Brief Communication

# Synaptic Transport of Human Immunodeficiency Virus–Tat Protein Causes Neurotoxicity and Gliosis in Rat Brain

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**Neurodegeneration, synaptic alterations, and gliosis are prominent features of human immunodeficiency virus (HIV) encephalitis, but HIV encephalitis is distinct from other viral encephalitides because neurodegeneration occurs in uninfected neurons at anatomical sites that are often distant from the site of viral replication. The HIV protein Tat is both neurotoxic and proinflammatory; however, its contribution to HIV-related synaptic dysfunction remains unknown. To determine the consequences of continuous Tat production in brain, we genetically engineered rat C6 glioma cells to stably produce Tat and stereotaxically infused these cells into the rat striatum or hippocampus. We discovered that HIV–Tat protein could be transported along anatomical pathways from the dentate gyrus to the CA3/4 region and from the striatum to the substantia nigra, resulting in behavioral abnormalities, neurotoxicity, and reactive gliosis. This demonstrates a unique neuronal transport property of a viral protein and establishes a mechanism for neuroglial dysfunction at sites distant from that of viral replication. Tat may thus be an important participant in brain dysfunction in HIV dementia.**

*Key words:* **AIDS dementia; basal ganglia; hippocampus; inflammation; neurodegeneration; synaptic transport**

## Introduction

Neuronal alterations and dementia occur in a significant number of human immunodeficiency virus (HIV)-infected individuals (Janssen et al., 1989), but the mechanisms whereby HIV damages brain are not fully understood. Clinically, HIV dementia (HIVD) is characterized by progressive motor, cognitive, and behavioral alterations (Heaton et al., 1994), whereas histopathologically, many HIVD brains are characterized by decreased synaptic density, neuronal loss, astrocytosis, and microglial nodules with multinucleated giant cells (Everall et al., 1993; Masliah et al., 1997). Because neurons are not permissive for HIV infection, most evidence suggests that HIV damages neurons through toxic substances released from infected cells. Microglial cells and macrophages are the predominant cells in the brain that undergo productive infection (Gabuzda et al., 1986), although there is evidence that astrocytes can be infected with HIV and can support limited viral replication (Tornatore et al., 1994).

Because HIV infection in the brain is thus relatively restricted, an unresolved issue is how low viral load in brain causes such pronounced neuronal dysfunction. HIV encephalitis is unlike other viral encephalitides in that degeneration occurs in neurons, which are not receptive to infection. Additionally, markers of neurodegeneration can frequently be detected at anatomical sites that are distant from sites of viral replication. An intriguing possibility is that the deleterious effects of HIV infection in the brain are mediated by secreted viral proteins, and a viral protein that has been repeatedly implicated in the pathogenesis of HIVD is the *trans*-activating regulatory protein Tat. Tat protein and mRNA can be detected in brains of patients with HIV encephalitis (Hofman et al., 1994; Wiley et al., 1996). Unlike many other HIV-1 proteins, Tat is actively released by infected lymphocytes (Ensoli et al., 1993) and glia (Tardieu et al., 1992). Because anti-Tat antibodies are frequently detected in the serum of AIDS patients (Aldovini et al., 1986), it is reasonable to hypothesize that secreted Tat has a role in the progression of HIVD. Although cell culture studies have demonstrated that Tat can cause inflammation (Sheng et al., 2000; Bruce-Keller et al., 2001) and excitotoxicity (New et al., 1998; Nath et al., 2000), the role of Tat in HIVD is not well resolved.

To determine whether continuous low-level production of Tat in rat brain could cause behavioral and histological abnormalities that mimic the characteristics of HIV dementia, we genetically engineered C6 rat glioma cells to stably produce and secrete Tat 1–86 and implanted these cells into either the striatum or hippocampus of adult male rats. Animals were then evaluated both behaviorally and histologically to document the neurotoxic consequences of continuous Tat production in the brain.

## Materials and Methods

*Plasmids and C6 cell transfection.* Tat 1–86 (HXB-2) was cloned from PGEX-Tat (provided by Dr. Mauro Giacca, International Center for Genetic Engineering and Biotechnology, Trieste, Italy) into pcDNA3 vector at *Bam*H1–*Eco*R1 driven by a cytomegalovirus (CMV) promoter. The Tat gene was cloned in-frame upstream of a green fluorescent protein (GFP) gene also driven by a CMV promoter in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). C6 (rat glioma) cells were transfected with either Tat or pcDNA3 alone using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the supplier's protocol using plasmid con-

Received March 17, 2003; revised July 24, 2003; accepted July 29, 2003.

This work was supported by grants from the National Institutes of Health (NIH) (NS39398; NS39253) and NIH/National Center for Research Resources (P20 RR15592). We are grateful to Carol Anderson and Sarah Martin for expert technical assistance and guidance and to Dr. Stephen Scheff for helpful input into data analysis and presentation.

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 0270-6474/03/238417-06$15.00/0

centrations ranging from 0.5 to 12 μg. Cells were grown for 72 hr and then subcultured in DMEM containing G418 (1.5 mg/ml) (Invitrogen) for 1 week. Cloning was done using trypsin-soaked filter disks, and cells were subcultured for 1–2 weeks under selection. All cells were then used within 30 passages and maintained in 500 μg/ml G418.

*Implantation of Tat-expressing cells in rat brain*. Twelve-week-old male Sprague Dawley rats were kept on a 12 hr light/dark cycle with *ad libitum* access to food and water. C6 cells ($7.5 \times 10^4$) were suspended in 1 μl of DMEM and unilaterally injected into either the neostriatum or the hilar area of the dentate gyrus using the following stereotaxic coordinates: striatum, 0.5 anterior and 2.5 mm lateral to bregma, and 4.0 mm ventral to the dura; hippocampus, 3.8 mm posterior and 1.5 mm lateral to bregma, and 3.5 mm ventral to the dura. All animals were killed by perfusion with normal saline followed by 4% paraformaldehyde 10 d after surgery.

*Behavioral testing*. Motor performance of rats that had received striatal injections was tested using a standard rodent rotorod (Columbus Instruments, Columbus, OH). To acclimate the animals to the device, rats were placed for 2 min on the wheel, which was moving at 0.3 revolutions per second, and this procedure was repeated three times daily for each animal on days 2 and 3 after surgery. Starting on postsurgical day 4, motor performance was tested by measuring the amount of time that rats were able to remain on the wheel, which was accelerating by 0.5 revolutions per second from a base speed of 0.3 revolutions per second. All animals were tested three times daily by an investigator blind to the treatment code.

*Histological analyses*. Sections (30 μm) were prepared from throughout the striatum, hippocampus, and substantia nigra and processed for Nissl stain or immunohistochemistry using the following primary antisera: monoclonal anti-Tat (1:1000; NIBSC centralized facility for AIDS reagents, UK catalog #ARP352); anti-tyrosine hydroxylase (TH) (1:5000; Chemicon, Temecula, CA), anti-CD11b (OX42; 1:500; Serotec, Raleigh, NC), anti-synaptophysin (1:1000; Sigma, St. Louis, MO), and anti-GFAP (1:1000; Chemicon) and the avidin–biotin immunoperoxidase system as described previously (Bruce-Keller et al., 1999).

For immunoabsorption of Tat antisera, recombinant Tat was made in *Escherichia coli* as described previously (Ma and Nath, 1997). The biotinylated Tat protein was bound to 0.5 ml streptavidin beads and washed five times with PBS. Tat antisera was added to the beads at 1:50 concentration in PBS and incubated at room temperature for 90 min. The beads were centrifuged, and the supernatant was used for immunohistochemistry. Streptavidin-coated beads without the biotinylated Tat protein were used as a control to exclude nonspecific binding of antisera to the beads.

*Quantification of neuronal damage*. Neuronal injury in the substantia nigra was determined by quantifying TH immunoreactivity in 4× digitized microscope images. The regions of interest (ROIs) were the pars compacta region of the substantia nigra and the ventral tegmental area (VTA). To ensure that comparisons between ipsilateral and contralateral sides were appropriate, it was confirmed that the brains were cut on an exact coronal plane by bilaterally identifying the medial accessory optic tract, which bisects the VTA from the pars compacta. Sections at this level and up to 300 μm anterior were analyzed for TH immunoreactivity, and at least three sections for each animal were averaged. Staining intensity was quantified using Scion Image software, and to ensure that the ROIs were drawn consistently, the ipsilateral side was measured by horizontally transposing the area drawn to measure the contralateral side. TH immunoreactivity values are presented as percentage contralateral side to control for variations in staining between sections, and as an additional control, TH immunoreactivity in the adjacent VTA, which is not synaptically coupled to the dorsal caudate putamen, is also presented.

Neuronal injury in hippocampus was measured by CA3 neuronal counts and lesion volume analyses. To determine pyramidal CA3 neuronal loss in the hippocampus, 40× magnification images of sections from the needle tract and 300, 600, and 900 μm posterior to the injection site were captured and digitized. Neurons within a $0.5 \times 0.5\ mm^2$ grid placed over the pyramidal cell layer directly adjacent to the lateral edge of the dentate gyrus were counted in ipsilateral and contralateral hippocampus. All Nissl-positive cells that retained their pyramidal morphology and were not rounded or pyknotic were counted.

Lesion volume was quantified in Nissl-stained sections by computer-assisted analysis of serial hippocampal sections, with the first section taken just posterior to the subfornical organ and the last section just caudal to the substantia nigra. The lesion was identified morphologically within the inner portion of the apical dendrites of CA3 pyramidal neurons as the area of increased Nissl staining caused by immune cell infiltration and proliferation within the stratum radiatum (see Fig. 2*A*). The area from each section was multiplied by intersectional distance (0.3 mm) to calculate the subvolume, and subvolumes were summed to yield the total volume for each animal.

*Statistical analyses*. Behavioral data were analyzed using one-way ANOVA, followed by Scheffe's *post hoc* analysis to determine statistical significance. TH immunoreactivity and neuronal lesion measurements were analyzed using unpaired *t* tests. $p$ values $<0.05$ or $<0.01$ were designated as statistically significant and are indicated in the text as * or **, respectively.

## Results

### Tat production in striatum decreases motor performance

HIV dementia is a subcortical disease with maximal viral load in hippocampus (Wiley et al., 1996) and striatum (Kure et al., 1990); hence we initially determined whether chronic Tat production in the striatum could alter motor function. Our previous studies show that C6 cells stably transfected with Tat release physiological levels of bioactive Tat that can both injure cocultured neurons and transactivate the HIV long-term repeat domain in cocultured lymphocytes or astrocytes transfected with a reporter LTR–GFP gene(Chauhan et al., 2003). Seventy-five thousand C6-Tat or control C6-pcDNA cells were thus implanted into the dorsal caudate putamen of adult male rats, and motor performance was tested on days 4–10 after surgery using a rotorod. All animals had similar rotorod times on days 4–7 (Fig. 1*A*); however, vehicle-treated and C6-pcDNA animals consistently improved their rotorod times compared with C6-Tat rats, so that by days 8, 9, and 10, the C6-Tat animals had significantly shorter rotorod times compared with C6-pcDNA or vehicle controls (Fig. 1*A*).

### Tat production in striatum alters neuronal and glial histology in substantia nigra

To determine the effects of chronic Tat production on basal ganglia histology, sections encompassing the rostrocaudal extent of the substantia nigra were prepared from the animals that had received striatal infusions of C6-Tat or pcDNA C6 cells. Selected sections were probed for TH immunoreactivity to identify nigral dopaminergic neurons. TH immunoreactivity was evaluated in the pars compacta region of the substantia nigra (Fig. 1*C*), which has direct projections to the caudate region of the striatum, and in the VTA, which contains dopaminergic cells associated with ventral forebrain limbic structures. Tat production in the striatum significantly decreased TH immunoreactivity in the ipsilateral pars compacta when compared with the contralateral side (Fig. 1*B*,*C*); however, there was no effect on TH immunoreactivity in the VTA (Fig. 1*B*) and no effect of striatal C6-pcDNA cells on TH immunoreactivity in either pars compacta or VTA (Fig. 1*B*,*C*). Conversely, immunoreactivity for both OX42 and GFAP was dramatically increased in the ipsilateral substantia nigra of rats injected with C6-Tat cells but not C6-pcDNA cells (Fig. 1*C*).

### Tat production in the hilus alters neuronal and glial histology in CA3

To determine the effects of chronic Tat production on neuronal histology in the hippocampus, animals that had received C6-Tat or C6-pcDNA cells into the hilus were killed 10 d after surgery. Infusion of C6-pcDNA cells into the hilus caused damage to den-

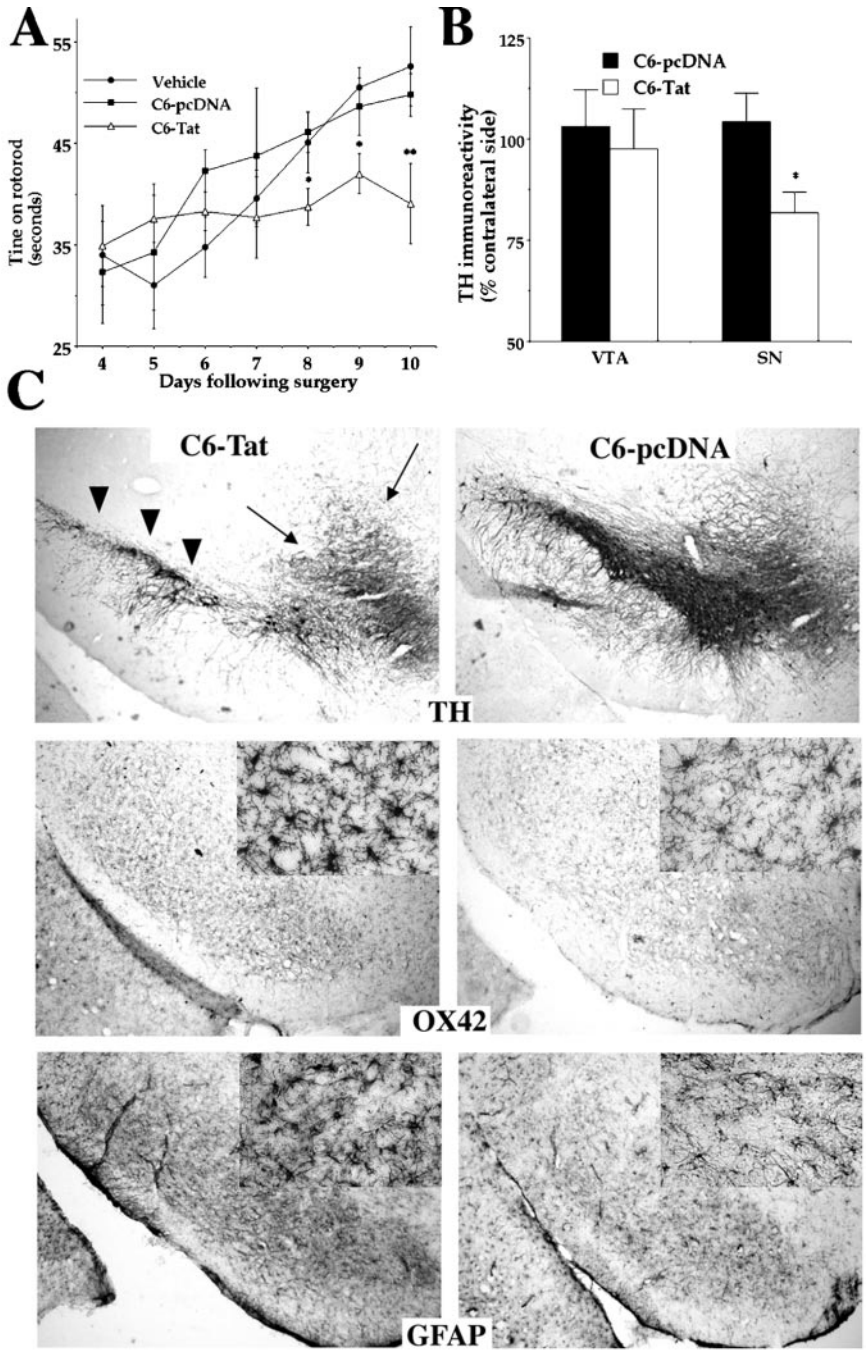


**Figure 1.** Implantation of C6-Tat cells in striatum decreases motor performance and causes neurotoxicity in substantia nigra. *A*, Motor performance was tested using a rotorod as described in Materials and Methods, and data are means and SEM of three trials per animal, with 12 animals per group. Asterisks indicate statistically significant (*$p < 0.05$, **$p < 0.01$) decreases in rotorod times for C6-Tat animals compared with both C6-pcDNA and vehicle controls. *B*, Levels of tyrosine hydroxylase (TH) immunoreactivity were quantified in the ventral tegmental area (VTA) and in the pars compacta region of the substantia nigra (SN) of animals that had received striatal infusions of either C6-Tat or C6-pcDNA cells. Data are TH intensity of the injected side represented as percentage contralateral side and are the means and SEM of immunoreactivity measured in triplicate from 12 animals per group. The asterisk indicates a statistically significant ( $p < 0.05$) decrease in nigral TH immunoreactivity in C6-Tat rats as compared with C6-pcDNA rats. *C*, Immunoreactivity for TH, OX42, and GFAP in the ipsilateral substantia nigra of animals that had received striatal infusions of C6-pcDNA or C6-Tat cells. Images were taken from 4× and 32× (corner insets) microscope fields and are representative of three separate experiments of four to six animals per group each. Arrows and arrowheads in the top panels illustrate the VTA and pars compacta, respectively, that were analyzed for quantification.

tate gyrus granule neurons that was mainly localized to the injection site (Fig. 2*A*). CA3 pyramidal neurons were counted in Nissl-stained sections from the needle track 300, 600, and 900 $\mu$m posterior to the injection site to determine the rostrocaudal extent of neuronal loss. Although infusion of C6-Tat cells into the hilus caused CA3 pyramidal cells to appear shrunken and darkly stained (Fig. 2*A*), cell counts did not reveal gross neuronal loss in rats injected with either pcDNA or Tat-producing C6 cells at any rostrocaudal level (data not shown). However, C6-Tat-injected rats were typified by a lesion within the CA3/4 stratum radiatum dendritic fields demarked by increased Nissl staining caused by inflammatory infiltrates and proliferation (Fig. 2*A*). Lesion volumetric analyses (see Materials and Methods) demonstrated that C6-Tat animals had significantly (fourfold) larger lesions as compared with C6-pcDNA rats (Fig. 2*B*). Synaptic density and reactive gliosis in the hippocampus of C6-Tat and C6-pcDNA-treated rats was determined via immunoreactivity for synaptophysin and OX42/GFAP, respectively, in sections 300 $\mu$m posterior to the injection site. Injection of C6-Tat cells into the hilus markedly decreased synaptophysin immunoreactivity in the dentate gyrus and in CA3/4 dendritic fields (Fig. 2*C*). Conversely, OX42 and GFAP immunoreactivity were dramatically increased in the dentate gyrus and CA3/4 synaptic fields of rats injected with C6-Tat cells but not with C6-pcDNA cells (Fig. 2*C*).

### Localization of Tat proteins

To verify the viability of implanted C6 cells and to determine the distribution of Tat proteins in the brain, animals that had been injected with either pcDNA or Tat-producing C6 cells were examined histologically for Tat immunoreactivity. Surprisingly, Tat proteins were detected in the substantia nigra of animals that had been infused with C6-Tat cells in the striatum (Fig. 3*A*). Likewise, examination of CA3/4 in brain sections 300 $\mu$m posterior to the injection site revealed extensive Tat immunoreactivity in animals that had been implanted with C6-Tat cells but not with C6-pcDNA cells (Fig. 3*A*). Tat proteins were associated with stratum radiatum dendritic fields rather than with CA3/4 pyramidal cell bodies (Fig. 3*B*,*C*), whereas Tat immunoreactivity at the needle tract in the hilus was localized to the implanted C6-Tat cell population (Fig. 3*B*). To control for nonspecific binding of primary and secondary antibodies to sites of inflammation in the hippocampus, the immunohistochemistry procedure was repeated without the primary antibody (data not shown) or using a primary anti-Tat antibody that had been preabsorbed with excess Tat protein (Fig. 3*D*). Thus, these data suggest that Tat proteins were not only produced by the C6 cells, but they could be secreted and transported to adjacent brain areas.

## Discussion

This study documents the effects of continuous Tat production in brain on neuronal morphology and function and shows that low-level production of Tat causes multiple behavioral and histological parameters of damage that model aspects of HIV dementia. Tat production in rat striatum results in impairment of motor performance with decreased tyrosine hydroxylase expression and increased glial activation in the ipsilateral substantia nigra. Likewise, Tat production in the hilar area of the hippocampus causes extensive neuronal damage, synaptic alterations, and glial activation both in dentate gyrus and in CA3/4 areas. Last, patterns of Tat immunoreactivity indicate that not only do C6-Tat cells survive and continue to produce Tat when implanted into rat brain, but Tat proteins can be transported to adjacent neuronal populations.

Tat has repeatedly been implicated in the pathogenesis of HIV dementia and has potent effects on cultured neurons (New et al., 1998; Nath et al., 2000) and microglial cells (Sheng et al., 2000; Bruce-Keller et al., 2001). Corroborating these studies, infusion of Tat proteins into the brains of experimental animals causes extensive neuronal damage (Hayman et al., 1993; Jones et al., 1998; Zauli et al., 2000); however, supraphysiological amounts (10–100 $\mu$g) of Tat were necessary to induce brain lesions in these studies. Because serum concentrations of Tat in HIV patients is in the order of 1 ng/ml (Westendorp et al., 1995) and concentrations of only 4 ng/ml have been reported in conditioned medium of HIV-infected cells (Albini et al., 1998), it may be that single or even repeated injections of microgram amounts of Tat are not an accurate reflection of the changes that occur in brains of HIV-infected patients. Our studies clearly demonstrate that chronic Tat production in the brain causes significant alterations in histological markers of inflammation, synaptic density, and most importantly in behavioral performance that are not associated with extensive neuronal loss in the striatum or hippocampus. Hence, it is likely that the role of Tat is more related to synaptic derangement and dysfunction than to abject neuronal loss.

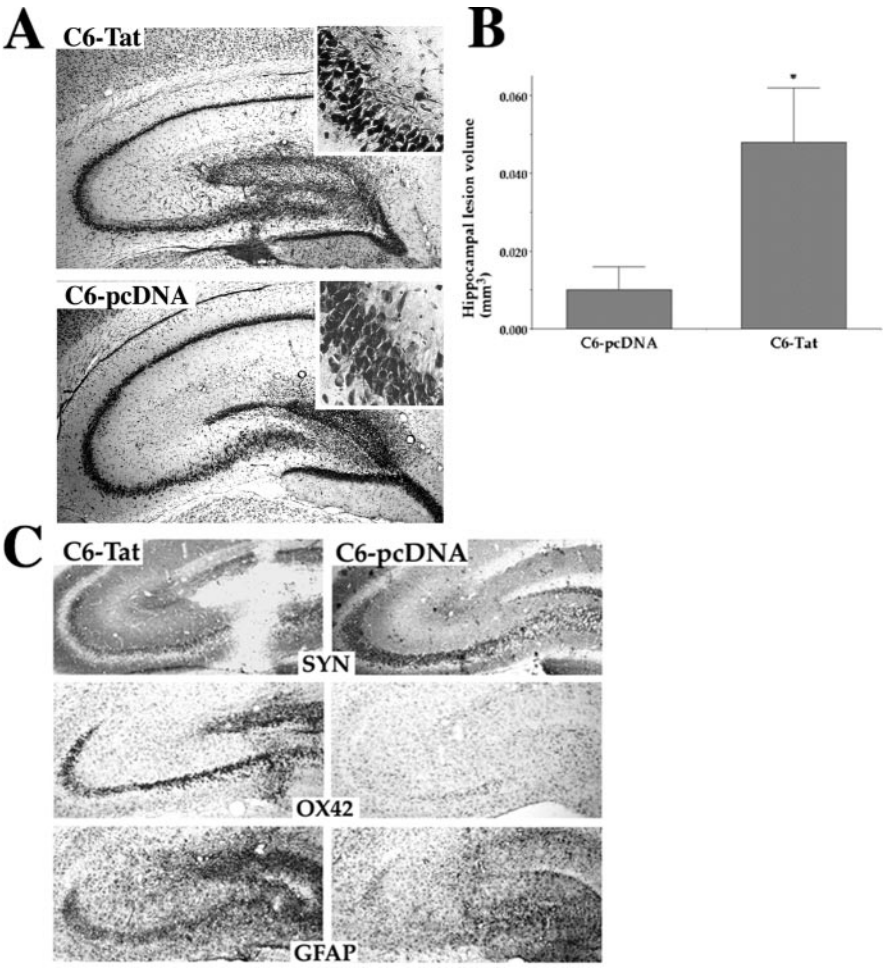


**Figure 2.** Implantation of Tat-producing C6 cells in hippocampus is neurotoxic to dentate granule and CA3 pyramidal neurons. *A*, Images from 4× and 32× (corner insets) microscope fields depict hippocampal pyramidal neurons 300 $\mu$m posterior to the injection site and show the characteristic lesion and inflammation in Tat-C6 rats. Images are representative of results obtained from three separate experiments with four animals per group. *B*, Lesion volume within CA3/4 apical dendrites was calculated in serial coronal sections, as described in Materials and Methods. Values represent the means and SEM of three experiments, with four animals per group, and the asterisk indicates significantly ( $p < 0.05$) increased lesion volume in C6-Tat rats. *C*, Synaptophysin (SYN), OX42, and GFAP immunoreactivity in the hippocampus of animals that had received hilar infusions of C6-pcDNA or C6-Tat cells. Images depict hippocampal neurons 300 $\mu$m posterior to the injection site and are representative of three separate experiments of four animals per group each.

The ability of Tat to be transported along anatomical pathways to adjacent neuronal populations is a novel observation. Previous cell culture studies have shown that when presented extracellularly, Tat can be taken up by various cell types, where it gets rapidly transported to the nucleus (Ensoli et al., 1993; Ma and Nath, 1997); however, this is the first documentation of its transport along anatomical pathways. Demonstration of microglial and astrocyte activation at adjacent synaptic sites further supports the notion of anatomical transport of Tat proteins and suggests that Tat either induces damage at the synapse or is released and then available to interact with glial cells. The reasons for the apparent lack of Tat immunoreactivity in other areas, such as the cortex, are not entirely clear, but it is possible that Tat proteins are present diffusely in the cortex and other brain areas, with immunohistochemical techniques not sensitive enough to detect it. Alternatively, Tat transport may preferentially select particular pathways. To determine the exact distribution of Tat proteins, increasingly sensitive techniques for Tat detection are under development and will be used in the future to map out the particulars of Tat transport.

Although transport of retrovirus particles has not been reported previously, several viruses such as herpes simplex, varicella zoster, rabies, and polio can be transported along axons and can be released trans-synaptically (Kelly and Strick, 2000; Smith et al., 2001). Transport of these viruses requires complete viral particles, however, although our data suggest the transport of a single early viral protein. Our data also indicate that Tat is able to escape cellular proteolytic pathways and enter neuronal transport systems. Consistent with the scenario, Tat can inhibit the activity of the 20S proteasome (Seeger et al., 1997; Huang et al., 2002),

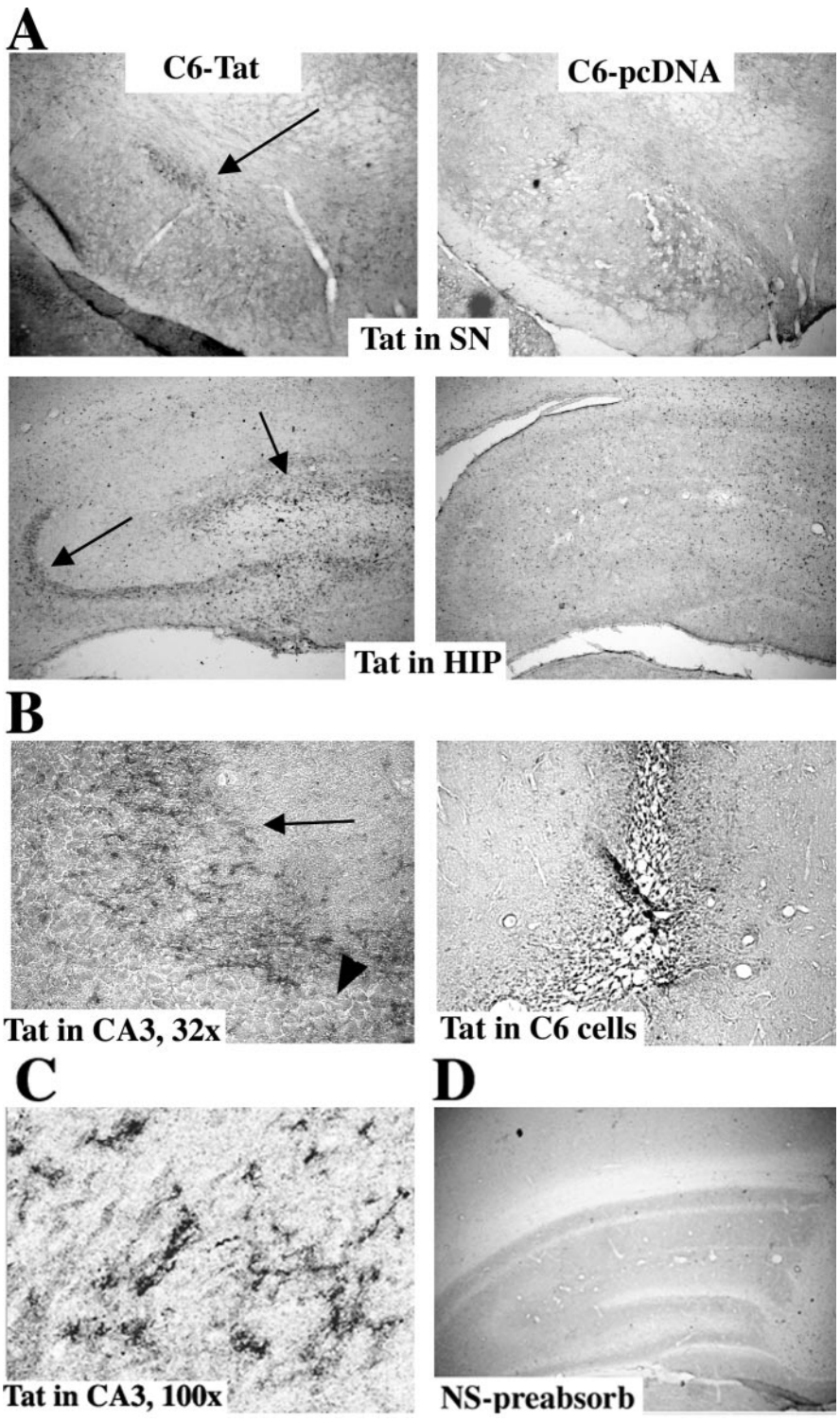


**Figure 3.** Tat-producing C6 cells induce Tat immunoreactivity in distal brain regions. *A*, Tat immunoreactivity in 4× microscope images at the level of the pars compacta in animals that had received striatal infusions (top row) and 300 $\mu$m posterior to the injection site in animals that had received hilar infusions of C6-pcDNA or C6-Tat cells (bottom row). Arrows indicate Tat immunoreactivity in rats that had received C6-Tat cell infusions, and images are representative of results obtained from three separate experiments of four animals per group. *B*, Higher magnification (32×) images depict hippocampal Tat immunoreactivity both in CA3 dendritic fields (left side) and in C6 cells injected into the hilar region (right side). Arrows depict Tat immunoreactivity in stratum radiatum of CA3, whereas the arrowhead indicates the position of CA3 pyramidal cells. *C*, Images (100×) of Tat immunoreactivity in CA3 stratum radiatum suggest that Tat proteins are confined to presynaptic terminals. *D*, No immunoreactivity is evident when the primary anti-Tat antibody is preabsorbed with excess Tat protein.

which may explain its ability to avoid proteolysis in neurons. Furthermore, our data suggest that Tat can be transported both retrogradely (from the striatum to the pars compacta region of the substantia nigra) and anterogradely (from the hilus to CA3/4). These observations may have important implications for neuropathogenesis of retroviral infections, suggesting that Tat-induced neuronal damage and glial cell activation may occur at sites distant from the cells infected with the virus, thus implicating Tat in neuronal, glial, and synaptic manifestations of HIV dementia.

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