

# Spatially distinct and functionally independent mechanisms of axonal degeneration in a model of HIV-associated sensory neuropathy

Giorgia Melli,<sup>1</sup> Sanjay C. Keswani,<sup>1</sup> Angela Fischer,<sup>1</sup> Weiran Chen<sup>1</sup> and Ahmet Höke<sup>1,2</sup>

Departments of Neurology<sup>1</sup> and Neuroscience<sup>2</sup>, Johns Hopkins University School of Medicine, Baltimore, MD, USA

Correspondence to: Ahmet Höke, MD, PhD, Department of Neurology, Johns Hopkins University, 600 N. Wolfe Street, Path 509, Baltimore, MD 21287, USA

E-mail: ahoke@jhmi.edu

**Sensory polyneuropathies are the most frequent neurological complication of human immunodeficiency virus (HIV) infection. Distal symmetric polyneuropathy (DSP), associated with HIV infection, is characterized by length-dependent axonal degeneration of sensory fibres. In rodent dorsal root ganglia (DRG) cultures, HIV viral envelope protein gp120 results in neurotoxicity and axonal degeneration. Since it is unknown whether the axonal degeneration is a consequence of neuronal death or whether it is due to a direct toxic effect on axons, we investigated gp120-induced axonal toxicity using compartmentalized cultures of sensory neurons. Our results show that gp120 causes neuronal apoptosis and axonal degeneration through two, independent and spatially separated mechanisms of action: (i) an indirect insult to cell bodies, requiring the presence of Schwann cells, results in neuronal apoptotic death and subsequent axonal degeneration; (ii) a direct, local toxicity exerted on axons through activation of mitochondrial caspase pathway that is independent of cell body. This local axonal toxicity is mediated through binding of gp120 to axonal chemokine receptors and can be prevented by chemokine receptor blockers. In conclusion, we propose a novel pathway of axonal degeneration mediated by gp120 that is dependent on local activation of caspases in the axon. This observation suggests that axonal protection is a relevant therapeutic target for HIV-associated sensory neuropathy. Furthermore, chemokine receptor inhibitors, which are currently being developed as HIV entry inhibitor drugs, may also have a therapeutic role in HIV-associated peripheral neuropathies by preventing axonal degeneration.**

**Keywords:** HIV-neuropathy; gp120; axonal degeneration; apoptosis; caspase; chemokine receptor inhibitors

**Abbreviations:** ATN = antiretroviral toxic neuropathy; DSP = distal symmetric polyneuropathy; DRG = dorsal root ganglia; HIV = human immunodeficiency virus

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## Introduction

Human immunodeficiency virus (HIV) associated sensory neuropathies are the most common neurological complications in HIV infection, affecting one-third of patients with AIDS (Estanislao *et al.*, 2004). They include distal symmetric polyneuropathy (DSP), secondary to HIV infection, and antiretroviral toxic neuropathy (ATN) associated with the use of antiretroviral agents, particularly nucleoside reverse transcriptase inhibitors. They are characterized by painful dysaesthesias in a length-dependent stocking-and-glove distribution and are clinically indistinguishable from each other. Neuropathic pain in these neuropathies can significantly impair the quality of life and is a major cause of

morbidity in this patient population (Verma *et al.*, 2005). Currently, there is no effective therapy to reverse the axonal degeneration in these patients. Furthermore, symptomatic control of neuropathic pain is severely limited by adverse effects or drug interactions.

The pathophysiology of DSP is not completely understood and the most commonly accepted pathogenic mechanism involves neuronal damage secondary to immune activation, similar to what is observed in HIV-associated dementia (Gonzalez-Scarano and Martin-Garcia, 2005; Hoke and Corblath, 2005). This concept is supported by pathological studies that failed to show HIV replication in neurons, but

demonstrated abundant macrophage activation and viral replication within macrophages of peripheral nerve and dorsal root ganglia in approximately 50% of cases (Kolson and Gonzalez-Scarano, 2001; Pardo *et al.*, 2001)

The pathological changes associated with DSP are axonal degeneration of sensory fibres and prominent loss of unmyelinated fibres (reviewed by Keswani *et al.*, 2002 and Hoke and Cornblath, 2004). The axonal damage follows the ‘dying back’ pattern of degeneration. ‘Dying back’ degeneration is the underlying pathological change in many systemic polyneuropathies such as diabetic and alcoholic neuropathies. This length-dependent dying-back phenomenon is probably also involved in degenerative diseases affecting the central nervous system, like Alzheimer’s and Parkinson’s diseases (Iseki *et al.*, 2001) and motoneuron disease (Azzouz *et al.*, 1997). In ‘dying back’ degeneration, the axon of a chronically injured neuron slowly degenerates from the distal end in a centripetal manner. However, the nature and the site of initial injury to the neuron are unknown; in particular it is unclear if the process starts in the axons itself or in the neuronal cell body. Raff *et al.* (2002) proposed the intriguing idea that ‘dying back’ degeneration is a stereotyped response of axons, which under some circumstances activate a self-destruct programme, similar to what happens in the cell bodies during apoptosis.

Recently, we demonstrated that DSP could be modelled *in vitro* using dorsal root ganglion sensory neurons (Keswani *et al.*, 2003a, b) and used it to study mechanisms of neuronal injury (Keswani *et al.*, 2003a, 2004a). To investigate the axonal degeneration underlying HIV-associated neuropathy we expanded on our previous observations by using a compartmentalized cell culture system, known as Campenot chambers (Campenot, 1977). In compartmentalized chambers, axons grow across a silicone grease barrier and enter a separate fluid environment within a distal compartment, which allows pharmacological manipulation of distal axons independently from cell bodies. This powerful system has been successfully used to study the formation and maintenance of neuronal projections (Campenot, 1982; Bertrand *et al.*, 2005) and the role of neurotrophins in development of the sympathetic nervous system (Riccio *et al.*, 1997; MacInnis and Campenot, 2002; Kuruvilla *et al.*, 2004). Using this system, we show that HIV viral envelope protein gp120 causes toxicity in sensory neurons through two independent mechanisms, one acting on cell bodies, mediated by Schwann cells, and the other one, exerted directly on axons, involving chemokine receptors and activation of the caspase pathway.

## Material and methods

### Pharmacological reagents

Recombinant HIV-1 gp120<sub>MN</sub> (>95% pure) was obtained from ImmunoDiagnostic (Woburn, MA); cytosine arabinoside and recombinant rat nerve growth factor from Sigma-Aldrich (St Louis, MO); Hoechst 33258 from Molecular Probes, Invitrogen (Carlsbad, CA); zVAD-fmk from Calbiochem (San Diego, CA); anti-cytochrome c antibody from Abcam (Cambridge, MA);

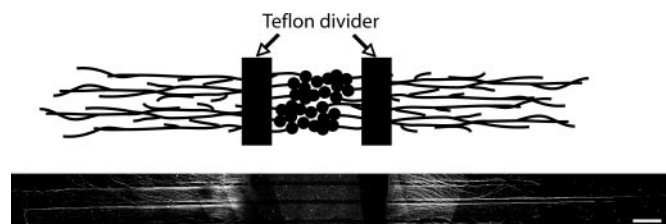
anti-human/rat activated caspase-3 antibody and anti-CCR5 monoclonal antibody from R & D Systems (Minneapolis, MN); anti-CXCR4 and anti-CCR5 polyclonal antibodies from Cell Sciences (Norwood, MA); anti-CXCR4 (clone 12G5) antibody from BD Pharmingen (San Diego, CA); and anti-HIV-1 (gp120) from Fitzgerald (Concord, MA).

### Sensory neuronal culture

All experiments involving animals were conducted according to protocols approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Spinal dorsal root ganglia were dissected from decapitated embryonic age Day 15 wild-type Sprague–Dawley rats and enzymatically dissociated with 0.25% trypsin in L-15 medium. The cells were plated onto collagen-coated tissue culture dishes in compartmentalized chambers. Neurons were maintained in Neurobasal medium (Gibco Invitrogen, Grand Island, NY) supplemented with 1% fetal bovine serum, penicillin (1 U/l), streptomycin (1 U/l) and nerve growth factor (10–100 ng/ml). For some experiments, the compartmentalized cultures were maintained from Day 2 to Day 7 in growth medium containing cytosine arabinoside (10 µM) in the centre chamber to eliminate Schwann cells and all non-neuronal, dividing cells.

### Preparation of compartmentalized chambers

Compartmentalized cultures were prepared as described previously (Campenot, 1982). After the application of silicone grease (Dow Corning, Midland, MI), a Camp-10 Teflon divider (Tyler Research, Alberta, Canada) was seated into a collagen-coated 35 mm tissue culture dish (Becton Dickinson Labware, Franklin Lakes, NJ). Before setting the divider, the collagen in the middle region of the dish was scraped with a pin rake in order to keep the axons growing on the collagen between the scratches. One drop of medium with 1% methylcellulose was placed in the scratched region, in order to prevent the silicone grease directly adhering to the collagen. This procedure allows the growth of the axons under the silicon grease barrier, without flow of medium or significant diffusion of medium component between compartments (Campenot, 1982). The integrity of the seal was checked by filling only the side compartments with medium and incubating the chambers at 37° overnight; only the cultures without leakage were used for the experiment (Tsui-Pierchala and Ginty, 1999). Dissociated cells (50 000/chamber) were plated in the middle compartment, and after 7 days many neurites had sprouted in the side compartments (Fig. 1). The



**Fig. 1** Compartmentalized neuronal culture system. Top panel depicts a schematic view of a three-compartment sensory neuronal culture system. Dissociated sensory ganglia cells were plated in the middle compartment and the axons projected under the two Teflon dividers into the side compartments. Bottom panel shows an example of a compartmentalized sensory neuronal culture, fixed and stained with anti-βIII-tubulin antibody in the side chambers, after 10 days in culture. Scale bar, 500 µm.

side chambers also contained migrating Schwann cells, which follow the axons through the barriers. In the absence of any axon growth, we did not observe any Schwann cell migration into the side chambers. In cases where we treated the centre compartment with cytosine arabinoside from Day 2 on (as explained above), we did not see any Schwann cells in the centre or side chambers. The experimental conditions labelled as ‘without Schwann cells’ did not have any Schwann cells either in the centre or side chambers and the conditions labelled as ‘with Schwann cells’ had Schwann cells in both the centre and side chambers.

### Axonal toxicity assay

After 7 days in culture, sensory neurons were exposed to gp120<sub>MN</sub> (10 ng/ml) either in the cell body compartment or in the distal axon compartment. The lengths of the axons in the side chambers were measured before treatment and after 72 h of exposure to gp120. The experiments were repeated in a similar manner in Campenot chambers treated with cytosine arabinoside, which eliminated the Schwann cells from the cultures. This allowed us to examine the role of glial cells in gp120 toxicity. In neuroprotection assays, the caspase inhibitor, zVAD (20  $\mu$ M), was added to the centre or side chambers treated with gp120. In experiments involving chemokine receptor blockers, anti-CCR5 or anti-CXCR4 monoclonal antibodies (1 : 200 dilution) were applied to the side chambers of cultures treated with cytosine arabinoside. Photographs of distal axon compartment were taken using a digital camera attached to a phase contrast, inverted Zeiss microscope. The lengths of individual axons were measured using the Openlab software (Improvision, Lexington, MA, USA). An average of 20 axons was measured in each condition and the experiment was repeated a minimum of 3 times. The results from each experiment were normalized and analysed using Student's *t*-test for unpaired data in Statview (Macintosh version 5.0.1) (the critical  $\alpha$ -level set at  $P = 0.005$ ) and corrected for multiple comparisons. The same method was used for the statistical analysis of other data (see below).

### Apoptosis assay

Cultures of pure sensory neurons in compartmentalized chambers, in which glia cells were previously eliminated as described before, were exposed to gp120<sub>MN</sub> (10 ng/ml) or vehicle for 72 h either in the cell body compartment or in the distal axon compartment. In other experiments probing the role of Schwann cells in mediating gp120 neurotoxicity, gp120<sub>MN</sub> was added to mixed DRG neuronal and Schwann cell cultures in compartmentalized chambers in a similar manner. The cells were then fixed and incubated with Hoechst 33258 dye (10  $\mu$ g/ml) to identify apoptotic cells. Apoptotic cells show bright, condensed and often fragmented nuclei (Ye *et al.*, 2003). The apoptotic cells were counted and the number expressed as percentage of the total number of cells present in each culture. The experiments were repeated three times and the results of each experiment were normalized and analysed as described above.

### Immunocytochemistry

Expression of chemokine receptors in the sensory axons was assessed by triple immunostaining of the DRG sensory axons in the side chambers using antibodies directed against CXCR4, CCR5 and  $\beta$ III-tubulin using standard methods. Cultures were fixed with 4% paraformaldehyde for 20 min, blocked with the appropriate serum and incubated in the primary antibody at a dilution of

1:200 (CXCR4), 1 : 200 (CCR5) and 1 : 1000 ( $\beta$ III-tubulin), followed by incubation in fluorescently tagged secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Expression of cytochrome c and activated caspase-3 was analysed in DRG sensory axons in the side chambers after exposure to gp120<sub>MN</sub>. Twelve or 24 h after exposure to gp120<sub>MN</sub>, axons were fixed and immunostained with anti-cytochrome c (1 : 200 dilution) or anti-activated caspase-3 (1 : 200 dilution). Co-labelling was done with anti- $\beta$ III-tubulin antibody as above.

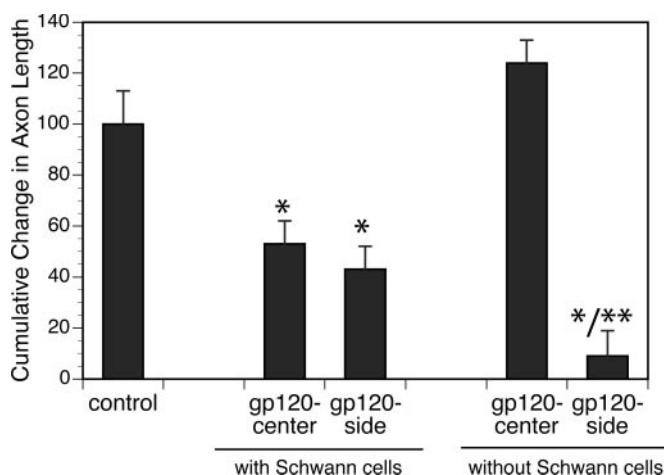
### Immunoprecipitation and western blotting

In experiments involving immunoprecipitation and western blotting, we used an immortalized rat sensory neuronal cell line (B50-11). Differentiated sensory neurons were treated with gp120<sub>MN</sub> for 1 h. Cell lysates were then subjected to immunoprecipitation with anti-gp120 antibody according to standard protocols. The immunoprecipitates were then blotted with anti-CCR5 and anti-CXCR4 antibodies on Polyvinylidene difluoride (PVDF) membranes.

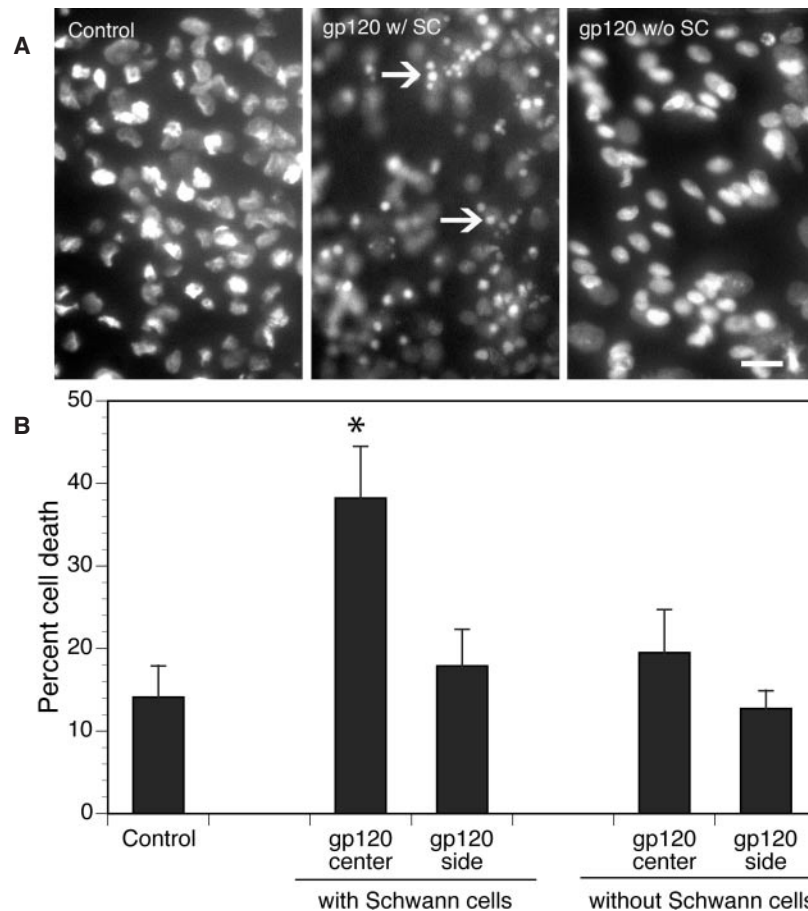
## Results

### Gp120 induced axonal toxicity

Gp120 caused significant axonal toxicity compared with control cultures, both when applied directly to axons (side chambers) and when applied to DRG neuronal cell bodies (middle chambers) in mixed cultures with Schwann cells. In contrast, in the absence of glial cells, gp120 caused prominent axonal toxicity only when it was applied directly on sensory axons in side chambers, but not when applied to cell bodies (Fig. 2). These results suggested that gp120 causes direct axonal toxicity that is independent of Schwann cells. In contrast, gp120 neurotoxicity on the neural cell bodies requires the presence of Schwann cells and is likely to be



**Fig. 2** Gp120-induced axonal toxicity. Gp120 applied to the side or centre chamber, in mixed cultures with Schwann cells, caused axonal toxicity as measured by axonal length over three days. In contrast, gp120, in the absence of Schwann cells, caused axonal toxicity only when applied to the side chamber but not when applied to the centre chamber. (\*Gp120 versus control,  $P < 0.05$ ; \*\*gp120 without Schwann cells versus gp120 with Schwann cells,  $P < 0.05$ ).



**Fig. 3** Gp120 causes apoptotic neuronal death. **(A)** Cultures exposed for 72 h to gp120 in the middle compartment were stained with Hoechst 33258 dye to identify apoptotic neurons. Arrows indicate apoptotic cells with shrunken, bright and condensed nuclei (middle panel). **(B)** Gp120 induced apoptosis when it was applied to the cell bodies in mixed cultures with Schwann cells. In contrast, gp120 did not induce apoptosis in pure neuronal cultures when glia cells were eliminated as described in the Material and methods section (\*gp120 versus control,  $P < 0.05$ ). Scale bar, 20  $\mu\text{m}$ .

mediated through activation of Schwann cells (Keswani *et al.*, 2003b).

Of note, when glia cells were eliminated from the axonal compartment, local application of gp120 resulted in an increased toxicity. These findings suggest that local glia cells associated with axons may play a partial neuroprotective role by counteracting the direct toxicity of gp120 (Keswani *et al.*, 2004b).

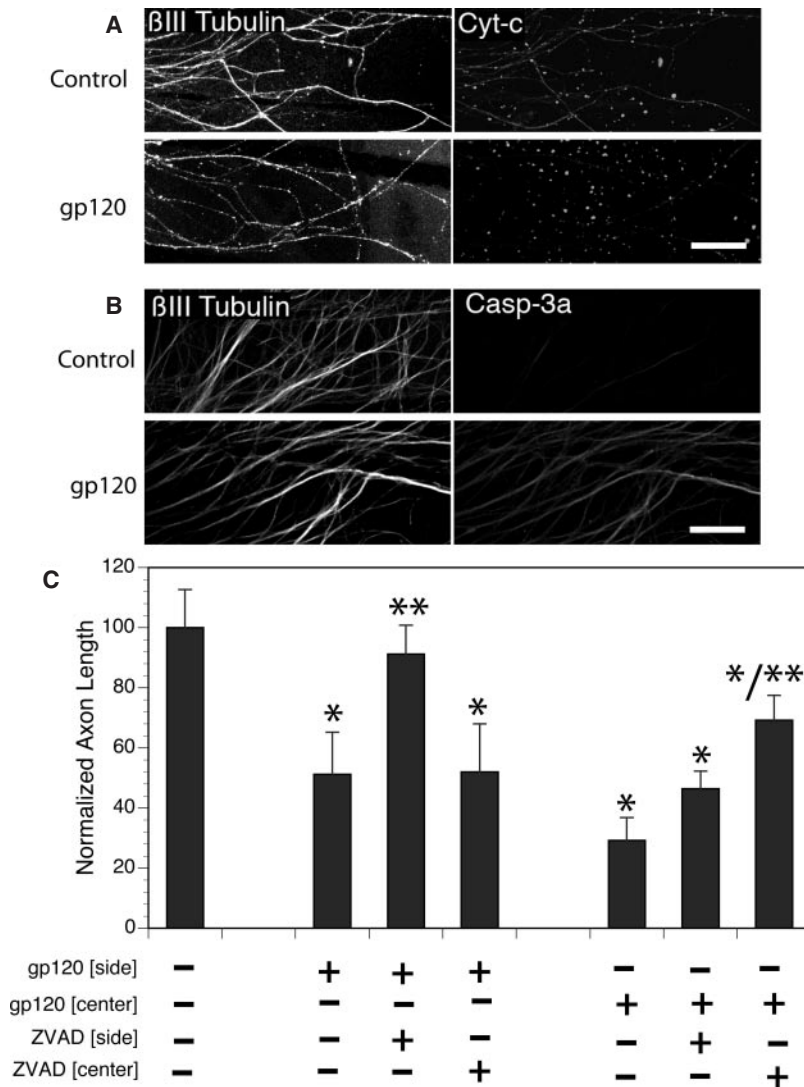
### Gp120-induced local toxicity in DRG sensory neuronal cell bodies requires presence of Schwann cells

Under normal culture conditions using the Campenot chambers, only a small percentage of DRG neurons are positive for apoptotic nuclei using the Hoechst staining. When applied directly in the centre chambers at the same concentration that induced axonal growth inhibition, gp120 resulted in a substantial increase in the number of apoptotic cells (Fig. 3). In contrast, there was no apoptosis of neuronal cell bodies in the centre chamber when gp120 was applied to the side axonal chambers, even after 72 h of exposure. This neurotoxicity on

the DRG sensory neuronal body required the presence of Schwann cells. When Schwann cells in the culture were killed by successive applications of ara-C, there was no increase in apoptotic nuclei in the centre chamber, even when higher concentrations of gp120 were applied in the centre chamber. The increase in apoptotic nuclei was not due to Schwann cell death, because even with very high concentrations of gp120 no glia cell death was detected (Keswani *et al.*, 2003b).

### Gp120-induced direct axonal degeneration is caspase-dependent, but is independent from cell body apoptosis

In order to elucidate the mechanisms underlying direct toxicity of gp120 on sensory axons, we incubated the side chambers with gp120 and added zVAD, a general caspase inhibitor. As shown in Fig. 4C, zVAD prevented axonal toxicity caused by direct application of gp120 in the side axonal chambers. These data suggested that, in addition to activation of apoptotic pathway in the cell body compartment, there is activation of the caspase family by gp120, locally in axons. This conclusion is supported by the fact that there was loss of



**Fig. 4** Gp120 induces caspase activation locally at the axonal level. **(A)** After exposure to gp120 for 24 h, distal axons show loss of immunofluorescence for mitochondrial cytochrome c and **(B)** increased expression of activated caspase-3 (both manifestation of apoptosis). All the pictures were taken at the same image acquisition settings on the confocal microscope. **(C)** Caspase inhibitor zVAD prevented gp120 toxicity when applied locally on the axons. However, application of zVAD to the cell body when gp120 was applied to the axons did not prevent axonal degeneration. In contrast, caspase inhibitor zVAD only partially prevented axonal degeneration when it was applied to the neuronal cell bodies in the centre chamber (\*gp120 versus control,  $P < 0.05$ ; \*\*gp120 on the axons versus gp120 + zVAD on the axons,  $P < 0.05$ ). Scale bar, 80  $\mu$ m.

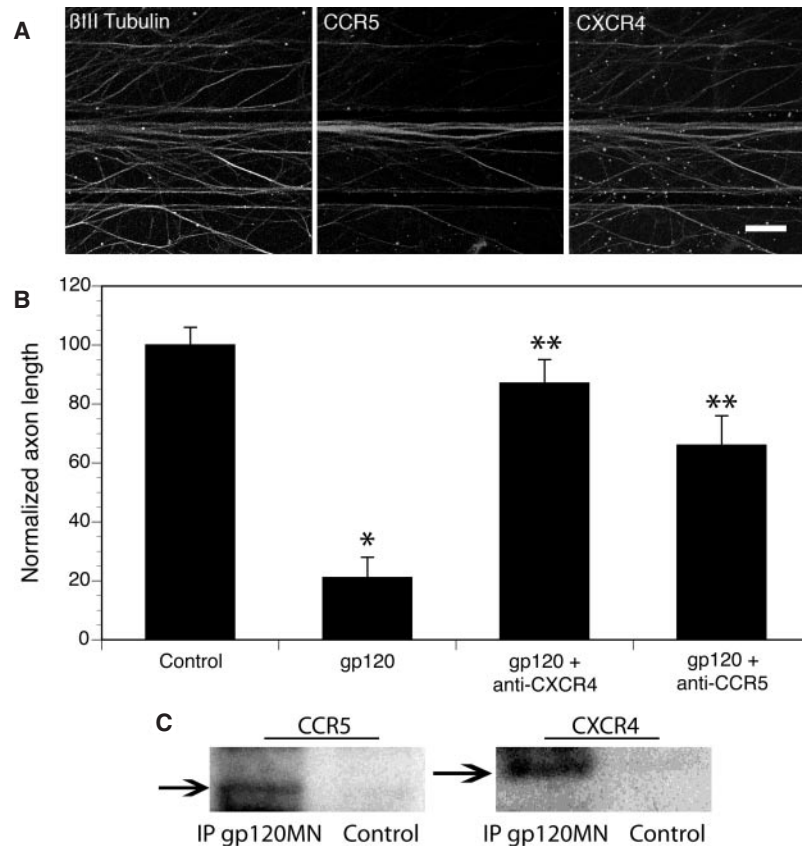
cytochrome c staining from axons, followed by activation of caspase-3, as demonstrated by specific antibody staining against activated caspase-3 (Fig. 4B). Activation of the apoptotic cascade at the level of neuronal cell body, following the administration of gp120, had been reported previously (Keswani *et al.*, 2003b). In addition, our results suggest that gp120-induced local activation of the caspase pathway in axons was not due to caspase activation in neuronal cell bodies. In fact, as shown in Fig. 4C, when zVAD was added to the centre chamber (neuronal cell bodies) at the time of application of gp120 into the side axonal chamber, there was no effect on gp120-induced local axonal toxicity.

As expected, the axonal toxicity induced by gp120 acting on cell bodies in the presence of Schwann cells was prevented

by the application of caspase inhibitor to the middle compartment, suggesting that it is resulting from neuronal apoptosis. No significant effect was observed when zVAD was added to the side chambers and gp120 was acting on cell bodies, suggesting that once the apoptotic pathway is initiated in cell bodies, it results in axonal degeneration and that it is not dependent on activation of the caspase pathway locally in the axons (Fig. 4C).

**Gp120-induced local axonal degeneration is mediated directly by chemokine receptors on axons**

HIV uses chemokine receptors, CXCR4 and CCR5, as co-receptors for entry into cells. Since we observed direct



**Fig. 5** Gp120-induced local axonal degeneration is mediated directly by chemokine receptors on axons. **(A)** Distal axons express both chemokine receptors CCR5 and CXCR4 as shown by triple immunostaining with anti- $\beta$ III tubulin, anti-CCR5 and anti-CXCR4 antibodies (63% of axons express CCR5 receptor and 94% express CXCR4). **(B)** Monoclonal antibodies anti-CXCR4 and anti-CCR5 prevented gp120 axonal toxicity when applied to the distal axons without Schwann cells (\*gp120 versus control,  $P < 0.05$ ; \*\*gp120 + aCXCR4/aCCR5 versus gp120,  $P < 0.05$ ). **(C)** Gp120<sub>MN</sub> binds to both CCR5 and CXCR4 on neurons. Immortalized sensory neuronal cell line was treated with gp120<sub>MN</sub>, and cell lysates were subjected to immunoprecipitation with antibodies anti-gp120. The immunoprecipitates were western blotted with antibodies against CCR5 and CXCR4. Scale bar, 200  $\mu$ m.

toxicity of gp120 on axons, we explored the expression pattern of chemokine receptors on axons in Campenot chambers. Triple immunolabelling of axons in the side chamber, in absence of any Schwann cells, showed that 63% of axons expressed CCR5 and 94% expressed CXCR4 (Fig. 5A). Immunoprecipitation experiments showed that both chemokine receptors could be co-immunoprecipitated by gp120 (Fig. 5C). Further examination of the role of these co-receptors for gp120 showed that both play a role in mediating gp120-induced local axonal toxicity. When blocking antibodies against each of the chemokine receptors were added to the side axonal chambers at the time of application of gp120, the toxicity of gp120 was partially blocked (Fig. 5B). The different level of protection observed with the chemokine receptor inhibitors, 87% with anti-CXCR4 and 65% with anti-CCR5, can be explained by the different expression of the chemokine receptors on axons (94% for CXCR4 and 63% of CCR5) and by the preferential selectivity of gp120<sub>MN</sub> for CXCR4.

## Discussion

As the most common neurological complication of the illness, HIV-associated sensory neuropathies result in impaired quality of life and limit the choices of available antiretroviral therapy regimens. The pathogenic mechanisms underlying these neuropathies are poorly understood. In an *in vitro* model of DSP, gp120 causes toxicity in DRG sensory neurons through two independent mechanisms of action: neuronal apoptosis mediated by the activation of Schwann cells and subsequent secondary axonal degeneration and a primary local effect on axons, by activation of caspases, resulting in axonal degeneration.

Most of the known pathways of HIV neurotoxicity in CNS imply an active role for glia cells or macrophages. In HIV dementia, production of cytokines and chemokines by astroglia and macrophages results in neuronal dysfunction and cell death. In the PNS, this indirect neurotoxicity is partially mediated by Schwann cells and involves chemokine receptors and regulated upon activation, normal T-cell expressed and

secreted (RANTES) (Keswani *et al.*, 2003b). In the current study, we describe a novel direct toxicity caused by gp120 locally on axons.

Gp120-induced axonal toxicity involves release of cytochrome c from mitochondria and activation of caspase pathway locally in the axon. However, the caspase-dependent cascade in the axon, triggered by gp120, is independent of the cell body. The axonal activation of caspase pathway by gp120 may be responsible for the clinical observation that patients infected with HIV develop a distal axonopathy and there is minimal neuronal loss in dorsal root ganglia at autopsy (Pardo *et al.*, 2001).

It has been shown that Wallerian degeneration and localized axonal degeneration induced by local deprivation of neurotrophins do not involve caspases (Finn *et al.*, 2000; Raff *et al.*, 2002; Whitmore *et al.*, 2003). However, in an animal model of brain traumatic axonal injury, cytochrome c release and caspase activation have been shown in the injured axons (Buki *et al.*, 2000). Furthermore, there is evidence of caspase activation at the axonal level in models of neurodegenerative diseases. In an *in vitro* model of Alzheimer's disease, after exposure to the pro-apoptotic agent  $\beta$ -amyloid, neurites show caspase activation and undergo degeneration before cell bodies show any sign of toxicity (Ivins *et al.*, 1998). The release of cytochrome c and activation of caspase-3 suggest that mitochondria play an essential role in this axonal degeneration process. Dysfunction in axonal mitochondria has already been implicated to play an important role in the pathogenesis of diabetic neuropathy (Feldman, 2005) and neuropathy associated with antiretroviral therapy (Dalakas *et al.*, 2001). Our findings have implications that extend beyond the mechanisms for HIV-associated neuropathies. If confirmed to be present in other distal axonopathies, such as diabetic or toxic neuropathies, local activation of apoptotic machinery in the axons may imply a more generalized role in cellular destruction pathways for members of the caspase family.

Axonal damage has been recently recognized as an important predictor of outcome in many CNS diseases, and, consequently, it is a critical target of therapeutic strategies (Medana and Esiri, 2003). In the pmn/pmn mouse, an animal model of motoneuron disease (Schmalbruch *et al.*, 1991), after the introduction of the anti-apoptotic factor, bcl-2 transgene, the motoneuron cell death is prevented, but no effect is seen on axonal degeneration, progressive weakness and final death of the animals (Sagot *et al.*, 1995).

Our results show that local glial cells associated with axons may play a neuroprotective role by counteracting the direct toxicity of gp120. Schwann cells, and glial cells in general, have an important trophic, supportive and protective role for neurons and their axons (Thippeswamy *et al.*, 2005). These observations, however, are in conflict with the role Schwann cells play in mediating the apoptotic toxicity on neuronal cell bodies. Our previous data in dissociated DRG neuronal cultures had suggested that gp120 neurotoxicity was mediated through the Schwann cells (Keswani *et al.*, 2003b). At that

time we did not know if the axonal degeneration and apoptosis of the sensory neurons were linked or not. In the current study we confirmed this observation with the experiments in the central compartment of the Campenot chambers and showed that, in response to gp120, axonal degeneration can occur via both direct axonal toxicity and indirectly through apoptosis. One potential caveat of our experiments is that cytosine arabinoside kills all dividing cells and would not differentiate between Schwann cells and macrophages or other cells that may be present in the culture system. However, we do not believe that cells other than Schwann cells are involved in this indirect neurotoxicity in this culture system because only DRG sensory neurons and Schwann cells survive in these cultures owing to the manner in which they are prepared. Previously, we had evaluated these co-cultures for the presence of macrophages and other non-neuronal cells and found none (Keswani *et al.*, 2003b).

The PNS contains several distinct glia cells, each associated with different parts of the neuron: neurons in dorsal root ganglia are covered by flattened sheet-like cells, satellite cells, which are not only morphologically but also embryogenically different from the Schwann cells ensheathing axons in nerve trunks (Jessen and Mirsky, 2005). Consequently, it is plausible to hypothesize that gp120 may activate different signalling pathways in glial cells surrounding neuronal cell bodies and in axonal Schwann cells. Elaboration of these signalling pathways can lead to new insights into disease mechanisms and perhaps novel therapeutic targets for diseases of the PNS.

In this study, we also show that gp120 protein alone, in absence of the whole infective virus, is toxic to sensory axons and that this toxicity involves chemokine receptors CXCR4 and CCR5. Of note, it has been shown that binding of gp120 to CXCR4, in the absence of CD4 signal, induces cytochrome c release and caspase activation (Roggero *et al.*, 2001), similar to what we observed in axons after exposure to gp120. Others have shown that sensory neurons in humans express CXCR4 and CCR5 receptors and that activation of these receptors can occur independently of association with CD4 (Hesselgesser *et al.*, 1997; Klein *et al.*, 1999; Boutet *et al.*, 2001). Activation of chemokine receptors on sensory neurons by gp120 produces neuronal excitation and pain (Oh *et al.*, 2001). Binding of natural ligands to chemokine receptors on hippocampal neurons has a pro-survival effect, whereas binding of HIV-1 to these receptors induces apoptosis (Meucci *et al.*, 1998). In fact, SDF-1 $\alpha$ , the natural ligand of CXCR4, and gp120 activate different cellular pathways; both are potent activators of MAP kinases, but only SDF-1 $\alpha$  stimulates Akt and anti-apoptotic factors (Khan *et al.*, 2004). Recently, it has been shown that gp120-induced apoptosis in cerebellar granular cells is prevented by AMD3100, a CXCR4 inhibitor (Bachis and Mocchetti, 2004). Similarly, in our system, blocking the CXCR4 chemokine receptor by monoclonal antibody prevented the axonal toxicity induced by gp120.

We also observed a partial prevention of axonal toxicity induced by gp120<sub>MN</sub> by monoclonal antibodies directed against the CCR5 chemokine receptor. HIV expressing gp120<sub>MN</sub> is a T-tropic virus, and uses CXCR4 as co-receptor in association with CD4 to enter the host cell. Co-receptor selectivity is an important determinant of viral diversity and pathogenesis (Shaheen and Collman, 2004). Our results raise the possibility that in neurons, in the absence of entry receptor CD4, gp120 loses the selectivity for chemokine receptors, and becomes capable of interacting with both chemokine receptor types. This conclusion is supported by the fact that co-immunoprecipitation experiments with anti-gp120 show binding of gp120 to both chemokine receptors.

In recent years, several chemokine receptor antagonists have been developed as promising HIV entry inhibitor drugs. Our results in peripheral neuropathy and the data of Bachis and Mocchetti in cerebellar cells show that chemokine inhibitors can have potential therapeutic utility in treating some of the major complications of AIDS, beyond their ability to prevent HIV infection.

In summary, our results suggest that in a model of HIV-associated peripheral neuropathy, the viral envelope protein gp120 causes axonal injury by binding to chemokine receptors. This toxicity involves two distinct pathways; one engages apoptotic neuronal cell death and the other uses direct local axonal toxicity, independent from the cell body, by activation of the caspase pathway. Further examination of this pathway may yield novel targets for preventing axonal degeneration in peripheral neuropathies. Our results also support the development and potential use of chemokine receptor inhibitors for prevention of HIV-associated neuropathies.

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