**Journal of NeuroVirology,** 15: 238–248, 2009

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280902939748

informa
healthcare

# Nef and cell signaling transduction: a possible involvement in the pathogenesis of human immunodeficiency virus–associated dementia

Valeria Bergonzini,[1] Arianna Calistri,[1] Cristiano Salata,[1] Claudia Del Vecchio,[1] Elena Sartori,[1] Cristina Parolin,[2] and Giogio Palù[1]

[1]*Department of Histology, Microbiology, and Medical Biotechnologies, Division of Microbiology and Virology; and* [2]*Department of Biology, University of Padova, Padova, Italy*

**Although the introduction of highly active antiretroviral therapy (HAART) has resulted in a significant decrease of acquired immunodeficiency syndrome (AIDS) morbidity and mortality, the prevalence of human immunodeficiency virus (HIV)-associated dementia (HAD) has actually risen, due to the increasing life expectancy of the infected subjects. To date, several aspects of the HAD pathogenesis remain to be dissected. In particular, the viral-cellular protein interplay is still under investigation. Given their specific features, two viral proteins, Tat and Nef, have been mainly hypothesized to play a role in HIV neuropathology. Here we show that HIV-1 Nef has an effect on the transcriptional levels of a cellular protein, anaplastic lymphoma kinase (ALK), that is preferentially expressed in the central and peripheral nervous system at late embryonic stages. By its overexpression along with Nef, the authors demonstrate ALK ability to influence, at least in the U87MG astrocytic glioma cells, the mytogen-activated protein kinase (MAP-K)–dependent pathway. Moreover, although in the absence of a physical direct interaction, Nef and ALK activate matrix metalloproteinases (MMPs), which are likely to contribute to blood-brain barrier (BBB) damage in HAD. Finally, in the *in vitro* model of glioblastoma cells adopted, Nef and ALK show similar effects by increasing different cytochines/chemokines that may be relevant for HAD pathogenesis. If confirmed *in vivo*, these data may indicate that, thanks to its ability to interfere with specific cellular pathways involved in BBB damage and in central nervous system (CNS) integrity, Nef, along with specific cellular counterparts, could be one of the viral players implicated in HAD development.** *Journal of NeuroVirology* (2009) **15,** 238–248.

**Keywords:** Nef, ALK, signaling pathways, MAP-K, MMP

## Introduction

Human immunodeficiency virus (HIV)-1 enters the brain at an early stage after systemic infection and resides primarily in macrophages/microglia and astrocytes (Kaul *et al*, 2001). HIV-1 entry in the central nervous system (CNS) determines a local immune system activation but neurological symptoms are usually absent. Moreover, the neuronal dysfunction present during seroconversion is frequently transient and not lethal. A variety of HIV-induced lesions of the CNS have been described, including axonal damage and neuronal loss of variable severity (Adle-Biassette *et al*, 1995; An *et al*, 1996; Gray *et al*, 2001; Kitayama *et al*, 2008). However, in contrast with other viral encephalites, HIV-1 is rarely present in neurons and the infection is not productive (Bagasra *et al*, 1996; Torres-Muñoz

Address correspondence to Giorgio Palù, Department of Histology, Microbiology, and Medical Biotechnologies, University of Padova, Via A. Gabelli 63, 35121 Padova, Italy. E-mail: giorgio.palu@unipd.it

This work was supported by grants from Regione Veneto, MIUR (PRIN-2005), Istituto Superiore di Sanità (Rome AIDS projects grant no. 30G.24 and 40F.57), and University of Padova (Ex 60% and ATENEO 2006) to A.C., C.P. and G.P.

Received 22 December 2008; revised 26 Feb 2009; accepted 21 March 2009.

*et al*, 2001; Trillo-Pazos *et al*, 2003). Thus, the mechanism behind the neuronal loss is still under investigation, and because HIV-1 neuronal infection in HIV-1–associated dementia (HAD) has not been documented, other mechanisms must be implicated in the neuropathological damage (Bergonzini *et al*, 2004; Fischer-Smith and Rappaport, 2005; Gonzales-Scarano and Martin-Garcia, 2005; Peruzzi *et al*, 2005). It has been reported that after introduction of the highly active antiretroviral therapy (HAART), the annual incidence of HAD started to decrease, but due to the increased numbers of individuals living with HIV/AIDS (acquired immunodeficiency syndrome), the prevalence of HAD is overall rising (McArthur, 2004).

The HIV-1 entry in the CNS is probably helped by the blood-brain barrier (BBB) damage present in HAD patients. In particular, during HAD, the expression levels of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs) are altered (Conant *et al*, 1999; Suryadevara *et al*, 2003). Indeed, in HAD patients, high levels of MMPs can be detected in the cerebrospinal fluid (Sporer *et al*, 1998; Conant *et al*, 1999). On the other hand, the TIMP-1 levels are significantly reduced, thus resulting in an impairment of the astrocytes ability to control the negative effects of MMPs on BBB damage (Suryadevara *et al*, 2003). For this reason, cytokines and chemokines that can control the MMP activation, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), may play a crucial role in HAD pathogenesis (Chakraborti *et al*, 2003).

Among HIV-1 proteins, Nef has been reported to induce BBB disruption in the rat model through the activation of MMPs (Sporer *et al*, 2004). Nef is a *N*-myristoylated accessory protein, expressed early and abundantly during HIV-1 infection. Nef localizes at the plasma membrane in infected cells and within the virion (Fackler *et al*, 1997; Hanna *et al*, 2004). Nef, thanks to the homology to SH3 domain–containing cellular proteins such as tyrosine kinases, transcription factors, and adaptor cellular molecules, affects different cell signaling pathways (Greenway *et al*, 2003). In particular, Nef has been shown to interact with p53 (Greenway *et al*, 2002), thus decreasing the half-life of this cellular protein and its DNA-binding ability. Moreover, binding to the phosphoinositide 3-kinase (PI-3K) (Linnemann *et al*, 2002) and activation of the mitogen-activated protein (MAP) kinase (MAP-K) pathway have been also reported (He *et al*, 2004). In this way, Nef is likely (i) to promote HIV-1 evasion from the immune system; (ii) to extend the life span of infected cells through the control of apoptosis; (iii) to regulate the expression of the key cellular factors necessary for virus replication; and (iv) to increase viral infectivity. Furthermore, Nef is toxic to neurons and glia, thus potentially contributing to the neuronal damage detected in HAD (Trillo-Pazos *et al*, 2000; van Marle *et al*, 2004). Persistently HIV-1–infected astrocytes have been reported to express Nef protein *in vivo* and *in vitro* (Ranki *et al*, 1995; van Marle *et al*, 2004). Furthermore, among HIV-1 proteins, Nef is preferentially expressed in infected astrocytes, which would thus represent an additional viral reservoir during rapidly progressive dementia (Ranki *et al*, 1995).

It has been reported in a large-scale expression array study in astrocytic cells (Kramer-Hämmerle *et al*, 2005) that Nef is able of activating different cell signaling pathways involved in CNS homeostasis, such as the signal transducer and activator of transcription 3 (Stat3). Thus, we decided to analyze different cellular proteins that share with Nef these transduction pathways. Among them, we focused our attention on the anaplastic lymphoma kinase (ALK), which plays a significant role in the brain tissue homeostasis and development. ALK is a putative receptor-type tyrosine kinase that is expressed preferentially in the central and peripheral nervous systems at late embryonic stages (Iwara *et al*, 1997; Morris *et al*, 1997). ALK plays different roles during neuronal development, such as cell proliferation, differentiation, cell survival, and synapse formation (Pulford *et al*, 2004). ALK was originally identified as an oncogene activated in a subtype of a non-Hodgkin lymphoma, named anaplastic large cell lymphomas (ALCLs), characterized by the chromosomal translocation t(2;5) (Morris *et al*, 1995; Shiota *et al*, 1994, 1995). In this context, ALK coding sequence is fused to the 3′ end of nucleophosmin (NPM), generating a fusion gene product, $p80^{NPM\text{-}ALK}$ (Morris *et al*, 1995; Shiota *et al*, 1995). ALK is also expressed in other tumors, such as neuroblastoma (Lamant *et al*, 2000). In a model of human neuroblastoma cell line, SK-N-SH (Souttou *et al*, 2001; Motegi *et al*, 2004), ALK has been shown to transmit both mitogenic and differentiation signals (neurite outgrowth) through the activation of the MAP-K transduction pathway. Furthermore, the ALK overexpression induces the constitutive phosphorylation of Stat-3, both in transfected cells and in human primary ALCL cells (Zamo *et al*, 2002). This feature is extremely important in the CNS because Stat3 is highly expressed in this compartment and can also control MMP activation (Tsareva *et al*, 2007).

In the present work, in order to contribute to the dissection of HAD pathogenetic mechanisms and taking into consideration cell signaling transduction pathways, we intended to analyze whether Nef and ALK could influence each other's activities, thus contributing to the HAD development and/or progression. Our data show that Nef and ALK have a synergic effect on MAP-K pathway, which is important for the regulation of MMP activation (Wang *et al*, 2002; Jung *et al*, 2006) and for maintaining CNS integrity (Morooka and Nishida, 1998;

Kaplan and Miller, 2000). Moreover, we show that Nef and ALK have a cumulative effect in the modulation of different cytochines/chemokines, such as the stromal cell–derived factor-1α (SDF-1α) and the regulated on activation normal t expressed and secreted (RANTES) molecule, that are altered in HAD patients (Kelder *et al*, 1998; Cota *et al*, 2000), along with IL-1β and TNF-α, that can control MMP activation (Chakraborti *et al*, 2003; Tyor *et al*, 2003; Brabers and Nottet, 2006). We also demonstrate that Nef and ALK do not physically interact, but that ALK expression could be influenced by Nef, at the transcriptional level. Overall, our data indicate that, thanks to its ability to interfere with specific cellular pathways involved in BBB damage and CNS integrity, Nef could be one of the viral players implicated in HAD development.

## Results

*Nef and ALK do not physically interact but ALK expression is influenced by Nef*

It has been previously demonstrated in a large-scale expression array study performed in astrocytic cells (Kramer-Hämmerle *et al*, 2005) that Nef is able of altering cell signaling pathways involved in CNS homeostasis, in a way similar to ALK. Hence, to verify whether Nef and ALK could cross-interfere on each other common signaling pathways, we overexpressed the two proteins in U87MG astrocytic glioma cells. Firstly, we analyzed the subcellular localization of ALK and Nef using confocal microscopy. U87MG cells were transfected with plasmid expressing Nef (pSRα/Nef-HA) and ALK (pcDNA3.1/ALK-Flag) proteins, fused to hemagglutinin (HA) and Flag epitopes, respectively. As shown in Figure 1A (panel 3), Nef and ALK colocalize on the U87MG cell membrane. This finding is in agreement with data obtained by expressing the two proteins alone, thus indicating that they do not interfere with each other's subcellular localization (Fackler *et al*, 1997; Morris *et al*, 1997).

On the other hand, because Nef and ALK colocalize on the U87MG cell surface, we checked whether they could physically interact. To this aim, we transfected U87MG cells with the constructs expressing Nef-HA and ALK-Flag and we performed coimmunoprecipitation assays. However, we were not able to detect a significant interaction between the two proteins, in any of the experimental conditions tested (data not shown).

Thus, we asked whether Nef could influence ALK expression in Nef-transfected cells. Indeed, we were able to show that, in the presence of Nef, the ALK transcript levels are enhanced (Figure 1B). This result seems to suggest that Nef can alter the expression of cellular proteins involved in the control of CNS integrity.

*Nef and ALK overexpression effect on MAP-K–dependent signaling pathway*

Because our data suggest that Nef can alter ALK expression and given the ability of Nef and ALK to use common signal transduction pathways (Souttou *et al*, 2001; Zamo *et al*, 2002; He *et al*, 2004; Kramer-Hämmerle *et al*, 2005), we hypothesized that these two proteins might interfere with each other's function (Figure 2). Moreover, it has been demonstrated that Nef and ALK are capable of positively influencing MAP-K phosphorylation (Souttou *et al*, 2001; He *et al*, 2004), thus we decided to test the effect of the combination of these two proteins on the MAP-K phosphorylation in the U87MG cell line. To this aim, cells were opportunely transfected and the presence in the lysates of the MAP-K phosphorylated forms was investigated by Western blot analysis (Figure 3A). In parallel, the correct expression of ALK and Nef in the same samples was checked by employing antibodies directed against the Flag and HA epitopes, respectively (Figure 3B and C). In our *in vitro* model, although ALK by itself does not alter MAP-K phosphorylation levels, compared to the control (Figure 3A, lanes 1 and 2), these are slightly enhanced by Nef expression (Figure 3A, lane 4) and increased even more when both proteins are coexpressed (Figure 3A, lane 3). These findings suggest a possible ALK/Nef cumulative effect on MAP-K signal transduction pathway in glioblastoma cells, maybe mediated by the positive effect of Nef on endogenous ALK transcriptional levels.

*Nef and ALK overexpression effect on gelatinase activation*

It is well known that the MMPs are one of the downstream substrates of MAP-K activity (Wang *et al*, 2002; Chakraborti *et al*, 2003; Jung *et al*, 2006). Interestingly, MMPs are involved in BBB damage, which has a relevant role in the HAD pathogenesis. Thus, because our data show that MAP-K pathway is altered by Nef and ALK combined overexpression, we analyzed, in this context, the gelatinase functionality, which is a marker of MMP activation. To this end, we performed a zymography assay, in which the gelatinase activity is visualized by the degradation of specific substrates in a gelatin-containing sodium dedecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. As reported in Figure 4A lane 2, gelatinases resulted active in U87MG cells transfected with pSRα/Nef-HA. In order to verify whether MMP activation was specific for neural cell lines, we expressed Nef in the SK-N-SH neuroblastoma cell line, and in the epithelioid 293T cell line. In this context, whereas Nef-mediated gelatinase activity was still present in SK-N-SH (Figure 4B, lane 4), in the case of 293T cell line, no difference could be observed between mock-transfected and Nef-expressing cells (Figure 4C, lanes 5 and 6, respectively). Moreover, when U87MG cells were

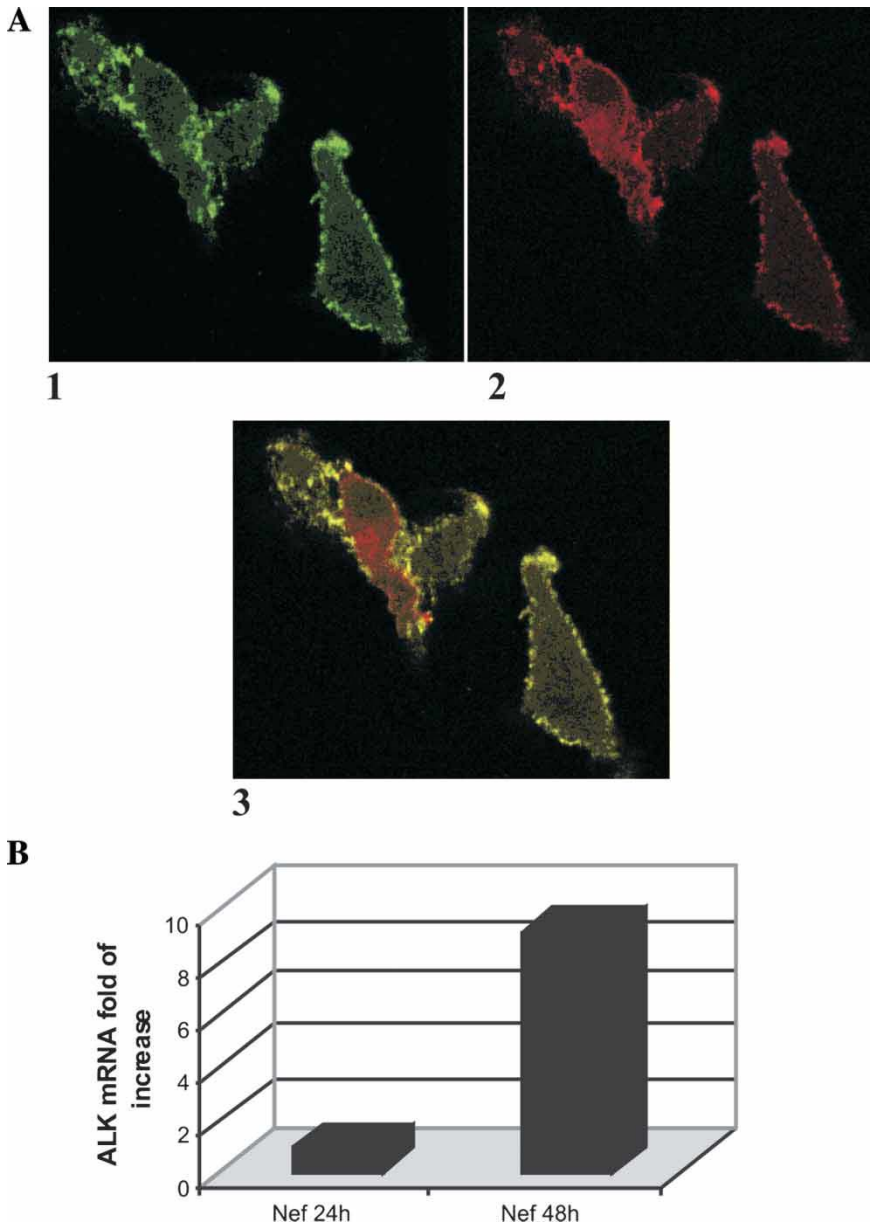


**Figure 1** Nef and ALK expression in U87MG cells. (**A**) In order to analyze Nef and ALK intracellular localization, immunofluorescence confocal laser scanning analysis was performed in U87MG cells transfected with pSRα/Nef-HA in combination with pcDNA3.1/ALK-Flag. Briefly, 48 h post transfection, cells were fixed in paraformaldeyde 4% to assess the cellular localization and incubated with primary antibodies directed against Flag and HA tags; secondary antibody were FITC and TRITC conjugated, respectively. (1) ALK-Flag (*green*), (2) Nef-HA (*red*), (3) overlay (*yellow*) of ALK-Flag and Nef-HA. Original magnification 63× . (**B**) Real-time PCR analysis of ALK mRNA expression in U87MG cells 24 and 48 h post transfection with pSRα or pSRα/Nef-HA. RNA was extracted by Trizol. Results are expressed as fold change calculated with respect to pSRα-transfected cells, after normalization against RNA polymerase II expression levels. These data are representative of three independent experiments showing similar results.

transfected with pcDNA3.1/ALK-Flag alone (Figure 4D, lane 2) or in combination with the pSRα/Nef-HA plasmid (Figure 4D, lane 3), gelatinase activity was clearly visible in both cases, with a slight cumulative effect when both proteins were present, as shown by the densitometric analysis (Figure 4E). Overall, our data indicate that the effect of Nef and ALK on gelatinase is specific for cells of CNS origin.

*Nef and ALK overexpression effect on cytochine/chemokine mRNA expression*

During chronic inflammation processes, such as HAD, cytokine and chemokine regulation may play an important role, by altering MMP activation. Thus, we investigated the effect of Nef and ALK, alone or in combination, on the expression levels of IL-1β and TNF-α. Indeed, both these cytokines are capable of inducing gelatinase expression (Chakraborti *et al*,

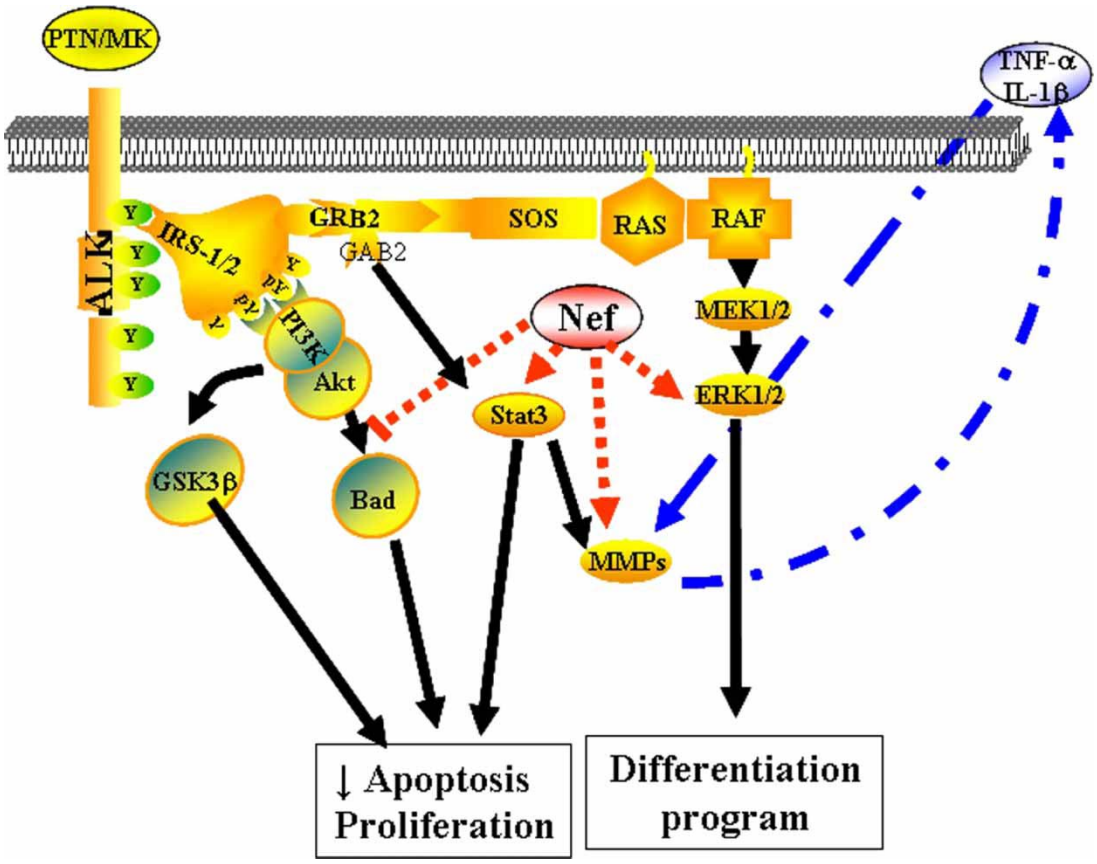


**Figure 2** Schematic representation of the ALK signal transduction pathway and possible common pathways to Nef.

2003). Furthermore, we analyzed the mRNA levels of SDF-1α and RANTES, which are altered in HAD patients and whose receptor expression is influenced by Nef (Kelder *et al*, 1998; Cota *et al*, 2000). To this end, U87MG cells were transfected with plasmids expressing Nef-HA and/or ALK-Flag. The empty vector pSRα was employed as a control. Fourty-eight hours later, the cells were lysed and the total mRNA was extracted and assayed by a real-time polymerase chain reaction (PCR) analysis. Our data show that IL-1β and TNF-α mRNAs were modulated by ALK and Nef (Figure 5). In general, a cumulative effect on the total amount of the mRNAs expression was found when ALK and Nef were expressed in combination. It is worthy to note that RANTES mRNA did not seem to be up-regulated by Nef, but the cumulative effect due to the presence of both ALK and Nef was still observed. By contrast, coexpression of ALK and Nef did not influence mRNA level of SDF-1α (Figure 5). Altogether, our data indicate that some of the cytokines/chemokines that are involved in CNS integrity and HAD pathogenesis are positively influenced by ALK and Nef expression.

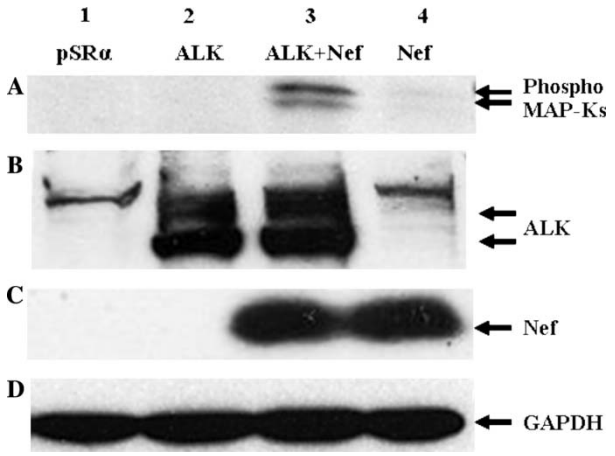


**Figure 3** Effect of Nef and ALK expression on MAP-K phosphorylation. U87MG cells were transfected with either pSRα (lane 1), pcDNA3.1/ALK-Flag (lane 2), pcDNA3.1/ALK-Flag along with pSRα/Nef-HA (lane 3), or pSRα/Nef-HA (lane 4). Twenty-four hours post transfection, 40 μg of whole-cell lysates were loaded on a SDS-PAGE gel and a Western blotting analysis against (**A**) phospho MAP-Ks, (**B**) Flag tag to detect ALK expression, (**C**) HA tag to detect Nef expression, and (**D**) GAPDH, as loading control, was performed.

## Discussion

HIV-1 enters the brain early after systemic infection, and resides primarily in macrophages/microglia and astrocytes. In a subset of patients, CNS HIV-1 infection evolves into encephalitis during the late stages

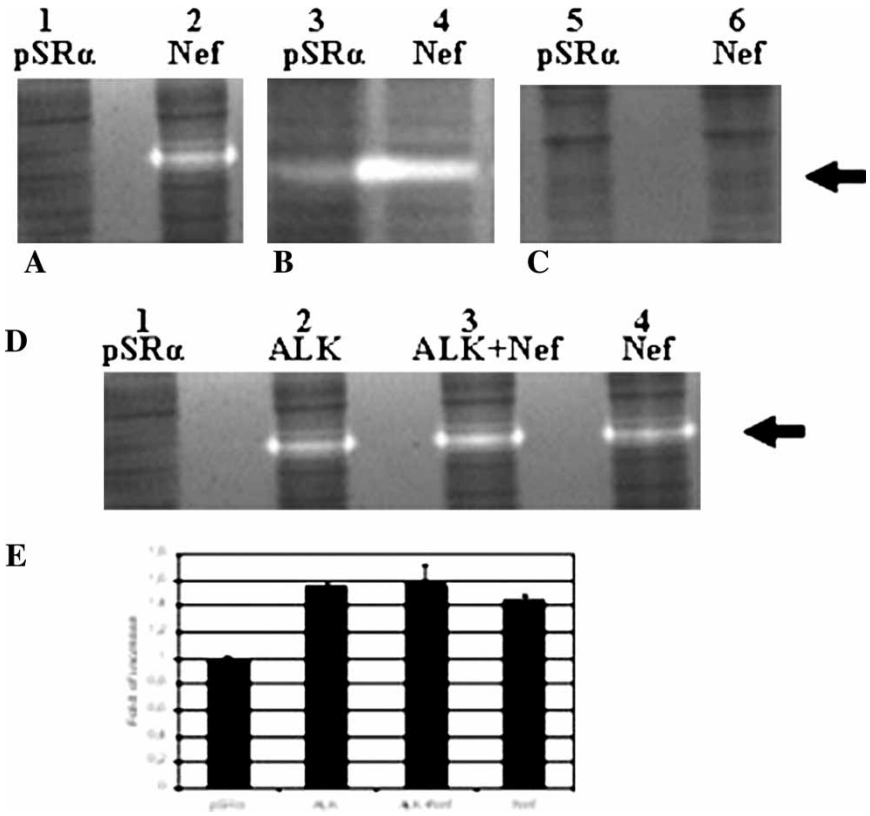


**Figure 4** Detection of gelatinase activity by zymography performed on protein extracts from (**A**) U87MG, (**B**) SK-N-SH, and (**C**) 293T cells, with pSRα (lanes 1, 3, and 5) or a construct expressing an HA-tagged version of Nef (lanes 2, 4, and 6). Forty micrograms of whole-cell lysates were loaded. (**D**) The result of a representative zymography assay performed in order to analyze the gelatinase activity in protein extracts from U87MG cells transfected with either pSRα (lane 1), pcDNA3.1/ALK-Flag (lane 2), pcDNA3.1/ALK-Flag along with pSRα/Nef-HA (lane 3), or pSRα/Nef-HA (lane 4). Forty micrograms of whole-cell lysates were loaded. (**E**) Densitometric analysis of three independent zymography assays. The mean of the fold of increase with respect to the pSRα-transfected cells are reported along with the relative standard deviations.

of systemic infection, a status clinically known as HAD. HAD is characterized by CNS alterations, and in particular by damage and loss of neuronal function, as a consequence of molecular mechanisms that are mainly unknown. The present study was addressed to contribute to the understanding of HAD pathogenesis, taking into consideration viral protein–mediated alteration of cell signaling transduction pathways that are known to be involved in CNS integrity. In this context, we focused our attention on the viral protein Nef. Indeed, it has been reported that Nef is present in the astrocytes of patients with rapidly progressive HAD (Ranki *et al*, 1995; van Marle *et al*, 2004). Furthermore, Nef is capable of altering the activation of MAP-K (He *et al*, 2004; Kramer-Hämmerle *et al*, 2005), MMPs (Sporer *et al*, 2004), and Stat3 (Kramer-Hämmerle *et al*, 2005), which are important for brain tissue homeostasis and integrity. These features are shared by ALK, a protein that may play a role as a direct or indirect mediator of Nef-induced alterations in the brain, contributing to HAD pathogenesis. Indeed, ALK is involved in neuronal differentiation and in CNS normal development and function (Morris *et al*, 1997; Pulford *et al*, 2004), through the modulation of the MAP-K transduction pathway (Souttou *et al*, 2001; Motegi *et al*, 2004). Furthermore, ALK induces Stat3 phosphorylation (Zamo *et al*, 2002), which is important for neuronal regeneration, and can control MMP expression (Tsareva *et al*, 2007), as reported for Nef in a rat model (Sporer *et al*, 2004).

In order to test the hypothesis that ALK and Nef could cooperate in HAD pathogenesis, firstly we selected the U87MG astrocytic glioma cell line as an *in vitro* model for our study, then we analyzed a possible direct interaction between the two proteins. We demonstrated that although Nef and ALK colocalize at the level of the plasma membrane, they do not physically interact. However, a possible cross-interference on each other's cell signaling transduction pathways could not be excluded. Indeed, we were able to show that Nef can alter ALK expression at the transcriptional level. Because Nef does not appear to affect the amount of ALK when the protein is expressed under the human cytomegalovirus intermediate-early (HCMV IE) promoter (pcDNA3.1/ALK-Flag), it seems likely that the viral protein acts at the level of ALK natural promoter. In order to clarify

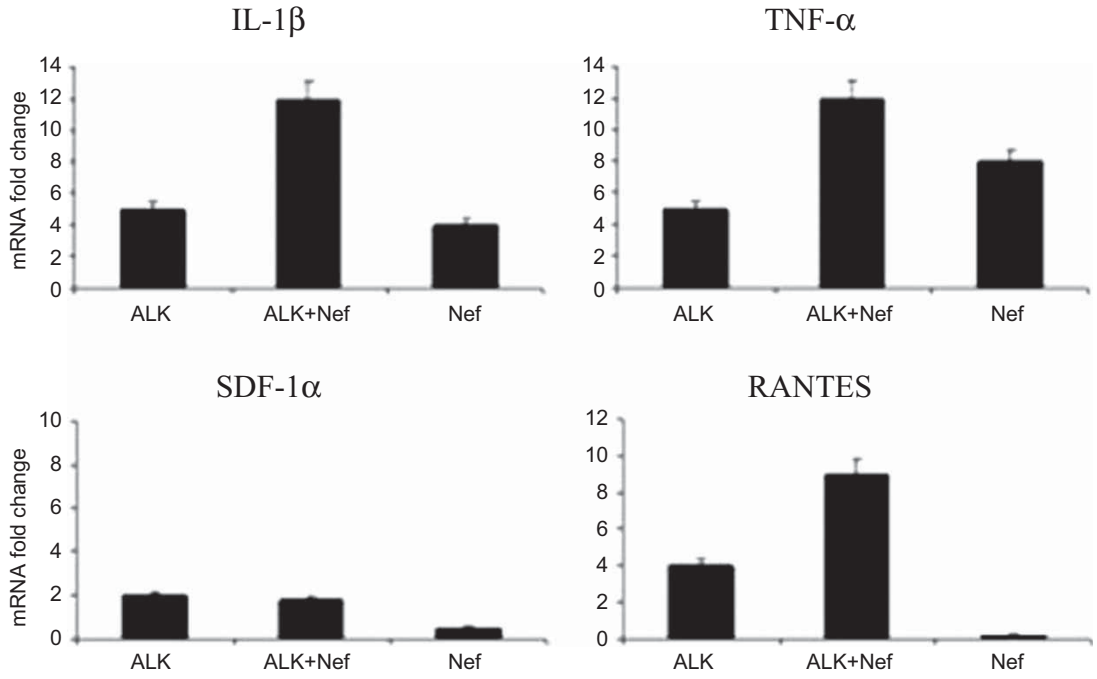


**Figure 5** Nef and/or ALK effects on cytokine and chemokine expression. U87MG cells were lysed 48 h post transfection with either pSRα, pcDNA3.1/ALK-Flag, pcDNA3.1/ALK-Flag along with pSRα/Nef-HA, or pSRα/Nef-HA. After retrotranscription, a real-time PCR analysis was performed as described in Materials and Methods. Results are expressed as mean fold change calculated with respect to pSRα-transfected cells, after normalization against RNA polymerase II expression levels. Error bars indicate standard deviations.

whether Nef could have a role in HAD pathogenesis and whether this phenomenon could be mediated by ALK, we focused our attention on signaling pathway that control the CNS integrity. Firstly, we analyzed the level of MAP-K phosphorylation in Nef- and/or ALK-expressing U87MG cells. A clear effect on this respect was observed in cells overexpressing both proteins. This finding is interesting in that MAP-K phosphorylation can modulate MMP activity, which is related to the BBB damage. Indeed, by zymography assay, we demonstrated a direct effect of ALK and Nef on gelatinase activation (Figure 4D, lanes 2 and 4 respectively), which is slightly enhanced when both proteins are present (Figure 4E). Furthermore, the Nef-mediated gelatinase induction is present also in the neuroblastoma SK-N-SH cell line, whereas it is absent in epithelioid cells, such as the 293T cells. This finding seems to suggest that Nef-related effect on gelatinase activity may be specific for neural cells. It is worthy to note that 293T cells do not express endogenous ALK (data not shown). Thus Nef, by modulating specific cellular proteins such as ALK, could influence important signaling pathways in neural cells. Indeed, the ability of ALK to activate Stat3 has been previously reported (Zamo *et al*, 2002) and it has been suggested that this effect could mediate gelatinase activation (Tsareva et al, 2007). Noteworthy, this feature is detectable *in vivo* only when ALK is overexpressed, for example in anaplastic large cell lymphoma (ALCL). In fact, it has been shown that in the latter context, ALK's fine mechanism of transcriptional regulation is lost due to the chromosomal translocation t(2;5), which generates a fusion gene product between ALK and nucleophosmin (NPM) (Morris *et al*, 1995; Shiota *et al*, 1995; Pulford *et al*, 2004), making ALK expression under the control of a constitutively activated promoter. Our data would suggest that the same loss of control mechanism could be induced in cells expressing the viral protein Nef and this may affect cell signaling pathways that control the CNS homeostasis. It has to be mentioned that from our results, the contribution of Nef-mediated ALK overexpression in U87MG cells transfected with the cellular protein–expressing construct does not appear to be strong. Indeed, a cumulative effect of the two proteins is clearly visible only in the case of MAP-K phosphorylation and of some cytokines/chemokines (see below). However, this finding may be due to a saturation of the system linked to the overexpression of exogenous ALK under the transcriptional control of a strong promoter (HMCV IE promoter). Because our data and literature reports (Souttou *et al*, 2001) demonstrate that ALK is capable of interfering with cellular pathways influenced also by Nef, if the ability of Nef to influence the expression of this specific cellular protein would be further confirmed, it could represent a mechanism by which Nef contributes to some of the HAD hystopathological findings. Indeed, it has already been demonstrated in podocytes that Nef is able to induce

a de-differentiation program, mediated by MAP-K phosphorylation, which can at least partly explain the failure of normal kidney functions, such as the glomerular filtration (Lu *et al*, 2007). Because there is a significant homology in the cytoplasm structure of podocytes and neurons (Kobayashi, 2002; Kobayashi *et al*, 2004) and the same cell signaling pathway is clearly altered by Nef in neural cells, a similar mechanism could be envisioned also in the case of the latter cell type. These conclusions are further supported by our evidence that Nef and ALK are capable of stimulating cytokines/chemokines that can control MMP activation.

Although our results need to be further confirmed in other neural cell models as well as *in vivo,* overall our data seem to suggest that the Nef-mediated alteration of specific cell signaling transduction pathways may contribute to HAD progression. Moreover, cellular proteins such as ALK could be involved in this phenomenon, through the resulting overexpression in Nef-positive cells, and are capable of interfering, like Nef, with pathways that control CNS integrity.

## Materials and methods

*Cell lines*
U87MG and 293T cells were grown in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA), whereas SK-N-SH cells were grown in minimum essential medium a (MEMα) (Invitrogen), both supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Invitrogen).

*Plasmids and cell culture transfections*
We performed transient transfections using Lipofectamine 2000 reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, cells were plated at a density of $1.5 \times 10^5$ cells/well in 6-well plates 24 h before transfection. Each well received 1.5 μg of exogenous DNA. Five hours later, the medium was changed to fresh medium with FBS and cells were lysed 24 or 48 h post transfection.

In addition to the appropriate empty vector (pSRα), the following plasmids were employed:

- pSRα/Nef-HA, kindly provided by Dr. Göttlinger, University of Massachusetts Medical School, Worcester, MA, USA, expresses an HA-tagged version of HIV-1 LAI (subtype B) Nef (Dorfman *et al*, 2002);
- pcDNA3.1/ALK-Flag was obtained by cloning into the XhoI/EcoRI sites of the pcDNA3.1 plasmid the Flag-tagged ALK coding sequence amplified by PCR starting from the plasmid pBluescript II KS (+)/ALK, kindly provided by Dr. Morris, St. Jude Children's Research Hospital, Memphis, TN, USA (Morris *et al*, 1997).

*RNA extraction and real-time PCR analysis*
At the appropriate time post transfection, cells were harvested and total RNA was extracted using the Qiagen Total RNA Kit (Qiagen, Milan, Italy). After treatment with RNase-free DNase (Roche Diagnostics, Manneheim, Germany), 3 μg of total RNA were PCR amplified, after retrotranscription, using random primers (PE Applied Biosystem, Milan, Italy). The reverse transcription reaction was carried out on 100 μl of final volume according to the manufacturer's instructions (PE Applied Biosystem). The real-time PCR reaction was carried out using fluorogenic probes or SYBR green as previously described (Salata *et al,* 2009) and employing an ABI PRISM 7700 sequence detection system. To normalize the RNA amount of the extracted samples, a real-time PCR analysis of the human RNA polymerase II cDNA was carried out. Gene expression was measured by quantification of cDNA corresponding to the target gene relative to a calibrator sample serving as a physiological reference. The calibrator sample was the cDNA obtained from the cells transfected with the vector control (pSRa). All the quantifications were also normalized to the endogenous control (RNA polymerase II), to account for the variability in the initial concentration, the quality of the total RNA, and the conversion efficiency of the reverse transcription reaction. All the experiments were conducted at least in triplicate and final quantification was carried out as previously described (Salata et al, 2009). The primers and probes employed are reported in Table 1.

*Protein extraction and Western blot analysis*
Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% [*w/v*] IGEPAL CA-630 [Sigma-Aldrich, St Louis, MO], 0.1% [*w/v*] sodium deoxycholate [Sigma-Aldrich], 4 mM EDTA), with protease inhibitors (Complete; Roche, Basel, Switzerland) and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 2.5 mM $Na_3OV_4$). The cell lysates were precleared by centrifugation at $13{,}000 \times g$ for 15 min at 4°C to remove nuclei and unlysed cells. Proteins were resolved by 10% SDS-PAGE using the Protean II System (BioRad Laboratories, Hercules, CA). After electrophoresis, proteins were blotted onto Hybond-C Extra membranes (Amersham, GE Healthcare, UK) using a Trans-Blot Electrophoretic Transfer Cell (BioRad Laboratories) apparatus set at 50 V for 2 h. After transfer, membranes were blocked using 5% *w/v* milk/TBS-T (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% *v/v* Tween 20) or bovine serum albumin (BSA)/TBS-T (for phospho-antibodies) for 30 min at room temperature. Blocked membranes were incubated overnight at 4°C with the rabbit anti-Flag (Santa Cruz Biotechnology, Santa Cruz, CA), the HA.11 mouse anti-HA antibody (Covance, Princeton, NJ), the mouse anti-GAPDH (Santa Cruz Biotechnology), the rabbit anti-ALK (kindly provided by

**Table 1** Real-time PCR primers and probes

| Primer/probe | Sequence | Tm | Detection |
|---|---|---|---|
| ALK Fw | 5′ CCA GAC AAC CCA TTT CGA GT 3′ | 62°C | SYBR green |
| ALK Rev | 5′ ACT GAG GAG TGT GGG GTG AC 3′ | 62°C | SYBR green |
| RNA polymerase II (RP) Fw | 5′ GAA ACG GTG GAC GTG CTT AT 3′ | 63°C | SYBR green |
| RP Rev | 5′ GGT GTC ATG GCA GGA GAG AT 3′ | 63°C | SYBR green |
| TNF-α Fw | 5′ CCC AGG GAC CTC TCT CTA ATC 3′ | 60°C | Fluorogenic probes |
| TNF-α Rev | 5′ ATG GGC TAC AGG CTT GTC ACT 3′ | 60°C | Fluorogenic probes |
| TNF-α probe | 5′ 6FAM-TGG CCC CAG GCA GTC AGA TCA TC-TAMRA 3′ | 60°C | Fluorogenic probes |
| IL-1β Fw | 5′ ACA GAT GAA GTG CTC CTT CCA 3′ | 60°C | Fluorogenic probes |
| IL-1β Rev | 5′ GTC GGA GAT TCG TAG CTG GAT 3′ | 60°C | Fluorogenic probes |
| IL-1β probe | 5′ 6FAM-CTC TGC CCT CTG GAT GGC GG-TAMRA 3′ | 60°C | Fluorogenic probes |
| SDF-1α Fw | 5′ TCA GCC TGA GCTA CAG ATGC 3′ | 60°C | SYBR green |
| SDF-1α Rev | 5′ CTT TAG CTT CGG GTC AAT GC 3′ | 60°C | SYBR green |
| RANTES Fw | 5′ AGC TAC TCG GGA GGC TAA GG 3′ | 60°C | SYBR green |
| RANTES Rev | 5′ CAA AGC CAG AGC TCA GAA CC 3′ | 60°C | SYBR green |

Dr. Morris, St. Jude Children's Research Hospital, Memphis, TN, USA), and the anti-phospho MAP-K and anti-total MAP-K (Cell Signaling Technology, Danvers, MA) diluted at the appropriate working concentration. After incubation with primary antibody, membranes were washed 3 times in TBS-T for 10 min at room temperature, incubated for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit immunoglobulin G antibody (Amersham). After chemioluminescence development with ECL Plus Western blotting detection system (Amersham), blots were exposed in Kodak BioMax Light Films (Kodak, Rochester, NY).

*Immunofluorescence*

U87MG cells were cultured on glass coverslides coated with poly-D-lysine in 6-well plates (BD Biosciences, Falcon). Forty-eight hours post transfection, cells were fixed with 4% (*w/v*) paraformaldeyde at room temperature. After blocking with 5% (*w/v*) BSA/PBS (phosphate-buffered saline) for 30 min at room temperature, cells were reacted with primary antibodies diluted at the appropriate working concentration in PBS for 2 h at 37°C, washed 4 times with PBS, and incubated with secondary antibodies, either a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Abcam, Cambridge, UK) or a Texas Red–conjugated goat anti-rabbit antibody (Abcam). Coverslips were mounted on glass slides and staining was visualized with a Leica TCS SP2 laser-scanning confocal microscope. Nef and ALK were detected with the HA.11 mouse anti-HA (Covance) and the rabbit anti-Flag (Santa Cruz Biotechnology), respectively.

*Detection of gelatinase activity*

The detection of gelatinase activity was performed by SDS-PAGE zymography. Briefly, cells were lysed in RIPA buffer, as described for the Western blot analysis, then applied on a 12% polyacrylamide gel (10×10 cm) that had been copolymerized with 0.1% (*w/v*) gelatin (Sigma-Aldrich). Stacking gel contained 5.4% polyacrylamide. Electrophoresis was carried out at 4°C for approximately 4 h at 80 V. After electrophoresis, the gel was washed 2 times for 30 min in Triton X-100 2.5% (*v/v*) in $H_2O$ (washing buffer), in order to remove SDS and to reactivate the enzyme, then incubated overnight in renaturing buffer (50 mM Tris-HCl pH7.5, 5 mM $CaCl_2$, 2.5% [*v/v*] Triton X-100). The following day, the gel was incubated in reaction buffer (50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 1 M $ZnCl_2$, 0.01% [*v/v*] sodium azide) overnight at 37°C. For the evaluation of the enzyme activity, the gel was extensively washed in distilled water, stained with Coomassie brilliant blue R-250 for 4 h and then destained for 1 h in 45% (*v/v*) methanol and 10% (*v/v*) acetic acid. Gelatinase activity was detected as a white band on a blue background and was quantified by computerized image analysis by two-dimensional scanning densitometry.

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

## References

Adle-Biassette H, Levy Y, Colombel M, Poron F, Natchev S, Keohane C, Gray F (1995). Neuronal apoptosis in HIV infection in adults. *Neuropathol App Neurobiol* **21:** 218–227.

An SF, Giometto B, Scaravilli T, Tavolato B, Gray F, Scaravilli F (1996). Programmed cell death in brains of HIV-1-positive AIDS and pre-AIDS patients. *Acta Neuropathol (Berl)* **91:** 169–173.

Bagasra O, Lavi E, Bobroski L, Khalili K, Pestaner JP, Tawadros R, Pomerantz RJ (1996). Cellular reservoirs

of HIV-1 in the central nervous system of infected individuals: identification by the combination of in situ polymerase chain reaction and immunohistochemistry. *AIDS* **6:** 573–585.

Bergonzini V, Delbue S, Wang JY, Reiss K, Prisco M, Amini S, Khalili K, Peruzzi F (2004). HIV-Tat promotes cellular proliferation and inhibits NGF-induced differentiation through mechanisms involving Id1 regulation. *Oncogene* **23:** 7701–7711.

Brabers NA, Nottet HS (2006). Role of the pro-inflammatory cytokines TNF-alpha and IL-1beta in HIV-associated dementia. *Eur J Clin Invest* **36:** 447–458.

Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T (2003). Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem* **253:** 269–285.

Conant K, McArthur JC, Griffin DE, Sjulson L, Wahl LM, Irani DN (1999). Cerebrospinal fluid levels of MMP-2, 7, and 9 are elevated in association with human immunodeficiency virus dementia. *Ann Neurol* **46:** 391–398.

Cota M, Kleinschmidt A, Ceccherini-Silberstein F, Aloisi F, Mengozzi M, Mantovani A, Brack-Werner R, Poli G (2000). Upregulated expression of interleukin-8, RANTES and chemokine receptors in human astrocytic cells infected with HIV-1. *J NeuroVirol* **1:** 75–83.

Dorfman T, Popova E, Pizzato M, Göttlinger HG (2002). Nef enhances human immunodeficiency virus type 1 infectivity in the absence of matrix. *J Virol* **76:** 6857–62.

Fackler OT, Kienzle N, Kremmer E, Boese A, Schramm B, Klimkait T, Kücherer C, Mueller-Lantzsch N (1997). Association of human immunodeficiency virus Nef protein with actin is myristoylation dependent and influences its subcellular localization. *Eur J Biochem* **3:** 843–51.

Fischer-Smith T, Rappaport J (2005). Evolving paradigms in the pathogenesis of HIV-1-associated dementia. *Expert Rev Mol Med* **7:** 1–26.

Gonzales-Scarano F, Martin-Garcia J (2005). The neuropathogenesis of AIDS. *Nat Rev Immunol* **5:** 69–81.

Gray F, Adle-Biassette H, Chretien F, Lorin de la Grandmaison G, Force G, Keohane C (2001). Neuropathology and neurodegeneration in human immunodeficiency virus infection. Pathogenesis of HIV-induced lesions of the brain, correlations with HIV-associated disorders and modifications according to treatments. *Clin Neuropathol* **4:** 146–155.

Greenway AL, Holloway G, McPhee DA, Ellis P, Cornall A, Lidman M (2003). HIV-1 Nef control of cell signaling molecules: multiple strategies to promote virus replication. *J Biosci* **28:** 323–335.

Greenway AL, McPhee DA, Allen K, Johnstone R, Holloway G, Mills J, Azad A, Sankovich S, Lambert P (2002). Human immunodeficiency virus type 1 Nef binds to tumor suppressor p53 and protects cells against p53-mediated apoptosis. *J Virol* **76:** 2692–702.

Hanna Z, Priceputu E, Kay DG, Poudrier J, Chrobak P, Jolicoeur P (2004). In vivo mutational analysis of the N-terminal region of HIV-1 Nef reveals critical motifs for the development of an AIDS-like disease in CD4C/HIV transgenic mice. *Virology* **327:** 273–286.

He JC, Husain M, Sunamoto M, D'Agati VD, Klotman ME, Iyengar R, Klotman PE (2004). Nef stimulates proliferation of glomerular podocytes through activation of Srcdependent Stat3 and MAPK1,2 pathways. *J Clin Invest* **114:** 643–651.

Iwahara T, Fujimoto J, Wen D, Cupples R, Bucay N, Arakawa T, Mori S, Ratzkin B, Yamamoto T (1997). Molecular characterization of ALK, a receptor tyrosine kinase expressed specifically in the nervous system. *Oncogene* **14:** 439–449.

Jung H, Woo MS, Kim SY, Kim WK, Hyun JW, Kim EJ, Kim DH, Kim HS (2006). Ginseng saponin metabolite suppresses phorbol ester-induced matrix metalloproteinase-9 expression through inhibition of activator protein-1 and mitogen-activated protein kinase signaling pathways in human astroglioma cells. *Int J Cancer* **118:** 490–497.

Kaplan DR, Miller FD (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* **10:** 381–391.

Kaul M, Garden GA, Lipton SA (2001). Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* **410:** 988–994.

Kelder W, McArthur JC, Nance-Sproson T, McClernon D, Griffin DE (1998). chemokines MCP-1 and RANTES are selectively increased in cerebrospinal fluid of patients with human immunodeficiency virus–associated dementia. *Ann Neurol* **44:** 831–835.

Kitayama H, Miura Y, Ando Y, Hoshino S, Ishizaka Y, Koyanagi Y (2008). Human immunodeficiency virus type 1 Vpr inhibits axonal outgrowth through induction of mitochondrial dysfunction. *J Virol* **5:** 2528–2542.

Kobayashi N (2002). Mechanism of the process formation; podocytes vs. neurons. *Microsc Res Tech* **57:** 217–223.

Kobayashi N, Gao SY, Chen J, Saito K, Miyawaki K, Li CY, Pan L, Saito S, Terashita T, Matsuda S (2004). Process formation of the renal glomerular podocyte: is there 71 common molecular machinery for processes of podocytes and neurons? *Anat Sci Int* **79:** 1–10.

Kramer-Hämmerle S, Hahn A, Brack-Werner R, Werner T (2005). Elucidating effects of long-term expression of HIV-1 Nef on astrocytes by microarray, promoter, and literature analyses. *Gene* **358:** 31–38.

Lamant L, Pulford K, Bischof D, Morris SW, Mason DY, Delsol G, Mariame B (2000). Expression of the ALK tyrosine kinase gene in neuroblastoma. *Am J Pathol* **156:** 1711–1721.

Linnemann T, Zheng YH, Mandic R, Peterlin BM (2002). Interaction between Nef and phosphatidylinositol-3-kinase leads to activation of p21-activated kinase and increased production of HIV. *Virology* **294:** 246–55.

Lu TC, He JC, Klotman PE (2007). Podocytes in HIV-associated nephropathy. *Nephron Clin Pract* **106:** 67–71.

McArthur JC (2004). HIV dementia: an evolving disease. *J Neuroimmunol* **157:** 3–10.

Morooka T, Nishida E (1998). Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC12 cells. *J Biol Chem* **38:** 24285–24288.

Morris SW, Kirstein MN, Valentine MB, Dittmer KG, Shapiro DN, Saltman DL, Look AT (1995). Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. *Science* **267:** 316–317.

Morris SW, Naeve C, Mathew P, James PL, Kirstein MN, Cui X, Witte DP (1997). ALK, the chromosome 2 gene locus altered by the t(2;5) in non-Hodgkin's lymphoma, encodes a novel neural receptor tyrosine kinase that is highly related to leukocyte tyrosine kinase (LTK). *Oncogene* **15:** 2883–2896.

Motegi A, Fujimoto J, Kotani M, Sakuraba H, Yamamoto T (2004). ALK receptor tyrosine kinase promotes cell growth and neurite outgrowth. *J Cell Sci* **117:** 3319–29.

Peruzzi F, Bergonzini V, Aprea S, Reiss K, Sawaya BE, Rappaport J, Amini S, Khalili K (2005). Cross talk between growth factors and viral and cellular factors alters neuronal signaling pathways: implication for HIV-associated dementia. *Brain Res Rev* **50:** 114–25.

Pulford K, Morris SW, Turturro F (2004). Anaplastic lymphoma kinase proteins in growth control and cancer. *J Cell Physiol* **3:** 330–58.

Ranki A, Nyberg M, Ovod V, Haltia M, Elovaara I, Raininko R, Haapasalo H, Krohn K (1995). Abundant expression of HIV Nef and Rev proteins in brain astrocytes in vivo is associated with dementia. *AIDS* **9:** 1001–1008.

Salata C, Curtarello M, Calistri A, Sartori E, Sette P, de Bernard M, Parolin C, Palù G (2009). vOX2 glycoprotein of human herpesvirus 8 modulates human primary macrophages activity. *J Cell Physiol* **219:** 698–706.

Shiota M, Fujimoto J, Semba T, Satoh H, Yamamoto T, Mori S (1994). Hyperphosphorylation of a novel 80kDa protein-tyrosine kinase similar to Ltk in a human Ki-1 lymphoma cell line, AMS3. *Oncogene* **9:** 1567–1574.

Shiota M, Nakamura S, Ichinohasama R, Abe M, Akagi T, Takeshita M, Mori N, Fujimoto J, Miyauchi J, Mikata A, Nanba K, Takami T, Yamabe H, Takano Y, Izumo T, Nagatani T, Mohri N, Nasu K, Satoh H, Katano H, Fujimoto J, Yamamoto T, Mori S (1995). Anaplastic large cell lymphomas expressing the novel chimeric protein p80NPM/ALK: a distinct clinicopathologic entity. *Blood* **86:** 1954–1960.

Souttou B, Carvalho NB, Raulais D, Vigny M (2001). Activation of anaplastic lymphoma kinase receptor tyrosine kinase induces neuronal differentiation through the mitogen-activated protein kinase pathway. *J Biol Chem* **23:** 9526–9531.

Sporer B, Koedel U, Paul R, Kohleisen B, Erfle V, Fontana A, Pfister HW (2004). Human immunodeficiency virus type-1 Nef protein induces blood-brain barrier disruption in the rat: role of matrix metalloproteinase-9. *J Neuroimmunol* **102:** 125–130.

Sporer B, Paul R, Koedel U, Grimm R, Wick M, Goebel FD, Pfister HW (1998). Presence of matrix metalloproteinase-9 activity in the cerebrospinal fluid of human immunodeficiency virus-infected patients. *J Infect Dis* **178:** 854–857.

Suryadevara R, Holter S, Borgmann K, Persidsky R, Labenz-Zink C, Persidsky Y, Gendelman HE, Wu L, Ghorpade A (2003). Regulation of tissue inhibitor of metalloproteinase-1 by astrocytes: links to HIV-1 dementia. *Glia* **44:** 47–56.

Torres-Muñoz J, Stockton P, Tacoronte N, Roberts B, Maronpot RR, Petito CK (2001). Detection of HIV-1 gene sequences in hippocampal neurons isolated from postmortem AIDS brains by laser capture microdissection. *J Neuropathol Exp Neurol* **9:** 885–892.

Trillo-Pazos G, Diamanturos A, Rislove L, Menza T, Chao W, Belem P, Sadiq S, Morgello S, Sharer L, Volsky DJ (2003). Detection of HIV-1 DNA in microglia/macrophages, astrocytes and neurons isolated from brain tissue with HIV-1 encephalitis by laser capture microdissection. *Brain Pathol* **2:** 144–154.

Trillo-Pazos G, McFarlane-Abdulla E, Campbell IC, Pilkington GJ, Everall IP (2000). Recombinant nef HIV-IIIB protein is toxic to human neurons in culture. *Brain Res* **12:** 315–326.

Tsareva SA, Moriggl R, Corvinus FM, Wiederanders B, Schutz A, Kovacic B, Friedrich K (2007). Signal transducer and activator of transcription 3 activation promotes invasive growth of colon carcinomas through matrix metalloproteinase induction. *Neoplasia* **9:** 279–291.

Tyor WR, Glass JD, Griffin JW, Becker PS, McArthur JC, Bezman L, Griffin DE (1992). Cytokine expression in the brain during the acquired immunodeficiency syndrome. *Ann Neurol* **31:** 349–360.

van Marle G, Henry S, Todoruk T, Sullivan A, Silva C, Rourke SB, Holden J, McArthur JC, Gill MJ, Power C (2004). Human immunodeficiency virus type 1 Nef protein mediates neural cell death: a neurotoxic role for IP-10. *Virology* **329:** 302–318.

Wang X, Mori T, Jung JC, Fini ME, Lo EH (2002). Secretion of matrix metalloproteinase-2 and -9 after mechanical trauma injury in rat cortical cultures and involvement of MAP kinase. *J Neurotrauma* **19:** 615–625.

Zamo A, Chiarle R, Piva R, Howes J, Fan Y, Chilosi M, Levy DE, Inghirami G (2002). Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. *Oncogene* **21:** 1038–1047.

This paper was first published online on iFirst on 19 May 2009.