

**Increased vulnerability of ApoE4 neurons to HIV proteins and opiates: protection
by diosgenin and L-deprenyl**

Jadwiga Turchan-Cholewo[¶], Yiling Liu^{*}, Suzanne Gartner^{*}, Chunfa Jie[†], Xuejun Peng[‡], Kuey Chu C. Chen[‡], Ashok Chauhan^{*}, Norman Haughey^{*}, Roy Cutler[§], Mark Mattson[§], Carlos Pardo^{*}, Katherine Conant^{*}, Ned Sacktor, Justin C. McArthur^{*}, Kurt F. Hauser[¶], Chandra Gairola^{||}, Avindra Nath^{*}

Department of ^{*}Neurology and [†]Institute of Genetic Medicine, Johns Hopkins University, Baltimore, MD; [‡]Microarray core, [¶]Departments of Anatomy and Neurobiology, and ^{||}Pharmacy, University of Kentucky, Lexington, KY, [§]National Institute of Aging, Baltimore, MD

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Running Head: HIV dementia with ApoE4 and drug abuse

Corresponding author: A. Nath MD, Department of Neurology, 600 N. Wolfe St., 509 Pathology, Johns Hopkins University, Baltimore MD, 21287; Tele: 443-287-4656; Fax:410-502-8075; e-mail: anath1@jhmi.edu

ABSTRACT:

Human immunodeficiency virus (HIV) infection continues to rise in drug abusing populations and causes a dementing illness in a subset of individuals. Factors contributing to the development of dementia in this population remain unknown. We found that HIV infected individuals with the E4 allele of Apolipoprotein E (ApoE) or history of intravenous drug abuse had increased oxidative stress in the CNS. In vitro studies showed that HIV proteins, gp120 and Tat, Tat+morphine but not tumor necrosis factor- α (TNF- α), caused increased neurotoxicity in human neuronal cultures with ApoE4 allele. Microarray analysis showed a differential alteration of transcripts involved in energy metabolism in cultures of ApoE3 and 4 neurons upon treatment with Tat+morphine. This was confirmed using assays of mitochondrial function and exposure of the neurons to Tat+morphine. Using this in vitro model, we screened a number of novel antioxidants and found that only L-deprenyl (L-deprenyl) and diosgenin protected against the neurotoxicity of Tat+morphine. Further, Tat-induced oxidative stress impaired morphine metabolism which could also be prevented by diosgenin. In conclusion, opiate abusers with HIV infection and the ApoE4 allele may be at increased risk of developing dementia. L-deprenyl and a plant estrogen, diosgenin, may have therapeutic potential in this population.

Key Words: drug abuse; genetics; HIV; Tat; opiates; neuroprotection; ApoE4; oxidative stress

INTRODUCTION

Human immunodeficiency virus (HIV) infection continues to rise in drug abusing populations (Davies et al. 1997) and some drug abusers with HIV infection have a severe encephalitis (Bell et al. 1998). Both drugs of abuse and HIV target the basal ganglia and cortex; areas rich in opioidergic receptors. Yet only 20% of patients with HIV infection develop dementia and in some patients encephalitis may occur without dementia (Sacktor 2002). These observations suggest that genetic factors may predispose to neuronal toxicity following HIV infection. The E4 allele of Apolipoprotein E (ApoE) has been associated with Alzheimer disease and with poor recovery following brain injury (Koponen et al. 2004) but not with Parkinson disease (Eerola et al. 2002) or Creutzfeldt-Jakob disease (Chapman et al. 1998). While ApoE4 was not associated with higher risk of developing multiple sclerosis, it was associated with disease activity and accumulation of disability (Fazekas et al. 2001). The role of ApoE in pathogenesis of HIV dementia (HIVD) remains unclear. In a previous cross sectional study, no association between ApoE4 and HIVD was found (Dunlop et al. 1997); however, a subsequent longitudinal study found that the presence of ApoE4 allele resulted in a two fold increase in the risk of developing HIVD (Corder et al. 1998). Recent studies from our laboratory suggest that HIVD patients with an ApoE4 genotype show a dysregulated lipid and sterol metabolism (Cutler et al. 2004). We have also shown that patients with HIVD have massive increases in oxidative stress as measured by immunostaining for lipid peroxidation product 4-hydroxynonenol (HNE) in brain tissue and by semiquantitative analysis of protein carbonyls in cerebrospinal fluid (CSF) (Turchan et al. 2003a). No information about drug abusing populations was available from these studies and they also did not determine if ApoE4 influenced the severity of dementia or oxidative stress in the central nervous system (CNS).

ApoE normally plays a role in the distribution of cholesterol for repairing nerve cells during development and after injury. There are three common isoforms of ApoE namely, E2, E3, and E4 as a result of nucleic acid substitutions at codons 112 and 158. ApoE is a major serum lipoprotein involved in cholesterol metabolism. Lipoproteins containing ApoE4 are cleared more efficiently from blood than those containing ApoE3 and ApoE2. ApoE does not cross the blood-brain barrier but is synthesized in the brain by astrocytes and neurons. In the brain, astrocytes are the major source of ApoE which maintains lipid homeostasis in the central nervous system(Huang et al. 2004). In the brain, ApoE is thought to be involved in the mobilization and redistribution of cholesterol and phospholipid during membrane remodeling associated with plasticity and synaptogenesis. Neurite extension and branching is more extensive in ApoE3-treated cells compared with ApoE4-treated cells (Qiu et al. 2004).

To further investigate the role of ApoE in the pathogenesis of HIVD, we initially measured HNE levels by mass spectroscopy in autopsy brain tissue and CSF of patients characterized for ApoE alleles and history of intravenous drug abuse (IVDA) respectively. To confirm these observations and to determine the underlying mechanisms involved in the differential susceptibility of neurons of different ApoE alleles to the combined effects of HIV and drugs of abuse, we used an in vitro human brain culture model. We chose a human system, because only humans and not rodents have all the ApoE alleles. Mixed human neuronal cultures derived from the different ApoE alleles were exposed to HIV proteins Tat and gp120 that have been previously implicated in HIV neuropathogenesis {Nath, 2002 #1720} in combination with morphine which is the major metabolite of heroin. The cultures were compared by gene arrays and confirmed by neurotoxicity and other functional assays. Our observations

suggest that the presence of ApoE4 allele predisposes neurons to toxicity via viral proteins and opiate drugs, and suggest that the neurotoxicity is mediated by increased oxidative stress.

METHODS.

Patient populations

All human samples were obtained following approval of the Johns Hopkins University institutional review board for research on human subjects. Human brain tissue from the pre-highly active antiretroviral therapy (HAART) era (pre-1996) were obtained from the Johns Hopkins AIDS brain bank. All brain tissues used had histopathological evidence of encephalitis as indicated by the infiltration of macrophages or presence of multinucleated giant cells and were free of opportunistic infections as determined by clinical evaluation prior to death and by histopathological examination of the tissues at autopsy. All patients were African American homosexual men aged 38 ± 5 years. Drug abuse histories were not documented in the charts, but these patients belonged to a predominantly heroin and cocaine abusing cohort in Baltimore. Cognitive status was determined within 6 months prior to death. All patients had dementia with Memorial Sloan Kettering (MSK) scores >1.0 . Patients were categorized by ApoE genotype into two groups, ApoE3/3 (n=6) or ApoE3/4 and 4/4 (n=4). CSF specimens were obtained from patients enrolled in the North Eastern AIDS Dementia cohort. Drug abuse status was carefully documented for these patients. There were 10 intravenous drug abusers (IVDA) and another 8 with no exposure to drugs of abuse (NDA). Both groups were matched for MSK scores (≤ 0.05), CD4 cell counts (mean+SEM for IVDA group = 201 ± 44 ; NDA group = 186 ± 60); CSF viral load (mean+SEM for IVDA = 7265 ± 4220 ; NDA group = 3762 ± 1748). All patients were African American males and all were on antiretroviral therapy. All IVDA patients had a history of heroin and

cocaine use, only two patients reported heroin use within the previous week prior to the spinal tap and four patients were on active methadone treatment.

Lipid extraction and measurements of lipid peroxides in brain tissue and CSF

Lipid extractions were performed with tissues from the indicated brain regions by homogenizing the tissue in 10 volumes of ice-cold water, then in 3 volumes of 100% methanol containing ammonium formate (53 mM). Similarly lipids were also extracted from 500ul of cell free CSF from each patient. After vortexing, four volumes of chloroform were added and the mixture was again vortexed and centrifuged at 1000g for 10 min. The chloroform layer was removed and all lipids were initially identified by a Q1 mass scan using electrospray ionization tandem mass-spectrometry (ESI/MS/MS). Samples were injected for 3 minutes, allowing for accumulation of mass counts and lipid peroxides were identified by precursor ion scanning or neutral loss scanning of purified standards. 4-hydroxynonenol (4-HNE) and adducts were purchased from Cayman Chemicals. The sum of the total counts under each peak was used to quantify each species and was normalized according to total protein content of the sample. Data was analyzed using Prizm InstatTM software. Comparisons were made using a 2 tailed Student T test.

Cultures of human brain cells.

Brain specimens were obtained from human fetuses of 12-14 weeks gestational age with consent from women undergoing elective termination of pregnancy. Neuronal cultures were prepared as described previously (Magnuson et al. 1995; Nath et al. 1996). Briefly, the cells were mechanically dissociated, suspended in OPTI-minimal essential media (MEM) with 5% heat-inactivated fetal bovine serum (FBS), 0.2% N2 supplement (GIBCO) and 1% antibiotic solution (Sigma) and plated in flat bottom 96

well plates. The cells were maintained in culture for at least one month before conducting the toxicity assay and mitochondrial experiments. For culturing astrocytes, the brain specimens following dissociation were plated in flat bottom flasks in DMEM with 10% heat-inactivated fetal bovine serum and 1% antibiotic solution. The cells were maintained in culture for one month until used. Cells differentiate into astrocytes and nearly 100% cells stain for glial fibrillary acidic protein (GFAP).

Recombinant proteins and stably transfected cell lines.

Recombinant Tat protein was prepared as described previously from the first exon of Tat (tat1-72) (Ma and Nath 1997). The protein was treated with Detoxi-gel (Pierce) to remove any trace of endotoxin and showed a single peak by reverse phase HPLC analysis. The protein was >99% pure. Recombinant gp120 was obtained from the NIH AIDS Reagent program and tumor necrosis factor- α (TNF- α) was obtained from Calbiochem. We used C6 (rat glioma cell line) and SVGA (a human astrocytic subclone of the SVG cells)(Major et al. 1985) to establish doxycycline -inducible SVGA-Tat-gfp (SVGA-Tat), SVGA-pcDNA which has the vector only (SVGA-Neo) and stable-inducible C6-Tat-gfp (C6Tat), C6- Δ Tat-gfp which has a deletion mutant of Tat-86 from which amino acids 48-56 were deleted (C6dTat), C6-pcDNA (C6-neo), SVGA-Tat stable (SVGATat-s) in our laboratory as previously described (Chauhan et al. 2003). The expression of Tat was verified by immunofluorescence for Tat using a monoclonal antibody (APR 352, National Institute for Biological Standards and Control, Herts, UK) and the functional properties confirmed by transfecting the cells with HIV-long terminal repeat region (LTR) conjugated to either green fluorescent protein (GFP) or chloramphenicol acetyltransferase (CAT) to measure Tat activity (Chauhan et al. 2003).

Co-culture studies.

Primary neurons were plated in the bottom of 24 well tissue culture plates SVGA-Tat cells or SVGA-neo, C6Tat, C6dTat, C6-neo cells were plated in the upper chamber of the transwells. The cells were maintained in OPTI-MEM medium containing with 1.0% FBS for 3-5 days prior to analysis. In SVGA-Tat cells, Tat expression was induced with doxycycline (1µg/ml/day) for the duration of the culture. Control cells were similarly exposed to doxycycline. Human fetal astrocytes, rat C6 glioma cells, SVGA cells were maintained in DMEM, 10% FBS and antibiotic solution.

Measurement of mitochondrial membrane potential activity.

To determine the effect of the opioids and/or viral proteins on mitochondrial function, we used the fluorescent dye JC-1 to measure changes in mitochondrial membrane potential as previously described (Turchan et al. 2003b). Mitochondrial function was monitored following treatment with the above compounds at different concentrations after incubation for 6h or after 72h in the co-culture studies. For monitoring mitochondrial function, the cells were incubated for 30 min at 37°C in a 5% CO₂ incubator in the presence of 10 µM of the JC-1 and then washed in Locke's solution (in mM) 154 NaCl, 5.6 KCl, 2.3 CaCl₂, 1 MgCl₂, 3.6 NaHCO₃, 5 glucose, 5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.2). Optical measurements were acquired at $\lambda_{ex} = 485\text{nm}$, and $\lambda_{em} = 527\text{nm}$ and 590nm. The levels of fluorescence at both emission wavelengths were quantified and ratio of measurements were assessed. The values are expressed as percent of the mean control values \pm S.E.M from three experiments done in eight replicates and analyzed using ANOVA. To investigate the neuroprotective properties of antioxidants and antiretroviral drugs, the cells were incubated with diosgenin (10µM), L-deprenyl (1µM), ebselen (5µM), Euk8 (100µM), trolox (1µM), didox (100µM), trimidox (100µM), imidate (1µM) followed by exposure to Tat and/or the morphine in Locke's buffer and mitochondrial membrane potential monitored as described. Dosages for the pharmacological

compounds were chosen from previously published studies (Turchan et al. 2003b). Following an extensive dose response for Tat and morphine (not shown) we used a dosage at which each of the compounds was individually subtoxic (Tat 80nM; morphine 1 μ M) but when combined, caused neurotoxicity. Similarly, we chose a subtoxic dose of gp120 (30pM) which when combined with Tat (80nM) was toxic. Extensive dose responses of Tat and gp120 have been previously published {Nath, 2000 #766}. Since TNF- α was alone, we chose a toxic dose (10ng/ml). In select experiments, 3-nitropropionic acid (3NP; 4mM) was used as a positive control (Sigma).

Glutathione assay.

Neuronal cultures in 24 well plates were exposed to SVGA-Tat cells or SVGA-neo cells for 3 days. Staurosporine (1 μ M) was used as a positive control. Glutathione in its reduced form acts as an endogenous buffer against oxidative stress hence we measured total glutathione levels using a cell lysates with a Glutathione Detection Kit (Chemicon) as per manufacturers instructions. The assay uses monochlorobimane (MCB), a dye that has a high affinity for glutathione (MCB binds other thiols in the cell with lower affinity). The unbound dye is minimally fluorescent, where as the thiol-bound dye fluoresces blue (λ_{ex} =380nm; λ_{em} =461nm). Florescence was quantified using a fluorescent plate reader. Results were analyzed in each sample against a standard curve and expressed as mM concentrations. The data were calculated as mean \pm S.E.M. from three experiments and analyzed by ANOVA (Tuckey-Kramer post-hoc comparisons test).

Mitochondrial oxidation assay.

Human neuronal cell cultures were washed with Locke's buffer, and incubated with Tat 1-72 (80nM) + morphine (1 μ M) in the presence or absence of the above neuroprotective compounds in Locke's buffer for 6 hours, washed again and then incubated with 150nM Mitotracker Green (λ_{ex} =490nm and λ_{em} =516nm) (Molecular Probes) and 500nM Mitotracker Red (λ_{ex} =579nm and λ_{em} =599nm) (Molecular Probes) at 37°C for 30 min and then washed extensively with buffer. Mitotracker Green is a cell-permeant, mitochondrial-specific dye which becomes fluorescent only on sequestration and association with lipids within the mitochondria without requiring oxidation or reduction. The dye measures cellular mitochondria content and distribution. Mitotracker Red is also cell-permeable and sequesters in the mitochondria, but does not emit fluorescence unless it is oxidized, implying that actively responding mitochondria are required to obtain fluorescence. Ratio of red/green fluorescence was calculated and expressed as % of control. The data were calculated as mean \pm S.E.M. for mean optical measurements from three experiments and analyzed by ANOVA (Tuckey-Kramer post-hoc comparisons).

Mitochondrial reactive oxygen species.

The Dihydrorhodamine (DHR) was used to quantify relative levels of mitochondrial reactive oxygen species as described previously (Kruman et al. 1998). DHR localizes to mitochondria and fluoresces when oxidized by mitochondrial ROS. Briefly, following treatment with Tat+morphine with and without L-deprenyl or diosgenin, the cells were incubated in the presence of 10 μ M DHR for 30 min at 37°C in a 5% CO₂ incubator, washed three times with Locke's solution, and confocal images of cellular fluorescence were acquired using Zeiss 510 CLSM (λ_{ex} =488nm and λ_{em} =510nm). The average pixel intensity in individual cell bodies was determined using software supplied by the

manufacturer and expressed as mean \pm S.E.M. All images were coded and analyzed without knowledge of experimental treatment history of the cultures.

Apolipoprotein E genotyping.

Apolipoprotein E genotyping was performed as follows. PCR reactions were carried out in a 25ul volume containing 0.2-1ug template DNA, 100uM each dNTP, 1 μ M each primer, 3mM MgCl₂, 10% DMSO and 0.5u Taq polymerase (PE-Applied Biosystems) in PCR buffer II (PE-Applied Biosystems). The primers were 5'-TCCAAGGAGCTGCAGGCGGCGCA-3' and 5'-ACAGAATTCGCCCCGGCCTGGTACTACTGCCA-3'; these generate an amplicon 227bp in length. The thermocycler program included a progressive lowering of the annealing temperature over the first 20 cycles, followed by an additional 20 cycles at constant conditions, for a total of 40 cycles. The synthesis and denaturation conditions were identical for each of the 40 cycles. The program consisted of an initial denaturation at 94°C for 5 minutes followed by 10 cycles of a 30 second annealing step at 65°C, a 90 second synthesis at 70°C and then a 30 second denaturation at 94°C. The annealing temperature for the next 5 cycles was 62°C, for the 5 cycles following that was 60°C, and for the last 20 cycles was 55°C. A final extension step was performed for 10 min at 70°C. The PCR reactions were performed in duplicate. DNA was precipitated from pooled duplicates and subjected to restriction enzyme digestion and analysis using the enzymes Hha I, Hae II and Afl III. A total 54 human fetal brain samples were used for this study.

Gene expression patterns in neuronal cultures: microarray analysis.

Neuronal cultures genotyped for ApoE3/3 and ApoE3/4 were treated with Tat (80nM) and morphine (1 μ M) for 6h. Cultures from two different fetuses with each of the ApoE alleles were used for these

experiments. Each experiment was performed in triplicates. RNA extracts were prepared using RNAwiz (Invitrogen). Quality of RNA was checked by Agilent Bionalayzer. Controls were included in gene chip kit: added to mix RNA and shown as a gene chip hybridization controls and gene expression patterns by monitored by Affymetrix oligonucleotide microarrays (HG_U133A chip). Radiolabeled cDNA was synthesized and probed against the oligos on each array. With the expectation that only a small fraction of genes is differentially expressed between samples under different treatments, the brightness of chips for the samples was adjusted to comparable level by normalizing the CEL file of signal values and the probe pair (perfect match and mismatch) level data of the Affymetrix expression chips, with the method of “invariant set normalization” (Hakak et al. 2001). The normalized CEL data were than used with estimated S.E.M. for the probes sets, leading to the further computation of the fold changes and their 90% confidence intervals (Hakak et al. 2001). A 90% confidence interval, a conservative estimate of the fold change, was then used to identify differentially expressed genes. The computing was performed with DCHIP1.2.

Method of Data Analysis.

The mRNA samples were analyzed with Affymetrix GeneChip HG-U133A. To adjust the brightness between the samples to comparable level, the CEL values, the probe pair (*PM* and *MM*) level data of the Affymetrix expression chips, were normalized with the method of “Quantile Normalization”, which makes the *PM*, *MM* quantiles of all arrays agree (Bolstad et al. 2003). After the normalization of the *PM* and *MM* data, summary expression measures for the probe sets, were computed as $\text{Avlog}(\text{PM}-\text{BG})$ with the method of Robust MultiChip Analysis, in which a linear model is taken to summarize the log transformed *PM* values while carrying out a global background adjustment (Irizarry et al. 2003). The computing work on the normalization and expression measures was done with the

RMA package in R environment available at www.bioconductor.org. With the expression measures on a log scale, the following split-plot mixed model was taken to analyze the effects of the drug treatment and the genotype on the expression of each gene.

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \eta_{ij} + \gamma_k + \delta_{ik} + \varepsilon_{ijk}$$

where, Y_{ijk} : the log-transformed version of the observed expression level for the k th treatment applied to the i th genotype in the j th batch of samples

α_i : the effect of the i th genotype on the expression level, $i=1(\text{ApoEIII}), 2(\text{ApoEIV})$

γ_k : the effect of the j th treatment on the expression level (on log scale), $j=1(\text{control}), 2(\text{drug})$

δ_{ik} : the effect of the combination of the i th genotype and the k th treatment on the expression level (on log scale)

β_j : the random effect of the j th batch on the expression level (on log scale), assuming on an independent and identical normal distribution $N(0, \sigma^2_{\beta})$

η_{ij} : the random whole plot effect, assuming on an independent and identical normal distribution $N(0, \sigma^2_w)$

ε_{mijk} : random error, assuming an independent and identical normal distribution $N(0, \sigma^2_e)$, $k=1, \dots, 3$, designating the replicates

Based on the model above, ANOVA analysis was taken to test whether the individual linear contrasts of the parameters of interest (α, γ, δ) are equal to zero, leading to the identification of differentially expressed genes. With the contrast of $(\delta_{22} - \delta_{21}) - (\delta_{12} - \delta_{11})$, a cutoff of <0.01 for the P-values derived from the tests above identifies a list of differentially expressed genes due to the combination effect of the drug treatment and the genotype. Among the remaining genes without the combination effect, tests for the contrasts of $\gamma_2 - \gamma_1$ and $\alpha_2 - \alpha_1$ were taken to identify the differentially expressed genes attributed to the effects of the drug treatment and the genotype. A cutoff of <0.001 was taken for the tests based on the contrast of $\gamma_2 - \gamma_1$, leading to the differentially expressed gene list for the effect of

the drug treatment. For the genotype effect, the differentially expressed genes based on the tests for the contrasts of $\alpha_2 - \alpha_1$ have p-values of <0.01 while their P-values for the combination effect are shown to insignificant (>0.1). The ANOVA analysis is implemented with *lms* in *Splus*. The three differentially expressed gene lists were pooled the clustering analysis with the software package of *GeneSpring*. The pooled expression measure data on the original scale were normalized with the medians across the samples for each probe set before hierarchical clustering analysis was taken for pattern recognition. For the hierarchical clustering analysis, uncentered correlation was taken with such normalize data. Biological function classification of the lists of the differentially expressed genes was made with the Gene Ontology database, using the feature in *GeneSpring*.

RESULTS:

Influence of ApoE allele on lipid peroxidation in brain of patients with HIV encephalitis.

To determine if ApoE allele could influence the amount of oxidative stress in the brain of HIV infected patients, we chose brain samples from patients with HIVD from the pre-HAART era where the course of dementia was subacute in all patients. We measured 4-HNE levels bound to lysine or histidine in extracts of autopsy brain tissue which is formed as a result of lipid peroxidation. Patients with ApoE4 allele had increased levels of HNE in the middle frontal gyrus, parietal lobe and the cerebellum when compared to those with the ApoE3 allele ($P<0.05$). HNE levels were greatest in the frontal lobe in patients with either allele compared to the parietal region and the cerebellum. In patients with ApoE4 allele, histidine HNE levels were markedly increased in the frontal lobe (Fig.1A). The course of HIVD in the post-HAART era is variable and includes inactive, chronic active and subacute dementia. Hence to determine if IVDA may be associated with increased oxidative stress and to exclude the possibility that the differences observed maybe due to the variable course of HIVD, we analyzed CSF from

prospectively followed HIV infected patients. We found a trend for increased lysine and histidine HNE products in the CSF of the IVDA patients although the differences were not statistically significant (Fig 1B).

Influence of ApoE allele on neurotoxicity of viral proteins, TNF- α and morphine.

To further determine the role of ApoE alleles and drug abuse on brain cells, we used in vitro human brain cultures from 54 different fetuses that had been genotyped for ApoE alleles and exposed them to HIV proteins (gp120 and Tat), TNF- α and opiates. These cultures were monitored for neurotoxicity by measuring changes in mitochondrial potential. The ApoE allele distribution of the 54 samples used in this study were as follows. ApoE 2/3, n=6, ApoE 2/4, n=3, ApoE 3/3, n=30, ApoE 3/4, n=13, ApoE 4/4, n=2. The distribution of ApoE alleles in fetal specimens was similar to that found in adult populations (Roses 1996).

Interestingly, we found that TNF- α caused similar amounts of toxicity irrespective of the ApoE genotype, however, ApoE3/4 and ApoE4/4 cultures were significantly more susceptible to toxicity with gp120+ Tat or Tat+ morphine ($P < 0.05$). Maximal toxicity with Tat+gp120 was seen in the cultures with the ApoE4/4 allele (Fig 2). All subsequent experiments were performed using cultured cells with an ApoE3/4 genotype and Tat+morphine were used to induce the toxicity.

Effect of Tat and morphine on gene expression in neurons.

To determine if exposure to Tat+morphine altered the expression of genes implicated in mitochondrial function and apoptotic pathways, we treated neuronal cultures with ApoE3/3 and ApoE3/4 alleles with Tat+morphine and monitored them by microarray analysis. In the untreated cultures, there was no

significant difference in gene expression between the ApoE3/3 and ApoE3/4 alleles. Comparing the treated to untreated cultures, we found that there was a combination effect of genotype and treatment. Treatment with Tat+morphine caused a significant change in 280 probe sets representing 179 genes in both genotypes. Of these genes 126 were up regulated and 53 were down regulated by treatment (provided in appendix for review only. The tables will be deposited on the NIH website and the web address will be provided in final version of manuscript). Although these genes represented a wide variety of cellular functions most of the genes perturbed have been implicated in mitochondrial or energy pathways suggesting an increased metabolic stress on the cells (Table). This suggests that the baseline differences in gene expression between ApoE 3/3 and ApoE3/4 are small if any but are brought out by treatment with Tat+morphine. Thus in subsequent experiments we used a variety of functional assays to confirm if Tat+morphine treatment could induce oxidative stress.

Disruption of mitochondrial membrane potential with chronic Tat and morphine treatment.

To assess the effect of Tat and morphine on mitochondrial function, we measured mitochondrial membrane potential in neuron enriched cultures following exposure to Tat (80nM) + morphine (1 μ M) daily for 5 days. A significant decrease in mitochondrial membrane potential was noted in the treated cultures at 6 hours ($P<0.05$) and at 24 hours ($P<0.05$) following the last exposure to treatment suggesting that the neurotoxic effects are long lasting (Fig.3a). However, no change in mitochondrial potential was noted in pure astrocyte cultures suggesting that the observed toxic effects were in neurons. To determine if the mitochondrial toxicity could be prevented by antioxidant drugs, we pre-exposed the cultures to a panel of novel antioxidant drugs some of which had been previously shown to block neurotoxicity induced by CSF from HIV infected patients (Turchan et al. 2003b). We found

that only diosgenin and L-deprenyl showed significant protection against Tat + morphine ($P < 0.05$; Fig.3a).

To further confirm the effect of chronic exposure of Tat and morphine on neurons, we generated astrocytic cell lines stably expressing Tat (SVGA-Tat). Neuronal cultures were exposed to the SVGA-Tat cells in transwells for three days and morphine ($1\mu\text{M}$) was added daily. A significant decrease in mitochondrial membrane potential was noted in the treated cultures at 6 hours ($P < 0.05$) and at 24 hours ($P < 0.05$) following the last exposure to morphine (Fig.3b). No effect was noted when SVGA-neo cells were substituted for the SVGA-Tat cells. Treatment of the neurons with diosgenin or L-deprenyl on days 1 and 3 showed protection against the neurotoxic effects (Fig.3b). Similar results were also obtained when C6Tat cells were used instead of the SVGA-Tat cells and C6dTat and C6neo cells were used as controls (data not shown)

Effect of Tat and morphine on glutathione levels and reactive oxygen species.

To further determine the effect of Tat and morphine on oxidative pathways, we measured glutathione levels (Fig.4a) and mitochondrial toxicity using a mitotracker probe (Fig.4b) in neurons following exposure to SVGA-Tat cells in transwells for three days or reactive oxygen species following incubation with Tat + morphine for 1 day (Fig.4c). A significant decrease in glutathione levels ($P < 0.05$) was noted suggesting an impairment of the endogenous buffering capacity of the cells against oxidative stress. This was accompanied by an increase in mitochondrial staining for reactive oxygen species was noted in the treated cultures. No effect was noted when SVGA-neo cells were substituted for the SVGA-Tat cells. In both instances, the neurotoxic effects could be prevented by diosgenin or L-

deprenyl suggesting that the neuroprotective effects of these drugs is mediated via antioxidative mechanisms.

Neuroprotection by diosgenin and L-deprenyl against prooxidants.

To further explore the antioxidant properties of these drugs, we treated the neuronal cultures with 3-nitropropionic acid (3NP) for 6 hours in the presence or absence of diosgenin or L-deprenyl and measured the mitochondrial membrane potential. Diosgenin and L-deprenyl were added either 15 min prior to adding 3NP or 15 min post incubation with 3NP. While diosgenin protected the neurons in both instances, L-deprenyl protected the neurons only if preincubated (Fig.5).

Effect of Tat on morphine metabolism.

To determine if Tat altered morphine levels, we co-cultured SVGA-Tat cells or SVGA neo-cells with neurons in transwells. The cells were exposed to morphine (1 μ M) daily for three days. Morphine levels were measured in the culture supernatants 6 hours following the last treatment. A significant increase in morphine levels were noted in the SVGA-Tat + morphine treated cultures suggesting that Tat interfered with morphine metabolism. This increase could be prevented by diosgenin and L-deprenyl suggesting that Tat induced oxidative stress was at least in part responsible for the altered morphine metabolism (Fig.6).

DISCUSSION

Even though the HIV epidemic is being driven by drug abusers in North America and several other regions in the world, clinical studies to determine the combined effects of HIV and drugs of abuse have been few and hard to interpret. These patients are often polydrug abusers and have other comorbidities such as hepatitis C infection. Despite these caveats a longitudinal study in HIV infected drug abusers failed to show and differences in cognitive function compared to the non-drug abusers (Concha et al. 1997). Hence we considered the possibility that genetic factors may play a role. ApoE4 allele has been implicated in the pathogenesis of several neurodegenerative diseases. In a previous study we found that patients with HIVD had massive increases in oxidative stress in the brain and in CSF (Turchan et al. 2003b). In that study we showed increased immunostaining for HNE in the brains of patients with HIVD and measured protein carbonyls in the CSF. HNE is produced by oxidation of polyunsaturated fatty acids. Brain polyunsaturated fatty acids are partially vulnerable to free radicals leading to the production of multiple aldehydes with different carbon lengths including HNE (Esterbauer et al. 1991). HNE forms adducts with proteins by covalent bonding to histidine and lysine residues. Further HNE may itself impair mitochondrial function at multiple sites leading to a positive feedback loop of oxidative stress (Picklo et al. 1999). In the present study, we used tandem mass spectroscopy to quantify HNE levels in brain and CSF of patients with HIV infection. This technique provides a highly reproducible, very sensitive and quantifiable measure of HNE. Since some of the antiretroviral drugs may themselves cause mitochondrial toxicity, we used brain tissues of patients from the pre-HAART era. Although these patients belonged to a heroin and cocaine-abusing cohort in Baltimore, the precise drug abuse histories were not available. Nonetheless, ApoE4 allele was associated with an increase in HNE adducts of both, lysine and histidine indicative of increased oxidative stress. Maximal increases were present in the frontal lobe consistent with previous

pathological studies showing prominent neuronal loss in this region (Masliah et al. 1992) suggesting that the increased oxidative stress and neuronal injury maybe related. To further determine if drug abuse could impact HNE production, we tested CSF from a well characterized HIV infected IVDA cohort and compared them to a matched control population of NDA patients. Even though the sample size of the patients was small due to the stringent criteria used for matching the two groups, a trend for increased HNE adducts of both lysine and histidine was noted in the IVDA group.

Several substances have been implicated in the pathogenesis of HIVD (reviewed in (Nath 1999) hence we determined if neurotoxicity induced by some of these substances was also ApoE allele dependent. Using human neuronal cultures that had been genotyped for ApoE, we found that while TNF α induced neurotoxicity was independent of the ApoE alleles, HIV proteins and HIV protein plus opiate toxicity was more prominent in the ApoE4 neurons. This stimulus specific sensitivity of ApoE4 neurons has been noted in previous studies where amyloid beta peptide produced increased toxicity in ApoE4 neurons (Sadowski et al. 2004) while NMDA and staurosporine produced toxicity independent of the ApoE genotype (Jordan et al. 1998).

Tat is a neurotoxic protein released extracellularly by an energy dependent pathway(Chang et al. 1997) and morphine is the major metabolite of heroin (Meissner et al. 2002). To explore the molecular basis of increased toxicity with these substances in the ApoE4 cultures, we determined gene expression by microarray in neuronal cultures exposed to Tat plus morphine. Interestingly, we did not find any significant difference in baseline levels of gene transcripts in the ApoE3/3 and ApoE3/4 cultures. We found that several genes involved in oxidative pathways were dysregulated in ApoE3/4 cultures compared to ApoE3/3 cultures upon treatment with Tat plus morphine. Further, the lack of and mitochondrial functional changes in astrocytes, suggest that the effects were specific for neurons. We found that the ApoE4 cultures had increased mitochondrial dysfunction and decreased levels of

glutathione confirming that there was increased oxidative stress. Our observations are consistent with a previous study, where cell lines created with each of the ApoE genotypes were exposed to hydrogen peroxide to induce oxidative stress and maximum cytotoxicity was seen in the ApoE4 cells (Kitagawa et al. 2002). The mechanism by which ApoE genes are linked to oxidative pathways needs to be further explored. Amyloid beta peptide also produces increased toxicity in ApoE4 neurons (Sadowski et al. 2004). Interestingly, both Tat and amyloid beta peptide bind to the low density lipoprotein receptor related protein (LRP) which is an important receptor that mediates the uptake of ApoE in neurons (Liu et al. 2000). Further, the different ApoE lipoproteins bind with variable affinity to LRP (Raffai et al. 2000). These observations may in part explain the ApoE dependent neurotoxicity with Tat.

Although we used highly purified recombinant Tat protein for our studies, we further confirmed our observations of Tat-induced effects on neurons by creating an astrocytic cell line that was stably transfected with Tat and releases Tat into the supernatant upon treatment with doxycycline (Chauhan et al. 2003). The same cells without doxycycline treatment failed to show neurotoxicity. We have previously shown that the toxicity can be blocked by treating the culture supernatants with Tat antisera and that cells stably transfected with a Tat deletion mutant from which amino acids 48-56 are deleted failed to cause neurotoxicity (Chauhan et al. 2003). Using these experimental systems, we evaluated the ability of several novel antioxidants to block the mitochondrial toxicity induced by Tat plus morphine. Several of the antioxidant compounds tested had been previously shown by us to block neurotoxicity induced by CSF from patients with HIVD (Turchan et al. 2003b). Of these compounds, only diosgenin and L-deprenyl showed significant protection. These compounds protected against an acute single as well as chronic exposure to Tat plus morphine. They also protected against a known pro-oxidant 3-NP which inhibits succinate dehydrogenase (Page et al.

1998). However, diosgenin was able to rescue neurons even after post-exposure to 3NP. It was also protective against valinomycin that is known to uncouple oxidative phosphorylation (Furlong et al. 1998) and staurosporine a potent inhibitor of calcium dependent protein kinase (Persaud et al. 1993). Diosgenin is a plant-derived steroid with anti-inflammatory properties (Yamada et al. 1997). Its antioxidant and neuroprotective properties have not been studied before. This compound is found in yams (Yang et al. 2003) and in a spice, fenugreek (Taylor et al. 2002). L-deprenyl prevents accumulation of reactive oxygen species in response to glutamate induced neurotoxicity (Pereira and Oliveira 2000). Further, it has been shown to improve motor and cognitive function in patients with HIVD (Consortium 1998; Sacktor et al. 2000) and currently a larger multicenter clinical trial is underway to confirm its efficacy in a larger population. However, drug abusers have been excluded from this and other previous clinical trials of HIVD.

Interestingly, we observed that morphine levels were elevated in the culture supernatants of astrocytes when co-incubated with Tat. This may be at least one mechanism by which synergism between Tat and morphine may occur. It is likely that the oxidative stress induced by Tat interferes with the metabolism of morphine, since both diosgenin and L-deprenyl were able to reverse the effect.

In summary, our observations suggest that individuals with ApoE4 genotype may be more susceptible to the developing neurodegeneration in a setting of HIV infection, and this inherent vulnerability is likely to be exacerbated by opiate drug abuse. The neurotoxicity is likely mediated via oxidative pathways. Further, L-deprenyl and a novel compound, diosgenin are worthy of further exploration for potential therapeutic benefit in this patient population.

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Figure legends and table:

Fig.1. Influence of Apo E allele on lipid peroxidation in the brain of HIV-1 infected patients.

Levels of 4-hydroxynonenal (HNE) adducts, 2-pentylpyrrole lysine-HNE and 2-pentylpyrrole histadine-HNE were determined by ESI/MS/MS in (A) three brain regions medial frontal gyrus (MFG), parietal lobe and cerebellum (CBLM) of each patient with HIV infection by ESI/MS/MS. Data represents mean+SEM. *** = $P < 0.001$. (B) CSF of IVDA and NDA

Fig.2. Influence of ApoE allele in response to neurotoxic agents. Mitochondrial membrane potential was measured after 6 hours in mixed neuronal cultures treated with $\text{TNF}\alpha$ (10ng/ml), Tat (80nM) + gp120 (30pM) and Tat (80nM) + morphine (1 μ M). Data represents mean \pm S.E.M * $P < 0.05$ compared with control.

Fig.3. Neuroprotective properties of novel antioxidants following exposure to Tat + morphine.

(a) Human fetal neurons (ApoE3/4) were exposed daily for five days to either diosgenin (10 μ M), L-deprenyl (L-deprenyl) (1 μ M), ebselen (5 μ M), trolox (10 μ M), Euk8 (100 μ M), didox (100 μ M), trimidox (100 μ M) or imidate (1 μ M) in the presence or absence of Tat (80nM) + morphine (1 μ M). (b) Neuronal cultures were exposed to SVGA-Tat (astrocytes expressing Tat under a doxycycline promoter) or SVGA-neo cells in transwells and 1 μ M morphine was administered daily for 3 days. 3NP was used as a positive control. In each instance mitochondrial membrane potential was measured at 6 or 24 hours following the last exposure. Data represents mean \pm S.E.M of three experiments. Only diogenin and L-deprenyl showed significant protection * $P < 0.05$.

Fig.4. Effect of Tat+morphine on glutathione and reactive oxygen species. (a) Glutathione levels were measured in neuronal cultures (ApoE3/4) exposed to SVGA-Tat or SVGA-neo cells. Morphine (1 μ M) was added daily for 3 days. Staurosporine (1 μ M) was used as a positive control. (b) Oxidization of mitochondria were measured by using mitotracker red and green in neuronal cultures exposed to SVGA-Tat or SVGA-neo cells. Morphine (1 μ M) was added daily for 3 days. (c) Mitochondrial reactive oxygen species were measured using Dihydrorhodamine (DHR) in neuronal cultures treated 24h with Tat (80nM), morphine (1 μ M), diosgenin (10 μ M) or L-deprenyl (1 μ M).

Fig.5. Neuroprotection by diosgenin and L-deprenyl against prooxidant 3-nitropropionic acid (3NP). Mitochondrial membrane potential was measured in neuronal cultures (ApoE3/4) following either pre-incubation with diosgenin (10 μ M), L-deprenyl (1 μ M) for 15 min followed by 3NP (diosgenin+3NP; L-deprenyl + 3NP) or preincubated with 3NP (4mM) for 15 min followed by diosgenin (3NP+diosgenin) or seligiline (3NP+L-deprenyl). Data represents mean \pm S.E.M of three experiments. *P<0.05 (control versus 3NP); #P<0.05 (3NP versus treatment).

Fig.6. Tat interferes with morphine metabolism. Morphine level was measured in culture supernatants of SVGA-neo and SVGA-Tat following morphine (1 μ M) treatment daily for three days in the presence or absence of diosgenin (10 μ M) and L-deprenyl (1 μ M).

Table 1. Fold expression of mitochondrial and apoptosis genes expression in ApoE3/4 compared to ApoE3/3 cultures treated with Tat+morphine

