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Acute exposure to ethanol potentiates human immunodeficiency virus type 1 Tat–induced Ca^{2+} overload and neuronal death in cultured rat cortical neurons

Eugen Brailoiu,[1] G Cristina Brailoiu,[1] Giuseppe Mameli,[2,3] Antonina Dolei,[3] Bassel E Sawaya,[2] and Nae J Dun[1]

[1]*Department of Pharmacology and* [2]*Department of Neuroscience and Center for Neurovirology, Temple University School of Medicine, Philadelphia, Pennsylvania, USA;* [3]*Department of Biomedical Science, University of Sassari, Sassari, Italy*

A significant number of human immunodeficiency virus type 1 (HIV-1)–infected patients are alcoholics. Either alcohol or HIV alone induces morphological and functional damage to the nervous system. HIV-1 Tat is a potent transcriptional activator of the viral promoter, with the ability to modulate a number of cellular regulatory circuits including apoptosis and to cause neuronal injury. To further evaluate the involvement of alcohol in neuronal injury, the authors examined the effect of ethanol on Tat-induced calcium responses in rat cerebral cortical neurons, using microfluorimetric calcium determination. HIV Tat protein (10 or 500 nM) elicited two types of calcium responses in cortical neurons: a fast-onset, short-lasting response and a slow-onset, sustained response. The responses were concentration-dependent and diminished in calcium-free saline. A short exposure to ethanol (50 mM) potentiated both types of calcium response, which was markedly decreased when the cells were pretreated with BAPTA-AM (20 μM). In addition, an increase in the neurotoxic effect of Tat, which was assessed by trypan blue exclusion assay, was observed. The result led the authors to conclude that alcohol exposure significantly potentiates Tat-induced calcium overload and neuronal death. *Journal of NeuroVirology* (2006) **12,** 17–24.

Keywords: HIV-1; neurotoxicity; Tat protein

Introduction

Alcohol is one of the leading drugs of abuse. Epidemiological studies show that a significant number of human immunodeficiency virus (HIV)-infected subjects consume alcohol (Samet *et al*, 2004). Alcohol abuse can negatively affect the progression of HIV infection and acquired immunodeficiency syndrome (AIDS) (Dingle and Oei, 1997). For example, alcohol consumption impairs the immune system and facilitates HIV infection. Alcohol exposure enhances HIV replication and infection in human lymphocytes (Cook *et al*, 1997; Liu *et al*, 2003), macrophages (Haorah *et al*, 2004), and oral epithelial cells (Chen *et al*, 2004; Zheng *et al*, 2004). Further, alcohol abuse in HIV-infected individuals is associated with an impairment of neuropsychological functions (Meyerhoff, 2001; Rothlind *et al*, 2005) and metabolic abnormalities (Pfefferbaum *et al*, 2005). Similarly, alcohol administration to rhesus monkeys infected with simian immunodeficiency virus (SIV) produced cognitive and behavioral deficits greater than those produced by alcohol or SIV alone (Winsauer *et al*, 2002).

Address correspondence to Eugen Brailoiu, MD, Department of Pharmacology, Temple University School of Medicine, 3420 N. Broad Street, Philadelphia, PA 19140, USA. E-mail: ebrailou@temple.edu

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HIV-1 enters the brain early in the disease (Resnick *et al*, 1988), altering neuronal functions, and inducing HIV-associated dementia (HAD) (Gonzalez-Scarano and Martin-Garcia, 2005). In the absence of productive neuronal infection, neurotoxicity is largely attributed to viral proteins and host factors (Kaul *et al*, 2001; Mattson *et al*, 2005). The demonstration that HIV-1 proteins including Tat, gp120, and gp41 are neurotoxic leads to the term "virotoxins" (Nath and Geiger, 1998). Tat is neurotoxic when injected intracerebroventricularly to mice *in vivo* (Sabatier *et al*, 1991) and in cultured human and rat neurons *in vitro* (Nath *et al*, 1996; New *et al*, 1997). Tat-induced neurotoxicity is presumed to be associated with an increase in cytosolic calcium levels (Kruman *et al*, 1998; Haughey *et al*, 1999; Bonavia *et al*, 2001).

Results from a number of studies suggest that interactions of alcohol and HIV proteins may result in greater neuronal injury than either agent alone. For example, long-term exposure to ethanol potentiates gp120-induced apoptosis in human (Chen *et al*, 2005) and enhances the cytotoxic effect of Tat in hippocampal neurons (Self *et al*, 2004), via a *N*-methyl-D-aspartate (NMDA)-sensitive pathway. Interactions between ethanol and viral proteins might also be responsible for increased HIV-1 neuroinvasion. Alcohol potentiates apoptosis induced by HIV-1 proteins in human brain endothelial cells, which are an important component of the blood-brain barrier (Acheampong *et al*, 2002). In addition, Tat and alcohol amplify their proinflammatory effects in mice hippocampus (Flora *et al*, 2005).

In the present study, we show for the first time that acute alcohol exposure potentiated HIV Tat–induced Ca^{2+} overload and neuronal death in rat cortical neurons.

Results

Measurement of cytosolic Ca^{2+} concentrations

$[Ca^{2+}]_i$ response in normal Ca^{2+} saline: Administration of HIV-1 Tat (10 nM) to rat cortical neurons induced an elevation of $[Ca^{2+}]_i$ characterized by two distinct profiles. In 34 of 50 neurons tested, we noticed a fast (less than 1 s) and transitory increase in $[Ca^{2+}]_i$ by 543 ± 28 nM (Figures 1A1 and 4). In 11 neurons, Tat elicited a slow and sustained rise of $[Ca^{2+}]_i$, with an average of 397 ± 16 nM (Figures 1A2 and 4). HIV-1 Tat had no significant effects on $[Ca^{2+}]_i$ in 5 neurons. Treatment with high K^+ (KCl 25 mM) of neurons unresponsive to Tat produced an increase in $[Ca^{2+}]_i$ by 217 ± 4.3 nM ($n = 5$). Administration of Tat (10 nM) to neurons pretreated with BAPTA-AM (20 μM) for 45 min induced a slow and short-lasting elevation of $[Ca^{2+}]_i$ of 36 ± 3.9 nM (Figures 1A3 and 4). Heat-inactivated HIV-1 Tat had no significant effects on $[Ca^{2+}]_i$; a representative recording is shown in Figure 1A4. Administration of glutathione *S*-transferase (GTS) (10 nM and 500 nM) to cortical neurons did not significantly change $[Ca^{2+}]_i$.

Similar profiles of calcium responses were observed for the high concentration of Tat (500 nM). At this concentration, the fast responses of $[Ca^{2+}]_i$ reached a peak of 1783 ± 94 nM ($n = 10$) (Figures 2A and 4), whereas the slow responses had an averaged increase of 1217 ± 88 nM ($n = 10$) (Figures 2B and 4). Administration of 500 nM Tat to neurons pretreated with BAPTA-AM (20 μM, 45 min) induced a transient increase of $[Ca^{2+}]_i$, averaging 181 ± 8.8 nM ($n = 8$) (Figures 2C and 4). Heat-inactivated HIV-1 Tat had no effects on $[Ca^{2+}]_i$; a representative trace is shown in Figure 2D.

Ethanol and $[Ca^{2+}]_i$: Administration of 50 mM ethanol alone induced a small, sustained elevation of $[Ca^{2+}]_i$, with an average of 183 ± 6.8 nM ($n = 8$); this effect was abolished by pretreatment with 20 μM BAPTA-AM for 45 min ($n = 8$); Figure 3B shows a representative experiment.

$[Ca^{2+}]_i$ to Tat and ethanol: In neurons responding to Tat with a fast, short-lasting rise of $[Ca^{2+}]_i$, addition of Tat (10 nM) to neurons pretreated with ethanol (50 mM, 2 min) changed the fast, short-lasting increase (Figure 1A1) to a fast, sustained rise of $[Ca^{2+}]_i$, averaging 1193 ± 64 nM ($n = 12$) (Figures 1B1 and 4). Administration of Tat (10 nM) to slowly responding neurons pretreated with 50 mM ethanol for 2 min elevated $[Ca^{2+}]_i$ by an average of 642 ± 37 nM (Figure 1B2), which was significantly higher than that caused by Tat alone (Figure 4). In neurons preloaded with BAPTA-AM (20 μM, 45 min), administration of Tat (10 nM) subsequent to ethanol (50 nM, 2 min) induced a transitory increase in $[Ca^{2+}]_i$ of 137 ± 6.2 nM (Figures 1B3 and 4). Heat-inactivated Tat administered to neurons pretreated with ethanol had no effect (Figure 1B4).

$[Ca^{2+}]_i$ response in Ca^{2+}-free saline: In Ca^{2+}-free saline supplemented with 2.5 mM EGTA, administration of Tat (10 nM or 500 nM) elevated $[Ca^{2+}]_i$ by 284 ± 8.2 nM and 547 ± 12.2 nM, respectively (Figures 5A, D and 6). Ethanol (50 mM) alone produced a small rise of $[Ca^{2+}]_i$, averaging 23 ± 3.1 nM (Figure 5B). Administration of Tat (10 nM) to neurons pretreated with ethanol (50 mM, 2 min) elevated $[Ca^{2+}]_i$ by 462 ± 9.6 nM (Figures 5C and 6).

Neurotoxicity assay

Neuronal cell death was measured by the trypan blue exclusion assay. In neurons treated with ethanol (50 mM, 12 h), the neuronal cell death was comparable to that of untreated cells, $3.1\% \pm 0.2\%$ versus $2.9\% \pm 0.3\%$ (Figure 7). Treatment with Tat alone (10 nM, 12 h) induced a cell death of $5.4\% \pm 0.4\%$. A short exposure (2 min) to ethanol (50 mM) significantly increased the cell death to $14.8\% \pm 1.6\%$ of neurons treated with Tat (10 nM). The cell death

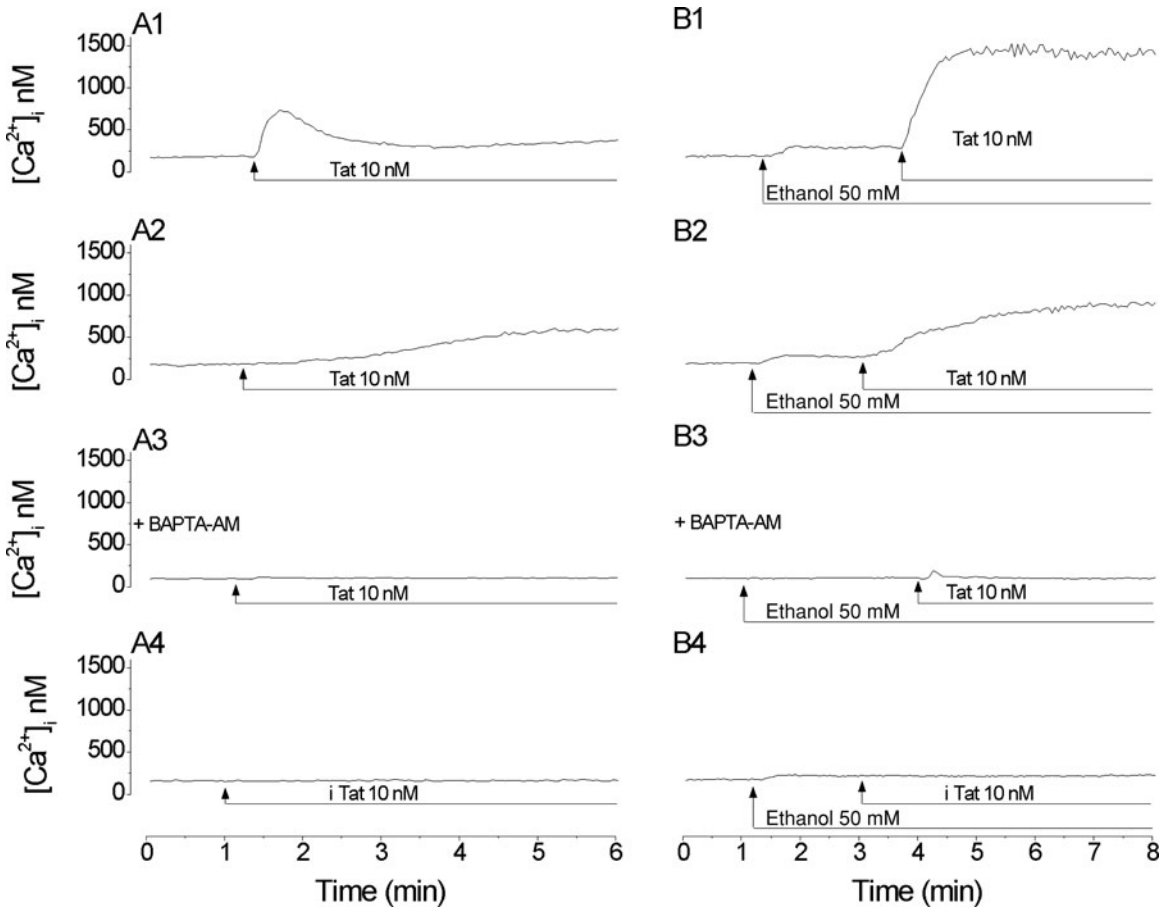

Figure 1 Potentiation by ethanol of HIV-1 Tat-induced increases of intracellular calcium concentrations in cultured rat cortical neurons. **A1**, A representative recording of a rapid onset, short-lasting elevation of $[Ca^{2+}]_i$ induced by Tat (10 nM). **A2**, A representative recording of a slow onset, sustained increase of $[Ca^{2+}]_i$ induced by Tat (10 nM). **A3**, The effect of Tat on $[Ca^{2+}]_i$ was markedly decreased by pretreatment with BAPTA-AM (20 μM). **A4**, Heat-inactivated Tat (iTat; 10 nM) caused no significant change of $[Ca^{24}]_i$. **B1**, Pretreatment with ethanol (50 mM, 2 min) increased $[Ca^{2+}]_i$ in fast responding neurons and changed the transitory calcium response to a sustained increase of $[Ca^{2+}]_i$. **B2**, Ethanol increased the amplitude of sustained calcium response. Ethanol alone produced a small increase of $[Ca^{2+}]_i$, as shown in **B1** and **B2**. **B3**, The potentiating effect of ethanol was markedly decreased in neurons pretreated with BAPTA-AM, which also abolished the increase of $[Ca^{2+}]_i$ induced by ethanol. **B4**, Ethanol produced a small increase of $[Ca^{2+}]_i$ but caused no significant change of the $[Ca^{2+}]_i$ following administration of heat inactivated Tat.

was reduced to 3.6% $\pm$ 0.4% in neurons treated with Tat (10 nM) subsequent to incubation with BAPTA-AM and ethanol (Figure 7). Treatment with a higher dose of Tat (500 nM) induced a statistically significant increase in cell death of 17.4% $\pm$ 1.3%, whereas pretreatment with BAPTA reduced the cell death to 4.2% $\pm$ 0.6% (Figure 7).

Discussion

HIV and alcohol can impair neuropsychological functions and their association has been shown to have additive effects (Rothlind *et al*, 2005; Schulte *et al*, 2005). The major goal of this study was to examine directly the effects of ethanol and HIV-1 Tat protein alone and in combination on calcium responses and neurotoxicity in rat cerebral cortical neurons. Our results show that acute exposure to ethanol increased Tat-induced calcium responses and cell death in rat cortical neurons.

HIV-1 Tat protein is one of the viral factors considered to be responsible for the pathogenesis of HIV-induced dementia. There is mounting evidence that HIV-1 Tat protein is neurotoxic, due largely to a calcium overload (Kruman *et al*, 1998; Haughey *et al*, 1999; 2001; Haughey and Mattson, 2002). Excessively high levels of intracellular calcium lead to cell death by apoptosis (Ghosh and Greenberg, 1995; Mattson and Chan, 2003). The calcium pool(s) mobilized by Tat is still a matter of debate. The Tat-induced increase in $[Ca^{2+}]_i$ is considered critically dependent on influx through voltage-gated calcium channels and ionotropic glutamate receptors in rat cortical and hippocampal neurons (Bonavia *et al*, 2001). However, Tat mobilizes calcium from both external and internal sources; i.e., calcium release from inositol triphosphate (IP_3)-sensitive intracellular stores and calcium

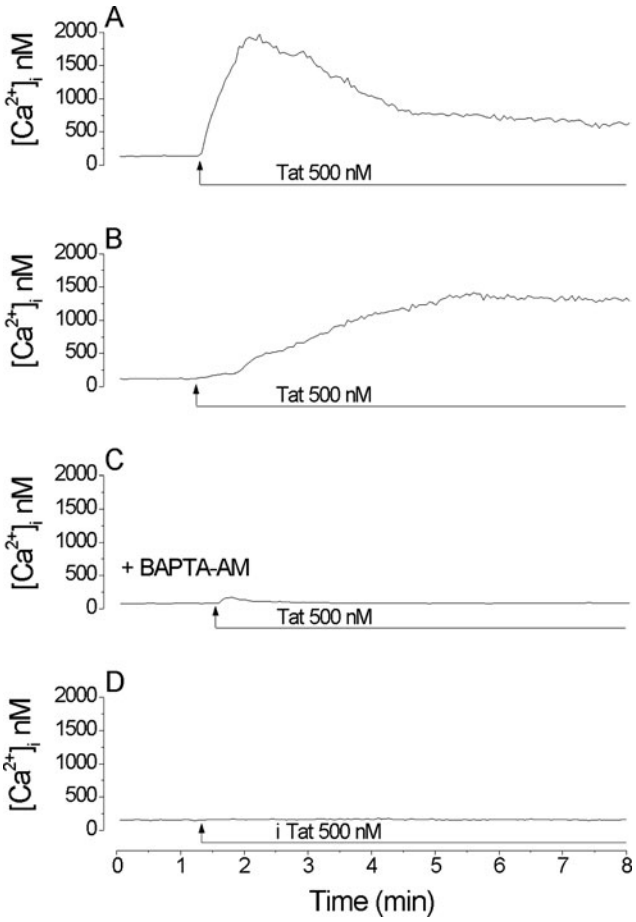

Figure 2 Two types of calcium responses induced by a higher concentration of Tat (500 nM). Calcium responses elicited by the high concentration had similar profiles as those elicited by a low concentration. **A**, A representative recording of a fast onset, short lasting increase of $[Ca^{2+}]_i$. **B**, A representative recording of a slow onset, sustained rise of $[Ca^{2+}]_i$. **C**, Pretreatment with BAPTA-AM (20 μM) markedly decreased the $[Ca^{2+}]_i$ induced by Tat; a small, transient response could be detected following Tat administration. **D**, No effect on $[Ca^{2+}]_i$ was noticed in neurons treated with heat inactivated Tat (500 nM).

influx through glutamate receptors, in human fetal neurons and astrocytes (Haughey *et al*, 1999). In contrast, Tat has been reported to reduce glutamate or adenosine triphosphate (ATP)-induced intracellular Ca^{2+} increase in rat cortical astrocytes and human glioblastoma cells (Koller *et al*, 2001).

To evaluate the direct effect of ethanol and/or Tat on neurons and to exclude glia-neuron interactions, we used rat cortical neurons in culture where glial cell growth was prevented by treatment with the mitotic inhibitor cytosine β-arabinofuranoside. In addition, cerebral cortical neurons are highly susceptible to the neurotoxic effect of Tat protein (Bonavia *et al*, 2001) and alcohol (Ikegami *et al*, 2003). Under this condition, HIV-1 Tat, irrespective of a low (10 nM) or high (500 nM) concentration, caused two types of calcium responses in rat cortical neurons: a fast-onset, short-lasting response and a slow-onset, sustained

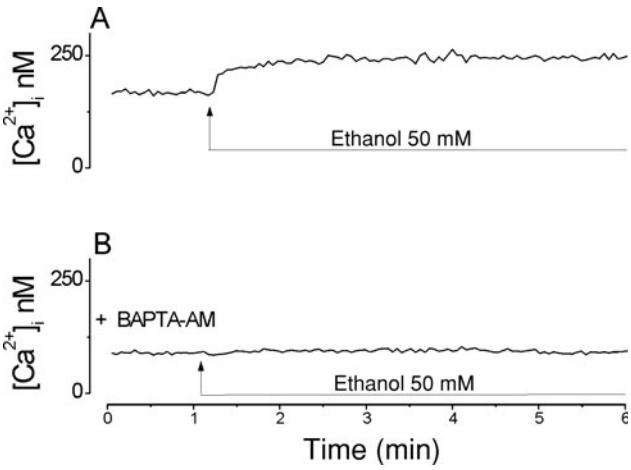

Figure 3 Ethanol-induced $[Ca^{2+}]_i$ in rat cortical neurons. **A**, Ethanol (50 mM) induced a small, but sustained increase in $[Ca^{2+}]_i$ of 183 ± 6.8 nM. **B**, The effect of ethanol was abolished by pretreatment with BAPTA-AM (20 μM) for 45 min.

response. The Tat-induced calcium response was diminished in a Ca^{2+}-free saline, indicating the participation of both intra- and extracellular sources in rat cortical neurons; a finding similar to that reported for human neurons (Haughey *et al*, 1999).

In our study, ethanol alone induced a small, but sustained increase in $[Ca^{2+}]_i$, which was completely abolished by pretreatment with BAPTA-AM, a potent membrane-permeable calcium chelator (Strayer

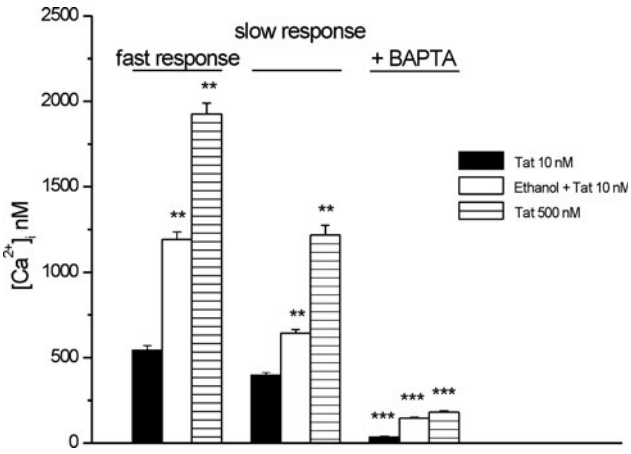

Figure 4 Comparison of two types of calcium responses in cortical neurons induced by a low or high concentration of Tat with or without ethanol pretreatment. In fast responding neurons treated with ethanol, Tat (10 nM) induced an increase of $[Ca^{2+}]_i$ of 1193 ± 64 nM, compared with an increase of 543 ± 28 nM in neurons treated with Tat alone. Tat (500 nM) induced an increase of $[Ca^{2+}]_i$ of 1783 ± 94 nM. In slow responding neurons, Tat (10 nM) alone induced an increase of $[Ca^{2+}]_i$ of 397 ± 16 nM; pretreatment with ethanol elicited an increase in $[Ca^{2+}]_i$ of 642 ± 37 nM. Tat (500 nM) increased $[Ca^{2+}]_i$ by 1217 ± 88 nM. In neurons pretreated with BAPTA-AM (20 μM), Tat (10 nM and 500 nM) induced an increase of $[Ca^{2+}]_i$ of 36 ± 6.2 nM, and 181 ± 8.8 nM, respectively; BAPTA also reduced $[Ca^{2+}]_i$ induced by ethanol and Tat (10 nM) to 137 ± 6.2 nM. ($^{**}P < .05$; $^{***}P < .01$).

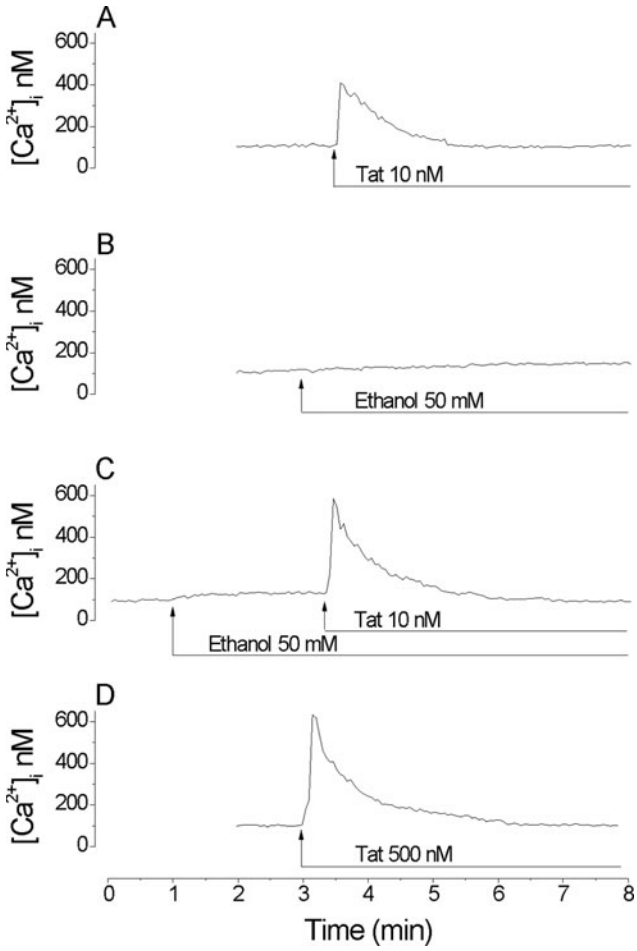

Figure 5 Calcium responses induced by Tat and ethanol in Ca^{2+}-free saline plus 2.5 mM EGTA in cultured rat cortical neurons. **A**, Administration of Tat (10 nM) elicited a fast onset, short lasting rise of $[Ca^{2+}]_i$ with an average of 284 ± 8.2 nM in a Ca^{2+}-free solution. **B**, Ethanol (50 mM) induced a negligible rise of $[Ca^{2+}]_i$ with an average of 23 ± 3.1 nM in a Ca^{2+}-free solution. **C**, Administration of Tat (10 nM) to neurons pretreated with ethanol (50 mM; 2 min) elevated $[Ca^{2+}]_i$ by 462 ± 9.6 nM, which is nearly double the response induced by Tat alone. **D**, A high concentration of Tat (500 nM) induced an increase of $[Ca^{2+}]_i$ of 547 ± 12.2 nM.

et al, 1999). The mechanism underlying ethanol-induced calcium release appears to vary in different cell models. For example, ethanol induces a release of Ca^{2+} from IP_3-insensitive stores in mouse brain microsomes (Daniell and Harris, 1989), and from IP_3-sensitive stores in hepatocytes (Hoek *et al*, 1987). In isolated canine basilar arteries, ethanol-induced contractions involve both Ca^{2+} influx through voltage-gated Ca^{2+} channels and release from internal stores (Yang *et al*, 2001). In the case of rat cortical neurons, the transient peak induced by Tat in the control medium was converted to a fast and sustained calcium response in the presence of ethanol. In slow-responding neurons, ethanol amplified the slow and sustained increase in $[Ca^{2+}]_i$. In both types of neurons, the potentiation by ethanol was markedly reduced, but not completely abolished

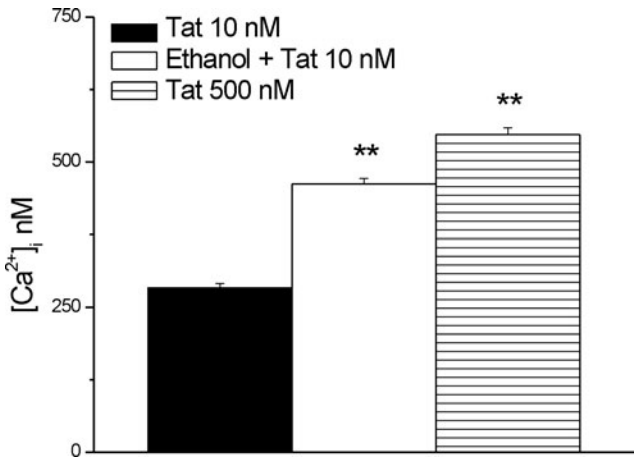

Figure 6 Comparison of calcium responses induced by a low concentration of Tat (10 nM) with or without pretreatment with ethanol and a high concentration of Tat (500 nM) in Ca^{2+}-free saline plus 2.5 mM EGTA ($^{**}P < .05$).

by BAPTA-AM, which has been reported to inhibit the Tat-induced calcium response in macrophages (Mayne *et al*, 2000). The potentiating effect of ethanol on Tat-induced calcium responses persists in a Ca^{2+}-free saline, implying ethanol mobilizes internal stores.

With respect to neurotoxicity, ethanol alone, at the concentration (50 mM) tested here, did not result

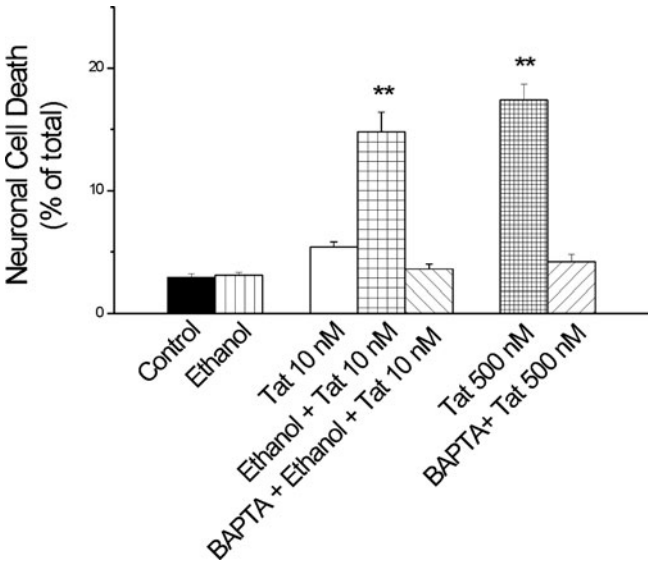

Figure 7 Potentiation by ethanol of Tat-induced neurotoxicity. Cultured cortical neurons were treated for 12 h with Tat (10 nM) or ethanol (50 mM) alone or a combination of Tat and ethanol, and the cell viability was determined by the trypan blue exclusion assay. Tat (10 nM) applied to neurons pretreated with ethanol significantly increased the neuronal death to $14.8\% \pm 1.6\%$ ($^{**}P < .05$). Tat (500 nM) alone significantly increased neuronal death to $17.4\% \pm 1.3\%$ ($^{**}P < .05$). Pretreatment with BAPTA-AM (20 μM) markedly lowered the neuronal death rate to $4.2\% \pm 0.6\%$ in cortical neurons exposed to Tat (10 nM)/ethanol (50 mM) and to a high concentration of Tat (500 nM).

in a significant number of neuronal deaths as compared to the control. Cell death was assessed by the trypan blue exclusion assay, which was used in a previous study to evaluate Tat-induced neurotoxicity (Haughey *et al*, 1999). A short (2-min) exposure to ethanol (50 mM) significantly increased the Tat-induced cell death; the latter was prevented by pretreatment with BAPTA-AM. The increase in neuronal death induced by a higher concentration of Tat (500 nM) was also prevented by BAPTA-AM. In a previous report, ethanol (16.7 and 50 mM) potentiated gp120-induced total neuronal death in a concentration-dependent manner, but failed to increase Tat-induced neuronal death, as evaluated by lactate dehydrogenase (LDH) release (Chen *et al*, 2005). In addition, a high concentration of ethanol (83 mM) did not influence the gp120- or Tat-induced neuronal death (Chen *et al*, 2005). Subchronic treatment of rat organotypic hippocampal-cortical slice cultures with ethanol at the concentrations (20 to 30 mM) comparable to that occurred during moderate drinking, reduced neurodegeneration due to gp120 (Collins *et al*, 2000), probably as a result of inhibiting glutamate uptake and calcium-mediated neurotoxicity (Belmadani *et al*, 2001). A higher concentration of alcohol (100 mM) (Belmadani *et al*, 2003) or long-term exposure to alcohol potentiated gp120- (Chen *et al*, 2005) or Tat-induced (Self *et al*, 2004) neurotoxicity.

Ethanol has been shown to induce direct neuronal injury in the developing rat brain (Ikonomidou *et al*, 2000; Green *et al*, 2002), the mouse hippocampus (Pawlak *et al*, 2002), and human cortex and hippocampus (Ikegami *et al*, 2003). Several mechanisms are proposed relative to the ethanol-induced neuronal injury (for review see Goodlett and Horn, 2001), and significant differences have been reported between acute and chronic exposure. Electrophysiological experiments indicated that ethanol depressed glutamatergic excitatory transmission in spinal cord slices (Wang *et al*, 1999) and cultured cerebellar granule cells (Popp *et al*, 1999), whereas chronic ethanol treatment potentiated NMDA-induced neurotoxicity on cultured cortical neurons (Nagy *et al*, 2001). Chronic but not acute ethanol exposure decreased the phospholipase C (PLC) activity in the rat brain (Pandey, 1996). Our finding that BAPTA significantly reduces the number of neuronal deaths exposed to ethanol and Tat is consistent with the notion that calcium overload is a critical event leading to neuronal death induced by ethanol-Tat interaction in rat cortical neurons.

Material and methods

Overexpression and purification of recombinant Tat proteins

GST and HIV-1 GST-Tat fusion proteins were prokaryotically expressed and purified as described previously (Amini *et al*, 2004). The integrity and purity of the GST fusion proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining. Known amounts of bovine serum albumin were included as controls on the same gel.

Neuronal cell culture

Neurons were isolated from the cerebral cortex of postnatal 1- to 3-days-old rats, as previously described (Brailoiu *et al*, 2005). Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Neurons were dissociated by enzymatic digestion with 0.5 mg papain/100 mg tissue. Cells were plated at a density of $10^3/mm^2$ in a Neurobasal-A medium, supplemented with 10% fetal calf serum, 20 mM glutamine, 100 units/ ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA), and maintained at 37°C in an atmosphere of 95% O_2 + 5% CO_2. Glial cell growth was inhibited by the mitotic inhibitor cytosine β-arabinofuranoside (1 μM) (Sigma, St. Louis, MO). Neurons were cultured for 5 days. Twelve hours prior to Ca^{2+} measurements or neurotoxicity assay, cells were transferred to a medium without fetal serum.

Measurement of cytosolic Ca^{2+} concentrations: The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by the microfluorimetric technique, as previously described (Brailoiu *et al*, 2005). Briefly, dissociated neurons were loaded onto the coverslips with the fluorescent Ca^{2+} indicator Fura 2-AM (3 μM) by incubation of the cells in Hank's balanced salt solution (HBSS) plus Fura 2-AM for 45 min and HBSS alone for an additional 15 to 60 min to allow deesterification of the dye. Coverslips were placed in a custom-designed bath and transferred to the stage of an inverted Nikon epifluorescence microscope equipped with a C & L Instruments Fluorimeter System (Brailoiu *et al*, 2005). Cells were perfused with HBSS at a flow rate of 2.5 ml/min and Fura-2 fluorescence (excitation wavelength = 340 and 380 nm, emission wavelength = 520 nm) of single cells was acquired at a frequency of 1 Hz. The ratio of the fluorescence signals obtained (340 nm/380 nm) was converted to Ca^{2+} concentrations according to Grynkiewicz *et al* (1985).

HIV-1 Tat inactivation

Repeated (10 times) heating (75°C for 30 s) and cooling (4°C for 1 min) was used to inactivate Tat.

Neurotoxicity assay

Cultured cortical neurons of 5-days-old rats were treated with HIV-1 Tat (10 nM or 500 nM) alone or after pretreatment with (i) ethanol (50 mM, 2 min), (ii) BAPTA-AM (20 μM, 45 min), or (iii) BAPTA-AM (20 μM, 45 min) and ethanol (50 mM, 2 min). Following 12 h of incubation, cell death

was determined using the trypan blue exclusion assay. Two hundred neurons from each treatment group were counted by an investigator unaware of the type of treatments. Each experiment was repeated six times.

Statistics

Paired *t* test followed by one-way analysis of variance (ANOVA) was used for evaluating statistically significant differences between controls and treated neurons; $P < .05$ being significantly different.

References

Acheampong E, Mukhtar M, Parveen Z, Ngoubilly N, Ahmad N, Patel C, Pomerantz RJ (2002). Ethanol strongly potentiates apoptosis induced by HIV-1 proteins in primary human brain microvascular endothelial cells. *Virology* **304:** 222–234.

Amini S, Saunders M, Kelley K, Khalili K, Sawaya SE (2004). Interplay between HIV-1 Vpr and Spl modulates $p21^{WAF1}$ gene expression in human astrocytes. *J Biol Chem* **279:** 46046–46056.

Belmadani A, Neafsey EJ, Collins MA (2003). Human immunodeficiency virus type 1 gp120 and ethanol coexposure in rat organotypic brain slice cultures: curtailment of gp120-induced neurotoxicity and neurotoxic mediators by moderate but not high ethanol concentrations. *J NeuroVirol* **9:** 45–54.

Belmadani A, Zou JY, Schipma MJ, Neafsey EJ, Collins MA (2001). Ethanol pre-exposure suppresses HIV-1 glycoprotein 120-induced neuronal degeneration by abrogating endogenous glutamate/Ca^{2+}-mediated neurotoxicity. *Neuroscience* **104:** 769–781.

Bonavia R, Bajetto A, Barbero S, Albini A, Noonan DM, Schettini G (2001). HIV-1 Tat causes apoptotic death and calcium homeostasis alterations in rat neurons. *Biochem Biophys Res Commun* **288:** 301–308.

Brailoiu E, Hoard JL, Filipeanu CM, Brailoiu GC, Dun SL, Patel S, Dun NJ (2005). NAADP potentiates neurite outgrowth. *J Biol Chem* **280:** 5646–5650.

Chen H, Zha J, Gowans RE, Camargo P, Nishitani J, McQuirter JL, Cole SW, Zack JA, Liu X (2004). Alcohol enhances HIV type 1 infection in normal human oral keratinocytes by up-regulating cell-surface CXCR4 coreceptor. *AIDS Res Hum Retroviruses* **20:** 513–519.

Chen W, Tang Z, Fortina P, Patel P, Addya S, Surrey S, Acheampong EA, Mukhtar M, Pomerantz RJ (2005). Ethanol potentiates HIV-1 gp120-induced apoptosis in human neurons via both the death receptor and NMDA receptor pathways. *Virology* **334:** 59–73.

Collins MA, Neafsey EJ, Zou JY (2000). HIV-I gpI20 neurotoxicity in brain cultures is prevented by moderate ethanol pretreatment. *Neuroreport* **11:** 1219–1222.

Cook RT, Stapleton JT, Dallas ZK, Klinzman D (1997). Effect of a single ethanol exposure on HIV replication in human lymphocytes. *J Invest Med* **45:** 265–271.

Dingle GA, Oei TP (1997). Is alcohol a cofactor of HIV and AIDS? Evidence from immunological and behavioral studies. *Psychol Bull* **122:** 56–71.

Daniell LC, Harris RA (1989). Ethanol and inositol 1,4,5-trisphosphate release calcium from separate stores of brain microsomes. *J Pharmacol Exp Ther* **250:** 875–881.

Flora G, Pu H, Lee YW, Ravikumar R, Nath A, Hennig B, Toborek M (2005). Proinflammatory synergism of ethanol and HIV-1 Tat protein in brain tissue. *Exp Neurol* **191:** 2–12.

Ghosh A, Greenberg ME (1995). Calcium signaling in neurons: molecular mechanisms and cellular conseqences. *Science* **268:** 239–247.

Goodlett CR, Horn KH (2001). Mechanisms of alcohol-induced damage to the developing nervous system. *Alcohol Res Health* **25:** 175–184.

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

Green JT, Tran T, Steinmetz JE, Goodlett CR (2002). Neonatal ethanol produces cerebellar deep nuclear cell loss and correlated disruption of eyeblink conditioning in adult rats. *Brain Res* **956:** 302–311.

Grynkiewicz G, Poenie M, Tsien RY (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450.

Haorah J, Heilman D, Diekmann C, Osna N, Donohue TM Jr, Ghorpade A, Persidsky Y (2004). Alcohol and HIV decrease proteasome and immunoproteasome function in macrophages: implications for impaired immune function during disease. *Cell Immunol* **229:** 139–148.

Haughey NJ, Holden CP, Nath A, Geiger JD (1999). Involvement of inositol 1,4,5-trisphosphate-regulated stores of intracellular calcium in calcium dysregulation and neuron cell death caused by HIV-1 protein tat. *J Neurochem* **73:** 1363–1374.

Haughey NJ, Mattson MP (2002). Calcium dysregulation and neuronal apoptosis by the HIV-1 proteins Tat and gp120. *J Acquir Immune Defic Syndr* **31:** S55–S61.

Haughey NJ, Nath A, Mattson MP, Slevin JT, Geiger JD (2001). HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *J Neurochem* **78:** 457–467.

Hoek JB, Thomas AP, Rubin R, Rubin E (1987). Ethanol-induced mobilization of calcium by activation of phosphoinositide-specific phospholipase C in intact hepatocytes. *J Biol Chem* **262:** 682–691.

Ikegami Y, Goodenough S, Inoue Y, Dodd PR, Wilce PA, Matsumoto I (2003). Increased TUNEL positive cells in human alcoholic brains. *Neurosci Lett* **349:** 201–205.

Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovska V, Horster F, Tenkova T, Dikranian K, Olney JW (2000). Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science* **287:** 1056–1060.

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

Koller H, Schaal H, Freund M, Garrido SR, von Giesen HJ, Ott M, Rosenbaum C, Arendt G (2001). HIV-1 protein Tat reduces the glutamate-induced intracellular Ca^{2+} increase in cultured cortical astrocytes. *Eur J Neurosci* **14:** 1793–1799.

Kruman II, Nath A, Mattson MP (1998). HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism involving caspase activation, calcium overload, and oxidative stress. *Exp Neurol* **154:** 276–288.

Liu X, Zha J, Nishitani J, Chen H, Zack JA (2003). HIV-1 infection in peripheral blood lymphocytes (PBLs) exposed to alcohol. *Virology* **307:** 37–44.

Meyerhoff DJ (2001). Effects of alcohol and HIV infection on the central nervous system. *Alcohol Res Health* **25:** 288–298

Mattson MP, Chan SL (2003). Calcium orchestrates apoptosis. *Nat Cell Biol* **5:** 1041–1043.

Mattson MP, Haughey NJ, Nath A (2005). Cell death in HIV dementia. *Cell Death Differ* **12:** 893–904.

Mayne M, Holden CP, Nath A, Geiger JD (2000). Release of calcium from inositol 1,4,5-trisphosphate receptor-regulated stores by HIV-1 Tat regulates TNF-alpha production in human macrophages. *J Immunol* **164:** 6538–6542.

Nagy J, Muller F, Laszlo L (2001). Cytotoxic effect of alcohol-withdrawal on primary cultures of cortical neurones. *Drug Alcohol Depend* **61:** 155–162.

Nath A, Geiger J (1998). Neurobiological aspects of human immunodeficiency virus infection: Neurotoxic mechanisms. *Prog Neurobiol* **54:** 19–33.

Nath A, Psooy K, Martin C, Knudsen B, Magnuson DS, Haughey N, Geiger JD (1996). Identification of a human immunodeficiency virus type 1 Tat epitope that is neuroexcitatory and neurotoxic. *J Virol* **70:** 1475–1480.

New DR, Ma M, Epstein LG, Nath A, Gelbard HA (1997). Human immunodeficiency virus type 1 Tat protein induces death by apoptosis in primary human neuron cultures. *J NeuroVirol* **3:** 168–173.

Pandey SC (1996). Acute and chronic ethanol consumption effects on the immunolabeling of Gq/11 alpha subunit protein and phospholipase C isozymes in the rat brain. *J Neurochem* **67:** 2355–2361.

Pawlak R, Skrzypiec A, Sulkowski S, Buczko W (2002). Ethanol-induced neurotoxicity is counterbalanced by increased cell proliferation in mouse dentate gyrus. *Neurosci Lett* **327:** 83–86.

Pfefferbaum A, Adalsteinsson E, Sullivan EV (2005). Cortical NAA deficits in HIV infection without dementia: influence of alcoholism comorbidity. *Neuropsychopharmacology* **30:** 1392–1399.

Popp RL, Lickteig RL, Lovinger DM (1999). Factors that enhance ethanol inhibition of *N*-methyl-D-aspartate receptors in cerebellar granule cells. *J Pharmacol Exp Ther* **289:** 1564–1574.

Resnick L, Berger JR, Shapshak P, Tourtellotte WW (1988). Early penetration of the blood–brain barrier by HTLV-III/LAV. *Neurology* **38:** 9–15.

Rothlind JC, Greenfield TM, Bruce AV, Meyerhoff DJ, Flenniken DL, Lindgren JA, Weiner MW (2005). Heavy alcohol consumption in individuals with HIV infection: effects on neuropsychological performance. *J Int Neuropsychol Soc* **11:** 70–83.

Sabatier JM, Vives E, Mabrouk K, Benjouad A, Rochat H, Duval A, Hue B, Bahraoui E (1991). Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J Virol* **65:** 961–967.

Samet JH, Phillips SJ, Morton NJ, Traphagen ET, Freedberg KA (2004). Detecting alcohol problems in HIV-infected patients: use of the CAGE questionnaire. *AIDS Res Hum Retroviruses* **20:** 151–155.

Schulte T, Mueller-Oehring EM, Rosenbloom MJ, Pfefferbaum A, Sullivan EV (2005). Differential effect of HIV infection and alcoholism on conflict processing, attentional allocation, and perceptual load: evidence from a Stroop Match-to-Sample task. *Biol Psychol* **57**: 67–75.

Self RL, Mulholland PJ, Harris BR, Nath A, Prendergast MA (2004). Cytotoxic effects of exposure to the human immunodeficiency virus type 1 protein Tat in the hippocampus are enhanced by prior ethanol treatment. *Alcohol Clin Exp Res* **28:** 1916–1924.

Strayer DS, Hoek JB, Thomas AP, White MK (1999). Cellular activation by Ca^{2+} release from stores in the endoplasmic reticulum but not by increased free Ca^{2+} in the cytosol. *Biochem J* **344:** 39–46.

Wang MY, Rampil IJ, Kendig JJ (1999). Ethanol directly depresses AMPA and NMDA glutamate currents in spinal cord motor neurons independent of actions on $GABA_A$ or glycine receptors. *J Pharmacol Exp Ther* **290:** 362–367.

Winsauer PJ, Moerschbaecher JM, Brauner IN, Purcell JE, Lancaster JR Jr, Bagby GJ, Nelson S (2002). Alcohol unmasks simian immunodeficiency virus-induced cognitive impairments in rhesus monkeys. *Alcohol Clin Exp Res* **26:** 1846–1857.

Yang Z, Wang J, Zheng T, Altura BT, Altura BM (2001). Importance of extracellular Ca^{2+} and intacellular Ca^{2+} release in ethanol-induced contraction of cerebral arterial smooth muscle. *Alcohol* **24:** 145–153.

Zheng J, Yang OO, Xie Y, Campbell R, Chen IS, Pang S (2004). Ethanol stimulation of HIV infection of oral epithelial cells. *J Acquir Immune Defic Syndr* **37:** 1445–1453.