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Short Communication

Human immunodeficiency virus type 1 Tat protein directly activates neuronal *N*-methyl-D-aspartate receptors at an allosteric zinc-sensitive site

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The human immunodeficiency virus type 1 (HIV-1) regulatory protein Tat is neurotoxic and may be involved in the neuropathogenesis of HIV-1 dementia, in part via *N*-methyl-D-aspartate (NMDA) receptor activation. Here, in acutely isolated rat hippocampal neurons, Tat evoked inward currents reversing near 0 mV, with a negative slope conductance region characteristic of NMDA receptor activation. Although the NMDA receptor antagonist ketamine blocked Tat's actions, competitive glutamate- and glycine-binding site antagonists were ineffective (AP-5 and 5,7-dichlorokynurenate, respectively). Evidence for Tat acting at a distinct modulatory site on the NR1 subunit of NMDA receptors was provided by findings that 1 μM Zn^{2+} abolished Tat-evoked responses in all neurons tested. Thus, Tat appears to excite neurons via direct activation of the NMDA receptor at an allosteric Zn^{2+}-sensitive site. *Journal of NeuroVirology* (2003) **9,** 399–403.

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Introduction

Excitatory synaptic transmission in the mammalian central nervous system (CNS) is mediated predominantly by ionotropic glutamate receptors. One receptor subtype, the *N*-methyl-D-aspartate (NMDA) receptor, plays important roles in CNS development, plasticity, and the excitotoxic injury that accompanies various acute and chronic neurological disorders. The presence of numerous ligands and binding sites on the NMDA receptor attests to an operation that is highly regulated, but one that may also provide for considerable pathological opportunism.

Frequently, patients infected with human immunodeficiency virus type 1 (HIV-1) develop a dementing illness, possibly related to toxin-induced neuronal death. Neurotoxicity may be induced indirectly through the release and consequent action of HIV-1 proteins from infected microglia or infiltrating monocytes (Nath and Geiger, 1998). HIV-1 Tat protein, a nonstructural regulatory protein that enhances viral gene transcription, is actively released from infected cells (Chang *et al*, 1997). Tat mRNA levels are elevated in patients with HIV encephalitis (Wesselingh *et al*, 1993; Wiley *et al*, 1996) and the detection of Tat protein in the brain correlates with the presence of HIV encephalitis (Hofman *et al*, 1994; Hudson *et al*, 2000). Tat protein is also present in abundance in macaques with encephalitis due to a chimeric strain of HIV and simian immunodeficiency virus (Nath and Geiger, 1998).

Previous electrophysiological studies demonstrated that exogenous application of Tat induced a depolarization of neurons in brain slices (Magnuson *et al*, 1995) or neuronal cell cultures (Cheng *et al*, 1998). In Tat-induced neurotoxicity studies, both NMDA and non-NMDA subtypes of glutamate receptors have been implicated (Magnuson *et al*, 1995; Hayman *et al*, 1993; New *et al*, 1998; Bonavia *et al*, 2001; Starling *et al*, 1999; Strijbos *et al*, 1995). From

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these studies, however, it remained uncertain as to whether Tat acted directly on glutamate receptors. In the present study, we used whole-cell recordings from acutely isolated rat hippocampal neurons to determine whether Tat has a direct action on excitatory amino acid receptors in neurons, and to identify the mechanism of glutamate receptor activation.

Materials and methods

Sprague-Dawley rats (8 to 14 days old) were anesthetized with ether, then the brains were removed and placed in cold (~4°C) artificial cerebrospinal fluid (ACSF) containing (in mM): NCl 125, KCl 2.5, glucose 25, MgCl 1, $CaCl_2$ 2, $NaHCO_3$ 26, NaH_2PO_4 1.25, and oxygenated with 95% O_2 and 5% CO_2. The hippocampus was isolated and sliced into ~300-μm thickness with a tissue chopper. The isolation procedure involved enzyme treatment (0.1% papain *w*/*v*, Sigma) at 22°C for 10 min and washes for 10 min in a Ca^{2+}-free HEPES-buffered solution containing (in mM); NaCl 150, KCl 5, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.3. The slices were then transferred into a dish containing HEPES-buffered solution also containing 2 mM $CaCl_2$, and triturated using fire-polished Pasteur pipettes. Whole-cell voltage-clamp recordings were made with glass microelectrodes filled with intracellular solution containing (in mM); CsF 140, EGTA 11, HEPES 10, KOH 35, $CaCl_2$ 1, pH 7.3. Neurons were identified by their characteristic morphology and early inward action current in response to depolarizing voltage steps.

Recombinant HIV-1 Tat protein was prepared as described previously (Ma and Nath, 1997). Tat protein and all other drugs were dissolved in a HEPES-buffered saline solution, loaded into glass pipettes (tip diameters of 1 to 2 μm), and placed ~50 μm from the recorded neuron. Tat and other drugs were applied to the recorded cells by pressure ejection (Picospritzer, PV820) ranging from 5 to 15 PSI and 50 to 2000-ms duration. Typically, two pipettes of equal tip diameter were positioned equidistant from the cell. Preliminary testing revealed near-identical cellular responses when both pipettes were filled with the same neuroactive agent. Hence, in single neurons, the membrane response generated by application of Tat in one ejection pipette was compared with the response evoked in the other pipette by Tat plus an additional substance. The concentration of Tat loaded in the pipette was 20 μM and typically 0.8 fmoles was ejected per pressure application. Substances coapplied with Tat were Zn^{2+} (1 μM), Mg^{2+} (1 mM), ketamine (10 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), 2-amino-5-phosphonopentanoic acid (AP-5; 50 μM), and 5,7-dichlorokynurenate (10 μM). All drugs were purchased from Sigma/RBI. Tat specificity was determined by treating Tat with 0.05% trypsin (Life Tech) for 30 min at 37°C followed by the addition of trypsin inhibitor (Sigma; final concentration 0.1%) for 30 min at 37°C, and this solution was used to determine if trypsin-treated Tat could evoke responses in dissociated neurons.

Results

Tat evoked inward currents at negative holding potentials in 74 of 94 isolated rat hippocampal neurons irrespective of the size and shape of the cells tested. Application of Tat pretreated with trypsin did not evoke inward currents. The actions of Tat on individual neurons are displayed in Figure 1**Aa**. Tat was applied to neurons in the presence (*left*, $n = 7$) or absence (*right*, $n = 12$) of 1 mM Mg^{2+}. Tat-evoked cell responses displayed outward rectification and were reversed near 0 mV. In a standard extracellular solution containing 1 mM Mg^{2+}, Tat-evoked responses were negligible at hyperpolarized holding potentials (~80 to ~60 mV), but increased nonlinearly in size at reduced hyperpolarized holding potentials (Figure 1**Aa**, *left*). Application of Tat in a nominally Mg^{2+}-free solution resulted in enhanced inward currents at hyperpolarized holding potentials (Figure 1**Aa**, *right*). The conversion of an N-shaped to a quasilinear currentvoltage (I-V) relationship following removal of Mg^{2+} (Figure 1**Ab**) suggests a voltage-dependent Mg^{2+} block of the Tat-evoked response, consistent with activation of NMDA receptors (Mayer *et al*, 1984; Nowak *et al*, 1984). Therefore, all experiments were conducted in nominally Mg^{2+}-free solutions to identify the mechanism(s) by which Tat activates NMDA receptors.

Supporting evidence for the involvement of NMDA receptors in the electrophysiological responses to Tat was provided using ketamine, a drug that reduces NMDA receptor activity at least in part via voltage-dependent block of the ionophore (MacDonald *et al*, 1991). Ketamine produced a voltage-dependent block of the Tat-evoked response (Figure 1**B**; $n = 8$). Moreover, CNQX, a selective μ-amino-3-hydroxy-5-methylisoxazole–4-proplonic acid (AMPA)/kainate receptor competitive antagonist, did not block Tat-induced effects ($n = 7$; not illustrated). Thus, Tat activated NMDA receptors.

We next attempted to determine the site on the NMDA receptor responsible for the actions of Tat. 5,7-Dichlorokynurenate, a potent and selective glycine-binding site blocker of NMDA receptor function (Baron *et al*, 1990), failed to block Tat-evoked responses ($n = 3$; not illustrated). Moreover, consistent with previous observations in hippocampal slices (Magnuson *et al*, 1995), application of AP-5 (50 μM), a competitive NMDA receptor antagonist (Davies *et al*, 1981), did not block Tat-induced responses ($n = 3$; not illustrated). In one cell, AP-5 actually enhanced evoked responses. On the other hand, Zn^{2+}, a cation that potently inhibits NMDA receptor–activated channels (Mayer and Vyklicky,

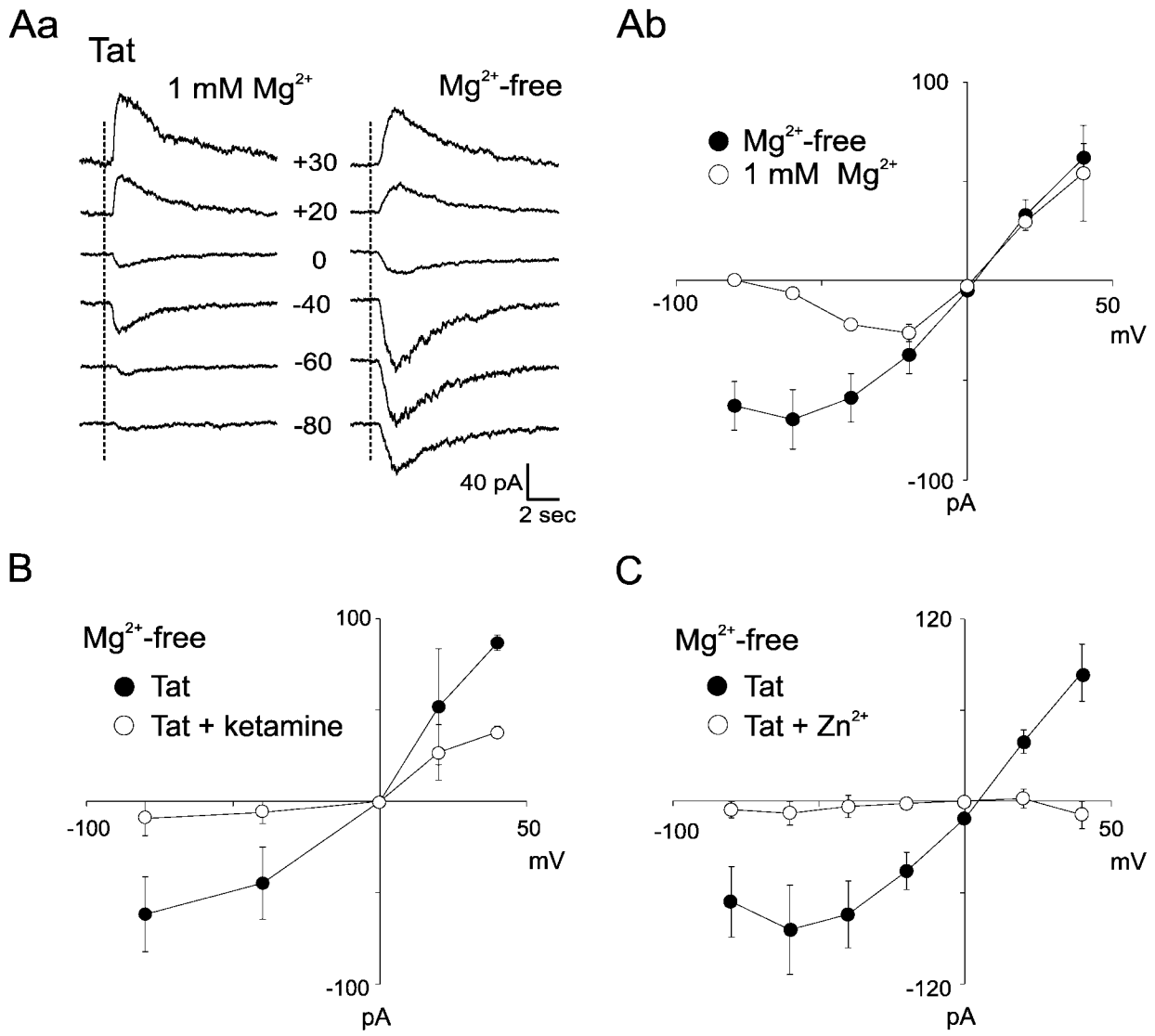

Figure 1 Effects of Tat consistent with activation of NMDA receptors. (**A**) Magnesium induces voltage-dependent block of Tat-evoked responses. **Aa**, Raw current traces showing Tat-evoked responses to a voltage step protocol from two different hippocampal neurons in the presence (*left*) and absence (*right*) of Mg^{2+}. Membrane holding potentials are indicated beside individual traces. Dotted vertical lines mark onset of Tat application. **Ab**, Summary I–V relation of Mg^{2+}-dependence of Tat-evoked responses (bars are SEM). (**B**) Ketamine blocks Tat-evoked responses predominantly at hyperpolarized holding potentials. (**C**) Neuronal responses to Tat are blocked in the presence of Zn^{2+} in a voltage-independent manner.

1989; Legendre and Westbrook, 1990), abolished Tat-evoked responses in all neurons tested independent of membrane voltage ($n = 5$; Figure 1**C**). At the concentration used (1 μM), Zn^{2+} does not interfere with the ionophore, but blocks NMDA receptor activity in a voltage-independent manner by binding to a distinct modulatory site on the NR1 subunit that is structurally associated with receptor modulation by polyamines and protons (Traynelis *et al*, 1998).

Discussion

Only a transient exposure to Tat is sufficient to cause immediate depolarizations of neurons, increase levels of intracellular calcium, and decrease neuronal viability (Haughey *et al*, 1999, 2001). Previous studies have implicated Tat-induced neurotoxocity as mediated in part via activation of NMDA receptors (Bonavia *et al*, 2001; Starling *et al*, 1999; Strijbos *et al*, 1995). We have now shown that each of these responses to Tat is mediated at least in part by activation of excitatory amino acid receptors. Our previous results using outside-out patches have shown clearly that Tat can act directly on the neuronal membrane (Cheng *et al*, 1998). These Tat-induced depolarizations are nondesensitizing (Cheng *et al*, 1998); hence, our previous data in conjunction with our present findings suggest that Tat-induced neurotoxicity may result from persistent neuronal NMDA

receptor activation. Because only 0.8 fmole of Tat was sufficient to provide a micromolar concentration, it suggests that a small number of molecules in restricted extracellular spaces can produce a concentration in brain needed for neuronal excitation, particularly when in close proximity of infected cells.

Our study provided several lines of evidence that suggested that Tat excited neurons via direct activation of the NMDA receptors. Firstly, Tat-evoked an I–V relationship that was identical to that observed for NMDA receptor activation, including the voltage-dependent block by Mg^{2+} at hyperpolarized potentials and a reversal potential of 0 mV. Secondly, ketamine, a NMDA receptor open-channel blocker, inhibited Tat-induced inward currents in a characteristic voltage-dependent manner. Thirdly, Zn^{2+}, a noncompetitive NMDA receptor blocker, reduced Tat-evoked responses. Finally, CNQX, a competitive non-NMDA receptor antagonist, had no effect on Tat-evoked responses.

Excitatory amino acid receptors are thought to be activated only by specific amino acids. However, an antibody to the GluR3 subunit can lead to activation of AMPA receptors (Twyman *et al*, 1995), and autoantibodies to GluR3 are strongly implicated in the disease process leading to Rasmussen's encephalitis (Rogers *et al*, 1994). We now demonstrate that the NMDA receptor is also capable of direct activation by a protein, in this case the viral protein Tat, with a molecular mass of 14 kDa. It remains to be determined if there are endogenous proteins capable of interacting with these receptors.

In addition to direct activation of the NMDA receptor, we previously showed that the Tat-induced neuronal cell death that occurs several hours after exposure to Tat could be prevented with CNQX, a non-NMDA ionotropic glutamate receptor antagonist (Nath *et al*, 1996). Others and we have also shown that Tat can induce tumor necrosis factor (TNF)-μ production in glial cells (Chen *et al*, 1997; New *et al*, 1998; Shi *et al*, 1998; Nath *et al*, 1999). Because TNF-μ has been shown to mediate neurotoxicity via non-NMDA receptors (New *et al*, 1998), it is likely that delayed neurotoxicity may be related to cytokine overproduction.

We thus propose that Tat-induced neurotoxicity occurs in two phases. The first early phase involves direct NMDA receptor stimulation, later phases involve indirect mechanisms that include phosphorylation of NMDA receptors (Haughey *et al*, 2001) and cytokine-mediated alterations in cell homeostasis and glutamate receptor function (e.g., Kruman *et al*, 1998; Nath *et al*, 1996; Nath and Geiger, 1998; Nath *et al*, 1999). Our observations that Tat can directly activate NMDA receptors suggest that therapeutic approaches using cytokine antagonists may not be sufficient for treatment of HIV dementia. Such treatment strategies may need to be combined with NMDA receptor antagonists to block direct actions of Tat and with antiviral drugs aimed at reducing viral products.

In this study, we show that Tat-evoked actions appear to occur by interacting with the identified voltage-independent Zn^{2+}/polyamine/H^+ binding site complex of the NR1 subunit (Traynelis *et al*, 1998) to cause activation by a novel mechanism. Zinc has a differential effect on NMDA receptors depending on their subunit composition. For example, there is a high-affinity voltage-independent inhibition by Zn^{2+} when NR1 is coexpressed with NR2A (IC_{50} of 5 nM), but a much lower affinity voltage-independent block by Zn^{2+} with NR1/NR2B coexpression (IC_{50} of 10 μM) (Chen *et al*, 1997). Both NR2A and NR2B subunits are expressed in the hippocampus (Monaghan and Buller, 1994) and the strong block by Zn^{2+} of Tat-evoked responses at 1 μM more closely resembles an action on NR1/NR2A receptors. An identification of the particular subunit(s) and site(s) of Tat action may provide new modes of treatment of HIV dementia using agents that modulate activity at these sites. Additionally, because Zn^{2+} is an antagonist of NMDA receptors, Zn^{2+}-deficient states could amplify Tat-induced neurotoxicity. Zn^{2+} deficiency frequently occurs in HIV-infected patients and the extent of the deficiency correlated with the progression of HIV infection (Allavena *et al*, 1995). However, its relationship to HIV dementia has yet to be determined.

In summary, we have demonstrated a novel action of the Tat protein of HIV-1 as an agonist of the NMDA receptor possibly acting at the allosteric Zn^{2+}-binding site. These observations may have important implications for future pharmacological approaches for treating HIV-1 dementia and will also further our understanding of the biology of NMDA receptors.

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