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HIV-1 Transactivator of Transcription Protein Induces Mitochondrial Hyperpolarization and Syncytic Stress Leading to Apoptosis

Seth W. Perry, John P. Norman, Angela Litzburg, Dabao Zhang, Stephen Dewhurst, and Harris A. Gelbard

Despite the efficacy of highly active antiretroviral therapy in reducing viral burden, neurologic disease associated with HIV-1 infection of the CNS has not decreased in prevalence. HIV-1 does not induce disease by direct infection of neurons, although extensive data suggest that intra-CNS viral burden correlates with both the severity of virally induced neurologic disease, and with the generation of neurotoxic metabolites. Many of these molecules are capable of inducing neuronal apoptosis in vitro, but neuronal apoptosis in vivo does not correlate with CNS dysfunction, thus prompting us to investigate cellular and synaptic events occurring before cell death that may contribute to HIV-1-associated neurologic disease. We now report that the HIV-1 regulatory protein transactivator of transcription protein (Tat) increased oxidative stress, ATP levels, and mitochondrial membrane potential in primary rodent cortical neurons. Additionally, a proinflammatory cellular metabolite up-regulated by Tat, platelet-activating factor, also induced oxidative stress and mitochondrial hyperpolarization in neurons, suggesting that this type of metabolic dysfunction may occur on a chronic basis during HIV-1 infection of the CNS. Tat-induced mitochondrial hyperpolarization could be blocked with a low dose of the protonophore FCCP, or the mitochondrial KATP channel antagonist, tolbutamide. Importantly, blocking the mitochondrial hyperpolarization attenuated Tat-induced neuronal apoptosis, suggesting that increased mitochondrial membrane potential may be a causal event in precipitating neuronal apoptosis in cell culture. Finally, Tat and platelet-activating factor also increased neuronal vesicular release, which may be related to increased mitochondrial bioenergetics and serve as a biomarker for early damage to neurons. The Journal of Immunology, 2005, 174: 4333–4344.

transactivator of transcription protein (Tat) is a small, nonstructural transcriptional regulator essential for the replication of the HIV type 1 (HIV-1). Tat has been detected in the brain of patients with HIV-1-associated dementia (HAD) by immunoblot analysis (1), and is unique among non-Env polypeptides because it is actively secreted by infected glial cells (2), thus increasing the probability that it will be found at higher concentrations in the extracellular space in brain parenchyma relative to other HIV gene products.

Tat is often described as pleiotropic because of its diverse effects in the periphery and the CNS. In keeping with this, Tat exerts transcriptional control over numerous cellular genes in a variety of cell types (1, 3), and it increases TNF-α expression in neuronal cells at the transcriptional level (3); this in turn leads to an increase in platelet-activating factor (PAF) production by mononuclear phagocytes (4). Furthermore, Tat has been shown to have a number of direct effects on neurons, including excessive depolarization and calcium influx (5) as well as apoptosis (5, 6).

Oxidative stress has been implicated as a potential cause of neuronal dysfunction in a number of neurological diseases, and may represent a final common mediator of both synaptic and somal stress in neurons afflicted during HAD and other neurodegenerative disorders (7). Tat has been reported to induce oxidative stress in the periphery and the CNS by a variety of mechanisms, including down-regulation of manganese-dependent superoxide dismutase and increased protein oxidation (8, 9). A particularly intriguing aspect of oxidative stress is the activation of proapoptotic pathways in the synapse that may be involved in plasticity and remodeling (10, 11). This has led to the concept of synaptic apoptosis, or deconstruction of established synapses. Because HIV-1-associated neurologic disease does not appear to correlate with neuronal apoptosis (12), but rather diminution of the dendritic arbor, this is a compelling phenomenon to study in the context of HIV-1-associated neurologic disease. Furthermore, it is particularly germane to investigate how Tat and other proinflammatory cellular metabolites may disrupt cellular energy metabolism under pathological conditions.
conditions of synaptic stress. We now report that Tat and the proinflammatory metabolite it up-regulates, PAF, are able to paradoxically increase mitochondrial membrane potential ($\Delta \psi_{m}$), ATP levels, and vesicle recycling before neuronal demise.

**Materials and Methods**

**Reagents**

Carbamyl-PAF (c-PAF or simply PAF in this work), a nonhydrolyzable form of PAF, was obtained from BIOMOL. HIV Tat$_{1-40}$ Protein and the biologically inactive mutant Tat$_{311-61}$ were received as generous gifts from the laboratory of A. Nath (Johns Hopkins University, Baltimore, MD), prepared as previously described (13).

**Cell culture**

**Primary neuronal cell cultures.** Primary neuronal cortical cultures were prepared from embryonic day 18 rats by modification of the protocol by Brewer et al. (14). In brief, cortices were dissected from a litter of E18 embryonic rats, dissected free of meninges and other tissue, and incubated in 2.0 ml of Ca$^{++}$/Mg$^{++}$-free HBSS (with 10 mM HEPES, pH 7.3) with PSN antibiotics (50 mg/mL penicillin, 50 mg/L streptomycin, 100 mg/L neomycin) plus 0.5 ml of 2.5% trypsin (for 0.25% final) for 15 min at 37°C per brain. After the 15-min incubation, trypsin was removed, cells were washed twice with HBSS (with Ca$^{++}$/Mg$^{++}$), then dissociated in growth medium (below) by Pasteur pipette trituration by 8–10 passages through a 0.9-mm bore 1000-μl blue pipette tip. Dissociated cells were counted by trypsin blue viability assay and plated in cell culture plates at 0.5–0.6 cells/cm$^2$, on poly(D-lysine)-coated cell culture plastic. The plating and maintenance medium used consisted of Neurobasal with B27 supplement (Invitrogen Life Technologies), as described by Brewer et al. (14), and as modified for antioxidant-free culture (14, 15). This medium formulation inhibits the outgrowth of glia, resulting in a neuronal population that is 98% pure (14); thus, glial inhibitors are unnecessary. Cells were cultured for 10–21 days at 37°C in a humidified atmosphere of 5% CO$_2$/95% air, changing medium every 4 days. Cells were used for experiments at days in vitro (DIV) 14–21, unless otherwise indicated.

**Assessment of neurite damage**

After experimental treatment of cortical cultures in 24-well plates, for each condition, neurons in 30 random $\times 30$ fields from three replicate wells/condition (10 random fields/well) were scored as either with (NwNR) or without (NwNR) neurite retraction by a trained observer blind to the experimental conditions. Neurons classified as with neurite retraction exhibited either truncated neurites or complete absence of neurites (see arrows, Fig. 1, B and C, for examples). All cells included in the analysis were still viable, as cells positive for cell death by TUNEL or trypsin blue dye exclusion assays were excluded from analysis (37). Percentage of NwNR per fields was determined by the equation: % NwNR/field = (number of NwNR/total number of neurons) $\times$ 100. These values were averaged for all fields per condition, and for each condition ($\pm$Tat treatment), data were expressed as mean percentage of neurons with neurite retraction per field $\pm$ SEM (NwNR/field $\pm$ SEM). Comparisons were made by unpaired $t$ tests with significance level of $p \leq 0.01$.

**Monitoring alterations in $\Delta \psi_{m}$**

Changes in $\Delta \psi_{m}$ in response to Tat treatment were assessed by the pH-sensitive cationic dyes tetramethylrhodamine ethyl and methyl ester (TMRE and TMRM, respectively). TMRE and TMRM are positively charged dyes that equilibrate across membranes in a Nernstian fashion, and therefore, will accumulate across the mitochondrial membrane and into the matrix space in inverse proportion to the $\Delta \psi_{m}$ (16). Due to the quenching properties of aggregated membrane-bound probe at higher dye concentrations, sensitivity to fluctuations in $\Delta \psi_{m}$ is maximized by using the lowest possible (i.e., subquenching) dye concentrations, because this ensures that the dye signal remains directly proportional to dye concentration and therefore, $\Delta \psi_{m}$. Moreover, because TMRE and TMRM are similar dyes must equilibrate across the plasma membrane before entering the mitochondria, in whole cell applications dye concentration in the mitochondria will be dependent on both plasma membrane potential ($\Delta \psi_{m}$) and $\Delta \psi_{m}$. At sufficiently low dye concentrations (i.e., high cell/dye ratio) and normal membrane potentials, $\geq$95% of the dye will be in the mitochondria, and $\leq$1% in the medium, which results in a $\pm$100% sensitivity of the dye to $\Delta \psi_{m}$, over $\Delta \psi_{m}$, given equivalent changes in plasma or $\Delta \psi_{m}$ (17). In other words, a 35% drop in $\Delta \psi_{m}$ would result in a $<1\%$ change in cell fluorescence, but an equivalent change in $\Delta \psi_{m}$ would result in a $>90\%$ change in cell fluorescence (17).

Thus, we used 1 nM TMRE or TMRM for these experiments, a dose that we confirmed was exponentially more sensitive to $\Delta \psi_{m}$, and did not exhibit quenching effects (17–19). At this concentration, dye signal emanated almost exclusively from mitochondria (see Results). TMRM has been reported to exhibit lower mitochondrial binding and toxicity (16, 20); thus, we used this probe exclusively in later experiments. However, our results did not vary whether TMRE or TMRM was used.

For these studies, primary rat cortical neurons were treated with reagent under normal culture conditions for the indicated time period, followed by removal of the medium, 1 $\times$ 2-min wash in prewarmed 37°C HBSS plus 10 mM glucose and 10 mM HEPES (HBSS$^+$), then incubation in HBSS$^+$ with 1 nM TMRE/M. Following equilibration of the dye for 20 min to ensure distribution across the mitochondrial membrane, the cells were imaged while remaining in the 1 nM TMRE/M solution, as is necessary for a careful dye equilibrium fixation state. Random field images were acquired with an Olympus IX-70 microscope and $\times$40 objective (fluorescent excitation, 545; emission, 610) and Sony DCX-9000 color charge-coupled device (CCD) camera, and the mean relative fluorescent unit (RFU) value (red channel only) of the neuronal soma and processes were quantified using Scansalytics IPLab software. This total neuronal (i.e., cytoplasmic, including soma and neurites) area was determined by thresholding to exclude cell- or mitochondria-deficient regions of the field. A mean neuronal fluorescence value (mean cell signal (MCS)) was acquired for this total mitochondria-containing neuronal area per field, equal to the sum of pixel values over the total included neuronal area, divided by the total number of pixels; the equation for this is as follows: for each field, the average pixel intensity value over the total cytoplasmic area, or field MCS (mCS) = sum of all cytoplasmic pixel values/total number of cytoplasmic pixels. By equally weighting all pixels over the total mitochondrial-containing neuronal area per field, this method is preferable to the more common method of taking the mean of the mean pixel value per cell (which is undesirable because it gives more weight to pixel values that occur more frequently).

To control for variations in signal intensity over time during the course of image acquisition, the integrated MCS from each treatment field (mCSx) was expressed as percentage of difference from the MCS of a time-matched control field (mCSctl), and percentage of differences for each pair was averaged over the total number of paired fields (at least 15 fields per condition) and expressed as mean percentage of difference from control $\pm$ SEM. The equation for this is: mean % in TMRE/M signal vs Ctl =

$$\left( \frac{\text{total uptake in individual culture wells was determined by}}{\text{the equation: ((mCSx) - mCSctl) \times 100}} \right)$$

$$-100\% / \text{no. time matched field pairs} \pm \text{SEM}$$

Significance between conditions was determined by Student’s $t$ test at a level of $p \leq 0.01$.

For some experiments, mitochondrial TMRE or TMRM signal was assessed by fluorometry, as described below; both methods produced equivalent fluorescence values for mitochondrial TMRE/M uptake (see Results and Fig. 3, G and H).

By taking average pixel intensity over a neuronal cell body, MCS values could be impacted by changes in cell size or morphology. To ensure that fluorescence signal changes were not caused by changes in cell size or morphology, total TMRE uptake in individual culture wells was determined by loading with 1 nM TMRE for 20 min, followed by removal of the dye solution, readdition of fresh dye-free HBSS$^+$, and assessment of the total accumulated dye signal minus background fluorescence (total uptake signal (TUS)) from the cells using a Bio-Rad Fluoromark plate reader (excitation, 544; emission, 605). For these experiments, TUS for each condition =

$$\sum_{R=1}^{\text{No Replicates}} \left( \frac{\text{well RFU value}_{R}}{\text{well RFU value}_{R} / \text{no. replicates} \pm \text{SEM}} \right)$$

TUS values were normalized to cell number and expressed as percentage of control $\pm$ SEM, and significance between conditions was determined by Student’s $t$ test at a level of $p \leq 0.01$. Compared with digital image analysis methods, by design fluorometers can read a TUS from a monolayer culture, solution, or both, without significant specimen depth or focal plane limitations (i.e., the fluorescence signal collected is more three dimensional
luminescence was negligible, but nonetheless was subtracted from all read-

Mitracker green (Molecular Probes), a largely potential independent mitochondrial marker (21), was also quantitated to ensure that changes in 

AFM-143 uptake assay

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wells with considerable cell clumping were not used for the assay. Averaging multiple replicates (≥3 for each condition) also helped to eliminate this potential source of error. Figures reflect representative data from multiple experiments. Significance was determined by Student’s t test at p ≤ 0.01.

Quantifying cell death

Cell death was assessed by visualizing fragmented DNA per the TUNEL method, as described previously (15). Briefly, after experimental treatment in 24-well plates, cells were fixed with Histochioce MB Tissue Fixative (AMRESCO), then TUNEL labeled with the ApopTag kit (Chemicon International), according to kit instructions. Cells were visualized under Hoffman modulation contrast optics using a ×40 objective, and images were taken of 10 random fields per well from 3 replicate wells per condition. Data were expressed as percentage of ApopTag-positive neurons (×100) per field, and then field values were averaged for a mean cell death value per well. Mean values from 3 wells were averaged for a final mean cell death value for each condition ± SEM; the analyses were made without knowledge of the treatment group. Significance of differences between conditions was determined by Student’s t test at p ≤ 0.01.

Statistical considerations

Statistical analyses were made by either unpaired Student’s t tests, or unbalanced ANOVA analysis using the PROC GLM procedure in the SAS statistical software package, as described under each Materials and Methods subsection. Differences between results were determined to be statistically significant if p < 0.01.

Results

Tat induces loss of neurites

We used primary rodent monolayer cortical cultures to investigate early effects of Tat on the integrity of synapses. In Fig. 1, we present data to demonstrate that 24-h application of 3.5 μg/ml Tat to cortical neuronal cultures that have already established synapses can alter synaptic connectivity, such that there is a 300% increase (23.1% Tat vs 7.7% control, p < 0.0001) in the percentage of neurons exhibiting neurite retraction (see Materials and Methods for description of analysis). The validity of the assessment criteria used to determine neurite retraction is further supported by noting the significant simplification of the neural network in Tat-treated wells (see boxed area). Arrows), and only a sparse neurite network (compare boxed areas in C and B, respectively).

From analysis), Nonetheless, this finding raises two intriguing questions: 1) does cell death begin at the synapse, and 2) do compensatory metabolic changes occur because of loss of synaptic connectivity?

Tat increases Δψm in a biphasic fashion

Mitochondrial function is essential for normal synaptic activity (33–35). Because Δψm is a sensitive indicator of mitochondrial function, and because collapse (i.e., depolarization) of the resting Δψm is commonly associated with apoptosis (36), we used the ethyl and methyl esters of the cationic dye tetramethylrhodamine (TMRE and TMRM, respectively) to quantitatively define changes in Δψm in response to Tat1–72. At low nM concentrations, TMRE and TMRM localize almost exclusively to the mitochondrial membrane, and are exponentially more sensitive to Δψm over Δψc. Thus, increased TMRE/M uptake reflects increased Δψm (i.e., hyperpolarization) and decreased dye uptake represents depolarization of Δψm.

Using this method, our data indicated that treatment of cortical neurons with Tat caused a biphasic increase in Δψm (i.e., hyperpolarization) that was both concentration and time dependent (Fig. 2). A low dose of Tat (100 ng/ml) caused a gradual increase in

FIGURE 1. Tat induces neurite retraction in rat cortical neurons. A, Treatment with 3.5 μg/ml Tat for 24 h resulted in a 3-fold (300%) increase in the percentage of neurons exhibiting retracted neuritic processes (*, p < 0.0001; unpaired t test). A neuron was judged to have retracted neurites based upon criteria described in Materials and Methods, and was only counted as having retracted processes if a clear judgment could be made. Fifteen ×30 fields (average field ~75 neurons × 15~1125 neurons total) per condition were scored by a blinded observer trained to recognize neurite retraction. Data were expressed as mean number of neurons with retracted processes ± SEM. Data are from one representative experiment replicated at least three times. B, Representative vehicle control subfield showing neurons with an extensive neurite network, including numerous long, largely intact processes forming a dense neurite mat (see boxed area). C, Representative 3.5 μg/ml Tat-treated subfield showing several neurons with truncated or retracted neurites (filled arrows), and only a sparse neurite network (compare boxed areas in C and B, respectively).

FIGURE 2. Tat causes a dose-dependent biphasic mitochondrial hyperpolarization in cortical neurons. Treatment of rat cortical neurons with 100 ng/ml or 2.5 μg/ml Tat for 1, 4, 10, 24, 26, 36, or 48 h resulted in a dose-dependent biphasic increase in Δψm over the time course. As described in Results, initial peaks for both doses were followed by periods of apparent Δψm stabilization, followed by increased Δψm again at later time points. For both doses, the later increase in Δψm persisted until the end of the analysis (48 h). A biologically inactive mutated Tat peptide (green) produced no effect. Curve fits are nonformulaic software interpolations of the data points. TMRE assay and analysis were performed, as described in Materials and Methods and Results.
Δψm, peaking with a 17% increase in mitochondrial TMRE uptake at 4 h (p < 0.0004), then declining to baseline by 14 h, before rising again (6% increase vs control vehicle) 26 h after application, followed by a plateau at 36 h that persists until the end of the analysis (48 h) (19% increase vs control vehicle) (Fig. 2, blue squares). In contrast, a higher dose of Tat (2.5 μg/ml) caused a sharp increase in Δψm vs control, peaking at a 39% increase over control by 1 h (p < 0.0003) before declining to control level by 10 h, then increasing again to a second peak of 27% vs control at 36 h, followed by a plateau of 23% vs control at 44 h that also persisted until the end of the analysis (48 h) (Fig. 2, red circles). Incubation of cortical cultures with the biologically inactive Tat mutant (Δ31–61) at either 1 or 26 h resulted in TMRE uptake values that were indistinguishable from control vehicle (green diamonds), demonstrating specificity of Tat’s effect on mitochondrial hyperpolarization. The mitochondria-depolarizing protonophore trifluoromethoxy carbonyl cyanide phenylhydrazone FCCP (5 μM), in contrast, substantially diminished mitochondrial TMRE/M signal (see Fig. 5C).

Fig. 3 provides visual representations of the increased TMRE signal seen with Tat treatment, and shows that the TMRE signal emanates primarily from mitochondria. Fig. 3A shows relatively faint uptake of TMRE in rodent cortical neurons under control conditions, while B shows a marked increase in TMRE uptake in response to 1 h of 2.5 μg/ml Tat treatment. In C, the uptake of TMRE in cortical neurons exposed for 1 h to an equimolar concentration of the biologically inactive Tat mutant (Δ31–61) is indistinguishable from A, establishing the specificity of Tat’s effect on TMRE uptake. D–F show ×40, ×60, and ×90 (respectively) magnifications of sample cultures to illustrate the subcellular distribution of TMRE in rodent cortical cultures, with pronounced uptake of TMRE into mitochondrial membranes in the perinuclear and process-bearing areas. Because of the very high density of mitochondria typically present in perinuclear regions of neurons, some portions of these images appear to have indiscriminate TMRE labeling within the perinuclear cytoplasm; however, the lack of cytoplasmic staining and distinctly punctate labeling of TMRE in cell body and neurite regions further from the nucleus emphasizes the specificity of dye uptake and its mitochondrial localization. Distribution, localization, and morphology of mitochondria and cells were not significantly affected by Tat treatment (see additional controls below).

**FIGURE 3.** Increased TMRE signal represents a verifiable increase in Δψm. After 1-h treatment, mitochondrial TMRE uptake was visibly lower in vehicle control cultures (A) as compared with 2.5 μg/ml Tat-treated cultures (B). Cultures treated with an equimolar amount of biologically inactive mutated Tat peptide, Δ31–61 (C), were no different from control. Furthermore, ×40 field of cortical neurons equilibrated with 1 nM TMRE (D) demonstrates that the TMRE signal is localized primarily to the mitochondria, as evidenced by the punctate signal in the cytoplasm and neuronal processes, but excluded from nuclear regions, and the relative absence of staining in the intermitochondrial cytoplasm. E, ×60 close-up of cultures in D further demonstrates mitochondrial localization of TMRE signal. Triangular arrows indicate mitochondrial TMRE labeling along clusters of neurites; square arrow indicates dense mitochondrial TMRE labeling of a cluster of neuronal cell bodies. F, ×90 high magnification field indicates punctate mitochondrial labeling (excluding the mitochondria-deficient nucleus) in a single neuronal cell body (square arrow) and along its neurite (triangular arrow). G, Comparing total cellular TMRE uptake with mitochondrial TMRE signal in control- vs Tat-treated cultures indicated that the increased signal seen with Tat treatment could not be accounted for by changes in cell morphology, nor changes in total mitochondrial mass or volume, as evidenced by Mitotracker green labeling analysis (H). I, Nor was the Tat-induced increase in mitochondrial TMRE staining an artifact of Tat-induced increases in Δψm, because Tat actually caused a slight depolarization of Δψm. Images were taken on an Olympus IX-70 microscope with ×40 and ×60 objectives (with ×1.5 inline slider lens) and 545 excitation and 610 emission filters, with a Sony DCX-9000 CCD camera in grayscale mode. Statistics were done by t test at p < 0.01.
Changes in cell morphology or mitochondrial mass do not account for increased TMRE signal

By taking average pixel intensity over a neuronal cell body, MCS values could be impacted by changes in cell size or morphology. To ensure that fluorescence signal changes were not caused by changes in cell size or morphology, total TMRE uptake in individual culture wells was also determined at matching time points by loading sample culture wells with 1 nM TMRE, and assessment of the total accumulated dye signal from the cells using a fluorescent plate reader (see Materials and Methods). Total dye uptake was normalized to cell number, expressed as TUS, and compared with MCS values. Quantification of TMRE uptake by both methods yielded equivalent values for TMRE signal intensity (Fig. 3G), indicating that increases in fluorescent TMRE signal intensity were not artifacts of changes in cell size, morphology, or three-dimensional mitochondrial localization.

To ensure that the increased TMRE signal we observed in neuronal mitochondria after Tat treatment was not due to an increase in mitochondrial mass or volume, Mitotracker green, a largely potential independent mitochondrial marker (21), was also quantitated by both MCS and TUS methods (Fig. 3H). Contrary to results with TMRE, Tat treatment did not increase Mitotracker green signal by either method, suggesting that mitochondrial mass and volume remained constant in Tat-treated cultures.

Together, these data show that the Tat-induced increase in mitochondrial TMRE signal was not an artifact of alterations in cell size, morphology, or total mitochondrial surface area, but rather reflects a verifiable increase in $\Delta \psi_m$.

Increased mitochondrial TMRE is not due to increased $\Delta \psi_m$.

By the Nernst equation, TMRE/M concentration in the mitochondria is dependent upon both $\Delta \psi_m$ and $\Delta \psi_m$. By increasing dye concentration in the cytoplasm, an increased $\Delta \psi_m$ will increase TMRE/M concentration in the mitochondria independent of any changes in $\Delta \psi_m$. As mentioned previously, this effect is greater at higher TMRE/M concentrations. Nonetheless, to further verify that the observed increased TMRE uptake following Tat treatment reflects hyperpolarization of the mitochondrial, but not the $\Delta \psi_m$, we performed additional experiments using the voltage-sensitive anionic fluorescent dye DiBAC$_4$(3) after a 1-h treatment with 2.5 $\mu$g/ml Tat or 100 mM KCl (a concentration known to induce strong depolarization of the $\Delta \psi_m$). In Fig. 3I, Tat induces a significant 11% increase in the relative intensity of the signal from DiBAC$_4$(3) fluorescence ($p < 0.001$), compared with a 22% increase after KCl ($p < 0.001$). These findings demonstrate that despite the ability of 2.5 $\mu$g/ml Tat to depolarize the $\Delta \psi_m$, thus decreasing the influx of cationic TMRE into the intracellular space relative to control vehicle, the mitochondrial TMRE signal is still significantly higher in Tat-treated vs control cells (Fig. 3, compare B and A, respectively).

Tat and PAF increase ROS in cortical neuronal cultures independently of $\Delta \psi_m$.

We then wondered whether mitochondrial membrane hyperpolarization represented a compensatory response to potential increases in reactive oxygen species (ROS) that may occur after exposure to Tat. Because Tat can exert some of its neurotoxic effects through increased production of the phospholipid mediator PAF (4), we also measured ROS production in neuronal cultures exposed to PAF, and mitochondrial TMRM uptake in rodent cortical neuronal cultures treated for 1 h with increasing doses of PAF. Data in Fig. 4A demonstrate a 50% increase in ROS after a 1-h exposure to 464 nM dose of cPAF, while Tat, at a dose of 2.5 $\mu$g/ml, produced a 110% increase in ROS relative to control vehicle. As a positive control, H$_2$O$_2$, at a dose of 500 $\mu$M, sufficient to induce neuronal apoptosis (37), induced a 90% increase in ROS relative to control (Fig. 4A). Data in B demonstrate that PAF also induced a dose-dependent increase in mitochondrial TMRM uptake, reflecting a dose-dependent mitochondrial hyperpolarization. However, in these same rodent cortical neuronal cultures, coinubcation of 2.5 $\mu$g/ml Tat with a congener of the endogenous antioxidant tauroursodeoxycholate, derived from bile salts (38, 39), or the potent antioxidant N-acetylcysteine (NAC), both failed to normalize $\Delta \psi_m$ to control values (C). Taken together, these data suggest that mitochondrial hyperpolarization is a generalized response to both the HIV-1 virotoxin Tat and its proinflammatory cellular mediator PAF, but that cellular ROS production does not directly cause the mitochondrial hyperpolarization.

**FIGURE 4.** Tat and PAF increase ROS in cortical neuronal cultures independently of $\Delta \psi_m$. A, Treatment of rat cortical neurons for 24 h with Tat or PAF induced elevated levels of ROS vs control, as measured with oxidizable dye indicator CM-H$_2$DCFDA (DCF). This effect was greatest with 2.5 $\mu$g/ml Tat, resulting in a 110% increase in ROS production ($*, p < 0.0001$ vs control; unpaired t test), whereas 464 nM PAF resulted in a 50% increase in ROS production, and the H$_2$O$_2$ positive control induced a 90% increase. B, Like Tat, its downstream mediator PAF also induced a dose-dependent rise in $\Delta \psi_m$ ($*, p < 0.002$ or $p < 0.0001$ vs control, respectively; unpaired t test). C, However, the antioxidants NAC (150 $\mu$M) and TUDCA (300 $\mu$M) failed to block the 2.5 $\mu$g/ml Tat-induced increase in $\Delta \psi_m$. NAC (150 $\mu$M) itself induced a rise in $\Delta \psi_m$, and augmented the increase in $\Delta \psi_m$ caused by 2.5 $\mu$g/ml Tat ($*, p < 0.01$; unpaired t test). These results suggest that ROS do not mediate Tat’s increase of $\Delta \psi_m$. Data acquisition and analysis were performed, as described in Materials and Methods, and comparable results were obtained over several experiments.
Possible mitochondrial loci for Tat-induced hyperpolarization of $\Delta \psi_m$

We next sought to identify mitochondrial agents that could block the Tat-induced mitochondrial hyperpolarization. As expected, our data demonstrate that coinubcation of Tat with a low dose (100 nM) application of the mitochondrial uncoupling protonophore FCCP completely abolishes low and high dose Tat-mediated increases in $\Delta \psi_m$ at both 1 and 24 h (Fig. 5A). FCCP decreases $\Delta \psi_m$ by increasing proton transport inward across the mitochondrial membrane, down the proton concentration gradient. However, it is important to note that at the FCCP concentration used in this study (100 nM), FCCP coinubcated with Tat did not result in a complete loss of $\Delta \psi_m$, but rather simply returned $\Delta \psi_m$ to approximately resting levels (i.e., baseline or control) levels (Fig. 5A). Importantly, at 1 and 24 h, 100 nM FCCP by itself had little effect on $\Delta \psi_m$ vs control (Fig. 5A). In stark contrast, however, 1 h of 5 μM FCCP alone resulted in a strong depolarization of $\Delta \psi_m$, reflected by significant loss of the TMRM signal (Fig. 5C).

Interestingly, some similar effects were observed when our cortical neuronal cultures were treated with both low and high dose Tat coinubcated with 100 μM tolbutamide (Fig. 5B). Tolbutamide is a potent antagonist for the ATP-sensitive K$^+$ channels in the inner mitochondrial membrane (40). As expected, 1-h incubation with 100 μM tolbutamide resulted in an apparent trend toward mitochondrial hyperpolarization, as would be expected by acutely blocking mitochondrial K$^+$ influx, although this trend was lost by 24 h (Fig. 5B). At 1 h, coinubcation of 100 μM tolbutamide with 2.5 μg/ml Tat resulted in a 75–80% attenuation of the Tat-mediated increase in $\Delta \psi_m$. At 24 h, tolbutamide blocked the hyperpolarizing effect of high-dose Tat by ~50%.

Neuronal ATP/ADP ratios and adenosine levels increase with prolonged exposure to Tat

Because mitochondrial membrane hyperpolarization might affect mitochondrial bioenergetics by numerous mechanisms, including alterations in the ability of cytochrome c oxidase to regulate mitochondrial energy metabolism (41, 42), we measured intracellular ATP and ADP content in response to Tat treatment. Changes in the ratio of ATP to ADP content is a key indicator of cells’ bioenergetic status, with rising ATP/ADP ratios indicating increased energy reserves, and declining ATP/ADP ratios indicating lower energy supplies (or increased ATP use). As shown in Fig. 6A, ATP/ADP ratios trended higher at most doses and time points analyzed, although this effect was only significant for 2.5 μg/ml Tat at 24 h (unbalanced ANOVA analysis, $p = 0.0053$). Equally important, however, adenosine (ATP and/or ADP) levels were significantly elevated under the majority of treatment conditions analyzed. We found that both low and high dose Tat were able to increase ATP and ADP levels relative to time-matched control vehicle after 24 h of exposure (Fig. 6B). After 48 h of either low or high dose Tat treatment, both ATP and ADP levels were still significantly increased vs control, but less so (Fig. 6B). Tat’s effects on adenosine levels at 1 h trended toward increases, but were not significant.

As additional controls, Tat’s downstream inflammatory mediator, PAF, which, like Tat, hyperpolarizes mitochondrial membranes (see Fig. 4B), also increased ATP and ATP levels in cortical neurons, while a $\Delta \psi_m$-depressing dose of the protonophore FCCP (5 μM; see Fig. 5C) abolished ATP production (data not shown). These results suggest that the Tat-induced rises in adenosine levels are not isolated or aberrant effects of Tat or the assay.

FIGURE 5. Tat-induced mitochondrial hyperpolarization is reversible with low-dose protonophore or KATP channel antagonist. A, One- or 24-h coinubcation of Tat (0.1 or 2.5 μg/ml) with a low dose (100 nM) of the protonophore FCCP normalized $\Delta \psi_m$ to approximately baseline values. A total of 100 nM FCCP by itself had minimal effect on $\Delta \psi_m$. High dose FCCP (5 μM) for 1 h, in contrast, induced a strong mitochondrial depolarization (see C) ($*, p < 0.001$; unpaired $t$ tests). B, Similarly, coinubcation of 100 μM tolbutamide with the same doses of Tat completely abrogated 2.5 μg/ml Tat’s increase of $\Delta \psi_m$ at 1 h, and partially abrogated (by 50%) this increase at 24 h ($*, p < 0.001$; $\#$, $p < 0.01$ at 1 h, $p < 0.1$ at 24 h; unpaired $t$ tests). C, High dose protonophore FCCP (5 μM) decreases mitochondrial TMRM uptake at 1 h ($*, p < 0.01$; unpaired $t$ test). This effect of 5 μM FCCP (which would induce strong mitochondrial depolarization) on TMRM uptake, when contrasted to the increased TMRM signal seen with 2.5 μg/ml Tat treatment, confirmed that Tat was increasing $\Delta \psi_m$ (and see other controls described in Materials and Methods).

FIGURE 6. Changes in neuronal ATP/ADP ratios and adenosine levels with chronic Tat exposure. Primary cortical neurons were treated for 1, 24, or 48 h with 0 (control), 0.1, or 2.5 μg/ml Tat. A, The ATP/ADP ratio was significantly increased at time = 24 h when the Tat dosage was 2.5 μg/ml ($*, p = 0.0053$ for 0 μg/ml vs 2.5 μg/ml Tat at 24 h; SAS PROC GLM procedure for unbalanced ANOVA), but there were no other significant changes, despite a general upward trend. All main effects and interaction effects of time (i.e., 1, 24, or 48 h) and dosage (i.e., 0, 0.1, or 2.5 μg/ml Tat) were included in the unbalanced ANOVA analysis. B, Importantly, both ATP and ADP levels were significantly increased by both doses of Tat at 24 and 48 h, even where ATP/ADP ratios were not significantly affected (at 24 and 48 h; $*, p < 0.0002$ for all changes in ATP and ADP levels vs time-matched 0 μg/ml control; ANOVA and unpaired $t$ tests).
but rather may more broadly represent a pathologic response seen in HAD.

Taken together, these results suggest that although cultures may not experience general energy deficits (as would traditionally be associated with a decline in ATP/ADP ratio, although these data do not exclude the possibility of local intracellular energy deficits), cultures appear to respond to Tat by increasing ATP levels, most likely by increased production rather than decreased consumption of ATP. Moreover, increases in ATP production may be a result of the Tat-induced mitochondrial hyperpolarization seen over a similar time scale (see Fig. 2), because increased $\Delta \mu_m$ will increase protonmotive driving force through the F$_1$F$_0$-ATPase, thus increasing ATP production. Together, these results may suggest that neurons exposed to varying concentrations of Tat respond by increasing cellular energy production (with a corresponding mitochondrial hyperpolarization), and this increased metabolic activity may contribute to the increased oxidative stress induced by Tat or its proinflammatory mediator PAF (see Fig. 4).

**Vesicle recycling increases with exposure to Tat or PAF**

To test the functional correlates of our bioenergetic data, and because both Tat and PAF have been shown to increase excitatory neuronal activity (5, 24, 27), we investigated changes in FM1-43 uptake in response to Tat and PAF. These experiments revealed punctate FM1-43 staining along neuronal processes (characteristic of synaptic vesicle labeling), with heavier punctate staining in areas of high neurite density, and preferential loss of punctate staining relative to background signal upon release in KCl (Fig. 7, A–C).

**FIGURE 7.** Tat and PAF cause an antioxidant-sensitive rise in vesicular activity. Images of Brightfield (A) and fluorescent FM1-43 (B) labeling show that FM1-43 uptake results in labeling that is punctate and oriented primarily along neuronal processes. C. Moreover, activity-dependent FM1-43 release by KCl depolarization preferentially reduces the punctate vesicular labeling vs background signal. D. In $\geq$14- and $\geq$14-day-old primary neurons, 2.5 μg/ml Tat and 4.25 μg/ml Tat induce an increase in spontaneous activity-dependent vesicular uptake, an effect that is both dose and culture age dependent. E. However, no effect is seen with mutated Tat protein, suggesting that Tat’s effect is due to biologically sp. act. of its functional region. F. A total of 464 nM PAF had an even greater effect on FM1-43 uptake at 24 h. G. The antioxidant TUDCA completely eliminated Tat’s effects on FM1-43 uptake for all doses of Tat, whereas the antioxidant (H) NAC partially reduced 2.5 μg/ml Tat’s effect on FM1-43 in an NAC dose-dependent fashion. (*, #, p < 0.01 for comparisons vs relevant control; unpaired t tests).

Twenty-four-hour application of Tat to primary rat cortical neurons, a time when cellular production of ATP is maximal (Fig. 6), caused a dose- and age-dependent increase in vesicular activity, as measured by FM1-43 uptake (Fig. 7D). In neurons aged greater than 14 DIV, Tat resulted in a 50% ($p < 0.02$) and 75% ($p < 0.002$) increase in FM1-43 uptake at doses of 2.5 and 4.25 μg/ml, respectively. Moreover, at each dose of Tat tested, relative to control, neurons aged greater than 14 DIV showed 40–50% more Tat-induced vesicular activity than neurons aged <14 days DIV (Fig. 7D). Tat’s effects on FM1-43 uptake were specific, as they could be blocked by both polyclonal Tat Abs (data not shown) and a mutated functionally deficient (functional region-deleted) Tat peptide (Fig. 7E).

Not surprisingly, PAF, the proinflammatory mediator of Tat, also increased FM1-43 uptake, to an even greater extent than Tat (6-fold vs 1.75-fold) (Fig. 7F). Interestingly, the antioxidant tauroursodeoxycholic acid (TUDCA) (Fig. 7G) was able to completely reverse Tat’s effects on FM1-43 uptake. The antioxidant NAC had a partial dose-dependent effect on reducing the Tat-induced increase in FM1-43 uptake (Fig. 7H). Thus, increased metabolic activity of nerve terminals, reflected by increased vesicle recycling, can be returned to basal or near-basal levels by treatment with antioxidants.

**Stabilizing the mitochondrial electrochemical gradient or blocking ATP-sensitive K$^+$ channels attenuates Tat-induced neuronal apoptosis**

A central question that remained unanswered in these studies was what perturbations in mitochondrial bioenergetics ultimately
mean for cell fate of neurons vulnerable to Tat. We speculated that prolonged exposure to even low levels of Tat, with a concomitant increase in $\Delta \phi_m$, energy production, and metabolic activity (reflected by increased vesicular uptake, recycling, and release), would ultimately result in increased levels of neuronal apoptosis. Thus, we used the mitochondrial uncoupling reagent FCCP, at doses known to reverse hyperpolarization of $\Delta \phi_m$ (Fig. 5A), to determine whether it was neuroprotective against increasing doses of Tat (Fig. 8A). A total of 100 nM FCCP was ineffective against low dose Tat, but partially reversed cell death from high dose Tat (Fig. 8A). Furthermore, the ATP-sensitive K⁺ channel blocker, tolbutamide, at a concentration that prevented Tat-induced mitochondrial hyperpolarization, also attenuated both low and high dose Tat-mediated neuronal apoptosis (Fig. 8B).

Parenthetically, low (i.e., 0.1 $\mu$g/ml) dose Tat exhibited moderately variable levels of neuronal apoptosis between replicate experiments (Fig. 8, compare A with B), which is a common occurrence for any cytotoxicity study, and most likely reflects intrinsic variability of culture systems. However, the dose-response relationship between low and high dose (2.5 $\mu$g/ml) Tat was always maintained. Although it is possible that this variability also accounts for the limited neuroprotective effect of FCCP observed at this dose of Tat, we believe it more likely that FCCP’s failure to provide neuroprotection under these conditions stems from the inherent toxicity of mitochondrial uncouplers. Indeed, while the FCCP paradigm demonstrated in this study suggests a controlled uncoupling effect (note in Fig. 5A, 100 mM FCCP returns $\Delta \phi_m$ to baseline, but not significantly below), and controlled mitochondrial uncoupling has been shown to have neuroprotective effects (reviewed in Ref. 33), mitochondrial uncouplers (at low, controlled doses), e.g., as weight loss agents, have shown only limited therapeutic potential due to toxic side effects.

In addition, it is important to note that FCCP and tolbutamide may be exerting neuroprotective effects through different mechanisms or subcellular sites of action. Both can act as mitochondrial uncouplers, but tolbutamide has additional effects as a KATP-channel antagonist; studies are currently underway to determine which mechanism(s) of action accounts for tolbutamide’s $\Delta \phi_m$-stabilizing and neuroprotective effects.

As a whole, these results suggest that preventing the Tat-induced increase in $\Delta \phi_m$ may serve to ameliorate a subsequent neuronal apoptosis. This finding may have critical implications for protecting against neuronal dysfunction and death in HAD and other neurodegenerative diseases.

Discussion

Our data bring to the forefront issues relevant to HIV-1-associated neurologic disease. Because highly active antiretroviral therapy (HAART) can decrease viral burden to very low or undetectable levels, florid HIV-1 encephalitis has become an uncommon neuropathologic outcome. Rather, HAART may have altered the phenotype of CNS disease by indirectly decreasing inflammatory burden in the CNS.

As a result, HAART has changed the presentation of HIV-associated neurologic disease from a rapidly progressive process to a more indolent pattern, characterized by minor cognitive and minor motor deficits (43–47). Thus, we now face the challenge of modeling a disease process that is characterized more by reversible, phenotypic changes in neuronal function and synaptic networks, rather than frank neuronal loss.

For these reasons, we have used the HIV-1 Tat and PAF in our experiments, because they induce multiple biologic effects important to HAD, and they represent physiologically relevant reagents that can induce both inflammatory and excitotoxic events at dose ranges that elicit both sublethal and lethal effects for vulnerable neurons. Moreover, several variants of Tat (Tat1–72, Tat1–86, Tat1–101) were assessed in our experiments, with similar results in the TMRE/M, FM1–43, ROS, ATP, and cell death assays (data for Tat1–86, Tat1–101 are not shown) (see Refs. 6 and 32).

Given this changing face of HAD, and because HIV-1-associated neurologic disease does not appear to correlate with neuronal apoptosis (12), but rather diminution of the dendritic arbor, we first studied neurite retraction as a suitable starting point to investigate how Tat might alter neurotransmission in a potential biologic model of HAD. We found that Tat did indeed induce simplification of the neuronal architecture, and have suggestive evidence that these changes might be linked to either increased synaptic activity and/or mitochondrial hyperpolarization. Studies are currently under way to more conclusively link aberrant synaptic activity and mitochondrial hyperpolarization with recoverable diminution of the dendritic arbor.

The ability of Tat and its cellular phospholipid metabolite PAF to hyperpolarize neuronal $\Delta \phi_m$ was more unexpected, given that collapse of the $\Delta \phi_m$ is frequently considered one of the penultimate events before neuronal apoptosis. Our findings in this study are not without support, however, as in recent years have others begun to report mitochondrial hyperpolarizations associated with neuronal apoptosis (19, 21, 48).

A potential clue to the biologic outcome of increased $\Delta \phi_m$, particularly as it relates to HAD, comes from a study of PBMCs in HIV-1-infected patients. In this study, the investigators reported that HIV-1-infected PBMCs have a hyperpolarized $\Delta \phi_m$, and behave like activated T cells, prone to apoptotic stimuli, while HAART protease inhibitors can inhibit this effect (49). To our knowledge, and contrary to the traditional dogma associating depolarization of $\Delta \phi_m$ with cell death, this was the first report showing that pharmacologically inhibiting a pathology-induced rise in $\Delta \phi_m$ can protect cells from death. We have extended that work in this study by showing similar results in a neuronal culture model.

FIGURE 8. Stabilization of the mitochondrial electrochemical gradient or blockade of ATP-sensitive K⁺ channels attenuates Tat-induced neuronal apoptosis. Cotreatment of 0.1 $\mu$g/ml or 2.5 $\mu$g/ml Tat-treated cortical cultures with A, a low (100 nM) dose of the protonophore FCCP, capable of returning $\Delta \phi_m$ to baseline (see Fig. 5A), or B, the mitochondrial KATP channel antagonist tolbutamide (100 $\mu$M), partially attenuated apoptotic cell death in response to Tat. FCCP was protective only against the high dose of Tat, which was not unexpected, whereas tolbutamide was protective against both doses of Tat and showed no toxicity when applied alone. ($^*$, +, #, p < 0.01 for all comparisons vs relevant control, and as indicated; unpaired t tests).
Mitochondrial bioenergetics during Tat-induced inflammation: effectors or the affected?

HAART, despite achieving effective concentrations in cerebrospinal fluid, may have relatively limited ability to achieve high concentrations in CNS parenchyma. This is evidenced in part by the fact that HIV-1 drug resistance mutations continue to occur in the brain of patients on HAART, a finding that has been attributed to the presence of ineffective or partially effective drug concentrations within CNS tissue (50). As a consequence, HAART may be unable to rescue all HIV-1-associated effects on vulnerable neurons, thereby allowing mitochondrial hyperpolarization, oxidative stress, and changes in energy metabolism to occur in brain neurons. This underscores the importance of finding alternative agents that may limit the mitochondrial membrane hyperpolarization in the nervous system, and our data show that a currently approved pharmacologic, tolbutamide, may be one agent capable of achieving this effect with consequent neuronal protection. Importantly, because we can achieve some neuronal protection by directly diminishing the mitochondrial proton gradient (the chief component of ΔΨm) with FCCP, this suggests that hyperpolarization of ΔΨm may be directly causal to neuronal apoptosis, and would be consistent with a paradigm of neuronal protection by controlled mitochondrial uncoupling (see Ref. 33 for review).

Mitochondrial hyperpolarization in neurons has been observed in other model systems, including 2-amino-5-hydroxy-5-methyl-4-isoxazolepropion acid-induced increases in ΔΨm in cerebellar Purkinje neurons with an acute time course (i.e., 30–90 min) similar to our findings after Tat treatment (Fig. 2) (51). Exposure of hippocampal neurons to staurosporine also induced elevations in ΔΨm followed by release of cytochrome c, ultimately leading to apoptosis (19). However, neither of these studies addressed the mitochondrial response to increasing doses of neurotoxic stimuli, including the ability of mitochondria to normalize their electrochemical gradient to more homeostatic values (i.e., a ΔΨm similar to control values) over time (see Fig. 2).

Despite this transient stabilization of ΔΨm after Tat treatment, it would appear that mitochondria are ultimately affected by a cellular milieu altered during Tat (and PAF)-induced inflammation. The increased ATP and ADP levels (and in some cases ATP/ADP ratio) we observe are consistent with, and most likely correspond to, the observed increases in ΔΨm, and would appear to reflect an increased activity of the metabolic pathways leading to ATP production, notably glycolysis and/or the TCA cycle. Although our current data do not support the hypothesis that neurons are dying from wholesale energy deprivation (because ATP/ADP ratios are maintained), nor do they eliminate the possibility that local energy deficits exist, for example, perhaps at hyperactive synapses. In addition, our data also strongly indicate that excessive ATP production may be equally detrimental, resulting in neuronal cell death by excess ROS levels as byproducts of oxidative phosphorylation. These concepts are discussed in greater detail below, and studies are currently underway to test these hypotheses. Either way, our data demonstrating that tolbutamide can normalize ΔΨm at 24 h (see Fig. 5), as well as ameliorate neuronal apoptosis (see Fig. 8), suggest a temporal window of potentially reversible dysfunction, and buttress the concept of reversible neuronal dysfunction in HAD.

Ultimately, it is difficult to determine precisely what intracellular pathways or mechanisms cause the mitochondrial hyperpolarization. In this study, we have shown correlative, although not conclusive, data that mitochondrial KATP channels may be involved in this effect, because blocking these channels with tolbutamide attenuates the mitochondrial hyperpolarization. This would seem contrary to well-documented evidence that, in the absence of other effects, blocking mitochondrial KATP channels frequently results in a slight increase in ΔΨm due to a decreased inward K+ gradient and consequent decreased K+ (out)/H+ (in) exchange, as has been shown for tolbutamide and other mitochondrial KATP antagonists (Ref. 40 and see Fig. 5); thus, other actions of tolbutamide, such as regulation of calcium or glycolytic pathways, may explain its ability to ameliorate Tat-induced rises in ΔΨm. For example, tolbutamide reduces glucose availability to the glycolytic pathway, which in turn reduces the supply of pyruvate to the Kreb’s cycle, resulting in decreased production of reduced NADH, and consequent decline in ΔΨm. Notably, one other report has demonstrated a neurotoxic effect that is induced via activation of neuronal KATP channels, and can be blocked by tolbutamide (52), which may be consistent with results shown in this work. Tat may also contribute to increased ΔΨm by other means, including inhibition of the mitochondrial F1F0-ATPase, or increasing electrochemical potential through increased production of (NADH) substrate.

Although it might be tempting to conclude that increased ΔΨm and ATP production represents a compensatory response to the increased energetic demands of Tat or PAF treatment, such as would be required for increased vesicle recycling or synaptic transmission, this may not be the interpretation most consistent with the data we have shown in this work. Were this a true compensatory response, i.e., neurons were increasing ΔΨm and ATP as a protective measure, or in response to energy demands, then contrary to what we found, we would expect that blocking the rise in ΔΨm would exacerbate cell death, and further, that at best ATP/ADP ratios would remain constant (if ATP supply met demand), or would decline (if ATP demand outstripped neurons’ production capability). To the contrary, cell death induced by 2.5 μg/ml Tat for 24 h was associated with increased ADP, ATP, and ATP/ADP ratio, suggesting that Tat-treated neurons were not ATP starved. Therefore, overall, it would appear that the condition of heightened ΔΨm and ATP production was not favorable for cell survival.

An alternate interpretation consistent with our findings would be that Tat or PAF treatment increases activity of the Kreb’s cycle, hence also elevating ΔΨm by increased production of NADH, leading to increased electron transport chain activity and consequent proton extrusion, and this enzymatic hyperactivity is detrimental to the cell. By this model, the increased ATP production that we observed, rather than being a compensatory response, would simply be a byproduct of the increased ΔΨm; this would also explain why ATP/ADP ratios did not decline (and in some cases even rose) with Tat treatment, suggesting stable or increasing ATP reserves. Studies are currently underway to pinpoint more specifically the mechanisms that lead to mitochondrial hyperpolarization and increased ATP production in Tat- and PAF-treated cells.

Regardless of whether neurons were responding to Tat (or PAF) by actively or by inadvertently increasing ΔΨm and ATP production, and regardless of cellular bioenergetic status, either scenario could be equally detrimental to the cell, because ROS production is directly linked to mitochondrial membrane polarization and ATP production. This relationship, i.e., limiting ROS byproducts of oxidative phosphorylation, is thought to underlie the neuroprotective properties of controlled mitochondrial uncoupling. These concepts are also consistent with our data indicating that Tat-mediated mitochondrial membrane hyperpolarization may be upstream of ROS production, because cotreatment with two different antioxidants failed to reverse Tat-mediated increases in ΔΨm (see Fig. 4C). This is in agreement with data indicating that increased ΔΨm will lead to increased ROS production from mitochondrial complex III, the cell’s chief superoxide (O2•-)-generating source,
and data that \( \Delta \text{O}_2 \) generation by complex 1 is proportional to ATP production (53). In summary, increased ROS production may be a result of, rather than causal to, increased \( \Delta \phi \text{m} \), and may link Tat’s neurotoxicity to its ability to enhance \( \Delta \phi \text{m} \) and ATP production.

Calcium’s contributions to Tat’s effects on \( \Delta \phi \text{m} \) also cannot be ignored, because Tat increases intracellular calcium levels in neurons (5), resulting in excitotoxic cell death (54, 55). A role for calcium would also be consistent with the possible model of mitochondrial hyperpolarization just described, as calcium can increase production of NADH by stimulating dehydrogenases. This may be a principal mechanism by which in our model, increased Ca\(^{2+}\) levels or altered Ca\(^{2+}\) signaling may lead to an increase in \( \Delta \phi \text{m} \) rather than the more frequently implicated model whereby excessive Ca\(^{2+}\) influx leads to mitochondrial Ca\(^{2+}\) uptake, invariably followed by a consequent decline in \( \Delta \phi \text{m} \), release of proapoptotic factors, and ultimately cell death. Alternatively, increased \( \Delta \phi \text{m} \) could be directly caused by diminished mitochondrial Ca\(^{2+}\) uptake, which in turn might result from either increased mitochondrial Ca\(^{2+}\) release or increased Ca\(^{2+}\) uptake by the endoplasmic reticulum. Studies are underway to determine the significance of Ca\(^{2+}\) altered handling, as well as Ca\(^{2+}\)-mediated downstream signaling events including pertussis-sensitive G protein-coupled events, protein kinase C, tyrosine kinases, and events modulated by zinc binding sites on the N-methyl-D-aspartate and other receptors, in our model of mitochondrial membrane hyperpolarization.

**Synaptic dysfunction during inflammation**

Data in Fig. 7 demonstrate that neuronal vesicular uptake actually increases after exposure to Tat or PAF, during the time that mitochondrial \( \Delta \phi \text{m} \) is hyperpolarized, and ATP production is maximal. This is consistent with proven excitatory activity of Tat and PAF (5, 24, 27), and also begs the question of whether the changes in mitochondrial function (i.e., increased \( \Delta \phi \text{m} \) and increased ATP production) are causal to, a result of, or independent of changes in vesicular activity. On the one hand, increased ATP production will signal adequate energy reserves to the ATP-sensitive plasma membrane KATP channels, thus closing these channels, blocking K\(^+\) efflux, and resulting in membrane depolarization and increased synaptic activity. This explanation may not be the one most consistent with the known excitatory actions of Tat and PAF, however. Rather, increased energetic demands from synaptic excitation by these molecules may stimulate increased ATP production, made possible by increased \( \Delta \phi \text{m} \). Alternatively, the potential failure in the efficiency of synaptic transmission (from excitotoxic stress) might elicit increased presynaptic vesicular supply of neurotransmitter as a compensatory mechanism. Ultimately, any of these mechanisms would most likely fail due to continued energy demands on the nerve terminal, and the eventual failure to maintain energy production sufficient for cellular functions relevant to neurotransmission, as would be consistent in the falloff in ATP/ADP ratio at 48 h shown in this work. Finally, we cannot exclude the possibility that increased vesicular activity is unrelated to changes in mitochondrial status and ATP levels; thus, future studies will attempt to dissect this relationship.

**Synaptic apoptosis: a pathophysiological substrate for HIV-1-associated neurologic disease in the era of HAART**

The concept of synaptic apoptosis has been advanced in other neurodegenerative diseases, particularly Alzheimer’s disease, by Gilman and Mattson (10), based on observations that caspases, the proapoptotic Bcl-2 protein Bax, mitochondrial factors cytochrome c, and apoptosis-inhibiting factor are present in neurites and synaptic terminals (56–58). Studies with synaptosomal preparations from hippocampal and cortical neurons have demonstrated that treatment with a caspase inhibitor prevents collapse of the \( \Delta \phi \text{m} \) and release of proapoptotic factors into the cytoplasm (53). Additional support for the phenomenon of synaptic apoptosis in neurodegenerative disease comes from a report that demonstrates amyloid \( \beta \)-mediated degeneration of primary hippocampal neurites by a caspase-mediated mechanism in compartmentalized cultures (11). Thus, our findings of Tat-mediated neurite retraction (Fig. 1), as well as our findings of an accompanying synaptic and metabolic stress, are not only consonant with the neuropathology of HIV-1-associated neurologic disease (31, 59), but also with the concept of synaptic apoptosis, and therefore, may represent a neurodegenerative mechanism similar to the type of neurite dissolution seen after exposure to A\(\beta\) (11). As reported in this work, our findings suggest numerous potential mechanisms by which the effects we describe might participate directly in synaptic apoptosis and neurite retraction, including toxic activation of neuronal plasma membrane ATP receptors; activation of proapoptotic pathways at the synapse by ROS or other factors; either intracellularly or extracellularly induced synaptic hyperactivity resulting in dying back of neurites and loss of synaptic function; or excitotoxic cell death, to name a few. Future studies will address these and other relevant in situ events that occur in nerve terminals and dendrites exposed to HIV-1 virotoxins such as Tat, or cellular metabolites such as PAF, including activation of caspases and other effector proteins and mechanisms potentially involved in mediating reversible synaptic apoptosis or dysfunction.

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