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73	Abstract	Human immunodeficiency virus type-1 (HIV) infection of the central nervous system promotes neuronal injury and apoptosis that culminate in HIV-associated neurocognitive disorders (HAND). Viral proteins, such as transactivator of transcription (Tat), have emerged as leading candidates to explain HIV-mediated neurotoxicity, though the mechanism remains unclear. To determine the effects of Tat, rat cortical neurons were exposed to nanomolar concentrations of Tat for various time points. Within a few hours, Tat induced the production of reactive oxygen species (ROS), and other indices of mitochondrial destabilization. In addition, we observed a significant induction of DNA double-strand breaks (DSBs) by Tat. We next investigated the neuroprotective activity of the pituitary adenylate cyclase-activating polypeptide 27 (PACAP27) against these cardinal features of Tat-induced neurodegeneration. PACAP27 (100 nM) inhibited all Tat-mediated toxic effects including DNA DSBs. Importantly, PACAP27 prevented the induction of neuronal loss induced by Tat. The neuroprotective effect of PACAP27 is correlated with its ability to release the anti-apoptotic chemokine CCL5. Our data support a mechanism of Tat neurotoxicity in which Tat induces mitochondrial destabilization, thus increasing the release of ROS, which causes DNA DSBs leading to cell death. PACAP27, through CCL5, mitigates the effects of Tat-induced neuronal dysfunction, suggesting that PACAP27 could be a new strategy for an adjunct therapy against HIV-associated neurocognitive disorders.

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PACAP27 is Protective Against Tat-Induced Neurotoxicity

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Abstract Human immunodeficiency virus type-1 (HIV) infection of the central nervous system promotes neuronal injury and apoptosis that culminate in HIV-associated neurocognitive disorders (HAND). Viral proteins, such as transactivator of transcription (Tat), have emerged as leading candidates to explain HIV-mediated neurotoxicity, though the mechanism remains unclear. To determine the effects of Tat, rat cortical neurons were exposed to nanomolar concentrations of Tat for various time points. Within a few hours, Tat induced the production of reactive oxygen species (ROS), and other indices of mitochondrial destabilization. In addition, we observed a significant induction of DNA double-strand breaks (DSBs) by Tat. We next investigated the neuroprotective activity of the pituitary adenylate cyclase-activating polypeptide 27 (PACAP27) against these cardinal features of Tat-induced neurodegeneration. PACAP27 (100 nM) inhibited all Tat-mediated toxic effects including DNA DSBs. Importantly, PACAP27 prevented the induction of neuronal loss induced by Tat. The neuroprotective effect of PACAP27 is correlated with its ability to release the anti-apoptotic chemokine CCL5. Our data support a mechanism of Tat neurotoxicity in which Tat induces mitochondrial destabilization, thus increasing the release of ROS, which causes DNA DSBs leading to cell death. PACAP27, through CCL5, mitigates the effects of Tat-induced neuronal dysfunction, suggesting that PACAP27 could be a new strategy for an adjunct therapy against HIV-associated neurocognitive disorders.

Keywords DNA damage · Oxidative stress · Mitochondria · CCL5 · HIV · gp120

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Introduction

Human immunodeficiency virus type-1 (HIV) causes HIV-associated neurocognitive disorders (HAND) in nearly one third of individuals (Heaton et al. 2011). Postmortem brains from subjects with the most severe form of HAND, HIV-associated dementia (HAD), exhibit neuronal loss accompanied by synaptic simplification, dendritic pruning, loss of spines, degradation of synaptic proteins (Crews et al. 2009) and neuronal apoptosis (James et al. 1999; Garden et al. 2002). These neurotoxic properties of HIV have been attributed to the combined effect of host cell-derived factors, including cytokines and glutamate, and other neurotoxins produced by activated microglia/macrophages (Kaul et al. 2001). Moreover, different viral proteins have been shown to directly cause this type of neuronal degeneration including transactivator of transcription (Tat) a 101 amino acid protein

that regulates transcription from the HIV promoter (Chen et al. 1997; Haughey et al. 2001; Bruce-Keller et al. 2003). In infected individuals, Tat is actively secreted from infected astrocytes, microglia, and macrophages and can be rapidly internalized by a variety of cell types, including neurons (Liu et al. 2000). This internalization has been reported to promote trimming of neurites, mitochondrial dysfunction, and cell death in neurons (reviewed in Pocernich et al. 2005), all of which correlate with the neurological and cognitive decline more highly than cell death or viral load (Ances and Ellis 2007). At present, there are no therapies that target Tat.

Tat-induced synaptic loss has been observed to differ both temporally and mechanistically from neuronal cell death (Kim et al. 2008); thus, synapse loss is not necessarily a step on the path to apoptosis. Additionally, Tat-induced synaptic loss has been observed to be reversible in vitro (Kim et al. 2008), suggesting the amelioration of Tat-induced toxicity may be a target for adjunct therapies and the reduction of cognitive deficits. Significant neurological improvement accompanies initiation of highly active antiretroviral therapy in patients with HAD (Bellizzi et al. 2006), consistent with the idea that cognitive impairment is due at least in part to reversible actions of the virus. Determining the specific mechanisms leading to neuronal dysfunction will enable the identification of an effective mechanism for prevention of the neurocognitive decline observed in most cases of HIV. Thus, a protective agent acting upstream of the neurotoxic Tat pathway, before synaptic loss and cell death events are activated, is necessary in order to improve both neuronal survival and connectivity. Previous explorations of compounds to protect against Tat toxicity have failed as viable options in part due to their widespread antagonistic properties (Pocernich et al. 2005). Thus, a new compound lacking broad adverse effects is necessary for adjunct therapeutic potential.

Pituitary adenylate cyclase-activating peptide (PACAP) is a member of the secretin/glucagon/vasoactive intestinal peptide (VIP) superfamily. PACAP is an endogenous peptide synthesized by all tissues in the body including the central nervous system and is expressed in two bioactive isoforms, the pituitary adenylate cyclase-activating polypeptides PACAP27 and PACAP38, differing only in amino acid length (reviewed in Vaudry et al. 2009). PACAPs signal through two G protein-coupled receptor subtypes, the low-affinity VPAC receptors (VPAC1 and VPAC2), which they share with VIP, and the high-affinity PACAP-specific receptor, PAC1R (May et al. 2010). Binding of the peptide to PAC1R initiates signaling through adenylyl cyclase and cAMP as well as, to a lesser extent, phosphatidylinositol 4,5-bisphosphate. Additionally, PACAP is able to cross the plasma membrane in a receptor-independent manner, initiating signaling in this fashion (Doan et al. 2012). Through these signaling cascades, PACAP supports a number of neuroprotective roles, protecting against excitotoxicity, stabilizing mitochondrial membrane potential, and reducing reactive oxygen species (ROS) production (Reglodi et al. 2011) and even preventing cortical neuron death through anti-inflammatory properties (Sanchez et al. 2009b). In the present study, we have investigated the protective effects of PACAP on Tat-induced neurotoxicity. We demonstrated that PACAP27 is able to mitigate the toxic effects of Tat that are believed to contribute to neuronal loss.

Materials and Methods

Reagents

The preparation of recombinant Tat 1-72 protein has been described previously (Ma and Nath 1997). Tat was also purchased from Immunodiagnostics (Woburn, MA). PACAP27 and PACAP38 were synthesized in the Department of Medical Chemistry, Szeged University, Hungary according to previous descriptions (Jozsa et al. 2005) or were purchased from R&D (Minneapolis, MN).

Cell Cultures

Cortical neuronal cultures were prepared from the cortex of embryonic (E17–18) Sprague–Dawley rats (Charles River, Gaithersburg, MD) following an established protocol (Avdoshina et al. 2010). Cells were seeded onto poly-L-lysine precoated plates in neurobasal medium (NBM) containing 2 % B27 supplement, 25 nM glutamate, 0.5 mM L-glutamine, and 1 % antibiotic–antimycotic solution (Invitrogen, Carlsbad, CA). Cultures were grown at 37 °C in 5 % CO_2/95 % air for 7–8 days. Cultures contained ~10 % of non-neuronal cells.

Astrocytes were prepared from the cerebral cortex of 1- to 2-day-old Sprague–Dawley rats according to an established protocol (Avdoshina et al. 2010). Cells were seeded on poly-L-lysine precoated tissue culture flasks in Dulbecco's modified eagle medium (DMEM, Invitrogen) containing 10 % fetal bovine serum, 2 % antibiotic–antimycotic and grown at 37 °C in 5 % CO_2/95 % relative atmosphere.

Cells were exposed to control medium (heat-inactivated Tat in 0.1 % bovine serum albumin, BSA), 10, 100, or 1,000 nM Tat (in 0.1 % BSA) and 100 nM PACAPs (in 0.1 % BSA) for various time points.

ROS Levels

Intracellular accumulation of ROS was determined with H2DCF-DA (Sigma-Aldrich, St. Louis, MO). This nonfluorescent compound accumulates within cells upon deacetylation. H2DCF then reacts with ROS to form fluorescent dichlorofluorescein (DCF). Following exposure to Tat and other compounds for 15 min alone or in combination,

cells were loaded with H2DCF-DA (5 μg/ml) at 37 °C for 45 min in a humidified 5 % CO_2/95 % air incubator. The free dye was washed away by several medium changes and fluorescence was measured with an excitation wavelength of 488 nm and emission wavelength of 525 nm (Synergy H4 hybrid reader, Biotek, Winooski, VT).

MTT Assay

The activity of mitochondrial dehydrogenases [3(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (MTT assay) was used to determine mitochondria viability. This assay was carried out according to the manufacturer's specifications (MTT Cell Grow Assay Kit, Millipore, Temecula, CA) as described previously (Bachis et al. 2003; Avdoshina et al. 2010).

Neutral Comet Assay

The CometAssay® kit (Trevigen, Gaithersburg, MD) was used with some modifications. Cells in 12-well plates were rinsed with ice-cold phosphate-buffered saline (PBS, Ca^{2+}/Mg^{2+}-free), gently scraped and transferred to a centrifuge tube where they were pelleted. Pellets were then washed in ice-cold PBS and cells were resuspended at 1×10^5 cells/ml in ice-cold PBS. Cells were combined with molten low melting-point agarose at 37 °C (LMAgarose, Trevigen) at a ratio of 1:10 (v/v), and 50 μl of the cells/LMAgarose mixture was spread onto CometSlides (Trevigen). After cooling at 4 °C for 10 min to allow LMAgarose to solidify, slides were placed in lysis buffer overnight. Following lysis, slides were washed with 1× TBE buffer (Cellgro Mediatech, Manassas, VA) and subjected to electrophoresis in TBE buffer. Electrophoresis was conducted at 1 V/cm for 30 min at 4 °C. The slides were washed twice with ddH_2O for 10 min and dehydrated with 70 % EtOH (Sigma-Aldrich) for 5 min. Slides were placed in a dry oven at 45 °C until dry (~15 min). Subsequently, cells were stained with SYBR Green (Trevigen) for 10 min, air-dried and stored in the dark with desiccating material until imaging. Images taken with Nikon eclipse Ni microscope were analyzed using ImageJ (National Institutes of Health, Bethesda, MD). Tail moment, the length from the center of the head of the comet to the end of the tail, was quantified as a measure of DNA double-strand breaks (DSBs).

Hoechst 33258/Propidium Iodide

The viability of primary cortical neurons was estimated by Hoechst 33258 and propidium iodide (Hoechst/PI; Sigma-Aldrich) co-staining and visualized using a fluorescence microscope. Briefly, cultures were incubated simultaneously with Hoechst 33258 and PI (both 5 μg/ml) for 30 min at 37 °C. Neurons were imaged in four microscopic fields in each well (three wells/treatment). Reaction was visualized with an Olympus IX71 (Tokyo, Japan) inverted fluorescence microscope. Hoechst/PI-positive cells were then counted using ImageJ and expressed as a percentage of the total number of neurons.

Enzyme-Linked Immunosorbent Assay

Levels of CCL5 were determined in the culture medium using the DuoSet enzyme-linked immunosorbent assay (ELISA) Development System Kits (R&D, Minneapolis, MN), according to the manufacturer's instructions and as described previously (Avdoshina et al. 2010).

Statistical Analysis

Statistical analysis was performed using ANOVA and Bonferroni's test for multiple comparisons (GraphPad Prism 5, La Jolla, CA).

Results

PACAP27 Prevents Tat-Induced Mitochondrial Destabilization

The neurotoxic effect of Tat and the potential neuroprotection of PACAP were first analyzed by the MTT colorimetric assay in rat cortical neurons. As shown in Fig. 1, Tat (100 nM) significantly decreased MTT when compared to control (medium containing heat-inactivated Tat in 0.1 % BSA), suggesting that Tat promotes mitochondrial damage. The neurotoxic effect of Tat was not seen using a lower concentration

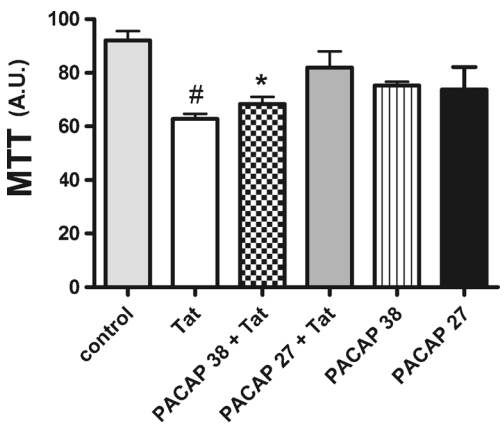


Fig. 1 Tat-induced mitochondrial impairment is attenuated by PACAP27. Neurons were exposed to control medium (heat-inactivated Tat in 0.1 % BSA) or medium containing Tat (100 nM), PACAP27 (100 nM) or PACAP38 (100 nM) alone or in combination. Cell viability was determined 24 h later by MTT assay. Data, expressed as arbitrary units (*A.U.*) are the mean±SEM of three independent experiments (n=24). $^{*}p<0.01$ vs. control; $^{\#}p<0.05$ vs. control

(10 nM), whereas a higher concentration (1 μM) elicited a quantitative effect similar to that obtained with 100 nM concentration (data not shown). Both PACAP38 (100 nM) and PACAP27 (100 nM) alone did not significantly change MTT (Fig. 1). However, PACAP27 pretreatment was able to prevent Tat toxicity (Fig. 1). PACAP38 was significantly less potent than PACAP27 (Fig. 1). Therefore, for the continuation of this study, we used PACAP27.

Mitochondrial damage leads to the increased production and release of reactive oxygen species (ROS) into the cytoplasm and extracellular space. To further assess the toxic effects of Tat and the neuroprotective effect of PACAP27, we quantified ROS accumulation in neuronal cultures. Congruent with the results observed in the mitochondrial viability assay, ROS levels were significantly increased in neurons following a 15-min exposure to Tat (Fig. 2). PACAP27 pretreatment attenuated this increase in ROS accumulation (Fig. 2), confirming the ability of this neuropeptide to prevent mitochondrial destabilization.

Tat, PACAP27, and DNA Damage

The overproduction of free radicals can induce oxidation of DNA bases, and consequently, DNA damage including DNA double-strand breaks (DSBs), the most severe type of DNA damage. If not repaired properly, DSB damage can lead to long-term neuronal injury. DSBs can be quantified using single-cell gel electrophoresis (Neutral Comet Assay). Neurons exposed to Tat for varying time points exhibited a greater number of DNA fragments migrating out of the nuclei to form the "comet tail" than control (heat-inactivated Tat in 0.1 % BSA) neurons, indicating a significantly greater number of DNA DSBs (data not shown). This effect was seen as early as 15 min after Tat exposure (Fig. 3). PACAP27 prevented Tat-induced DNA DSBs. In fact, cultures exposed to PACAP27 15 min prior to Tat exhibited significantly less DNA DSBs than Tat alone (Fig. 3), suggesting that PACAP27 may either prevent DNA DSB damage or facilitate the DSB repair process.

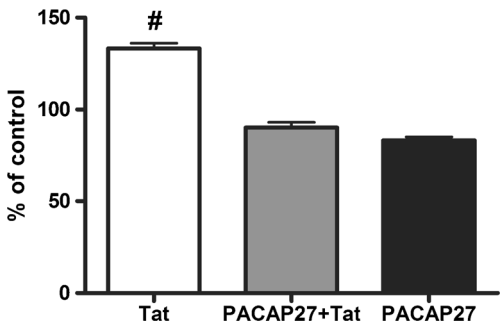


Fig. 2 Tat-induced ROS release is attenuated by PACAP27. Cortical neurons were exposed to control medium, Tat (100 nM) or PACAP27 (100 nM) alone or in combination for 15 min. ROS production was determined by H2DCF-dA fluorescence as described in "Materials and Methods" section. Data, expressed as percent of control, are the mean± SEM from three independent experiments (n=2 each experiment). $^{\#}p<0.05$ vs. control

Astrocytes are more resistant than neurons to Tat-induced toxicity (Pocernich et al. 2004; Eugenin et al. 2007). To determine whether DNA DSBs underlie Tat neurotoxicity, we assessed DNA DSBs in cultured astrocytes following exposure to Tat. This viral protein did not cause a significant change in DNA DSBs in astrocytes (Fig. 4), indicating that this mechanism of Tat-induced damage may be neuron-specific.

PACAP27 Inhibits Tat-Induced Neuronal Cell Death

Mitochondrial health and DNA damage are acceptable proxies for neuronal health and viability; nevertheless, our data so far has failed to demonstrate the causal relationship between mitochondrial alteration and neuronal death. Thus, we assessed cell death at time points beyond the observed mitochondrial impairment and DNA DSB accumulation by using Hoechst/PI. Neurons were exposed to Tat for several time points up to 72 h. Control cells were exposed to heat-inactivated Tat in 0.1 % BSA for the same time points. While cell death was not significantly different from control at 24 and 48 h after Tat exposure, there was a significant increase in cell death 72 h after exposure (Fig. 5). Cultures exposed to PACAP27 before Tat had a similar proportion of cell death, as compared to those treated with PACAP27 alone and untreated controls (Fig. 5), indicating PACAP27 is effective in protecting neurons from Tat-induced death.

Potential Mechanisms of Neuroprotection

PACAP has been shown to activate a number of neuroprotective pathways (Reglodi et al. 2011). Relevant for Tat toxicity is the fact that PACAP38 and related neuropeptide VIP can also induce the release of CCL5 from astrocytes (Brenneman et al. 2002). Released CCL5, in turn, is neuroprotective against neurotoxins (Sanchez et al. 2009a) including the HIV viral protein gp120 (Brenneman et al. 1988; Avdoshina et al. 2010). Therefore, we tested whether PACAP27 releases CCL5 in our neuronal cultures. We observed that PACAP27 promotes the release of CCL5 from our cultures that contain ~10 % of astrocytes (data not shown). However, the effect of PACAP on CCL5 release was significantly more robust in primary cultures of astrocytes (Fig. 6). Thus, we confirm previous data that PACAP enhances the release of CCL5 mainly from astrocytes (Brenneman et al. 2002).

To establish whether CCL5 prevents Tat toxicity in our experimental system, cultures were then exposed to CCL5 (20 nM) 15 min prior to Tat. MTT assay revealed that CCL5 inhibits the mitochondrial impairment induced by Tat (Fig. 7).

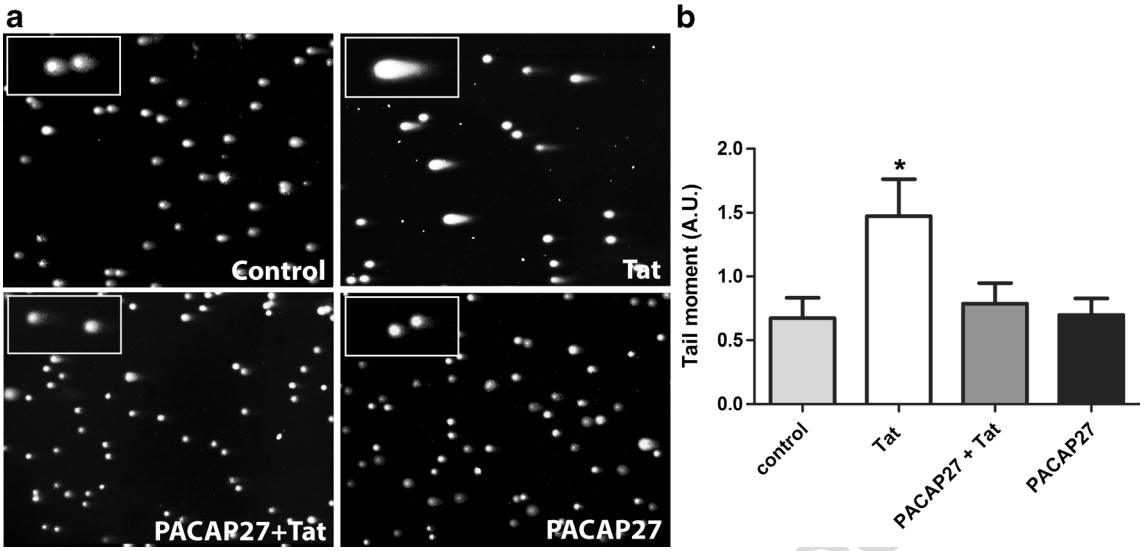


Fig. 3 PACAP27 pretreatment protects against Tat-induced DNA DSBs in neurons. **a** Representative images of neurons exposed to control medium (heat-inactivated Tat in 0.1 % BSA), Tat (100 nM), PACAP27 (100 nM) alone or in combination for 15 min showing tail moment. *Insets* are enlargements to show "tails." **b** Quantification of tail moment representing DNA DSBs. Data, expressed as arbitrary units, are the mean of 100 cells/treatment randomly selected from four fields. The experiment was repeated four times. $^{*}p<0.01$ vs. control

Thus, it appears that CCL5 prevents the toxic effect of not only gp120 but also Tat.

Discussion

The ability of Tat to induce neuronal damage and dysfunction in vitro and in vivo has been established (reviewed in Li et al. 2009). Several mechanisms have been suggested to underlie the neurotoxic effect of Tat. These include activation of NMDA receptors (Haughey et al. 2001; Eugenin et al. 2007; Li et al. 2008), impairment of mitochondria physiology (Chen et al. 2002; Norman et al. 2007), and production of reactive oxygen species (Kruman et al. 1998) which ultimately may result in apoptosis. Our study showed that Tat, in addition to the destabilization of mitochondria and production of ROS previously described (Hui et al. 2012), promotes accumulation of DNA DSBs, which can be lethal to cells. These events start as early as 15 min after Tat exposure and they occur at least a couple of days before neuronal cell death. Additionally, Tat caused a significant increase in the number of DNA DSBs in neurons, but not astrocytes, which are "resistant" to the toxic

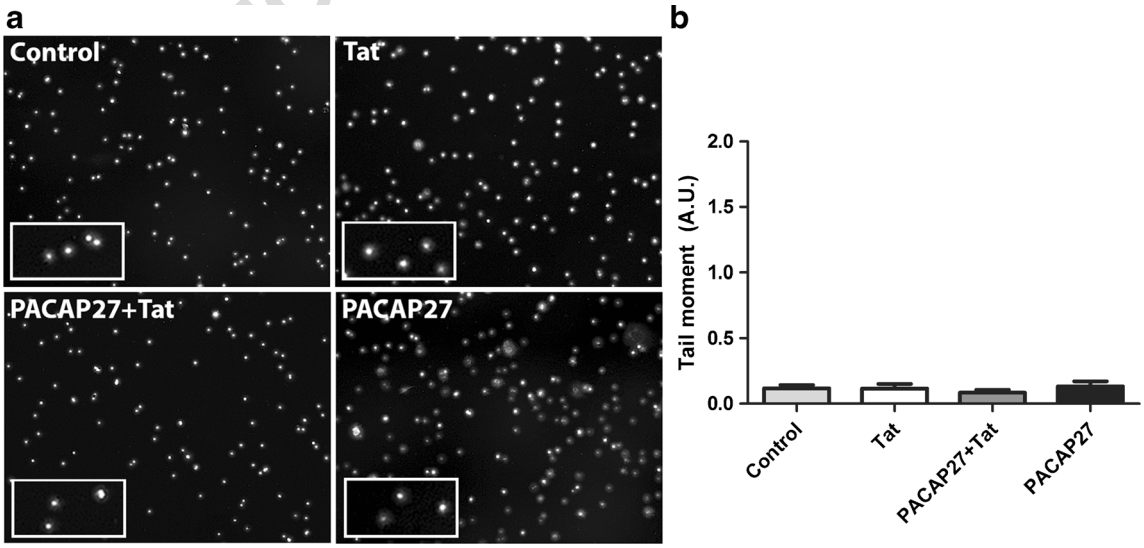
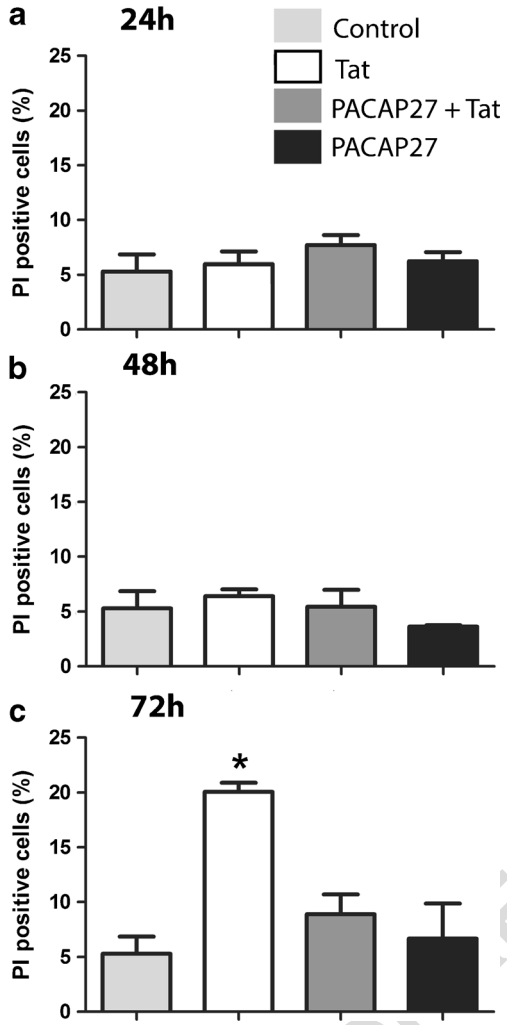


Fig. 4 Tat does not induce DNA DSBs in astrocytes. Cortical astrocytes were prepared as described in "Materials and Methods" section. **a** Representative images of astrocytes exposed to Tat (100 nM), PACAP27 (100 nM) alone or in combination for 15 min showing tail moment. Tail moment was quantified as described in "Materials and Methods" section. *Insets* are enlargements to show "tails." **b** Quantification of tail moment representing DNA DSBs. Data, expressed as arbitrary units, are the mean±SEM of 100 cells/treatment from four fields (from two independent experiments)



Q1 **Fig. 5** Tat-induced cell death is prevented by PACAP27. Neurons were exposed to control medium or Tat (100 nM) for the indicated time points. PACAP27 (100 nM) was added 15 min prior to Tat. Quantification of cell death was done using Hoechst and PI, as described in "Materials and Methods" section. Data are the mean±SEM from a total of 500 neurons from 12 randomly selected fields/treatment from two independent experiments. $^{*}p<0.01$ vs. control

effect of Tat. Importantly, all of these neurotoxic effects of Tat were lessened by a 15-min pretreatment with PACAP27.

Tat induces ROS production, rapid loss of mitochondrial membrane potential and increases mitochondrial uptake of intracellular calcium (Mattson et al. 2005). Tat injections into the frontal cortex of young adult mice lead to irregularly shaped and enlarged mitochondria (Norman et al. 2008). This aberrant morphology mirrors the mitochondrial irregularities observed in the cortex of patients with HIV encephalitis (Zhang et al. 2012), indicating the relevance of mitochondrial impairment to disease progression. Mitochondria are vital for cell function, wherein they supply up to 95 % of the required ATP and regulate intracellular calcium homeostasis. In neurons, mitochondria must travel extreme distances (e.g., axons) and maintain energy homeostasis in these highly metabolically active cells. In fact, synaptic activity and

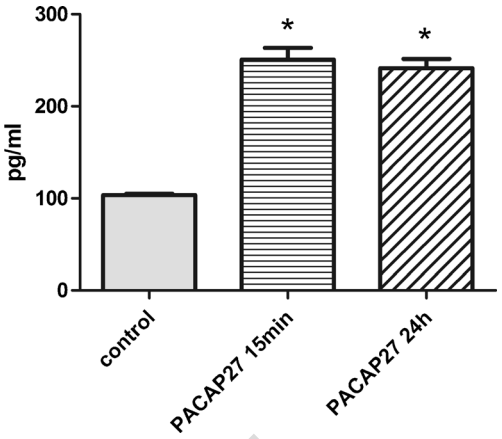


Fig. 6 PACAP27 induces the release of CCL5 from astrocytes. Rat primary astrocytes were exposed to control medium, or medium containing PACAP27 (100 nM) for 15 min and 24 h. The medium was collected and an aliquot was used to determine CCL5 levels by ELISA. CCL5 levels in control cells at 24 h were 104±5 picograms per milliliter). Data are the mean±SEM of three independent samples at each time point. $^{*}p<0.05$ vs. control

mitochondrial motility are highly positively correlated processes (Sun et al. 2013). Neuronal mitochondria are distributed to regions of high metabolic demand, including synapses, nodes of Ranvier, and myelination/demyelination interfaces. Thus, mitochondrial health is intimately tied with the functional status of neurons. Recently impairments to mitochondrial dynamics have been implicated in a causal role of neurodegenerative diseases including Parkinson's, Alzheimer's, and Huntington's diseases (Eckmann et al. 2013; Itoh et al. 2013). The prevalence of neuronal diseases associated with general mitochondrial impairment underscores the important functional relationship between neurons and mitochondria.

In this study, we observed a significant increase in ROS production in neurons exposed to Tat concomitantly to DNA DSBs. ROS can interact with a variety of cellular macromolecules, resulting in oxidative DNA damage, among other ill

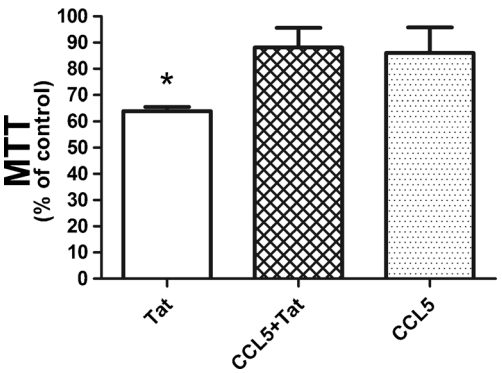


Fig. 7 CCL5 prevents Tat-induced mitochondrial destabilization. Cortical cultures were exposed to control medium, Tat alone or in combination with CCL5 (20 nM). CCL5 was added 15 min prior to Tat. Cell viability was determined 24 h after Tat exposure by MTT assay. Data, expressed as percent of control, are the mean±SEM of four independent samples. $^{*}p<0.05$ vs. control

effects. As postmitotic cells, neurons are particularly vulnerable to DNA DSBs. However, DNA damage even within neurons does not immediately induce apoptosis. The accumulation of DNA damage that is not repaired or is incorrectly repaired will lead to errors in protein translation, resulting in misfolded proteins and eventual cellular dysfunction and cell death (Brasnjevic et al. 2008; Jeppesen et al. 2011). The precise load of DNA damage any neuron can withstand remains unclear. Nevertheless, DNA damage has increasingly been observed in neurodegenerative diseases (Fishel et al. 2007) including HAND, where a significant accumulation of DNA damage has been observed in the post-mortem brain tissue of HIV patients with cognitive deficits (Zhang et al. 2012). Indeed, patients with HAND had an average of 45 % 8-oxoG positive cells, compared to only 30 % in HIV patients without HAND and 4 % in controls (Zhang et al. 2012). These results suggest that nuclear DNA damage exists at least in part due to the high levels of ROS, likely contributing to neuronal injury and cell death. The present study is the first implicating Tat in the development of DNA damage in neurons, preceding cell death.

Both PACAPs protect neurons from diverse insults such as excitotoxicity, oxidative stress, and ischemia (Vaudry et al. 2002; Ohtaki et al. 2010). Most of these injuries result in the production of ROS; though it is not clear how PACAPs regulate this oxidative stress. Some reports have raised the possibility of PACAPs' involvement in the production of antioxidants (Fabian et al. 2012). Our findings demonstrate for the first time that PACAP27 prevents Tat-induced neurotoxicity. The neuroprotective activity of PACAP27 on viral proteins is not surprising because previous studies have shown that both PACAP27 and PACAP38 attenuate neuronal death induced by the HIV envelope protein gp120 (Brenneman et al. 2002). Surprisingly, PACAP38 was unable to counteract the toxic effect of Tat. This appears to be a contradictory result because both PACAPs bind to same receptors and share a similar pharmacological profile. On the other hand, previous studies have shown that PACAP38 and PACAP27 have an opposite profile on the secretion of luteinizing hormone, most likely through a vasoactive intestinal peptide receptor, VPAC1 (Kantora et al. 2000). Thus, different receptors may mediate the neuroprotective effect against Tat. On the other hand, PACAPs can also be neuroprotective by the activation of anti-apoptotic chemokines such as CCL5. CCL5 exerts neuroprotective activity against other viral proteins such as gp120 (Kaul et al. 2007; Avdoshina et al. 2010). Indeed, both PACAP38 (Brenneman et al. 2002) and PACAP27 promote the release of CCL5 from astrocytes. Intriguingly, Brenneman et al. (2002) have shown that the ability of PACAP38 to induce the release of CCL5 from astrocytes is biphasic and concentration-dependent, with the maximal activity on the release of CCL5 in the low picomolar range. Thus, we may have used a concentration of PACAP38 that does not release sufficient amount of CCL5 to prevent Tat toxicity. Further studies are needed to confirm this hypothesis. Hence, while we cannot point at a specific mechanism of PACAP27 neuroprotection we cannot exclude the hypothesis that PACAP27 may be neuroprotective against Tat because of its antioxidant property combined with its ability to release CCL5. Future studies, using CCL5 knock-out animals or a CCL5 blocking antibody, will prove or disprove this hypothesis.

Whereas CCL5 is one mechanism by which PACAP27 can be neuroprotective, other mechanisms may also be implicated including the activation of cAMP-protein kinase A pathway which then activates cAMP-response element binding protein-mediated gene expression (Baxter et al. 2011). PACAPs have also been previously observed to provide neurotrophin-like protection to different populations of neurons (Reglodi et al. 2011). For instance, PACAP exhibits properties similar to nerve growth factor (NGF) in peripheral neurons (Lioudyno et al. 1998), and both PACAP forms activate TrkA and TrkB, the tyrosine kinase receptors for NGF and brain-derived neurotrophic factor (BDNF), respectively (Lee et al. 2002). TrkA is mostly localized in the basal forebrain; thus, TrkA most likely does not account for the neuroprotective effect of PACAP against Tat. Nevertheless, activation of TrkB could participate in fast-acting protection that we have observed in vitro. Indeed, BDNF is particularly potent as neuroprotective compound against Tat (Ramirez et al. 2001) and gp120 (Bachis et al. 2003). This would be in line with a recent study (Nath et al. 2012) showing that flavonoids, alkaloids present in many plants, prevent Tat-mediated mitochondrial dysfunction and neuritic damage by up-regulating the expression of BDNF. Thus, the ability of PACAP to activate TrkB signaling pathways could provide an additional neurotrophic effect against Tat. Our results do not exclude that CCL5 works in concert with BDNF or other trophic factors. In conclusion, our findings implicate PACAP27 as a potent neuroprotective peptide against Tat; however, more experiments are needed to further examine mechanisms underlying its neuroprotective effect. Such mechanisms may yield novel targets for preventing Tat-mediated neuronal injury and delay HAND.

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References

Ances BM, Ellis RJ (2007) Dementia and neurocognitive disorders due to HIV-1 infection. Semin Neurol 27:86–92

Avdoshina V, Biggio F, Palchik G, Campbell LA, Mocchetti I (2010) Morphine induces the release of CCL5 from astrocytes: potential

neuroprotective mechanism against the HIV protein gp120. Glia 58: 1630–1639

Bachis A, Major EO, Mocchetti I (2003) Brain-derived neurotrophic factor inhibits human immunodeficiency virus-1/gp120-mediated cerebellar granule cell death by preventing gp120 internalization. J Neurosci 23:5715–5722

Baxter PS, Martel M-A, McMahon A, Kind PC, Hardingham GE (2011) Pituitary adenylate cyclase-activating peptide induces long-lasting neuroprotection through the induction of activity-dependent signaling via the cyclic AMP response element-binding protein-regulated transcription co-activator 1. J Neurochem 118:365–378

Bellizzi MJ, Lu SM, Gelbard HA (2006) Protecting the synapse: evidence for a rational strategy to treat HIV-1 associated neurologic disease. J Neuroimmune Pharm 1:20–31

Brasnjevic I, Hof PR, Steinbusch HW, Schmitz C (2008) Accumulation of nuclear DNA damage or neuron loss: molecular basis for a new approach to understanding selective neuronal vulnerability in neurodegenerative diseases. DNA Repair (Amst) 7:1087–1097

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

Brenneman DE, Hauser JM, Spong C, Phillips TM (2002) Chemokine release is associated with the protective action of PACAP-38 against HIV envelope protein neurotoxicity. Neuropeptides 36:271–280

Bruce-Keller AJ, Chauhan A, Dimayuga FO, Gee J, Keller JN, Nath A (2003) Synaptic transport of human immunodeficiency virus-Tat protein causes neurotoxicity and gliosis in rat brain. J Neurosci 23: 8417–8422

Chen P, Mayne M, Power C, Nath A (1997) The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. J Biol Chem 272:22385–22388

Chen D, Wang M, Zhou S, Zhou Q (2002) HIV-1 Tat targets microtubules to induce apoptosis, a process promoted by the pro-apoptotic Bcl-2 relative Bim. EMBO J 21:6801–6810

Crews L, Patrick C, Achim CL, Everall IP, Masliah E (2009) Molecular pathology of neuro-AIDS (CNS-HIV). Int J Mol Sci 10:1045–1063

Doan ND, Chatenet D, Letourneau M, Vaudry H, Vaudry D, Fournier A (2012) Receptor-independent cellular uptake of pituitary adenylate cyclase-activating polypeptide. Biochim Biophys Acta 1823:940–949

Eckmann J, Eckert SH, Leuner K, Muller WE, Eckert GP (2013) Mitochondria: mitochondrial membranes in brain ageing and neurodegeneration. Int J Biochem Cell Biol 45:76–80

Eugenin EA, King JE, Nath A et al (2007) HIV-tat induces formation of an LRP-PSD-95-NMDAR-nNOS complex that promotes apoptosis in neurons and astrocytes. Proc Natl Acad Sci U S A 104:3438–3443

Fabian E, Reglodi D, Mester L et al (2012) Effects of PACAP on intracellular signaling pathways in human retinal pigment epithelial cells exposed to oxidative stress. J Mol Neurosci 48:493–500

Fishel ML, Vasko MR, Kelley MR (2007) DNA repair in neurons: so if they don't divide what's to repair? Mutat Res 614:24–36

Garden GA, Budd SL, Tsai E et al (2002) Caspase cascades in human immunodeficiency virus-associated neurodegeneration. J Neurosci 22:4015–4024

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

Heaton RK, Franklin DR, Ellis RJ et al (2011) HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors. J Neurovirol 17:3–16

Itoh K, Nakamura K, Iijima M, Sesaki H (2013) Mitochondrial dynamics in neurodegeneration. Trends Cell Biol 23:64–71

James HJ, Sharer LR, Zhang Q et al (1999) Expression of caspase-3 in brains from paediatric patients with HIV-1 encephalitis. Neuropathol Appl Neurobiol 25:380–386

Jeppesen DK, Bohr VA, Stevnsner T (2011) DNA repair deficiency in neurodegeneration. Prog Neurobiol 94:166–200

Jozsa R, Hollosy T, Tamas A, Toth G, Lengvari I, Reglodi D (2005) Pituitary adenylate cyclase activating polypeptide plays a role in olfactory memory formation in chicken. Peptides 26:2344–2350

Kantora O, Molnar J, Arimura A, Koves K (2000) PACAP38 and PACAP27 administered intracerebroventricularly have an opposite effect on LH secretion. Peptides 21:817–820

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

Kaul M, Ma Q, Medders KE, Desai MK, Lipton SA (2007) HIV-1 coreceptors CCR5 and CXCR4 both mediate neuronal cell death but CCR5 paradoxically can also contribute to protection. Cell Death Differ 14:296–305

Kim HJ, Martemyanov KA, Thayer SA (2008) Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death. J Neurosci 28:12604–12613

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

Lee FS, Rajagopal R, Kim AH, Chang PC, Chao MV (2002) Activation of Trk neurotrophin receptor signaling by pituitary adenylate cyclase-activating polypeptides. J Biol Chem 277:9096–9102

Li W, Huang Y, Reid R et al (2008) NMDA receptor activation by HIV-Tat protein is clade dependent. J Neurosci 28:12190–12198

Li W, Li G, Steiner J, Nath A (2009) Role of Tat protein in HIV neuropathogenesis. Neurotox Res 16:205–220

Lioudyno M, Skoglösa Y, Takei N, Lindholm D (1998) Pituitary adenylate cyclase-activating polypeptide (PACAP) protects dorsal root ganglion neurons from death and induces calcitonin gene-related peptide (CGRP) immunoreactivity in vitro. J Neurosci Res 51:243–256

Liu Y, Jones M, Hingtgen CM et al (2000) Uptake of HIV-1 tat protein mediated by low-density lipoprotein receptor-related protein disrupts the neuronal metabolic balance of the receptor ligands. Nat Med 6:1380–1387

Ma M, Nath A (1997) Molecular determinants for cellular uptake of Tat protein of human immunodeficiency virus type 1 in brain cells. J Virol 71:2495–2499

Mattson MP, Haughey NJ, Nath A (2005) Cell death in HIV dementia. Cell Death Differ 12(Suppl 1):893–904

May V, Lutz E, MacKenzie C, Schutz KC, Dozark K, Braas KM (2010) Pituitary adenylate cyclase-activating polypeptide (PACAP)/PAC1HOP1 receptor activation coordinates multiple neurotrophic signaling pathways: Akt activation through phosphatidylinositol 3-kinase gamma and vesicle endocytosis for neuronal survival. J Biol Chem 285:9749–9761

Nath S, Bachani M, Harshavardhana D, Steiner JP (2012) Catechins protect neurons against mitochondrial toxins and HIV proteins via activation of the BDNF pathway. J Neurovirol 18:445–455

Norman JP, Perry SW, Kasischke KA, Volsky DJ, Gelbard HA (2007) HIV-1 trans activator of transcription protein elicits mitochondrial hyperpolarization and respiratory deficit, with dysregulation of complex IV and nicotinamide adenine dinucleotide homeostasis in cortical neurons. J Immunol 178:869–876

Norman JP, Perry SW, Reynolds HM et al (2008) HIV-1 Tat activates neuronal ryanodine receptors with rapid induction of the unfolded protein response and mitochondrial hyperpolarization. PLoS One 3: e3731

Ohtaki H, Satoh A, Nakamachi T et al (2010) Regulation of oxidative stress by pituitary adenylate cyclase-activating polypeptide (PACAP) mediated by PACAP receptor. J Mol Neurosci 42:397–403

Pocernich CB, Sultana R, Hone E et al (2004) Effects of apolipoprotein E on the human immunodeficiency virus protein Tat in neuronal cultures and synaptosomes. J Neurosci Res 77:532–539

Pocernich CB, Sultana R, Mohmmad-Abdul H, Nath A, Butterfield DA (2005) HIV-dementia, Tat-induced oxidative stress, and antioxidant therapeutic considerations. Brain Res Brain Res Rev 50:14–26

Ramirez SH, Sanchez JF, Dimitri CA, Gelbard HA, Dewhurst S, Maggirwar SB (2001) Neurotrophins prevent HIV Tat-induced neuronal apoptosis via a nuclear factor-kappaB (NF-kappaB)-dependent mechanism. J Neurochem 78:874–889

Reglodi D, Kiss P, Lubics A, Tamas A (2011) Review on the protective effects of PACAP in models of neurodegenerative diseases in vitro and in vivo. Curr Pharm Des 17:962–972

Sanchez A, Tripathy D, Grammas P (2009a) RANTES release contributes to the protective action of PACAP38 against sodium nitroprusside in cortical neurons. Neuropeptides 43:315–320

Sanchez A, Rao HV, Grammas P (2009b) PACAP38 protects rat cortical neurons against the neurotoxicity evoked by sodium nitroprusside and thrombin. Regul Pept 152:33–40

Sun T, Qiao H, Pan PY, Chen Y, Sheng ZH (2013) Motile axonal mitochondria contribute to the variability of presynaptic strength. Cell Rep 4:413–419

Vaudry D, Pamantung TF, Basille M et al (2002) PACAP protects cerebellar granule neurons against oxidative stress-induced apoptosis. Eur J Neurosci 15:1451–1460

Vaudry D, Falluel-Morel A, Bourgault S et al (2009) Pituitary adenylate cyclase-activating polypeptide and its receptors: 20 years after the discovery. Pharmacol Rev 61:283–357

Zhang Y, Wang M, Li H et al (2012) Accumulation of nuclear and mitochondrial DNA damage in the frontal cortex cells of patients with HIV-associated neurocognitive disorders. Brain Res 1458:1–11

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