**Journal of NeuroVirology,** 9: 45–54, 2003
© 2003 Taylor & Francis ISSN 1355-0284/03 $12.00+.00
DOI: 10.1080/13550280390173409

Taylor & Francis
Taylor & Francis Group

# 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

A Belmadani, EJ Neafsey, and MA Collins

*Department of Cell Biology, Neurobiology and Anatomy, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois, USA*

**Human immunodeficiency virus type 1 (HIV-1) envelope protein gp120, implicated with other retroviral proteins in acquired immunodeficiency syndrome (AIDS)-related dementia, causes neuronal degeneration by inciting cascades of neurotoxic mediators from glia. It also may facilitate neuronal glutamate (*N*-methyl-D-aspartate, NMDA) receptor–mediated excitotoxicity by interacting at the glycine coagonist site. The authors reported that preconditioning rat organotypic hippocampal-cortical slice cultures subchronically with ethanol at concentrations occurring during moderate drinking (20 to 30 mM) prevented gp120's induction of neurotoxic mediators and intracellular calcium, as well as neuronal death. The authors now find that the acute copresence of ethanol in moderate as opposed to high concentrations similarly blocks the retroviral protein's neurotoxic effects in brain slice cultures, assessed with lactate dehydrogenase (LDH) release and propidium iodide (PI) labeling. As with ethanol preconditioning, neuroprotection against gp120 by moderate ethanol coexposure appears secondary to abrogation of the retroviral protein's early induction of arachidonic acid (AA), glutamate, and superoxide (but not nitric oxide) elevations/release. Additionally, experiments indicate that 30 mM ethanol is sufficient to inhibit the NMDA receptor, particularly in the presence of added glycine, thus hindering potential direct neuronal stimulation by gp120. However, in contrast to moderate ethanol, 100 mM ethanol, a concentration tolerated only in chronic alcoholics, potentiates gp120-dependent neurotoxicity (PI labeling) in the hippocampal CA1 region, augments LDH release, and fails to curtail gp120's actions on AA, glutamate, and superoxide—but does suppress nitric oxide induction. The results indicate dominant roles for AA, superoxide, and glutamate-mediated oxidative stress in gp120's neurotoxic mechanism, but perhaps a less important role for NMDA receptor stimulation, which would be constrained at both ethanol concentrations employed. We suggest that ethanol's concentration-dependent, two-edged sword behavior could alter the development of dementia in HIV-1–infected individuals during social consumption or abuse. Further studies are needed to elucidate the differing apparently glial effects of the two concentrations of ethanol.** *Journal of NeuroVirology* (2003) **9,** 45–54.

**Keywords:** alcohol; arachidonic acid; glutamate; hippocampus; neuroprotection; NMDA receptor; superoxide

Address correspondence to Michael A Collins, PhD, Division of Biochemistry, Department of Cell Biology, Neurobiology, and Anatomy, Loyola Medical School, 2160 S. 1st Avenue, Maywood, IL 60153, USA. E-mail: mcollin@lumc.edu

The support of NIH AA11723 is gratefully acknowledged. The authors thank the NIH AIDS Research and Reference Program for HIV-1 $gp120_{IIIB}$.

Received 18 January 2002; revised 19 February 2002; accepted 5 August 2002.

## Introduction

Neuronal degeneration, encephalitis, and cognitive impairment/dementia are key aspects of neuro–acquired immunodeficiency syndrome (neuroAIDS) that result largely from the actions of retroviral proteins shed by human immunodeficiency virus type 1 (HIV-1) during brain glial infection (Nath, 1999; Xiong *et al*, 2000). One of the most potent of these in mixed neuronal cultures is glycoprotein 120 (gp120) derived from the virus' envelope. Picomolar concentrations of gp120 cause significant neuronal loss and apoptosis in primary brain cultures (Brenneman *et al*, 1988; Aggoun-Zouaoui *et al*, 1996), and transgenic mice overexpressing the retroviral protein eventually demonstrate cerebrocortical damage and neurobehavioral impairment (Toggas *et al*, 1994; D'Hooge *et al*, 1999).

The robust neurotoxicity of gp120 is thought to be due ultimately to a combination of glutamatergic receptor activation, $[Ca^{2+}]_i$ elevations, and/or oxidative stress, but proinflammatory cytokine mechanisms might also be involved (Kaul *et al*, 2001; Bezzi *et al*, 2001). Microglia and astroglia are activated by gp120 to mobilize a range of potentially neurotoxic agents. These include extracellular glutamate, elevated because of increased release in association with decreased uptake. Arachidonic acid (AA), mobilized by gp120 from glia, not only inhibits glutamate uptake (Dreyer and Lipton, 1995), but also sensitizes the *N*-methyl-D-aspartate (NMDA) receptor toward agonists (Ushijima *et al*, 1995), and directly signals intracellular apoptotic pathways (Cao *et al*, 2000; Scorrano *et al*, 2001). Superoxide arising from AA metabolism and from mitochondrial processes is likely to be important (Viviani *et al*, 2001); it also is a glutamate uptake inhibitor (Piani *et al*, 1993) and, by combining with nitric oxide (NO) radical (induced by gp120 as well), produces peroxynitrite, a potent oxidizing/nitrosylating species. Other neurotoxic agents released or stimulated by gp120, such as quinolinic acid, cysteine, and unidentified amines, might also have deleterious roles. In addition to these mainly glial-mediated effects, at least in higher concentrations, gp120 may directly augment NMDA receptor responses via binding to the glycine coagonist site (Pattarini *et al*, 1998; Gemignani *et al*, 2000).

In view of the reported prevalence of polydrug use during HIV-1 infection (Tyor and Middaugh, 1999), it is important to understand how drugs of abuse impinge upon the neurotoxic actions of HIV-1 retroviral proteins and the development of AIDS dementia. In addition to impairing already compromised immune function, ethanol abuse can promote nonadherence to antiretroviral therapy and interfere with its pharmacodynamics (Watson, 1990; Fein *et al*, 1995; Bauer, 1998). However, the impact of moderate or social consumption on the central nervous system (CNS) complications of the disease is unclear, and the effects of ethanol on the actions of the HIV-1 retroviral proteins that are responsible for AIDS dementia have been little studied.

We previously reported that rat brain slice cultures, which undergo neuronal apoptosis due to picomolar amounts of gp120 (Aggoun-Zouaoui *et al*, 1996), were protected from the envelope glycoprotein but not from NMDA following subchronic preconditioning with low/moderate ethanol concentrations (20 to 30 mM; Collins *et al*, 2000). The "moderate ethanol preconditioning" abrogated the ability of gp120 to potentiate neurotoxic mediators—e.g., glutamate, AA, and superoxide, and intracellular $Ca^{2+}$ (Belmadani *et al*, 2001). However, prolonged treatment of brain slice cultures with high ethanol concentrations (100 mM) increases neuronal damage due to another neurotoxic HIV-1 protein, Tat (Prendergast and Nath, 2001; Belmadani, unpublished results).

We now report the effects on gp120-dependent neurotoxicity and early induced changes in glutamate, AA, superoxide, and NO of acute *coexposure* with either moderate (30 mM) and high (100 mM) ethanol concentrations in long-term rat organotypic hippocampal-entorhinal cortical (HEC) slice cultures. Neurotoxicity was quantitatively assessed as in previous studies with lactate dehydrogenase (LDH) release, and qualitatively confirmed with propidium iodide (PI) labeling as well as with Fluoro-Jade (FJ), a recently developed neuronal degeneration stain (Schmued and Hopkins, 2000). The possibility that moderate ethanol concentrations could directly inhibit the activity of NMDA receptors in HEC slice cultures, particularly with added glycine, was also studied. While this article was in preparation, Navarra *et al* (2001) reported that ethanol dose-dependently inhibited gp120's toxicity in human neuroblastoma cells and ascribed the inhibition in these glial-free cultures to NMDA receptor blockade by ethanol.

## Results

### *Neuronal degeneration in HEC slice cultures due to HIV-1 gp120 is suppressed by 30 mM ethanol but not 100 mM ethanol coexposure*

Rat HEC slice cultures treated with 200 pM gp120 for 24 h demonstrated significant increases in LDH release compared to control slice cultures (Figure 1**A**). Slice cultures treated for 24 h with a high concentration (1.5 $\mu$M) of an unrelated glycoprotein, albumin, showed no increase in LDH release above controls. Ethanol alone at 30 mM or 100 mM for 15 h, the duration used for coexposure, had no effect on LDH release in 24-h medium (prolonged exposures of these or similar slice cultures to the higher ethanol concentration are necessary to obtain neurotoxicity, e.g., Collins *et al*, 1998; Prendergast and Nath, 2001). Coexposure of gp120 with 30 mM ethanol blocked the retroviral protein's induction of LDH release,

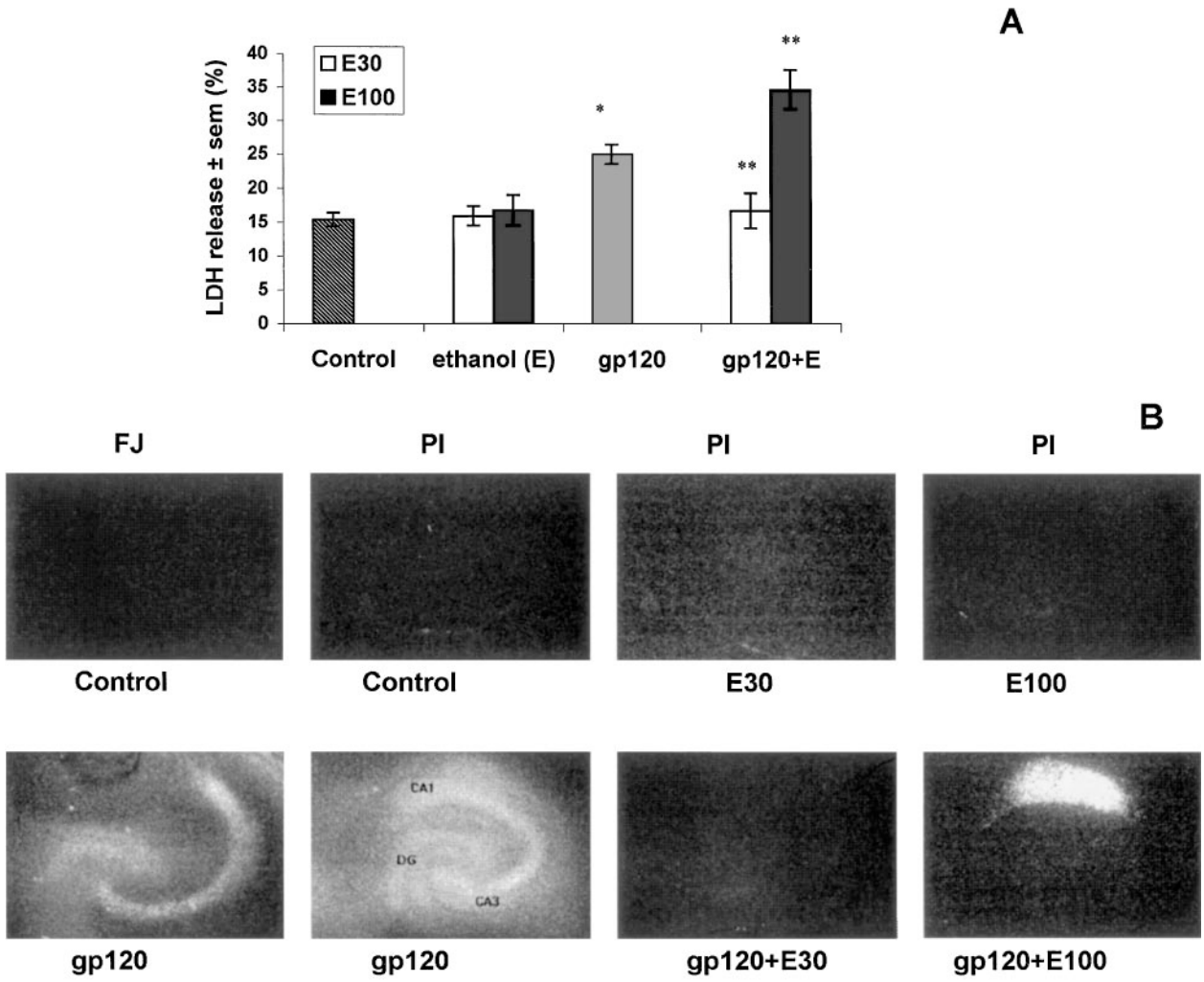


**Figure 1** Neurotoxicity of HIV-1 $gp120_{IIIB}$ in rat HEC slice cultures and effect of ethanol (E) coexposure. (**A**) Percent LDH released over 24 h in control, gp120, and/or ethanol-treated cultures (E30 = 30 mM; E100 = 100 mM). $N = 6$–12 wells/group, representative of three to four replicate experiments. Gp120, 200 pM. $^{\sim}P < .05$ versus control. $^{\approx}P < .05$ versus gp120. (**B**) Degenerating neurons labeled with Fluoro-Jade (FJ) in representative HEC control or gp120-treated slices, or labeled with propidium iodide (PI) in representative HEC control, gp120 (200 pM), and/or ethanol (E30 or E100)–treated slices, as described in Materials and methods.

indicating prevention of neurotoxicity. However, coexposure of gp120 with 100 mM ethanol potentiated LDH release above that due to gp120 alone.

The PI labeling of HEC slices (Figure 1**B**) revealed gp120-induced cellular degeneration in the CA1, CA3, dentate gyrus, and entorhinal cortex (last region not shown). That neurons were being labeled by PI was supported by comparable labeling with FJ, which has been shown to selectively stain degenerating neurons (Schmued and Hopkins, 2000). As shown in Figure 1, FJ cellular labeling in gp120-treated HEC slices was essentially identical to that obtained with PI. In agreement with LDH results, ethanol at either concentration did not cause obvious PI labeling when compared to control slices. When ethanol was copresent at 30 mM (E30) with gp120, the widespread PI labeling induced by gp120 was not seen. However, in coexposure experiments with gp120 and 100 mM ethanol (E100), PI labeling was only apparent in the CA1 field and tended to be more intense in this region than that induced by gp120 alone.

*Potentiation of extracellular glutamate levels by HIV-1 gp120 is prevented by 30 mM ethanol but not 100 mM ethanol coexposure*

Glutamate, the principal NMDA receptor agonist, is considered a critical determinant in gp120 neurotoxicity. We earlier had found that gp120 caused significant elevations in extracellular (medium) glutamate levels in HEC slice cultures within 5 min (Belmadani *et al*, 2001). Despite variability between experiments, glutamate potentiation by gp120 was replicated in these studies, but at a later timepoint (45 min). Compared to respective controls, ethanol by itself at either 30 mM or 100 mM did not change medium glutamate levels at this time point. Coexposure of gp120 with 30 mM ethanol completely prevented the retroviral protein-induced increases in medium glutamate

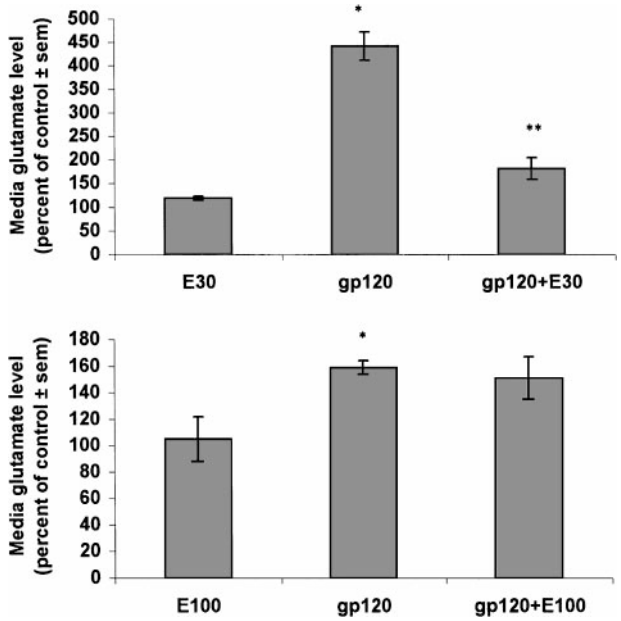


**Figure 2** Medium glutamate levels of rat HEC slice cultures 45 min following treatment with 200 pM HIV-1 gp120 and/or ethanol (*top*, E30 = 30 mM; *bottom*, E100 = 100 mM). $N$ = 6–9 wells/group. $^{\sim}P < .05$ versus control. $^{\sim\sim}P < .05$ versus gp120. Control means (pmoles/mg protein ≈ *SEM*) ranged from 77 ≈ 13 to 223 ≈ 22 in replicate experiments.

levels (Figure 2, *top*). However, gp120 coexposure with 100 mM ethanol failed to prevent the potentiation of medium glutamate levels by gp120 (Figure 2, *bottom*).

### *Mobilization of AA by HIV-1 gp120 is prevented by 30 mM ethanol but not 100 mM ethanol coexposure*

The effects of ethanol on gp120-induced stimulation of AA release were examined because initial mobilization of this key lipid mediator, ostensibly from glia, may be an important modulating event in the protein's overall neurotoxic mechanism. As shown (Figure 3), [$^3$H]-AA–preloaded HEC slices demonstrated significant release of AA after 15 min exposure to 200 pM gp120, consistent with the 5-min and 20-min results reported in our previous experiments (Belmadani *et al*, 2001). Ethanol alone did not significantly alter AA release. However, ethanol coexposure at 30 mM thwarted gp120-induced AA release, whereas 100 mM ethanol coexposure with gp120 did not prevent AA release due to gp120.

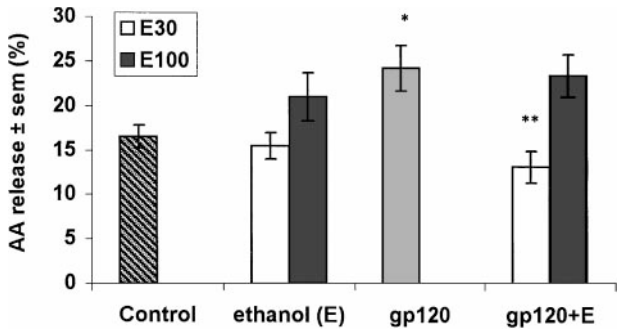


**Figure 3** Mobilization (percent released from tissue into media) of [$^3$H]-arachidonic acid ([$^3$H]-AA) in rat HEC slice cultures 15 min following treatment with 200 pM HIV-1 gp120 and/or ethanol (E30 or E100). $N$ = 6–9 wells/group, replicated twice. $^{\sim}P < .05$ versus control. $^{\sim\sim}P < .05$ versus gp120.

### *Induction of brain slice superoxide levels by gp120 is suppressed by 30 mM ethanol but not 100 mM ethanol coexposure*

Slightly higher gp120 concentrations were used in the superoxide and NO studies because the viral protein's toxic potency had diminished during storage. Levels of superoxide, another possible mediator of gp120-dependent neurotoxicity, were significantly enhanced in the HEC slices 45 min after treatment with gp120 at 250 pM (Figure 4).

Ethanol at 30 mM did not alter superoxide levels, but 100 mM ethanol increased tissue superoxide levels ≈20% over control ($P < .05$). Coexposure of gp120 and 30 mM ethanol completely blocked gp120-dependent induction of the oxygen free radical, whereas superoxide levels remained elevated during gp120 coexposure with 100 mM ethanol.

### *Potentiation of NO levels by HIV-1 gp120 is unaffected by 30 mM ethanol but is blocked by 100 mM ethanol coexposure*

Because NO is known to be increased by gp120 in brain preparations, we determined the effect of the two ethanol concentrations on NO levels (as total nitrite) in medium of gp120-treated cultures (Figure 5). Consistent with reports in dispersed neuronal cultures, the medium nitrite levels in HEC slice cultures were increased ≈120% over control 45 min after gp120 addition. Ethanol alone at either 30 mM and 100 mM also induced significant 50% to 60% increases in nitrite levels. Coexposure of gp120 with

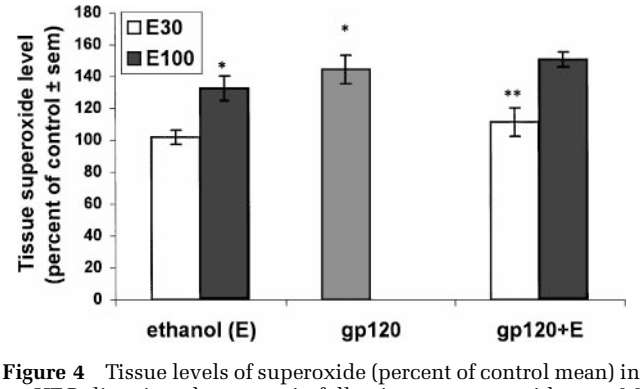


**Figure 4** Tissue levels of superoxide (percent of control mean) in rat HEC slices in culture 45 min following treatment with 250 pM HIV-1 gp120 and/or ethanol (E30 or E100). $N$ = 6–9 wells/group. $^{\sim}P < .05$ versus control. $^{\sim\sim}P < .05$ versus gp120. Control mean, 10.90 ≈ 0.31 nmoles/mg protein ≈ *SEM*); results replicated in three studies.

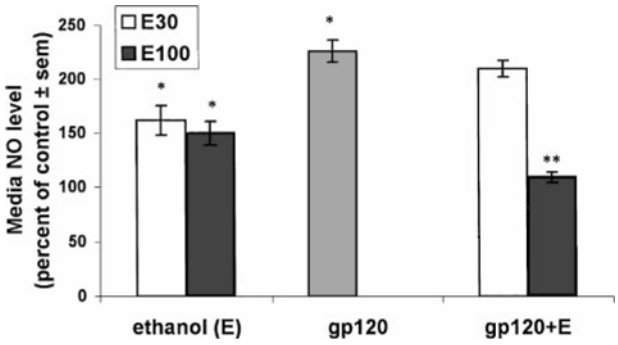


**Figure 5** Medium nitric oxide (total nitrite) levels (percent of control mean) in rat HEC slices in culture 45 min following treatment with 250 pM HIV-1 gp120 and/or ethanol (E30 or E100). $N =$ 6–9 wells/group. $^{\sim}P < .05$ versus control. $^{\sim\sim}P < .05$ versus gp120. Control mean, 68.0 ≈ 13.0 (ng/mg protein ≈ *SEM*).

30 mM ethanol did not alter the potentiation of medium nitrite by gp120. However, 100 mM ethanol coexposure with gp120 completely blocked the induction of medium nitrite levels.

*Neurotoxicity in HEC slice cultures caused by NMDA and added glycine is prevented by 30 mM ethanol*

To examine whether 30 mM ethanol was sufficient to directly inhibit neurotoxicity induced by activation of the NMDA receptor, HEC slice cultures were treated with either NMDA alone or NMDA plus glycine, with and without ethanol coexposure. The percent of LDH released in the 48-h medium was ≈160% of control in slice cultures treated 30 min with 100 $\mu$M NMDA, and ≈220% of control after 30-min treatment with a combination of 25 $\mu$M NMDA and 10 $\mu$M glycine, consistent with neurotoxicity in either treatment (Figure 6). Addition to cultures of 30 mM ethanol with agonists for 30 min and the following $14^1/_2$ h (total, 15 h) did not alter the increased LDH release due to NMDA alone, but it prevented the increase in LDH release due to treatment with NMDA and glycine.

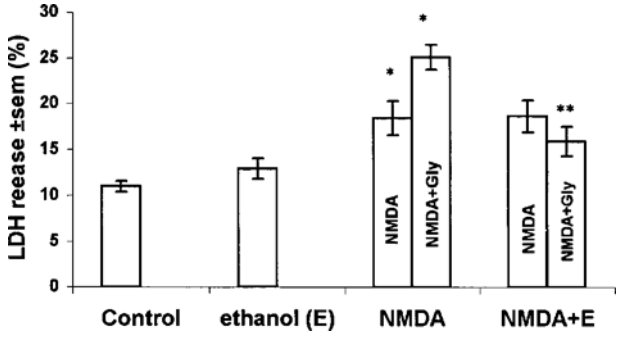


**Figure 6** Effect of 30 mM ethanol on percent LDH released over 48 h in rat HEC slice cultures following treatment for 30 min with 100 $\mu$M NMDA or 25 $\mu$M NMDA/10 $\mu$M glycine (gly), as described in Materials and Methods. $N = 6$–9 wells/group. $^{\sim}P < .05$ versus control. $^{\sim\sim}P < .05$ versus gp120.

## Discussion

The interactions of ethanol and the CNS have received relatively little attention in the context of HIV-1–associated dementia. This is somewhat surprising, because ethanol and HIV-1 gp120 share overlapping—sometimes antagonistic and sometimes complementary—mechanisms with respect to neuronal glutamatergic systems and glial cells (Tabakoff, 1994; Davis and Wu, 2001; Kaul *et al*, 2001). The intact regional nature of organotypic brain slices in long-term culture, as with other neuronal degeneration studies (e.g., Diekmann *et al*, 1994), permits transcellular studies with gp120 and ethanol. It should be noted that the HEC slices were allowed to mature a minimum of 3 weeks *in vitro* in order to permit recovery from dissection, adherence to membrane, and removal of nonviable slices. Slices used showed little if any "control" neuronal death with neuronal degeneration stains (Sytox or PI). In that sense they are different from the immature week-old organotypic rat hippocampal slice cultures used by Thomas and Morrisett (2000) that apparently exhibit considerable PI-labeled neurons before additions of NMDA receptor agonists or ethanol.

Our use of LDH as a quantitative measure of neuronal death is supported by the knowledge that the cytosolic protein is derived largely from neurons during excitotoxic challenges in a range of neuronal cultures, including organotypic brain slices (Vornov *et al*, 1991; Bruce *et al*, 1996), and that excitotoxicity is believed to constitute a central mechanistic route underlying neuronal degeneration induced by gp120 (Kaul *et al*, 2001). Furthermore, PI labeling was shown to correlate well with LDH release as well as with FJ labeling of neurons in organotypic slice cultures subjected to a number of differing neurotoxins (Zimmer *et al*, 2000). Recently, LDH release was used as a quantitative measure of neuronal death in primary mixed brain cultures exposed to HIV-1 Tat protein (Perez *et al*, 2001). Specifically concerning gp120, despite the fact that nanomolar concentrations of the retroviral protein caused gliotoxicity after prolonged (7 days) treatment of human brain cultures (Pulliam *et al*, 1993), similar gp120 concentrations did not increase LDH release from pure astroglial cultures (Boutet *et al*, 2000), and importantly, during neurotoxic treatment of HEC slice cultures with gp120, we observed no significant change in the number of glial acidic fibrillary protein (GFAP)-labeled astroglia (Belmadani *et al*, 2001). In summary, the evidence supports LDH assays as a valid marker of gp120-induced neuronal degeneration in brain slices.

Table 1 is a summary of the actions of HIV-1 gp120 on tissue AA mobilization and superoxide levels, medium glutamate and NO levels, and assessments of neurotoxicity—percent LDH release and PI-labeled neurons—in HEC slice cultures, and, moreover, how ethanol coexposure at 30 mM or 100 mM affects the

**Table 1** Summary: induction by HIV-1 gp120 of neurotoxic mediators/neurotoxicity in rat brain slice cultures and the effect of ethanol (e)

| | *Early effects* | | | | *24-h neurotoxicity* | |
|---|---|---|---|---|---|---|
| | *Extracell. glutamate* | *AA mobilization* | *Tissue superoxide* | *Medium nitric oxide* | *LDH release* | *PI staining* |
| gp120 | $\Delta$ | $\Delta$ | $\Delta$ | $\Delta$ | $\Delta$ | $\Delta$ |
| +30 mM E | $\Downarrow$ | $\Downarrow$ | $\Downarrow$ | $\Longleftrightarrow$ | $\Downarrow$ | $\Downarrow$ |
| +100 mM E | $\Longleftrightarrow$ | $\Longleftrightarrow$ | $\Longleftrightarrow$ | $\Downarrow$ | $\Uparrow$ | $\Uparrow$ (CA) |

$\Delta$ = induction by gp120 compared to control.
$\Downarrow$ = suppression of gp120 induction by ethanol coexposure.
$\Longleftrightarrow$ = no significant change in gp120 induction by ethanol coexposure.
$\Uparrow$ = potentiation of gp120 induction by ethanol coexposure.
CA = predominantly hippocampal CA1 field.

retroviral protein's actions. The two concentrations have divergent effects on gp120's early stimulation of the presumed glial mediators and subsequent neuronal degeneration. With regard to the physiological significance of the ethanol concentrations, brain ethanol concentrations are known to parallel ethanol in the blood (Fein and Meyerhoff, 2000), and a blood ethanol of 25 to 30 mM or 115 to 138 mg/dl is a moderately intoxicating concentration achieved after five to seven alcoholic drinks in most individuals. A blood ethanol concentration of 100 mM ($\approx$460 mg/dl) is attained only in chronically adapted active alcoholics and alcohol abusers (Adachi *et al*, 1991; Jones 1998), and is neurotoxic in HEC slice cultures during extended treatment (Collins *et al*, 1998).

That coexposure with the lower ethanol concentration provides neuroprotection against gp120 could reflect two different modes of action consonant with the mechanisms of the retroviral protein. The first, resembling the protection observed in HEC slice cultures preconditioned with moderate ethanol (Belmadani *et al*, 2001), targets glia, and specifically could be secondary to initial blockade of gp120-induced mobilization of largely astroglial-derived AA (Ushijima *et al*, 1995; Kaul *et al*, 2001). Released in excess from membranes by phospholipase A2 (PLA2) activation, this essential unsaturated fatty acid (a) inhibits astroglial glutamate uptake while promoting glutamate release directly or through prostaglandin PGE-2 (Bezzi *et al*, 1998), and possibly involving increased astroglial $Na^+/H^+$ exchange (Benos *et al*, 1994), (b) increases NMDA receptor responsiveness (Ushijima *et al*, 1995), (c) produces free radicals including superoxide anion, and (d) triggers apoptosis by increasing mitochondrial permeability (Scorriano *et al*, 2001). Reactive oxygen species (in particular superoxide, another neurotoxic mediator and possible inhibitor of glutamate reuptake [Piani *et al*, 1993]) also are induced in glia by gp120 (Viviani *et al*, 2001).

Consequently, high-performance liquid chromatography (HPLC) measurements of HEC slice culture medium shortly after gp120 addition indeed confirmed several-fold glutamate elevations above controls, consistent with increased release and inhibited re-uptake. Again paralleling the effect of subchronic 30 mM ethanol pre-exposure (Belmadani *et al*, 2001), cotreatment with 30 mM ethanol completely suppressed the glutamate increases. In addition, gp120 caused superoxide increases in the HEC slices that would augment oxidative stress, and we suggest the glutamate suppression by 30 mM ethanol could ensue from decreased AA mobilization. It is interesting that the gp120-dependent NO induction was unresponsive to the neuroprotective ethanol concentration, but superoxide suppression in theory would nevertheless tend to nullify peroxynitrite formation, protein nitrosylation, hydroxyl radical generation, and their contributions to gp120-mediated neuronal death.

A second mode of neuroprotection by ethanol against gp120, as indicated by our results in Figure 6, could involve direct suppression of glutamatergic (NMDA) receptor function, possibly at the glycine coagonist site. This interpretation is consistent with the evidence that ethanol reduces gp120 neurotoxicity in neuroblastoma cells by inhibiting NMDA receptor activity and $Ca^{2+}$ responses (Navarra *et al*, 2001). However, with neuroblastoma cultures that lack glia to supply AA, superoxide, and other possible toxic mediators, NMDA receptor-mediated toxicity directly induced by gp120 may be the principal route available for the induction of cell death.

If gp120 does interact via its V3 loop with the glycine site of the NMDA receptor (Pattarini *et al*, 1998), such receptor inhibition by ethanol might be important in the HEC slice cultures. Earlier demonstrations of ethanol's reduction of NMDA-induced neurotoxicity have utilized dispersed neuronal cell cultures (Takadera *et al*, 1990; Lustig *et al*, 1992; Chandler *et al*, 1993; Crews *et al*, 1998). In those cases, the $IC_{50}$ range for ethanol was 45 to 60 mM, somewhat higher than the concentration that is effective against gp120 in HEC slice cultures. Moreover, because NMDA receptor stimulation in mixed neuronal cultures has been reported to promote release of AA, glutamate, and superoxide (Lafon-Cazal *et al*, 1993; Hamada *et al*, 1998), an additional outcome of ethanol's inhibition of NMDA receptor activity in HEC cultures conceivably would be decreased

gp120-dependent release of these mediators from neuronal processes—although the evidence, again from dispersed brain cell cultures, indicates that glia are the primary source of these mediators during gp120 treatment (Kaul *et al*, 2001).

However, the fact that high "alcoholic" ethanol concentrations, well-established to inhibit NMDA receptor–mediated excitotoxicity, enhanced rather than prevented gp120 neurotoxicity indicates that NMDA receptor activation during gp120 treatment may be a less important path to neuronal death than previously believed. Rather, other glial derived toxic factors or pathways that are now promoted by 100 mM ethanol might now reinforce gp120's apoptotic mechanism(s). These include renewed augmentation of gp120-induced AA mobilization, and potentiation, as opposed to reduction, of tissue superoxide. Interestingly, in striatal cultures, glutamate-dependent AA release was inhibited by very brief exposure to a similar high ethanol concentration, but it was increased, as was neurotoxicity, by 48-h ethanol exposure (Navamani *et al*, 1997). These authors reasoned that increased AA mobilization was critical to the increased neuronal damage. Also, superoxide has been suggested to be fundamental to gp120's mechanism as well (Viviani *et al*, 2001). The amplification of extracellular glutamate, rather than promoting excitotoxic receptor-mediated pathways (inhibited by ethanol's presence even at 30 mM), could trigger oxidative stress via "oxidative glutamate toxicity" (Schubert and Piasecki, 2001). Overall, the neurotoxic mechanism of gp120 could be shifted by high ethanol to a greater extent toward oxidative stress, but this interpretation requires confirmation with measurement of intracellular players such as glutathione. Last, a further determinant in HEC cultures possibly contributing to the augmentation of gp120's neurotoxic effects by high ethanol could be blockade of gp120-induced elevations in NO; this free radical is neuroprotective in certain instances, particularly if generated in the brain vasculature (Lipton, 1999). Further studies are required to determine the role of NO in this model.

The molecular mechanisms engendered by moderate versus high ethanol concentrations that alter gp120's actions are speculative at this point. Aside from its possible interactions with neuronal NMDA receptors, gp120 signals via chemokine receptors on glia and perhaps neurons to elicit the above-mentioned neurotoxic cascades and apoptotic pathways, and it also alters brain chemokine ligand expression (Gabuzda and Wang, 2000; Miller and Meucci, 1999). Ethanol also has been shown to alter chemokine ligands and their receptors in nonneural cells (Bautista, 2000, 2001). Relevant to our CNS studies is the alcohol's suppression of gp120/chemokine receptor binding in liver macrophages, possibly through interference with postreceptor signal transduction. Furthermore, ethanol down-regulated the chemokines macrophage inflammatory protein (MIP)-1$\mu$ and monocyte chemoattractant protein (MCP)-1, while priming the cells for enhanced induction by gp120 of the chemokine, RANTES. In view of the evidence that RANTES exerts neuroprotection against gp120 (Brenneman *et al*, 1999; Meucci *et al*, 1998), one possibility is that ethanol's effects with respect to gp120 in our HEC slice cultures involves modulation of glial chemokines such as RANTES, as well as their receptors.

The significance of these findings to human health is also conjectural at this juncture. Exacerbation of gp120 neurotoxicity by a high ethanol concentration is interesting but not surprising, but neuroprotection against gp120 by the presence of moderate ethanol concentrations is unforeseen. Moderate or social alcohol (notably wine) consumption may be cardioprotective and, more recently, has been associated with a reduced risk of dementia in aging (Orgogozo *et al*, 1997; Zuccala *et al*, 2001); however, to suggest that HIV-1–infected individuals consume ethanol to ward off brain damage would not be prudent. Nevertheless, epidemiological studies might further explore whether similar associations exist between social drinking and cognitive impairments in HIV-1 subjects. In addition, clarifying ethanol's mechanisms could lead to insights in the control and treatment of brain encephalitis and dementia in AIDS. It also might eventually have a bearing on neuroprotective approaches in other CNS viral conditions.

## Materials and methods

Reagents were from Sigma Chemical Co. (St. Louis, MO) except where noted otherwise. Rat sacrifices for organotypic HEC slice cultures were done with minimal suffering and in accordance with National Institute of Health (NIH) guideline #80-23 and Loyola University Medical College (LUMC) Animal Care requirements. One-week-old Sprague-Dawley rat pups (Zivic-Miller Labs, USA), hypothermically anesthetized on ice for 2 min, were decapitated and the HEC complex was dissected free. Following Stoppini *et al* (1991), 350-$\mu$ transverse brain slices were taken with a tissue chopper and placed on interface membranes (Millicell-CM 0.4 $\mu$, Millipore Co., Bedford, MA), four to five slices per membrane, in 6-well plates. Covered plates containing HEC slices were cultured in an atmosphere of 95% $O_2$/5% $CO_2$ over modified Eagle's medium (MEM) (Gibco Corp., Rockville, MD) containing 25% horse serum, 1.3 mM $Ca^{2+}$, and 7.5 mg/ml glucose; medium was changed every 3 days. Slices were examined periodically and at the beginning of each experiment for viability using Sytox (Molecular Probes, Eugene, OR), a fluorescent dye for dead or dying cells. Following exposure to 20 nM Sytox for 20 min, slices were examined with a Nikon fluorescent microscope using a standard fluorescein filter, and those showing extensive fluorescent neurons were discarded.

*Treatment of HEC slice cultures*
After 3 to 4 weeks in culture, HEC slices on membranes in fresh medium were treated with 200 to 250 pM recombinant HIV-1 $gp120_{IIIB}$ (NIH AIDS Reagent & Reference Program, Rockville, MD), ethanol (30 mM or 100 mM), gp120 with ethanol (30 mM or 100 mM), or medium alone (control). Ethanol was removed after 15 h, and gp120 treatment was continued for a total of 24 h. As control for nonspecific neurotoxic effects of a glycoprotein, HEC slice cultures were treated for 24 h with 1.5 $\mu$M albumin (fraction V). In experiments studying the effect of moderate ethanol concentrations on NMDA receptor–mediated neurotoxicity, NMDA (100 $\mu$M) or NMDA (25 $\mu$M) plus glycine (10 $\mu$M) were added in MEM for 30 min, ethanol when present was continued for 15 h, and medium samples were taken at 48 h for neurotoxicity assays (below).

*Neurotoxicity assessment*
*LDH assays and PI or FJ labeling:* Neurotoxicity was determined after 24 or 48 h in aliquots (100 $\mu$l) of culture medium and homogenates of HEC slices by determining LDH activities with a Sigma LD-L50 kit. Activities were standardized per milligram tissue protein, measured in homogenates of slices with the BCA protein assay kit (Pierce Chemical Co., Rockford, IL), and were expressed as percent of total LDH released. Selected HEC slices in culture were stained for degenerating neurons by adding PI (5 $\mu$g/ml of medium) 1 h before addition of gp120 and/or ethanol (Kristensen *et al*, 1999). For FJ labeling, selected slices were fixed in 4% paraformaldehyde and stained with FJ according to Zimmer *et al* (2000).

*Glutamate assays:* Modifying the method of Bianchi *et al* (1999), glutamate concentration in HEC slice culture medium was determined by reverse-phase HPLC. Aliquots (50 $\mu$l) of medium from HEC slice cultures were taken 45 min after addition of 200 pM gp120 and/or ethanol (30 mM or 100 mM), and added to 50 $\mu$l 0.4 M sodium borate, pH 10.4, containing 375 ng/ml cysteic acid as internal standard. Mixtures were derivatized with 100 $\mu$l of an orthophthaldehyde-$\mu$-mercaptoethanol reagent solution for 2.5 min and injected into a gradient HPLC system fitted with a Varian Microsorb 5 $\mu$ C18 column (4.6 $\approx$ 250 mm). Gradient separation was done with 0.1 M potassium acetate (pH 5.2) as solvent A and HPLC grade methanol as solvent B (flow rate, 0.9 ml/min). Derivatized glutamate and cysteic acid were detected with a Milton-Roy fluorometer III detector at 340 nm excitation and 455 nm emission, quantitated with an HP 3390A integrator using a curve generated from standards, calculated as pmoles/mg protein, and expressed as percent above control means.

*Arachidonic acid:* For AA release, following Dreyer and Lipton (1995), HEC slices in culture were prelabeled overnight at 37$^{\circ}$C with 1 $\mu$Ci/ml [$^3$H]-AA (specific activity, 200 Ci/mmol; Moravek Biochemicals, Brea, CA) in fresh MEM and washed three times in Locke's buffer. Slices on membranes were then divided and treated with gp120 (200 pM) and/or ethanol (30 mM or 100 mM), or with fresh medium only. After 15 min, aliquots (100 $\mu$l) of the culture medium and tissue homogenates were taken for radioactive counting. Slice protein was determined (above), and results expressed as percent of released [$^3$H]-AA.

*Superoxide:* Superoxide in HEC slices was determined by the indirect method of the superoxide dismutase (SOD)-inhibitable reduction of iodonitrotetrazolium violet (INTV) (Podczasy and Wei, 1988). To slices treated with gp120 (250 pM) and/or ethanol (30 or 100 mM) in serum-free MEM at 37$^{\circ}$C, 0.75 mg/ml INTV and 18 units/ml SOD were added. After 45 min, slices were washed with phosphate-buffered saline (PBS) and solubilized by overnight incubation in DMSO containing 0.6 M HCl. The absorbance of the INTV reduced by SOD was calculated using a molar extinction coefficient of 19.31 $mM^{\approx 1}cm^{\approx 1}$ for INTV. After measuring tissue protein with the protein assay (above), results were calculated as nmol superoxide/mg protein and expressed as percent above control.

*Nitric oxide:* NO was estimated in the medium of HEC cultures 45 min after treatment with gp120 (250 pM) and/or ethanol (30 mM or 100 mM), or medium (control), by determining total nitrite concentrations with the Greiss reagent (Green *et al*, 1982). Media samples (75 $\mu$l) were taken and mixed with an equal volume of reagent consisting of 0.1% *N*-(1-naphthyl)ethylenediamine diHCl, 1% sufanilamide, and 2.5% $H_3PO_4$. After 15 min at room temperature, the absorbance at 492 nm (the maximum ascertained for our studies) was measured in a microplate reader. The concentration of NO in the medium was determined with a sodium nitrite standard curve, and with tissue protein values (BCA protein assay, above), was calculated in nmoles NO/mg protein and expressed as percent above control.

Mean values in all experiments were compared with analysis of variance (ANOVA) and appropriate posthoc tests, and $P < .05$ was considered significant.

## References

Adachi J, Mizoi Y, Fukunaga T, Ogawa Y, Ueno Y, Imamichi H (1991). Degrees of alcohol intoxication in 117 hospitalized cases. *J Stud Alcohol* **52:** 448–453.

Aggoun-Zouaoui D, Charriaut-Marlangue C, *et al* (1996). The HIV-1 envelope protein gp120 induces neuronal apoptosis in hippocampal slices. *NeuroRep* **7:** 433–436.

Bauer LO (1998). Effects of chronic opioid dependence and HIV-1 infection on pattern shift visual evoked potentials. *Drug Alcohol Depend* **50:** 147–155.

Bautista AP (2000). Impact of alcohol on the ability of Kupffer cells to produce chemokines and its role in alcoholic liver disease. *J Gastroenterol Hepatol* **15:** 349–356.

Bautista AP (2001). Acute alcohol intoxication and endotoxemia desensitize HIV-1 gp120-induced CC-chemokine production by Kupffer cells. *Life Sci* **68:** 1939–1949.

Belmadani A, Zou JY, *et al* (2001). Ethanol pre-exposure suppresses HIV-1 gp120-induced neuronal degeneration by abrogating endogenous glutamate/$Ca^{2+}$-mediated neurotoxicity. *Neuroscience* **104:** 769–781.

Benos DJ, Hahn BH, *et al* (1994). Envelope gp120 of HIV-1 alters ion transport in astrocytes—implications for AIDS dementia complex. *Proc Natl Acad Sci USA* **91:** 494–498.

Bezzi P, Carmignoto G, *et al* (1998). Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391:** 281–285.

Bezzi P, Domercq M, *et al* (2001). CXCR4-activated astrocyte glutamate release via TNFa: amplification by microglia triggers neurotoxicity. *Nat Neurosci* **4:** 702–710.

Bianchi L, Della Corte L, *et al* (1999). Simultaneous determination of basal and evoked output levels of aspartate, glutamate, taurine and 4-aminobutyric acid during microdialysis and from superfused brain slices. *J Chromatog* **723:** 47–59.

Boutet A, Altmeyer R, *et al* (2000). Direct role of plasma membrane-expressed gp120/41 in toxicity to human astrocytes induced by HIV-1-infected macrophages. *AIDS* **14:** 2687–2697.

Brenneman DE, Hauser J, *et al* (1999). VIP and D-ala-peptide T-amide release chemokines which prevent HIV-1 gp120-induced neuronal death. *Brain Res* **838:** 27–36.

Brenneman DE, Westbrook GL (1988). Neuronal cell killing by the envelope protein of HIV and its prevention by vasoactive intestinal polypeptide. *Nature* **335:** 639–642.

Bruce A, Malfroy B, *et al* (1996). $\mu$-Amyloid toxicity in organotypic hippocampal cultures: protection by EUK-8, a synthetic free radical scavenger. *Proc Natl Acad Sci U S A* **93:** 2312–2316.

Cao Y, Pearman AT, *et al* (2000). Intracellular unesterified arachidonic acid signals apoptosis. *Proc Natl Acad Sci U S A* **97:** 11280–11285.

Chandler LJ, Sumners C, Crews FT (1993). Ethanol inhibits NMDA receptor-mediated excitotoxicity in rat primary neuronal cultures. *Alc Clin Exp Res* **17:** 54–60.

Collins MA, Neafsey EJ, *et al* (2000). HIV-1 gp120-induced neurotoxicity in brain cultures is prevented by ethanol pre-treatment. *NeuroRep* **11:** 1219–1222.

Collins MA, Zou JY, *et al* (1998). Brain damage due to episodic alcohol exposure *in vivo* and *in vitro*: furosemide neuroprotection implicates key role for edema. *FASEB J* **12:** 221–230.

Crews FT, Steck JC, *et al* (1998). Ethanol, stroke, brain damage, and excitotoxicity. *Pharmacol Biochem Behav* **59:** 981–991.

Davis KM, Wu JY (2001). Role of glutamatergic and GABAergic systems in alcoholism. *J Biomed Sci* **8:** 7–19.

D'Hooge R, Franck F, *et al* (1999). Age-related behavioural deficits in transgenic mice expressing the HIV-1 coat protein gp120. *Eur J Neurosci* **11:** 4398–4402.

Diekmann S, Nitsch R, *et al* (1994). The organotypic entorhinal-hippocampal complex slice culture of adolescent rats. A model to study transcellular changes in a circuit particularly vulnerable in neurodegenerative disorders. *J Neural Trans (Suppl)* **44:** 61–71.

Dreyer EB, Lipton SA (1995). The coat protein gp120 of HIV-1 inhibits astrocyte uptake of excitatory amino acids via macrophage arachidonic acid. *Eur J Neurosci* **7:** 2502–2507.

Fein G, Biggins CA, *et al* (1995). Alcohol abuse and HIV infection have additive effects on frontal cortex function as measured by auditory evoked potential P3A latency. *Biol Psychiatry* **37:** 183–195.

Fein G, Meyerhoff DJ (2000). Ethanol in human brain by magnetic resonance spectroscopy: correlation with blood and breath levels, relaxation, and magnetization transfer. *Alc Clin Exp Res* **24:** 1227–1235.

Gabuzda D, Wang JB (2000). Chemokine receptors and mechanisms of cell death in HIV neuropathogenesis. *J NeuroVirol* **6(Suppl 1):** S24–S32.

Gemignani A, Paudice P, *et al* (2000). The HIV-1 coat protein gp120 and some of its fragments potently activate native cerebral NMDA receptors mediating neuropeptide release. *Eur J Neurosci* **12:** 2839–2846.

Green LC, Wagner DA, *et al* (1982). Analysis of nitrate, nitrite, and [$^{15}$N]nitrate in biological fluids. *Anal Biochem* **126:** 131–138.

Hamada T, Sonoda R, *et al* (1998). NMDA induced glutamate release from the suprachiasmatic nucleus: an in vitro study in the rat. *Neurosci Lett* **256:** 93–96.

Jones AW (1998). Lack of association between urinary creatinine and ethanol concentrations and urine/blood ratio of ethanol in two successive voids from drinking drivers. *J Anal Toxicol* **22:** 184–190.

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

Kristensen BW, Noraberg J, *et al* (1999). Excitotoxic effects of non-NMDA receptor agonists in organotypic corticostriatal slice cultures. *Brain Res* **841:** 143–159.

Lafon-Cazal M, Pietri S, *et al* (1993). NMDA-dependent superoxide production and neurotoxicity. *Nature* **364:** 535–537.

Lipton SA (1999). Neuronal protection and destruction by NO. *Cell Death Differ* **6:** 943–951.

Lustig HS, Chan J, Greenberg DA (1992). Ethanol inhibits excitotoxicity in cerebral cortical cultures. *Neurosci Lett* **135:** 259–261.

Meucci O, Fatatis A, *et al* (1998). Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Natl Acad Sci U S A* **95:** 14500–14505.

Miller RJ, Meucci O (1999). AIDS and the brain: is there a chemokine connection? *Trends Neurosci* **22:** 471–479.

Nath A (1999). Pathobiology of HIV dementia. *Sem Neurol* **19:** 113–127.

Navamani M, Morgan M, *et al* (1997). Ethanol modulates *N*-methyl-D-aspartate-evoked arachidonic acid release from neurones. *Eur J Pharmacol* **34:** 27–34.

Navarra M, Romano C, *et al* (2001). Ethanol exposure inhibits the cytotoxic effect induced by gp120 in CHP100 human neuroblastoma cells. *J Neurosci Res* **65:** 354–361.

Orgogozo JM, Dartigues JF, *et al* (1997). Wine consumption and dementia in the elderly: a prospective community study in the Bordeaux area. *Revue Neurol* **153:** 185–192.

Pattarini R, Pittaluga A, *et al* (1998). The human immunodeficiency virus-1 envelope protein gp120 binds through its V3 sequence to the glycine site of *N*-methyl-D-aspartate receptors mediating noradrenaline release in the hippocampus. *Neuroscience* **87:** 147–157.

Perez A, Probert AW, *et al* (2001). Evaluation of HIV-1 Tat induced neurotoxicity in rat cortical cell culture. *J NeuroVirol* **7:** 1–10.

Piani D, Frei K, *et al* (1993). Glutamate uptake by astrocytes is inhibited by reactive oxygen intermediates but not by other macrophage-derived molecules including cytokines, leukotrienes or platelet-activating factor. *J Neuroimmunol* **48:** 99–104.

Podczasy JJ, Wei R (1988). Reduction of iodonitrotetrazolium violet by superoxide radicals. *Biochem Biophys Res Commun* **150:** 1294–1301.

Prendergast MA, Nath A (2001). Synergistic effects of ethanol withdrawal and HIV-1 Tat protein on hippocampal damage in rat. *Alcohol Clin Exp Res* **27:** 9A.

Pulliam L, West D, *et al* (1993). HIV-1 envelope gp120 alters astrocytes in human brain cultures. *AIDS Res Hum Retrovirus* **9:** 439–444.

Scorrano Y, Penzo D, *et al* (2001). Arachidonic acid causes cell death through the mitochondrial permeability transition. *J Biol Chem* **276:** 12035–12040.

Schmued LC, Hopkins KJ (2000). Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res* **874:** 123–130.

Schubert D, Piasecki D (2001). Oxidative glutamate toxicity can be a component of the excitotoxicity cascade. *J Neurosci* **21:** 7455–7462.

Stoppini L, Buchs PA, *et al* (1991). A simple method for organotypic cultures of nervous tissue. *J Neurosci Methods* **37:** 173–182.

Tabakoff B (1994). Alcohol and AIDS: is it all in our heads? *Alcohol Clin Exp Res* **18:** 415–416.

Takadera T, Suzuki R, Mohri T (1990). Protection by ethanol of cortical neurons from NMDA-induced neurotoxicity is associated with blocking calcium influx. *Brain Res* **537:** 109–114.

Thomas MP, Morrisett RA (2000). Dynamics of NMDAR-mediated neurotoxicity during chronic ethanol exposure and withdrawal.*Neuropharmacology* **39:** 218–226.

Toggas SM, Masliah E, *et al* (1994). Central nervous system damage produced by expression of the HIV-1 coat protein gp120 in transgenic mice. *Nature* **367:** 188–193.

Tyor WR, Middaugh LD (1999). Do alcohol and cocaine abuse alter the course of HIV-associated dementia complex? *J Leukoc Biol* **65:** 475–481.

Ushijima H, Nishio O, *et al* (1995). Exposure to gp120 of HIV-1 induces an increased release of arachidonic acid in rat primary neuronal cell culture followed by NMDA receptor-mediated neurotoxicity. *Eur J Neurosci* **7:** 1353–1359.

Viviani B, Corsini E, Binaglia M, Galli CL, Marinovich M (2001). Reactive oxygen species generated by glia are responsible for neuron death induced by HIV-1 120 *in vitro*. *Neuroscience* **107:** 51–58.

Vornov JJ, Tasker RC, *et al* (1991). Direct observation of the agonist-specific regional vulnerability to glutamate, NMDA, and kainate neurotoxicity in organotypic hippocampal cultures. *Exp Neurol* **114:** 11–22.

Watson RR (1990). Immunomodulation by alcohol: a cofactor in development of AIDS after retrovirus infection. In: *Cofactors in HIV-1 infection and AIDS*. Watson RR (ed). CRC Press: New York, pp 47–53.

Xiong HG, Zeng YC, *et al* (2000). HIV-1 infected mononuclear phagocyte secretory products affect neuronal physiology leading to cellular demise: relevance for HIV-1-associated dementia. *J NeuroVirol* **6(Suppl 1):** S14–S23.

Zimmer J, Kristensen BW, *et al* (2000). Excitatory amino acid neurotoxicity and modulation of glutamate receptor expression in organotypic brain slice cultures. *Amino Acids* **19:** 7–21.

Zuccala G, Onder G, *et al* (2001). Dose-related impact of alcohol consumption on cognitive function in advanced age: results of a multicenter study. *Alcohol Clin Exp Res* **25:** 1743–1748.