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Activation of Egr-1 expression in astrocytes by HIV-1 Tat: new insights into astrocyte-mediated Tat neurotoxicity

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Abstract

Human immunodeficiency virus type 1 (HIV-1) Tat plays an important role in HIV-associated neuropathogenesis; the underlying mechanisms are still evolving. We have recently shown that HIV-1 Tat induces expression of glial fibrillary acidic protein (GFAP), a characteristic of HIV-1 infection of the central nervous system (CNS). We have also shown that the Tat-induced GFAP expression in astrocytes is regulated by p300, and that deletion of the early growth response 1 (Egr-1) *cis*-transacting element within the p300 promoter abolishes Tat-induced GFAP expression. In this study, we further examined the relationship between Tat and Egr-1 in astrocytes. We found increased Egr-1 protein expression in Tat-expressing human astrocytoma cells and mouse primary astrocytes. Using the Egr-1 promoter-driven firefly luciferase reporter gene assay and the site-directed mutagenesis, we demonstrated that Tat increased Egr-1 expression by transactivating the Egr-1 promoter and involving specific serum response elements (SRE) within the promoter. Consistent with these data, we showed that Tat transactivation of the Egr-1 promoter was abrogated when astrocytes were cultured in serum-reduced media. Taken together, these results reveal that Tat directly transactivates Egr-1 expression and suggest that Tat interaction with Egr-1 is probably one of the very upstream molecular events that initiate Tat-induced astrocyte dysfunction and subsequent Tat neurotoxicity.

Keywords

HIV-1 Tat; Egr-1; p300; transcription activation; SRE; astrocytes

Introduction

Infection of the central nervous system (CNS) by HIV-1 often causes neurological disorders such as dementia in AIDS patients (Price *et al*, 1988). The main neuropathologies are astrocytosis and cerebral atrophy in the early stage of infection, and formation of myelin pallor, multinucleated giant cells, neuron degeneration, and an abnormal blood-brain barrier in the later stage of the disease (Bell *et al*, 2006; Brew, 2009; Cohen and Gongvatana, 2009;

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Del Valle and Pina-Oviedo, 2006; Ellis *et al*, 2007; Sacktor, 2002). In the era of highly active anti-retroviral therapy, a mild form of neurological disorder called mild cognitive and motor disorder has become more prevalent among HIV-infected subjects (Brew, 2009; Cohen and Gongvatana, 2009; Sacktor, 2002). In the brain, the primary cell targets for HIV infection are macrophages/microglia and, to a lesser extent, astrocytes (Gorry *et al*, 1998; Lee *et al*, 1993; Liu *et al*, 2004; Saito *et al*, 1994; Schweighardt and Atwood, 2001; Tornatore *et al*, 1994). However, neurons that are mostly affected in the brain of HIV-infected individuals are rarely infected. Therefore, a number of indirect mechanisms have been proposed for HIV-associated neuropathogenesis. In general, these indirect mechanisms can be grouped into three main categories: improper immune activation, soluble factors of both viral and host origins, and astrocyte dysfunction.

Among the soluble factors is the HIV-1 Tat protein. Tat is present in the serum and the brain of HIV-infected individuals (Bratanich *et al*, 1998; Cupp *et al*, 1993; Kolson *et al*, 1993; Parmentier *et al*, 1992; Westendorp *et al*, 1995a; Xiao *et al*, 2000b). It causes direct neurotoxicity by various mechanisms including neuroexcitation, Ca^{2+} flux, and oxidative stress (Chen *et al*, 1997; Cheng *et al*, 1998; Conant *et al*, 1998; Hofman *et al*, 1999; Hofman *et al*, 1994; Hofman *et al*, 1993; Jones *et al*, 1998; Liu *et al*, 2000a; Sabatier *et al*, 1991; Westendorp *et al*, 1995b; Zidovetzki *et al*, 1998; Zocchi *et al*, 1998). It can also affect neuronal proliferation and differentiation through interaction with cell cycle-related proteins (Milani *et al*, 1993; Zhou and He, 2004a; Zhou *et al*, 2004). Moreover, Tat could also specifically affect neuronal functions through its uptake, followed by altering neuronal gene expression (Bonifaci *et al*, 1995; Frankel and Pabo, 1988; Green *et al*, 1989; Liu *et al*, 2000a; Mann and Frankel, 1991; Viscidi *et al*, 1989; Westendorp *et al*, 1995a; Xiao *et al*, 2000a). Furthermore, Tat itself possesses the chemoattractant activity and induces excessive CNS infiltration of neutrophils, macrophages and monocytes, which in turn affect neuron survival and function (Chen *et al*, 1997; Cheng *et al*, 1998; Conant *et al*, 1998; Jones *et al*, 1998; Kim *et al*, 2003; Liu *et al*, 2000b).

More evidence has begun to emerge to indicate that Tat interaction with astrocytes also contributes to Tat neurotoxicity. Tat alters astrocytes and neurons to form aggregates and the neuritic processes to coalesce into fascicles in cultures (Kolson *et al*, 1993). Tat induces MCP-1 expression in and release from astrocytes, and MCP-1 level in the cerebrospinal fluid correlates with AIDS dementia (Conant *et al*, 1998). In addition to MCP-1, Tat also induces expression of several other cytokines and chemokines, including IL-1 β , IL-6, Rantes, and CXCL10 (El-Hage *et al*, 2005; Nath *et al*, 1999; Williams *et al*, 2009). We have recently shown that Tat enhances GFAP expression in astrocytes and impairs astrocyte function and that GFAP dysregulation is directly involved in Tat neurotoxicity (Zhou and He, 2004b; Zhou *et al*, 2004; Zou *et al*, 2007). Furthermore, we have shown that the transcriptional co-activator p300 regulates Tat-induced GFAP up-regulation through transcription factor early growth response 1 (Egr-1) (Zou, et al. 2009, in preparation). In this study, we aimed to analyze the relationship between Tat and Egr-1 in astrocytes and the possible roles of Egr-1 in regulation of Tat interaction with astrocytes and Tat neurotoxicity.

Materials and Methods

Cells and cell cultures

293T cells, U373.MG and U87.MG were purchased from American Tissue Culture Collection (ATCC, Manassas, VA). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin in a 37°C, 5% CO_2 incubator. Primary astrocytes were isolated from 18.5 day old embryonic brain tissue of the Tat-inducible transgenic mice described previously (Zhou *et al*, 2004). Briefly, the cortex of the mouse embryonic brain

tissue was aseptically and mechanically separated and dissociated in $\text{Ca}^{2+}/\text{Mg}^{2+}$ free Hanks balanced salt solution (HBSS) with 0.035% sodium bicarbonate and 1 mM sodium pyruvate (pH7.4), and then treated with 0.25% trypsin at 37°C for 30 min. Trypsin digestion was halted by washing cells with $\text{Ca}^{2+}/\text{Mg}^{2+}$ free HBSS supplemented with 10% FBS, followed by low speed centrifugation to remove debris. Cells were suspended and plated at an appropriate density in F12-K medium (Cellgro, Manassas, VA) containing 10% FBS, 50 units/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin. Culture medium was changed 24 hr following the initial plating and then every 3-4 days in order to remove non-adherent cells; primary astrocytes were used after 3-4 passages.

Plasmids

pcDNA3, pGL3-basic and pCMV- β gal were purchased from Clontech (Mountain View, CA). pcTat.Myc was previously described (Zhou and He, 2004b). For pEgr-1p-Luc, the full-length human Egr-1 promoter (about 700 bp) was amplified from the genomic DNA of U87 cells and cloned into pGL3-basic using Mlu I and Xho I to produce pEgr-1p-Luc. pEgr-1p-Luc was then digested with Sca I to remove 468 bp at the 5' end of the Egr-1 promoter, the resulting DNA was ligated to generate the plasmid pEgr-1p Δ 468-Luc. Similarly, pEgr-1p Δ 260-Luc was made using Mlu I/Pvu II. pEgr-1p Δ EBS-Luc was constructed using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and pEgr-1p-Luc as the template and primers 5'-GCG ATA GAA **CCG CGG CCC** GAC TCG **CCG CGG** GTC TGG GCT TCC CCA GCC T-3' and 5'-AGG CTG GGG AAG CCC AGA CCC **GCG GCG** AGT CGG GCC **GCG GTT** CTA TCG C-3' (Sac II sites in bold). pEgr-1p Δ EBS Δ TRE was obtained by digesting pEgr-1p Δ EBS-Luc with Sac II, followed by ligation. All recombinant plasmids were verified by sequencing.

Transfections and Western blot analysis

U373.MG were plated in a 12-well plate at a density of $6 \times 10^4/\text{well}$ or a 6-well plate at a density of $2 \times 10^5/\text{well}$ and transfected with plasmids using the standard calcium phosphate precipitation methods. Cells were harvested 24 hr after transfection for RNA analysis and 72 hr after transfection for protein analysis and reporter gene assays. Cells were washed once in ice-cold phosphate-buffered saline (PBS) and lysed in a RIPA buffer [50 mM Tris.HCl pH7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM PMSF, and 1 $\times$ protease inhibitor cocktail (Roche, Indianapolis, IN)] on ice for 20 min to obtain whole cell lysates. For GFAP, cells were lysed in a Triton buffer [20 mM Tris.HCl pH7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 $\times$ protease inhibitor cocktail (Roche)] on ice for 20 min. The pellets were then extracted with 4 $\times$ SDS-PAGE loading buffer (0.2 M Tris.HCl, pH6.8, 8% SDS, 50 mM EDTA, 40% glycerol, 0.08% bromophenol blue, and 4% β -mercaptoethanol) and heated at 60°C for 30 min, then at 100°C for 10 min, and then used to determine for GFAP expression. Protein concentration was determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Cell lysates were subjected to 10% SDS-PAGE and Western blot analysis with appropriate primary antibodies, HRP-labeled secondary antibodies and a ECL chemiluminescence kit (Amersham, Piscataway, NJ). α -GFAP and α -actin antibodies were from Sigma (St. Louis, MO), and α -Egr-1 and α -Myc antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA).

RNA isolation and semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated using a Trizol Reagent kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed on a PE Thermocycler 9700 (PE Applied Biosystem, Foster City, CA) using a Titan One Tube RT-PCR kit (Boehringer Mannheim, Indianapolis, IN) with Tat primers 5'-GTC GGG ATC CTA ATG GAG CCA GTA GAT CCT-3' and 5'-TGC TTT GAT AGA GAA ACT TGA TGA GTC-3', GFAP primers 5'-AAG CAG ATG AAG CCA CCC TG-3' and 5'-GTC TGC ACG GGA ATG

GTG AT-3', and GAPDH primers 5'-CTC AGT GTA GCC CAG GAT GC-3' and 5'-ACC ACC ATG GAG AAG GCT GG-3'. All the reactions were performed with the program of 50°C for 30 min, 94°C for 2 min, followed by 10 cycles of 94°C for 15 sec, 55°C for 1 min and 72°C for 1 min, and 25 cycles of 94°C for 15 sec, 58°C for 1 min and 72°C for 1 min, then 1 cycle of 72°C for 7 min. The expected sizes for GFAP, Tat and GAPDH amplification products were 625, 216 and 500 bp, respectively. Control RT-PCR reactions containing no reverse transcriptase were included to ensure no genomic DNA contamination.

Preparation of pseudotyped HIV-GFP virus and infection of U373.MG cells

293T cells were transfected with pHIV-GFP plasmid and pHCMV-G plasmid using the standard calcium phosphate precipitation method. The culture supernatant was collected 48 hr after the medium change, briefly centrifuged to remove cell debris, and used as the virus stock. U373.MG cells were infected with VSV-G pseudo-typed HIV-GFP viruses in the presence of 8 µg/ml polybrene at 37°C for 2 hr. The cells were then thoroughly washed with culture medium to remove unbound viruses and cultured for additional 48 hr. The dosages of the viruses for infection were titrated for U373.MG to ensure minimal cell death and maximal infection efficiency.

Reporter gene assays

Cells were harvested in ice-cold PBS and collected by brief centrifugation. Cell lysates were obtained for the firefly luciferase assay according to the manufacturer's instruction (Promega, Madison, WI). The luciferase activity was quantitated using an Opticomp Luminometer (MGM Instruments, Hamden, CT). For β -galactosidase reporter gene assay, 30 µl cell extract was mixed with 3 µl 100× Mg²⁺ solution (0.1 M MgCl₂ and 4.5 M β -mecaptoethanol, 66 µl 4 mg/ml O-nitrophenyl- β -D-galactopyranoside in 0.1 M sodium phosphate, pH7.5 and 201 µl 0.1 M sodium phosphate, pH7.5). The mixture was incubated at 37°C for 30 min, the optical density at 405 nm was determined and expressed as the relative β -galactosidase activity. The β -galactosidase activity was then used to normalize the transfection variations for the luciferase reporter gene assay.

Data analysis

All experiment data were analyzed by two-tailed student's *t* test. A *p* < 0.05 was considered to be statistically significant and labeled “*”; a *p* < 0.01 was considered to be statistically highly significant and labeled “**”.

Results

Egr-1 up-regulation in Tat-expressing U373.MG, U87.MG and primary astrocytes

Our previous studies suggest that Egr-1 is likely directly involved in Tat-induced p300 and GFAP expression in astrocytes. Egr-1 gene belongs to a group of early response genes and regulates expression of a wide spectrum of cellular genes; it functions as a convergence point for many intracellular signaling cascades. Thus, we examined the relationship between Tat expression and Egr-1 expression. We first transfected Tat-expressing plasmid into human astrocytoma cells U373.MG and determined Egr-1 expression by Western blot analysis. The results showed that Tat expression in these cells was accompanied by increased Egr-1 protein expression (Fig. 1A). Similar results were obtained in another widely used human astrocytoma cells U87.MG (Fig. 1B). To further examine the phenomenon, we isolated primary astrocytes from the brain of inducible and astrocyte-specific Tat transgenic mice (Kim *et al*, 2003; Zou *et al*, 2007). We then cultured these cells with doxycycline (Dox) for 1, 2, 3, and 4 days. We also included primary astrocytes from

wild-type C57/BL6 mice, in which the Tat transgenic mice were bred as a control in these experiments. As shown previously, Tat expression was induced in these primary cells by Dox over time, as demonstrated by RT-PCR (top panel, Fig. 1C). In parallel, there was induction of Egr-1 protein expression in these cells (middle panel, Fig. 1C, shown by arrow). In contrast, Egr-1 expression showed no changes in Dox-treated wild-type primary astrocytes (Fig. 1D). We next determined whether HIV-1 infection would also lead to increased Egr-1 expression in astrocytes. To facilitate HIV entry into these cells, we took advantage of the vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped replication-defective HIV-GFP reporter virus. U373.MG cells were infected with this virus at different multiplicities of infection (MOI) as indicated. Cells were then harvested for Western blot analysis. The results showed that Egr-1 expression was gradually induced at a MOI lower than 1, and maximized at MOI of 1, and then decreased to the basal level when the MOI exceeded 1 (Fig. 1E). GFAP expression showed a similar expression kinetics in HIV infected astrocytes, which further support our previous finding that Egr-1 regulates GFAP expression (Zou, et al, in preparation). HIV infection was verified by p24 and GFP expression in these cells. Taken together, these results indicate that Egr-1 is induced in astrocytes either by Tat expressing alone or by HIV-1 infection.

Up-regulation of the Egr-1 promoter activity in astrocytes by Tat

Next, we wished to determine whether Tat enhanced Egr-1 expression at the transcriptional level. To address this possibility, we created a firefly luciferase (Luc) reporter gene under the control of the human Egr-1 promoter pEgr-1p-Luc. U373.MG cells were transfected with pEgr-1p-Luc and different amounts of Tat-expressing plasmid as indicated. pCMV- β gal was included in the transfection as a transfection efficiency control for normalization, while pcDNA3 was added to equalize the total amount of DNA among the transfections. The cells were harvested for cell lysates for the reporter gene assays as well as protein expression analysis. The results showed that Tat increased the Egr-1 promoter-driven Luc reporter gene expression when the input amount of Tat was below 0.2 μ g (top panel, Fig. 2). Further increase of the input amount of Tat led to decreases in the Luc reporter gene expression, which is likely due to the anti-proliferative effects of Tat on these cells (Zhou and He, 2004b). In agreement with the reporter gene data, Egr-1 protein expression exhibited a similar kinetics (bottom panel, Fig. 2). In addition, GFAP protein expression was in parallel with that of Egr-1. These results suggest that Tat-induced Egr-1 expression likely results from transactivation of the Egr-1 promoter transcription.

Analysis of the functional elements of the human Egr-1 promoter in response to Tat expression

To further examine the mechanism of Tat-induced Egr-1 up-regulation, we investigated the importance of the *cis*-acting elements of the Egr-1 promoter. There are several putative DNA binding sites for transcription factors including one Sp1 site, two cAMP response elements (CRE), one 12-O-tetradecanoyl phorbol 13-acetate response element (TRE), one Egr-1 binding site (EBS), and five serum response elements (SRE) within the human Egr-1 promoter (Aicher *et al*, 1999; Schwachtgen *et al*, 2000). Thus, we first constructed a deletion mutant Δ 468 that was deleted the 468 bp fragment of the Egr-1 promoter at the 5' end (left panel, Fig. 3) and determined its effects on Tat-induced Egr-1 transactivation. We then performed similar transfections as above with the reporter plasmid and 0.2 μ g Tat plasmid. Compared to the full-length Egr-1 promoter-driven reporter (FL), Δ 468 showed a significantly lower Luc activity (right panel, Fig. 3). Meanwhile, this deletion gave rise to no significant difference in the Luc activity between Tat and the cDNA3 control. In addition, we also constructed Δ 260, Δ EBS, and Δ EBS Δ TRE, which were deleted the 260 bp fragment at the 5' end, the EBS binding site, and both EBS and TRE DNA binding sites, respectively, and determined their effects on Tat-induced transactivation of the Egr-1 promoter.

Interestingly, compared to the full-length promoter, all deletion mutants showed a significant increase in the Luc activity, and the biggest increase was detected in Δ EBS-transfected cells. On the other hand, none of the mutants had significant changes in the Luc activity in Tat-expressing cells. Taken together, these results indicate that SRE1/2/3 are likely involved in Tat-induced Egr-1 transactivation. Moreover, these results also suggest that the Egr-1 promoter contains positive *cis*-elements SRE1/2/3 and negative *cis*-elements TRE and EBS for its transcriptional control.

Tat-induced Egr-1 up-regulation is modulated by SRE within the Egr-1 promoter

The SRE is the binding site for serum response factor (SRF), a ubiquitous 67 kDa protein whose binding is essential for response of the promoter to serum stimulation (Treisman, 1992). Thus, to ascertain the role of SRE in Tat-induced Egr-1 expression, we determined Tat effects on the Egr-1 promoter-driven reporter gene activity in the serum-starved cells. We transfected U373.MG cells with the full-length Egr-1 promoter-driven Luc reporter with or without Tat-expressing plasmid and cultured these cells first for 24 hr in the regular 10% FBS-containing medium and then in medium containing no FBS for 24 hr. Compared to the cells that were cultured in the regular 10% FBS-containing medium, serum starvation not only significantly decreased the basal activity of the Egr-1 promoter but also Tat-induced transactivation of the Egr-1 promoter (Fig. 4). In contrast, serum starvation exhibited little effects on the activity of the Δ 468 Egr-1 promoter. The slightly higher activity of the full-length Egr-1 promoter and the slightly lower activity of the Δ 468 Egr-1 promoter in the serum starved cells than the activity of the Δ 468 Egr-1 promoter in the 10% FBS-cultured cells are likely due to the presence of the other two SRE at the 3' end of the Egr-1 promoter. These results provide further evidence to support that the SRE play important role in regulating Egr-1 transcription but also Tat-induced Egr-1 transactivation.

Discussion

In this study, we showed that Egr-1 protein level was up-regulated in Tat-expressing human astrocytoma cell lines and mouse primary astrocytes and HIV-infected astrocytes. We then showed that Tat-induced Egr-1 up-regulation occurred at the transcriptional level. Moreover, we showed that SRE within the Egr-1 promoter were important for Tat-induced Egr-1 up-regulation.

HIV-1 Tat regulates expression of a plethora of cellular genes, including several genes in astrocytes, as discussed above. In this study, we identified Egr-1 as a Tat positive regulatory target gene in astrocytes, which functioned upstream of two other Tat positive regulatory genes p300 and GFAP in these cells. Egr-1 is induced in HIV-infected monocytic and human CD4⁺ T lymphocytes (Dron *et al*, 1999) and astrocytes that were infected with HIV as shown in this study and other viruses (Cai *et al*, 2006; Romagnoli *et al*, 2008). In contrast, two early studies have shown that HIV-1 Tat blocks nerve growth factor-induced Egr-1 expression in neurons (Darbinian *et al*, 2008; Darbinian-Sarkissian *et al*, 2006). The discrete effects of Tat on Egr-1 expression are likely due to the differences between the target cells, i.e., among neurons, lymphocytes, and astrocytes. Nevertheless, the current study adds the Egr-1/p300/GFAP to the increasing number of the Tat target genes and supports the notion that altered cellular gene expression is an important indirect mechanisms by which Tat contributes to HIV-associated neuropathologies in the brain.

HIV-1 Tat effects on host gene expression are thought to be mediated through indirect mechanisms, since it has no DNA-binding activity. Our studies showed that Tat activated Egr-1 expression at the transcriptional level. The Egr-1 promoter has a number of *cis*-regulatory elements including the major SRE regulatory sequences, the SRE 1/2/3 (Aicher *et al*, 1999; Herbison *et al*, 1999). In agreement with these findings, we showed that SRE 1/2/3

were the major regulator for Egr-1 expression as well as Tat-induced Egr-1 up-regulation. The SRE activity is highly responsive to a wide range of physiological stimuli and signals, mostly through various intracellular signaling pathways (Eugenin *et al*, 2007; Gineitis and Treisman, 2001; Khachigian and Collins, 1997; Peruzzi, 2006). On the other hand, both intracellular and extracellular Tat has been linked to intracellular signaling pathways to regulate cellular gene expression and subsequently cell proliferation and survival (Eugenin *et al*, 2007; Gineitis and Treisman, 2001; Khachigian and Collins, 1997; Peruzzi, 2006). Thus, it is likely that Tat-induced Egr-1 up-regulation results from cross talks with Tat-elicited intracellular signaling. Nevertheless, the exact mechanisms underlying the Tat-induced Egr-1 expression remain to be elucidated.

Egr-1, like other members of the immediate early gene family, functions as a convergence “gateway” for many signaling cascades and controls many biological functions by altering its target gene expression to couple extracellular signals to long-term responses. A growing list of genes have been identified to be Egr-1 regulatory targets. These include transcription factors such as Egr-1, cytokines such as TGF- β and IL-1, chemokines such as MIP-2, MCP-1, and neuronal genes (James *et al*, 2004; Petersohn *et al*, 1995; Treisman, 1992). Many of these genes clearly play key roles in cell proliferation and differentiation, pathological settings and neuronal responses such as neuronal plasticity. Interestingly, several recent studies have directly linked Egr-1 to astrocyte proliferation, glial scar formation, astrocyte function and neurite outgrowth and survival of neuronal cells (Beck *et al*, 2008; Mayer *et al*, 2009; Mittelbronn *et al*, 2009; Shin *et al*, 2009). Thus, based on our previous studies and the current study, we propose a Egr-1-mediated astrocyte transcriptional cascade for Tat neurotoxicity (Fig. 5). It begins with Egr-1 transactivation by Tat, produced by direct HIV infection of astrocytes or secreted by HIV-infected macrophages/microglia and taken up by astrocytes or direct HIV infection of astrocytes. Egr-1 activation leads to p300 expression, which in turn activates GFAP expression in astrocytes or astrocytosis. Elucidation of the molecular pathways by which Tat activates Egr-1 expression presents a new opportunity to further discern the neuroprotective from the neurotoxic functions of astrocytosis and might lead to new approaches to modulate the time span and severity of astrocytosis, thus fine-tuning neuronal tissue repair and regeneration in the context of HIV infection.

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References

- Aicher WK, Sakamoto KM, Hack A, Eibel H. Analysis of functional elements in the human Egr-1 gene promoter. *Rheumatol Int* 1999;18:207–14. [PubMed: 10399797]
- Beck H, Semisch M, Culmsee C, Plesnila N, Hatzopoulos AK. Egr-1 regulates expression of the glial scar component phosphacan in astrocytes after experimental stroke. *Am J Pathol* 2008;173:77–92. [PubMed: 18556777]
- Bell JE, Anthony IC, Simmonds P. Impact of HIV on regional & cellular organisation of the brain. *Curr HIV Res* 2006;4:249–57. [PubMed: 16842078]
- Bonifaci N, Sitia R, Rubartelli A. Nuclear translocation of an exogenous fusion protein containing HIV Tat requires unfolding. *Aids* 1995;9:995–1000. [PubMed: 8527095]
- Bratanich AC, Liu C, McArthur JC, Fudyk T, Glass JD, Mittoo S, Klassen GA, Power C. Brain-derived HIV-1 tat sequences from AIDS patients with dementia show increased molecular heterogeneity [In Process Citation]. *J Neurovirol* 1998;4:387–93. [PubMed: 9718130]
- Brew BJ. HIV, the brain, children, HAART and ‘neuro-HAART’: a complex mix. *AIDS* 2009;23:1909–10. [PubMed: 19550290]

- Cai Y, Liu Y, Zhang X. Induction of transcription factor Egr-1 gene expression in astrocytoma cells by Murine coronavirus infection. *Virology* 2006;355:152–63. [PubMed: 16908043]
- Chen P, Mayne M, Power C, Nath A. The Tat protein of HIV-1 induces tumor necrosis factor- α production. Implications for HIV-1-associated neurological diseases. *J Biol Chem* 1997;272:22385–8. [PubMed: 9278385]
- Cheng J, Nath A, Knudsen B, Hochman S, Geiger JD, Ma M, Magnuson DS. Neuronal excitatory properties of human immunodeficiency virus type 1 Tat protein. *Neuroscience* 1998;82:97–106. [PubMed: 9483506]
- Cohen RA, Gongvatana A. HIV-associated brain dysfunction in the era of HAART: reasons for hope, but continued concern. *Neurology* 2009;73:338–9. [PubMed: 19553591]
- Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, Gallo RC, Major EO. Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and elevation in AIDS dementia. *Proc Natl Acad Sci U S A* 1998;95:3117–21. [PubMed: 9501225]
- Cupp C, Taylor JP, Khalili K, Amini S. Evidence for stimulation of the transforming growth factor beta 1 promoter by HIV-1 Tat in cells derived from CNS. *Oncogene* 1993;8:2231–6. [PubMed: 8336945]
- Darbinian N, Darbinyan A, Czernik M, Peruzzi F, Khalili K, Reiss K, Gordon J, Amini S. HIV-1 Tat inhibits NGF-induced Egr-1 transcriptional activity and consequent p35 expression in neural cells. *J Cell Physiol* 2008;216:128–34. [PubMed: 18247371]
- Darbinian-Sarkissian N, Czernik M, Peruzzi F, Gordon J, Rappaport J, Reiss K, Khalili K, Amini S. Dysregulation of NGF-signaling and Egr-1 expression by Tat in neuronal cell culture. *J Cell Physiol* 2006;208:506–15. [PubMed: 16741963]
- Del Valle L, Pina-Oviedo S. HIV disorders of the brain: pathology and pathogenesis. *Front Biosci* 2006;11:718–32. [PubMed: 16146764]
- Dron M, Hameau L, Benboudjema L, Guymarho J, Cajean-Feroldi C, Rizza P, Godard C, Jasmin C, Tovey MG, Lang MC. Cloning of a long HIV-1 readthrough transcript and detection of an increased level of early growth response protein-1 (Egr-1) mRNA in chronically infected U937 cells. *Arch Virol* 1999;144:19–28. [PubMed: 10076506]
- El-Hage N, Gurwell JA, Singh IN, Knapp PE, Nath A, Hauser KF. Synergistic increases in intracellular Ca²⁺, and the release of MCP-1, RANTES, and IL-6 by astrocytes treated with opiates and HIV-1 Tat. *Glia* 2005;50:91–106. [PubMed: 15630704]
- Ellis R, Langford D, Masliah E. HIV and antiretroviral therapy in the brain: neuronal injury and repair. *Nat Rev Neurosci* 2007;8:33–44. [PubMed: 17180161]
- Eugenin EA, King JE, Nath A, Calderon TM, Zukin RS, Bennett MV, Berman JW. 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* 2007;104:3438–43. [PubMed: 17360663]
- Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 1988;55:1189–93. [PubMed: 2849510]
- Gineitis D, Treisman R. Differential usage of signal transduction pathways defines two types of serum response factor target gene. *J Biol Chem* 2001;276:24531–9. [PubMed: 11342553]
- Gorry P, Purcell D, Howard J, McPhee D. Restricted HIV-1 infection of human astrocytes: potential role of nef in the regulation of virus replication. *J Neurovirol* 1998;4:377–86. [PubMed: 9718129]
- Green M, Ishino M, Loewenstein PM. Mutational analysis of HIV-1 Tat minimal domain peptides: identification of trans-dominant mutants that suppress HIV-LTR-driven gene expression. *Cell* 1989;58:215–23. [PubMed: 2752420]
- Herbison CE, Sayer DC, Bellgard M, Allcock RJ, Christiansen FT, Price P. Structure and polymorphism of two stress-activated protein kinase genes centromeric of the MHC: SAPK2a and SAPK4. *DNA Seq* 1999;10:229–43. [PubMed: 10727080]
- Hofman FM, Chen P, Incardona F, Zidovetzki R, Hinton DR. HIV-1 tat protein induces the production of interleukin-8 by human brain-derived endothelial cells. *J Neuroimmunol* 1999;94:28–39. [PubMed: 10376933]
- Hofman FM, Dohadwala MM, Wright AD, Hinton DR, Walker SM. Exogenous tat protein activates central nervous system-derived endothelial cells. *J Neuroimmunol* 1994;54:19–28. [PubMed: 7523444]

- Hofman FM, Wright AD, Dohadwala MM, Wong-Staal F, Walker SM. Exogenous tat protein activates human endothelial cells. *Blood* 1993;82:2774–80. [PubMed: 7693046]
- James AB, Conway AM, Thiel G, Morris BJ. Egr-1 modulation of synapsin I expression: permissive effect of forskolin via cAMP. *Cell Signal* 2004;16:1355–62. [PubMed: 15381251]
- Jones M, Olafson K, Del Bigio MR, Peeling J, Nath A. Intraventricular injection of human immunodeficiency virus type 1 (HIV-1) tat protein causes inflammation, gliosis, apoptosis, and ventricular enlargement. *J Neuropathol Exp Neurol* 1998;57:563–70. [PubMed: 9630236]
- Khachigian LM, Collins T. Inducible expression of Egr-1-dependent genes. A paradigm of transcriptional activation in vascular endothelium. *Circ Res* 1997;81:457–61. [PubMed: 9314825]
- Kim BO, Liu Y, Ruan Y, Xu ZC, Schantz L, He JJ. Neuropathologies in transgenic mice expressing human immunodeficiency virus type 1 Tat protein under the regulation of the astrocyte-specific glial fibrillary acidic protein promoter and doxycycline. *Am J Pathol* 2003;162:1693–707. [PubMed: 12707054]
- Kolson DL, Buchhalter J, Collman R, Hellmig B, Farrell CF, Debouck C, Gonzalez-Scarano F. HIV-1 Tat alters normal organization of neurons and astrocytes in primary rodent brain cell cultures: RGD sequence dependence. *AIDS Res Hum Retroviruses* 1993;9:677–85. [PubMed: 8369172]
- Lee SC, Hatch WC, Liu W, Brosnan CF, Dickson DW. Productive infection of human fetal microglia in vitro by HIV-1. *Ann N Y Acad Sci* 1993;693:314–6. [PubMed: 8267290]
- Liu Y, Jones M, Hingtgen CM, Bu G, Laribee N, Tanzi RE, Moir RD, Nath A, He JJ. 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* 2000a;6:1380–7. [PubMed: 11100124]
- Liu Y, Jones M, Hingtgen CM, Bu G, Laribee N, Tanzi RE, Moir RD, Nath A, He JJ. 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* 2000b;6:1380–7. [PubMed: 11100124]
- Liu Y, Liu H, Kim BO, Gattone VH, Li J, Nath A, Blum J, He JJ. CD4-independent infection of astrocytes by human immunodeficiency virus type 1: requirement for the human mannose receptor. *J Virol* 2004;78:4120–33. [PubMed: 15047828]
- Mann DA, Frankel AD. Endocytosis and targeting of exogenous HIV-1 Tat protein. *Embo J* 1991;10:1733–9. [PubMed: 2050110]
- Mayer SI, Rossler OG, Endo T, Charnay P, Thiel G. Epidermal-growth-factor-induced proliferation of astrocytes requires Egr transcription factors. *J Cell Sci* 2009;122:3340–50. [PubMed: 19706684]
- Milani D, Zauli G, Neri LM, Marchisio M, Prevati M, Capitani S. Influence of the human immunodeficiency virus type 1 Tat protein on the proliferation and differentiation of PC12 rat pheochromocytoma cells. *J Gen Virol* 1993;74:2587–94. [PubMed: 8277265]
- Mittelbronn M, Harter P, Warth A, Lupescu A, Schilbach K, Vollmann H, Capper D, Goepfert B, Frei K, Bertalanffy H, Weller M, Meyermann R, Lang F, Simon P. EGR-1 is regulated by N-methyl-D-aspartate-receptor stimulation and associated with patient survival in human high grade astrocytomas. *Brain Pathol* 2009;19:195–204. [PubMed: 18489490]
- Nath A, Conant K, Chen P, Scott C, Major EO. Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *J Biol Chem* 1999;274:17098–102. [PubMed: 10358063]
- Parmentier HK, van Wichen DF, Meyling FH, Goudsmit J, Schuurman HJ. Epitopes of human immunodeficiency virus regulatory proteins tat, nef, and rev are expressed in normal human tissue. *Am J Pathol* 1992;141:1209–16. [PubMed: 1279980]
- Peruzzi F. The multiple functions of HIV-1 Tat: proliferation versus apoptosis. *Front Biosci* 2006;11:708–17. [PubMed: 16146763]
- Petersohn D, Schoch S, Brinkmann DR, Thiel G. The human synapsin II gene promoter. Possible role for the transcription factor zif268/egr-1, polyoma enhancer activator 3, and AP2. *J Biol Chem* 1995;270:24361–9. [PubMed: 7592648]
- Price RW, Brew B, Sidtis J, Rosenblum M, Scheck AC, Cleary P. The brain in AIDS: central nervous system HIV-1 infection and AIDS dementia complex. *Science* 1988;239:586–92. [PubMed: 3277272]

- Romagnoli L, Sariyer IK, Tung J, Feliciano M, Sawaya BE, Del Valle L, Ferrante P, Khalili K, Safak M, White MK. Early growth response-1 protein is induced by JC virus infection and binds and regulates the JC virus promoter. *Virology* 2008;375:331–41. [PubMed: 18353421]
- Sabatier JM, Vives E, Mabrouk K, Benjouad A, Rochat H, Duval A, Hue B, Bahraoui E. Evidence for neurotoxic activity of tat from human immunodeficiency virus type 1. *J Virol* 1991;65:961–967. [PubMed: 1898974]
- Sacktor N. The epidemiology of human immunodeficiency virus-associated neurological disease in the era of highly active antiretroviral therapy. *J Neurovirol* 2002;8(Suppl 2):115–21. [PubMed: 12491162]
- Saito Y, Sharer LR, Epstein LG, Michaels J, Mintz M, Louder M, Golding K, Cvetkovich TA, Blumberg BM. Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues. *Neurology* 1994;44:474–81. [PubMed: 8145918]
- Schwachtgen JL, Campbell CJ, Braddock M. Full promoter sequence of human early growth response factor-1 (Egr-1): demonstration of a fifth functional serum response element. *DNA Seq* 2000;10:429–32. [PubMed: 10826704]
- Schweighardt B, Atwood WJ. HIV type 1 infection of human astrocytes is restricted by inefficient viral entry. *AIDS Res Hum Retroviruses* 2001;17:1133–42. [PubMed: 11522183]
- Shin SY, Song H, Kim CG, Choi YK, Lee KS, Lee SJ, Lee HJ, Lim Y, Lee YH. Egr-1 is necessary for fibroblast growth factor-2-induced transcriptional activation of the glial cell line-derived neurotrophic factor in murine astrocytes. *J Biol Chem* 2009;284:30583–93. [PubMed: 19721135]
- Tornatore C, Chandra R, Berger JR, Major EO. HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* 1994;44:481–7. [PubMed: 8145919]
- Treisman R. The serum response element. *Trends Biochem Sci* 1992;17:423–6. [PubMed: 1455511]
- Viscidi RP, Mayur K, Lederman HM, Frankel AD. Inhibition of antigen-induced lymphocyte proliferation by Tat protein from HIV-1. *Science* 1989;246:1606–8. [PubMed: 2556795]
- Westendorp MO, Frank R, Ochsenbauer C, Stricker K, Dhein J, Walczak H, Debatin KM, Krammer PH. Sensitization of T cells to CD95-mediated apoptosis by HIV-1 Tat and gp120. *Nature* 1995a;375:497–500. [PubMed: 7539892]
- Westendorp MO, Shatrov VA, Schulze-Osthoff K, Frank R, Kraft M, Los M, Krammer PH, Droge W, Lehmann V. HIV-1 Tat potentiates TNF-induced NF-kappa B activation and cytotoxicity by altering the cellular redox state. *Embo J* 1995b;14:546–54. [PubMed: 7859743]
- Williams R, Yao H, Dhillon NK, Buch SJ. HIV-1 Tat co-operates with IFN-gamma and TNF-alpha to increase CXCL10 in human astrocytes. *PLoS One* 2009;4:e5709. [PubMed: 19479051]
- Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, Jeang KT. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc Natl Acad Sci U S A* 2000a;97:11466–71. [PubMed: 11027346]
- Xiao H, Neuveut C, Tiffany HL, Benkirane M, Rich EA, Murphy PM, Jeang KT. Selective CXCR4 antagonism by Tat: implications for in vivo expansion of coreceptor use by HIV-1. *Proc Natl Acad Sci U S A* 2000b;97:11466–71. [PubMed: 11027346]
- Zhou BY, He JJ. Proliferation inhibition of astrocytes, neurons, and non-glial cells by HIV-1 Tat protein. *Neuroscience Letters* 2004a;359:155–158. [PubMed: 15050687]
- Zhou BY, He JJ. Proliferation inhibition of astrocytes, neurons, and non-glial cells by intracellularly expressed human immunodeficiency virus type 1 (HIV-1) Tat protein. *Neurosci Lett* 2004b;359:155–8. [PubMed: 15050687]
- Zhou BY, Liu Y, Kim B, Xiao Y, He JJ. Astrocyte activation and dysfunction and neuron death by HIV-1 Tat expression in astrocytes. *Mol Cell Neurosci* 2004;27:296–305. [PubMed: 15519244]
- Zidovetzki R, Wang JL, Chen P, Jeyaseelan R, Hofman F. Human immunodeficiency virus Tat protein induces interleukin 6 mRNA expression in human brain endothelial cells via protein kinase C- and cAMP-dependent protein kinase pathways. *AIDS Res Hum Retroviruses* 1998;14:825–33. [PubMed: 9671211]
- Zocchi MR, Rubartelli A, Morgavi P, Poggi A. HIV-1 Tat inhibits human natural killer cell function by blocking L-type calcium channels. *J Immunol* 1998;161:2938–43. [PubMed: 9743356]

Zou W, Kim BO, Zhou BY, Liu Y, Messing A, He JJ. Protection against human immunodeficiency virus type 1 Tat neurotoxicity by Ginkgo biloba extract EGb 761 involving glial fibrillary acidic protein. *Am J Pathol* 2007;171:1923–35. [PubMed: 18055541]

Figure 1. Egr-1 up-regulation in Tat-expressing U373 cells, U87 cells and primary astrocytes
Cells were transiently transfected with pCDNA3 or pcTat.Myc and harvested after 72 h; Cell lysates of (A) U373 and (B) U87 cells were analyzed for Tat and Egr-1 expression, with β -actin included as a loading control. Primary astrocytes were prepared from brain-targeted inducible Tat transgenic mice and cultured in the absence or presence of 5 μ g/ml doxycycline (Dox) for up to 3 days (C) then analyzed for Tat and Egr-1 expression. Total RNA was isolated and subjected to RT-PCR using Tat-specific primers with GAPDH used as a loading control. As a control, primary astrocytes were also prepared from wild-type C57/BL6 mice, treated with Dox and analyzed for Egr-1 expression (D). Meanwhile, U373 cells were infected with increasing amounts of VSV-G pseudotyped HIV-GFP virus and harvested after 72 h. Cell lysates were analyzed for Egr-1, GFAP, and Tat expression (E), along with p24 and GFP to ensure infection efficiency. β -actin was included as a loading control. Data is representative of at least three independent experiments.

Figure 2. Egr-1 promoter activity and expression of Egr-1 protein increases in the presence of a specific amount of Tat

U373 cells were transiently transfected with Egr-1 promoter-driven luciferase reporter plasmid pEgr-1p-Luc or control plasmid pGL3 along with increasing amounts of pcTat.Myc and harvested 72h post-transfection. Half of the cells were to detect Egr-1 promoter activity (A) with pCMV-βgal used to normalize for variations in transfection efficiency. The remaining transfected cells were separated and used for Triton-soluble (TS) and Triton-insoluble (TIS) fractions and analyzed for Egr-1, GFAP and Tat expression, with β-actin included as a loading control (B).

Figure 3. Analysis of the functional elements of the human Egr-1 promoter in response to Tat expression

The full length human Egr-1 promoter was cloned from the genomic DNA of U87 cells and inserted into pGL3, and then used to create a panel of Egr-1 promoter mutations (A). Cells were transiently transfected each pEgr-1p-luc mutant plasmid and either pcTat.Myc or pcDNA3, then harvested 72h post-transfection. Cell lysates were used to determine luciferase activity (B), with pCMV-βgal included to normalize the transfection efficiency. Data is representative of at least three independent experiments.

Figure 4. Tat-induced Egr-1 up-regulation is modulated by the serum response element during starvation

U373 cells were transfected with either pEgr-1p-700-luc or pEgr-1p-200-luc along with pcTat.Myc or pcDNA3. At 16h post-transfection the media was replaced with DMEM containing reduced concentrations of serum and incubated for additional 48 hr. Cell lysates were then used to determine luciferase activity, with pCMV-βgal included to normalize the transfection efficiency. Data is representative of at least three independent experiments.

Figure 5. The Egr-1 transcriptional network altered by HIV-1 Tat in astrocytes

HIV-1 Tat protein in astrocytes can be derived from direct HIV infection of these cells themselves. It can also be from HIV-infected microglia/macrophages, followed by its uptake by astrocytes. Tat then transactivates Egr-1 expression, which in turn transactivates p300 expression, which in turn transactivates GFAP expression. Activation of GFAP expression or expression of other target genes of Egr-1 and p300 leads to changes in astrocyte proliferation, survival and function and as a result, impairs neuronal integrity and survival.