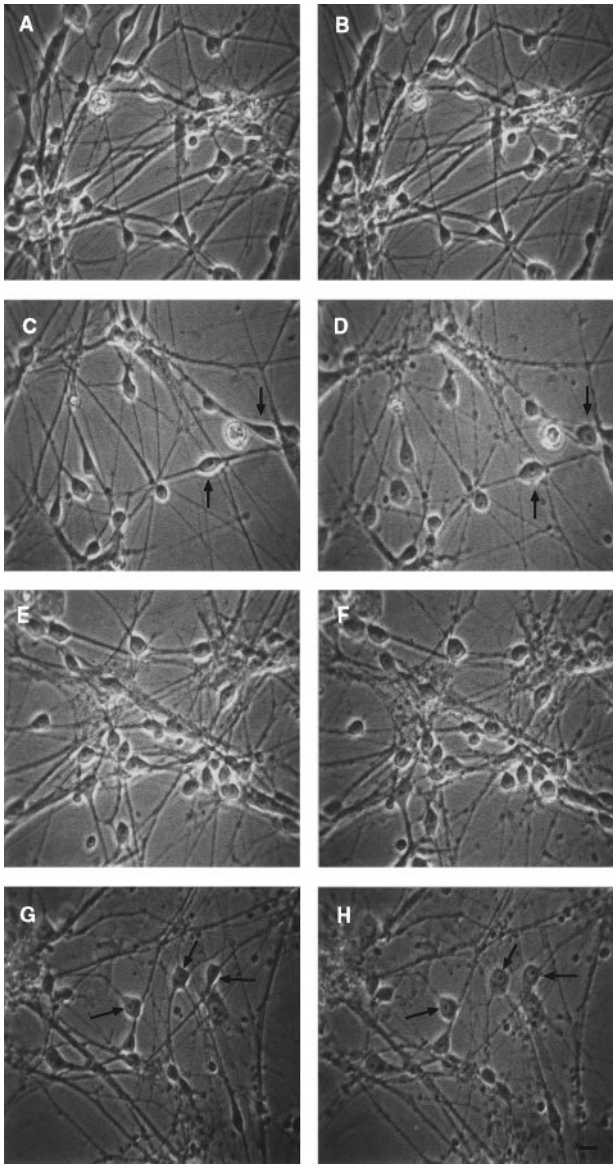


**Figure 2** Effects of FIV–PPR on neuron cell size. After a brief rinse, enriched neuronal cultures (DIV 17–22) were exposed to MPS at 0 min (A) and 15 min (B); to a whole virus preparation (diluted 1 : 5 final dilution with low $Mg^{2+}$ saline) at 0 min (C) and 15 min (D); to heat denatured virus at 0 min (E) and 15 min (F) and to heat inactivated virus (56°C for 1 h) at 0 min (G) and 15 min (H). Arrows in picture C showed the cell body before FIV exposure. Arrows in picture D indicated the same cell 15 min after virus application. Arrows in picture G showed the cell body before inactivated FIV exposure. Arrows in picture H indicated the same cell 15 min after inactivated virus application. Notice the soma swelling. Similar results were obtained in four separate experiments. Scale bar, 20 mm.

In addition to cell swelling, excitotoxicity is characterized by a breakdown of the cellular membrane and leakage of cytoplasmic enzymes such as LDH into the culture supernatant. Thus, as a second measure of virus-induced neurotoxicity, the level of LDH-release was assessed after a 24-h-incubation period. The results of these experiments are consistent with the cell size data, as the cultures that were exposed to the virus preparation had a significant ($P \leqslant 0.05$) increase in the amount of LDH in the supernatant relative to LDH levels in control cultures (Figure 3B).

In culture models of HIV toxicity, the HIV Env protein has been shown to induce neurotoxicity (Dawson *et al*, 1993; Lipton, 1991). Further, it was shown that the resulting neurotoxicity involved calcium influx through the NMDA subtype of glutamate receptors (Dawson *et al*, 1993; Lipton, 1991). Thus, we determined if FIV–PPR Env protein could produce similar effects. To address this issue, we examined the effect of FIV–PPR Env protein preparations on neuronal viability using cell size and LDH assays. The FIV–PPR Env protein was added to the cultures either alone or in the presence of glutamate, the endogenous ligand for NMDA receptors. Using fura-2 based microscopic calcium imaging, we also assessed the effect of FIV–PPR Env protein on resting calcium levels and the increase in intracellular calcium (i.e. calcium signal), that results from calcium influx through NMDA receptors. Cell size and intracellular calcium measurements were made at the end of a 30 min incubation period; whereas, LDH measurements were made after a 24-h-incubation period. For all studies, the culture medium was physiological saline with a reduced level of magnesium (30 $\mu$M) and supplemented with 10 $\mu$M glycine, conditions that enable expression of NMDA receptor activity. As shown in Figure 4, the addition of glutamate at 20 $\mu$M concentration to the culture saline had no effect on neuron cell size nor were there any effects when the baculovirus expressed FIV–PPR Env protein was separately added to the culture. However, when both 20 $\mu$M glutamate and the FIV–PPR Env protein were added to the same culture, a significant ($P \leqslant 0.05$) increase in cell size was observed, reaching the same degree of neuronal swelling as observed when a high concentration of glutamate (200 $\mu$M) was added to the cultures (Figure 4).

Although FIV–PPR Env protein alone, did not induce cell swelling in the 30 min test period, measurement of LDH levels after 24 h of exposure

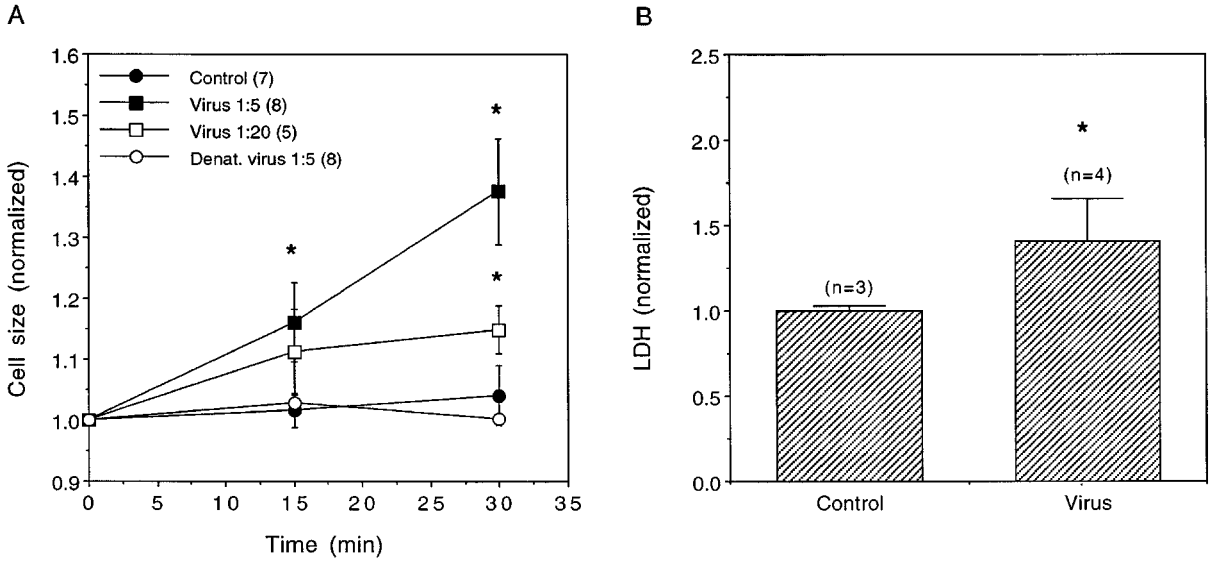


**Figure 3** (A) Quantitative analysis of FIV–PPR induced neuronal cell swelling. Images of neuronal cell cultures were collected at 0 min, 15 min and 30 min after the application of indicated treatments. Five to eight neurons were selected in each field and were measured at each time point. For each cell the mean cell size at 15 min or 30 min were normalized to the mean cell size at time 0. *Indicates a significant ($P \leqslant 0.05$) difference from time 0 (2-tailed paired *t*-test). (B) Effects of FIV–PPR on the LDH levels. PPR virus (at 1 : 5 final dilution) was applied to the dishes after a brief rinse. Supernatants were collected and LDH values were determined as described in Materials and methods. The LDH levels were normalized to the protein levels for each culture (basal level of LDH activity were $0.515 \pm 0.015$ U/mg protein/24 h for control and $0.726 \pm 0.174$ U/mg protein/24 h for the viral treatment). The mean results were expressed as a ratio of the mean control value. Number in the parenthesis indicated in the samples tested. *Indicates a significant difference from control at $P \leqslant 0.05$. (2-tailed *t*-test).

demonstrated its toxic effect, an effect that was not observed with heat denatured (100°C for 10 min) FIV–PPR Env. As shown in Figure 5, when applied separately to the cultures, both 20 $\mu$M glutamate and FIV–PPR Env resulted in a significant ($P \leqslant 0.05$) accumulation of LDH in the tissue culture supernatant. The combination of both 20 $\mu$M glutamate and FIV–PPR Env to the culture resulted in an increased release of LDH, but the LDH level for combined exposure was not significantly greater than the results obtained with the separate addition of either 20 $\mu$M glutamate or FIV–PPR Env to the cultures (Figure 5).

To assess the effect of FIV–PPR env on NMDA induced calcium signals, the peak amplitude of calcium signals produced by a brief (1 s) application of NMDA to the cultured neurons under control conditions was compared to that which was elicited after addition of FIV–PPR Env to the culture saline. Although the general form of the NMDA responses were similar between the control and FIV–PPR Env exposed cultures, the presence of FIV–PPR Env protein in the bath saline resulted in a dose-dependent enhancement of the calcium signal to NMDA (Figure 6A). Similar effects were seen when glutamate was used as the stimulus, applied either in a brief manner as for NMDA or for longer periods (min) by bath addition (data not shown). Heat denatured (100°C for 20 min) FIV–PPR Env did not significantly alter the calcium signal to NMDA (data not shown). FIV–PPR Env produced a significant enhancement of the calcium signal to NMDA at all culture ages studied (Figure 6B). However, the effect was greatest in young neurons (i.e. the enhancement was ~40% in 1-week-cultures and ~10% in 3-week-cultures). This age-dependent reduction in sensitivity could reflect developmental changes in the NMDA receptor function or other pathways involved in calcium homeostasis. The FIV–PPR Env induced increase in LDH levels did not show a similar age-dependency (data not shown), suggesting that calcium influx was sufficient to support the neurotoxic process at all culture ages. Resting calcium levels were not significantly altered by FIV–PPR Env at any of the ages studied.

## Discussion

As has been shown in other models of HIV infection, the results from the current study suggest that the cellular mechanisms responsible for neurotoxicity in the FIV model are mediated through excitotoxicity, involving interactions among viral protein, glutamate receptors, and increased intracellular calcium. These results are consistent with the interpretation that the cellular mechanisms mediating FIV induced neuropathology are similar to those that occur in HIV-1 infection.

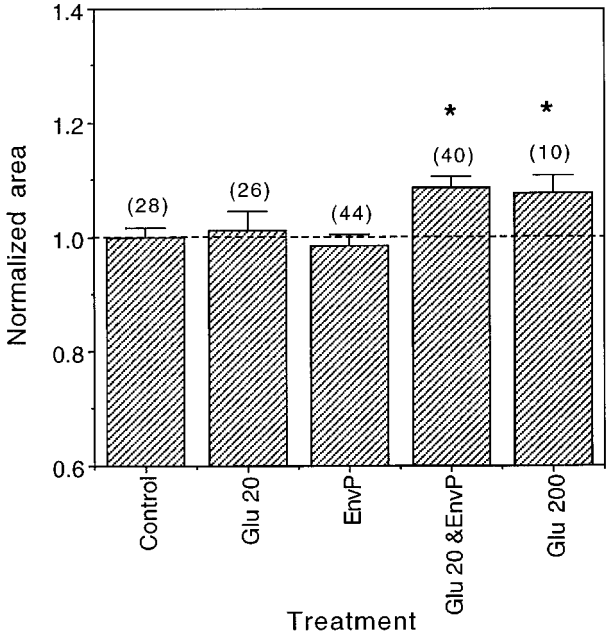


**Figure 4** Effect of glutamate and FIV–PPR Env protein on neuronal swelling. In two separate culture preparations between 6–14 days *in vitro* (DIV), cell size was measured under control conditions and after 30 min exposure to FIV–PPR Env protein (EnvP, 5 $\mu$g/ml), glutamate (glu; 20 or 200 $\mu$M) or Env protein plus glutamate (20 $\mu$M). The mean cell size after treatment was normalized to the mean cell size under control conditions and results from each field of neurons pooled. Number in the parenthesis indicated the number of cells tested. *Indicates a significant difference from control value at the $P \leqslant 0.05$ level (2-tailed paired *t*-test).

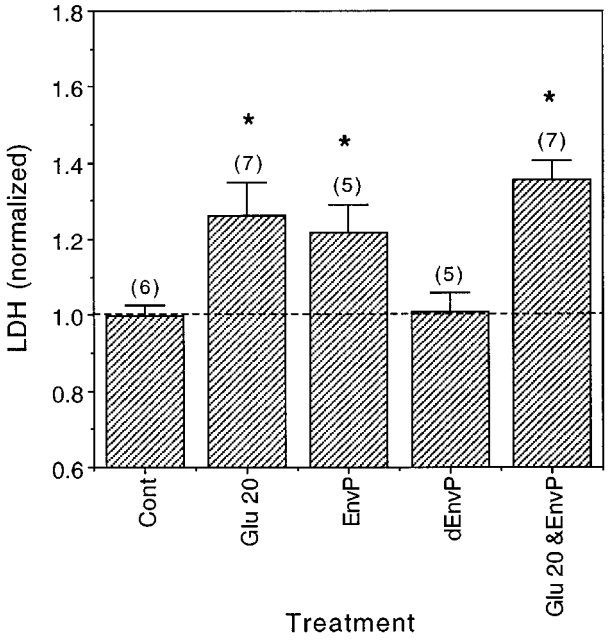


**Figure 5** Effects of recombinant FIV–PPR Env protein on LDH levels. Neuronal cultures were exposed for 24 h to the following reagents: FIV–PPR-Env protein (EnvP, 5 $\mu$g/ml), glutamate (glu; 20 $\mu$M), heat denatured FIV–PPR Env protein (dEnvP, 5 $\mu$g/ml), and a combination of FIV–PPR-Env Protein and glutamate. Supernatants were collected and LDH values were determined as described in Materials and methods. Results were expressed as the ratio of the mean LDH levels of the various treatment groups over the LDH levels of the control cultures. Number in the parenthesis indicated the number of samples tested. *Indicates a significant difference from control at the $P \leqslant 0.05$ level (2-tailed *t*-test).

These findings are important, because several therapeutic approaches that target the NMDA receptor have been suggested (Lipton, 1992; 1994b; Muller *et al*, 1992; Perovic *et al*, 1994). The results of this study help to validate the FIV/cat system as a potential predictive animal model for evaluating these and other therapeutic approaches that may prevent the excitotoxicity mechanism of lentivirus-induced CNS disease.

In the current study, we first examined the effects of whole viral preparations on mixed feline neuron cultures. Results showed that washed and resuspended viral pellets caused significant neuronal swelling within 15 min of exposure. Neuronal swelling has repeatedly been used as a measure of neurotoxicity (Choi, 1992; Ikeda *et al*, 1996; Peterson *et al*, 1989). Usually, the swelling occurs within minutes of the addition of the toxic agent (Ikeda *et al*, 1996). The neuronal swelling effect did not require infectious FIV virions, as heat inactivated virus (56°C for 60 min) induced a substantial neuronal swelling. However, the significant neuronal swelling was prevented by heat denaturation of the viral preparation (100°C for 20 min). The whole virus preparation also caused a significant increase in the amount of LDH released from these cultures. Thus, by two different measures, the FIV-PPR viral preparation demonstrated significant toxicity to feline neuron enriched cultures.

In studies with HIV-1, it was shown that Env proteins (gp120 and/or gp160) of the virus are responsible for the neurotoxic effects (Benos *et al*, 1994a,b; Dreyer and Lipton, 1995; Lannuzel *et al*, 1995; Lipton, 1994a; Lipton *et al*, 1991; Muller *et al*, 1992; Toggas *et al*, 1996; Yeung *et al*, 1995). Thus, the baculovirus preparations of the Env proteins of FIV were examined in this present study. Similar to the whole viral pellets, the FIV–PPR Env preparations caused an increased release of LDH from the mixed neuronal cultures. However, in the neuronal swelling assay, FIV–PPR Env did not, by itself, induce neuron swelling. When both FIV–PPR Env and a subtoxic concentration of glutamate (20 $\mu$M) were added together to the mixed feline neuron cultures, significant swelling was obtained. This sensitization of neurons to the toxic effect of glutamate has been previously reported for HIV-1 Env protein preparation (Lipton, 1991; Lipton *et al*, 1991).

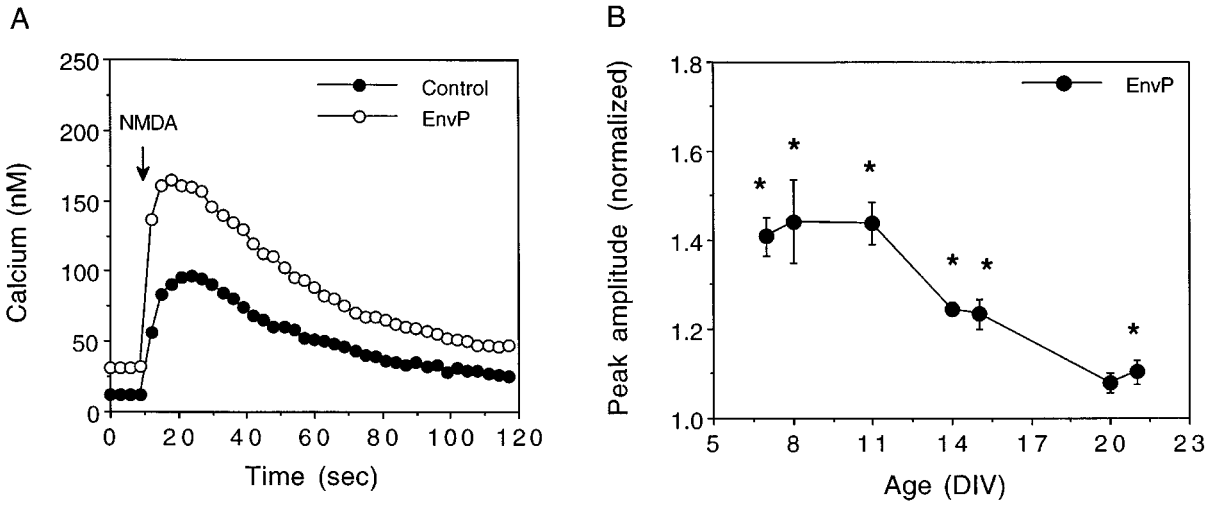


**Figure 6** Effect of FIV–PPR Env on the calcium signal to NMDA. (A) Representative calcium signals evoked by NMDA in cultured cat cortical neurons under baseline conditions and after addition of FIV–PPR Env protein (EnvP, 5 $\mu$g/ml) to the recording saline (recordings are from two different neurons). NMDA was applied at the arrow by a brief (1 s) microperfusion pulse from a pipette containing 200 $\mu$M NMDA dissolved in bath saline. The peak amplitude of the calcium signal to NMDA was enhanced by exposure (30 min) to FIV–PPR Env in the bath. (B) Age-dependent effect of FIV–PPR Env (EnvP, 5 $\mu$g/ml) on the peak amplitude of the calcium signal to NMDA. Mean values (50–280 neurons per each treatment group) ($\pm$S.E.M.) are shown for measurements made at different days *in vitro* (DIV). The enhancement of the calcium signal to NMDA by FIV–PPR Env was largest at the early culture ages. Peak amplitude measurements are normalized to the peak amplitude of the calcium signal to NMDA under baseline control conditions for the same culture set. *Indicates a significant difference from control value at the $P \leqslant 0.05$ level (ANOVA).

It is interesting to speculate as to why the intact FIV–PPR viral preparations caused neuronal swelling without the addition of glutamate, while the FIV–PPR Env preparation required the addition of a subtoxic concentration of glutamate to induce significant swelling. Possible reasons for this difference could be: (1) the difference in the glycosylation of the two FIV–PPR Env preparations as mammalian and insect protein expression systems are known to glycosylate proteins differently; (2) the Env protein conformation, within the confines of a virus particle, may differ from that of the free protein; or (3) additional viral proteins may be needed to achieve the complete toxic effect. Also, exposure to the complete virion may stimulate the release of endogenous glutamate within the cultures, thus, negating the necessity for adding glutamate exogenously. Additionally, although FIV–PPR Env did not, by itself, induce neuron swelling, it did cause a significant increase in the LDH assay. Thus, it is possible that cell damage was merely delayed, since cell swelling was examined at 15 min post-exposure and the LDH data were collected at 24 h post-exposure. Further studies will be needed to resolve this issue.

The acute neurotoxicity demonstrated by the addition of FIV–PPR to the neuron cultures is in contrast to the slow chronic progressive disease that is normally typical of lentiviral diseases. This difference can possibly be explained by the amount of virus that the cells were exposed to *in vitro*. The results of this present study demonstrated that neuronal swelling was related to the amount of virus added to the cultures, as greater swelling was shown with the addition of high concentrations of the virus. *In vivo*, it is unlikely that large numbers of neurons will simultaneously be exposed to such high levels of virus. However, individual neurons, on occasion, may have a high level of exposure based upon local conditions. At times of high levels of Env exposure, local conditions may impart an environment that could result in neuron damage through the excitotoxicity mechanism.

To further investigate the possible role of excitotoxicity in neuron damage within the feline system, the effects of a FIV–PPR Env preparation on calcium signaling, produced by activation of glutamate response, was examined. Similar to that which has been described for HIV-1 (Dreyer *et al*, 1990; Lannuzel *et al*, 1995; Lipton *et al*, 1991; Lo *et al*, 1992), neurons exposed to FIV–PPR Env prior to NMDA stimulation had a significantly enhanced intracellular response to NMDA, thus, demonstrating that FIV–PPR Env, similar to HIV-1 Env preparations, enhances NMDA receptor-linked calcium responses.

Given the data present in this study, it is not possible to determine if the effects of FIV–PPR Env on neuron toxicity are direct or indirect. However, with HIV-1, most of the evidence points to indirect mechanisms (Dewhurst *et al*, 1996; Gendelman *et al*, 1994; Genis *et al*, 1992; Giulian *et al*, 1986, 1996; Grimaldi *et al*, 1991; Heyes *et al*, 1991; Lipton *et al*, 1994). It is possible that FIV–PPR Env may bind

directly to a neuron surface protein or act indirectly by stimulating the release of factors from microglia/macrophages or astrocytes, as both cell types were present in the cultures. *In vitro*, a variety of microglia/macrophage and astrocyte products have been implicated to play a potential role in neuronal toxicity, such as: TNF-$\alpha$, IL-1$\beta$, quinolinate, eicosanoids, and Ntox (Genis *et al*, 1992; Giulian *et al*, 1986, 1996; Grimaldi *et al*, 1991; Heyes *et al*, 1991). The *in vivo* relevance of these products in lentivirus induced CNS pathogenesis awaits better elucidation.

Another mechanism of neuronal toxicity may operate through the accumulation of extracellular glutamate. We have recently demonstrated that FIV infection of feline astrocyte cultures significantly decreases the ability of these cells to remove glutamate from the extracellular fluid (Yu *et al*, 1998). The results of this present study demonstrated that FIV-Env protein sensitizes the neuron to the toxic effects of glutamate. Thus, the virus infection may inhibit the ability of astroycytes to remove extracellular glutamate, while the presence of Env may increase the sensitivity of neurons to the toxic effects of glutamate. The combination of these two events may result in neuronal death. This is a particularly attractive theory as a recent publication has demonstrated, through proton nuclear magnetic resonance spectroscopy, that a significant increase in glutamate levels occurs in FIV infected cats (Power *et al*, 1997). It is likely that multiple mechanisms are operating to cause lentivirus induced neuronal damage.

The results of this study demonstrate that whole virus and Env preparations of FIV–PPR induce neurotoxic effects in a manner that is very similar to the effects that have been described for HIV-1 on rodent and human cell preparations. Although further investigation is needed to determine the exact mechanism by which neuron toxicity is produced, the results of this study indicate that the FIV/cat system is a good model to investigate potential compounds *in vivo* that may prevent or ameliorate lentivirus induced excitotoxic effects on the CNS.

## Materials and methods

*Cell culture*

Specific Pathogen Free (SPF) pregnant cats were obtained from Harlan Sprague Dawley. Neuronal cultures were prepared from the cerebral cortex of embryonic or postnatal kittens ranging in age from 40-days of gestation through 2-days post-partum. Embryonic animals were obtained by cesarean section. The queens were given glycopyrolate (0.01 mg/kg), ketamine (8 mg/kg) and acepromazine (0.08 mg/kg) as preanesthetics and maintained in a deep plane of anesthesia with inhaled isoflurane during the surgery. Each fetus or new born was humanely euthanized by cervival luxation while in a deep plane of anesthesia. The brains were dissected from the skull and transported to the laboratory in ice cold $Ca^{2+}$-, $Mg^{2+}$-free saline (composition in mM: NaCl, 137; KCl, 5.4; $Na_2HPO_4$, 0.17; $KH_2PO_4$, 0.22; glucose 33; sucrose, 44; HEPES Buffer, 10, pH 7.3). The cerebral cortical cells were isolated by a standard enzyme treatment protocol based on the methods of Trenkner (Trenkner and Sturman, 1991). Briefly, the cerebral cortex was dissociated in $Ca^{2+}$-, $Mg^{2+}$-free saline, treated with trypsin and then DNase, the neurons collected by centrifugation, and then plated at a concentration of $2 \times 10^6$ cell/ml on MATRIGEL (Collaborative Biomedical Products, Bedford, MA) coated coverglasses placed in 35 mm culture dishes containing 1 ml of plating medium. The plating medium consisted of Minimum Essential Media with Earle's salts (MEM; GIBCO) supplemented with 10% fetal bovine serum, 10% horse serum, glucose (5 g/l) and 25 $\mu$M penicillin-streptomycin. After 3 days, the medium was changed to MEM with 10% horse serum, glucose (5 g/l) and 25 $\mu$M penicillin-streptomycin, which was used for the remainder of the culture period. Astrocyte contamination was minimized by treating the cultures with 5-fluorodeoxyuridine (20 $\mu$g/ml) for 3 days starting on the third day of culture. The cultures were maintained in a standard $CO_2$ incubator and survived for approximately 30 days.

*Intracellular calcium measurement*

Intracellular calcium levels were determined in individual cells using standard microscopic fura-2 digital imaging (Grynkiewicz *et al*, 1985) and previously published methods (Holliday and Gruol, 1993). Neurons were incubated with 3 $\mu$M Fura-2/AM and 0.02% pluronic F-127 (Molecular Probes) in physiological saline (composition in mM: NaCl, 140; KCl, 3.5; $KH_2PO_4$, 0.4; $Na_2HPO_4$, 1.25; $CaCl_2$, 2.2; $MgSO_4$, 2; glucose, 10; HEPES-NaOH, 10, pH 7.3) for 30 min to load the dye, followed by an additional 45 min incubation in dye-free saline solution at room temperature. The glass coverslip was then placed into the stage chamber of an inverted microscope equipped for fura-2 video images and containing low $Mg^{2+}$ saline (composition in mM: NaCl, 140; KCl, 3.5; $KH_2PO_4$, 0.4; $Na_2HPO_4$, 1.25; $CaCl_2$, 2.2; $MgSO_4$, 0.03; glucose, 10; HEPES-NaOH, 10, pH 7.3) with 5 $\mu$M glycine. Low $Mg^{2+}$ saline was used to prevent $Mg^{2+}$ blockade of the NMDA receptor; glycine is a co-agonist at the NMDA receptor. This modified physiological saline is referred to as MPS. Live video images of selected microscopic fields were recorded with SIT-66 video camera (DAGE-MTI) and digitized by computer. Video images were collected at 3-s intervals for at least 12 s before neuronal stimulation and 120 s after stimulation. To ensure that the peak of the response was detected, the first collection after

stimulation was made within 1 s of stimulation, with subsequent collections occurring at 3-s intervals. Ratio images (340 nm excited image/380 nm excited image) were formed by a pixel-by-pixel division. The somata of approximately 5–10 cells in each microscopic field were individually measured. Real time image acquisition and calcium measurements were made with MCID imaging software (Imaging Research, Inc.). Calibration was done using fura salt (100 $\mu$M) in solutions of known calcium concentration. All experiments were performed at room temperature.

*Drug application*
NMDA (200 $\mu$M) was dissolved in bath saline and applied by a standardized 1-s microperfusion pulse from drug pipettes (3 $\mu$M tip diameter) placed near the neurons of interest. In some studies, bath addition was used. Other reagents were applied by bath addition or bath exchange. In all experiments the bath saline was low $Mg^{2+}$ saline with glycine.

*LDH assay*
LDH activity was determined in the neuronal culture supernatant. Briefly, the neuronal culture was carefully washed with modified physiological saline (MPS) (see Methods for calcium imaging studies). One ml of fresh MPS was added (with or without the reagent(s) to be tested). The cultures were transferred to an incubator at 37°C. Twenty-four hours later, supernatants were collected and stored at −20°C for later LDH measurement. Cells were lysed with 1 ml of a cell lysis solution (10 mM NaOH, 0.1% Triton) for Bradford protein quantitation. LDH activity of a 100 ml sample was tested by a Cytotoxicity Detection Kit (Boehringer Mannheim). The LDH activity of beef muscle (Boehringer Mannheim) was used as the standard to calibrate the standard curve. LDH was normalized to the amount of cellular protein and was expressed as units of activity per mg of protein. The results were expressed as a percentage of the control.

*Neuronal swelling*
Neuronal swelling was determined by measuring morphological changes over time (0 min, 15 min, and 30 min). The neuronal cultures were prepared as for the LDH assays, which were run in parallel. The neurons were visualized on a Zeiss IM microscope under phase contrast optics and video images collected with a SIT-66 video camera (DAGE-MTI) and digitized by computer. Morphometric measurements (two-dimensional cell size) were made on the same microscopic field under baseline control conditions (0 min), 15 min and 30 min after addition of test reagents. The somata of approximately 6–10 cells in each microscopic field was individually measured. A new culture dish was used for each reagent tested. MCID imaging software (Imaging Research Inc.) was used for image acquisition and morphometric analysis.

*Virus*
Virus stock was prepared by transfecting CrFK cells with the pUC 119 plasmid containing the proviral sequence of the infectious molecular clone FIV–PPR and co-cultivating the transfecting CrFK cells with SPF-feline-PBMCs, as previously described (Phillips *et al*, 1990). To pellet the virus, 2 ml of FIV–PPR laden tissue culture supernatant, containing 169 000 RT counts/ml, was spun at 55 K at 20°C for 1 h in a Beckman TL 100 rotor. The supernatant was removed and drained from the viral pellet. The virus was then resuspended in 1 ml of DMEM (GIBCO) and used at the indicated dilutions.

*FIV–PPR Env protein preparation*
The preparation of FIV–PPR Env proteins has been previously described (Wickham *et al*, 1992; Wickham and Nemerow, 1993). Briefly, the envelope (Env) gene of FIV–PPR was cloned into Bluebac II vector (Invitrogen). After plaque purification, a high titer stock of baculovirus expressing recombinant FIV–PPR Env was produced in SF9 cells. This viral stock was then used to infect TN5-B cells (Wickham *et al*, 1992). After 72 h, the cells were harvested and cell debris was removed by centrifugation at 5 K for 20 min. The pH of the supernatant was raised to 8. The supernatant was then centrifuged at 5 K for 20 min and filtered through a 0.45 micron filter (Corning). A lentil lectin column (matrix from Sigma) was used to purify the FIV envelope protein from the supernatant. The glycoproteins were eluted with 2% methyl alpha-d-mannopyranoside (Sigma) in lentil lectin buffer (20 mM Tris pH 8, 2 mM $CaCl_2$, 1 mM NaCl). Fractions were collected and the presence of FIV envelope was confirmed by SDS–PAGE and Western Blot using anti-FIV polyclonal antisera. For all experiments the protein preparation was added at a concentration of 5 $\mu$g/ml.

*Immunohistochemistry*
Immunostaining was used to identify cell types in the cultures. Antibodies to feline proteins were not available, but two antibodies derived from proteins from other species were effective. An antibody to microtubular associated protein-2 (mouse monoclonal; Boehringer Mannheim), a neuronal cytoskeletal protein, was used to identify neurons. An antibody to glial fibrillary acidic protein (mouse monoclonal; Boehringer Mannheim) was used to identify astroyctes. A standard protocol used for rodent neuronal cultures was used. Briefly, cultures were fixed with a solution of 3% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.3) for 15 min. After rinsing, the cultures were treated with 0.05% Triton X-100 in phosphate-buffered saline and incubated overnight (4°C) in the primary

antibody. Immunoreactivity was detected the following day by an immunoperoxidase reaction using the materials and procedures provided in the Vectastain kit (Vector Laboratories).

Microglia were identified by overnight incubation of live cells in fluorescently-tagged DiI-acetylated low density lipoprotein (10 $\mu$g/ml; Biomedical Technologies Inc., Stoughton, MA).

*Data analysis*

Intracellular calcium responses were quantified by measurement of peak amplitude. Resting calcium levels were subtracted from all peak amplitude values on an individual cell basis. Cell size and LDH values were normalized to the control cell values for each data set, and data sets from several cultures were pooled for statistical analysis. Values are expressed as mean $\pm$ s.e.m. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Fisher post-hoc test for multiple comparisons or by the pair *t*-test. $P<0.05$ was considered statistically significant.

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