Mechanisms of HIV-tat-Induced Phosphorylation of N-Methyl-D-Aspartate Receptor Subunit 2A in Human Primary Neurons

Implications for NeuroAIDS Pathogenesis

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HIV infection of the central nervous system results in neurological dysfunction in a large number of individuals. NeuroAIDS is characterized by neuronal injury and loss, yet there is no evidence of HIV-infected neurons. Neuronal damage and dropout must therefore be due to indirect effects of HIV infection of other central nervous system cells through elaboration of inflammatory factors and neurotoxic viral proteins, including the viral transactivator, tat. We previously demonstrated that HIV-tat-induced apoptosis in human primary neurons is dependent on N-methyl-D-aspartate receptor (NMDAR) activity. NMDAR activity is regulated by various mechanisms including NMDAR phosphorylation, which may lead to neuronal dysfunction and apoptosis in pathological conditions. We now demonstrate that tat treatment of human neurons results in tyrosine (Y) phosphorylation of the NMDAR subunit 2A (NR2A) in a src kinase-dependent manner. *In vitro* kinase assays and *in vivo* data indicated that NR2A Y1184, Y1325, and Y1425 are phosphorylated. Tat treatment of neuronal cultures enhanced phosphorylation of NR2A Y1325, indicating that this site is tat sensitive. Human brain tissue sections from HIV-infected individuals with encephalitis showed an increased phosphorylation of NR2A Y1325 in neurons as compared with uninfected and HIV-infected individuals without encephalitis. These findings suggest new avenues of treatment for HIV-associated cognitive

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HIV infection causes varying degrees of cognitive impairment in a significant number of individuals.^{1,2} Damage to the central nervous system is a multifactorial process including early viral entry, neuroinflammation, and secretion of toxic factors.³ During this process, neuronal damage and apoptosis occur,⁴ but there is little evidence that neurons are infected with HIV.^{5,6} Therefore, the neuronal damage and death characteristic of neuroAIDS must be mediated through indirect mechanisms.³ HIV-tat, the transactivator of the virus, is one such toxic factor that causes apoptosis in cultured human neurons.^{7–11}

Binding of tat to the low density lipoprotein receptor-related protein (LRP) on neurons results in the formation of a macromolecular complex at the neuronal cell membrane between LRP and the N-methyl-D-aspartate receptor (NMDAR), mediated by the scaffolding protein PSD-95.⁹ In this complex, the NMDAR plays a critical role in the process of tat induced apoptosis in human primary neurons, as blocking with specific NMDAR inhibitors ab-

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rogated cell death completely.^{8,9} The specific mechanisms by which tat alters NMDAR activity are unknown.

NMDAR phosphorylation results in alterations in NMDAR activity,^{12–14} protein-protein interactions,¹⁵ and trafficking.^{15–17} In pathological conditions, overactivation of the NMDAR results in toxicity.¹⁸ Studies in rodent models indicate that postsynaptic density proteins facilitate the interaction of kinases, such as src,¹⁹ fyn,^{20,21} and pyk2^{22–24} with the NMDAR, resulting in phosphorylation of its different subunits. As PSD-95 is recruited to the NMDAR after tat treatment,⁹ we examined tat induced association of these kinases with the NMDAR subunit 2A (NR2A), the main subunit present in the macromolecular complex induced by tat,⁹ and subsequent changes in NR2A phosphorylation.

We demonstrate that tat induces tyrosine phosphorylation of the NR2A subunit by a mechanism that involves recruitment of active src to the receptor. We also identify *in vitro* three tyrosine (Y) residues phosphorylated on human NR2A in a src-dependent manner. One of these, Y1325, showed significantly enhanced phosphorylation in response to tat treatment of human neurons that was dependent on src activity. We also demonstrate *in vivo* that phosphorylation of Y1325 is significantly increased in neurons present in HIV encephalitic brain tissue sections as compared with those from uninfected and HIV-infected individuals without encephalitis.

Materials and Methods

Materials

Neurobasal media, N₂ supplement, penicillin/streptomycin (P/S) and trypsin-EDTA were from GibcoBRL (Grand Island, NY). NMDA, glycine, polyclonal antibody to NMDAR1, rabbit polyclonal antibody to MAP-2, anti-mouse Cy3, and anti-rabbit fluorescein isothiocyanate secondary antibodies were from Sigma Chemical (St. Louis, MO). Chicken polyclonal antibody to MAP-2 and nonimmune chicken IgY were from Aves Labs (Tigard, OR). Monoclonal antibodies to the NMDAR subunits NR2A, NR2B, and fyn were from Zymed (San Francisco, CA). Polyclonal and monoclonal antibodies to pyk2, as well as normal rabbit IgG, were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphotyrosine antibody, P-Tyr-100, src rabbit polyclonal antibody 36D10, and phosphor-specific antibody for active src rabbit polyclonal antibody were from Cell Signaling (Beverly, MA). A second phosphospecific antibody for active src was from Biosource. Control IgG1 and IgG2A purified mouse antibodies were from ICN/Cappel (Salon, OH). Anti-chicken Cy5 secondary antibody was from Abcam (Cambridge, MA). Anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 were from Invitrogen. Src kinase inhibitor I was from Calbiochem (Darmstadt, Germany). Src kinase was purchased from Upstate (Billerica, MA) and EasyTides gamma³² P-ATP from Perkin Elmer (Melville, NY). Isotrips for antibody isotyping and complete protease inhibitor tablet were from Roche Diagnostics (Manheim, Germany). NR2A peptides for the *in vitro* kinase assay were

synthesized by GenScript (Piscataway, NJ). Recombinant HIV-1 tat protein (1–72) was from Dr. Avindra Nath, Johns Hopkins Medical Center.

Primary Human Neuronal Cultures

Human fetal cortical tissue was used as part of an ongoing research protocol approved by the Albert Einstein College of Medicine. Brain tissue was cultured according to previously published protocols.⁸ After 7 to 10 days in culture, the cells were dissociated with trypsin-EDTA and plated onto 100-mm dishes, 35-mm culture dishes, or 24-well tissue culture plates with cover slips in Neurobasal media plus N₂ supplement, 0 to 1% fetal bovine serum, and 1% Pen/Strep. Media was replaced every 5 days. This resulted in mixed cultures of neurons and astrocytes with no evidence of microglial contamination as determined by immunohistochemical staining for CD68, CD11b, and CD14 (data not shown). To test for neuronal maturation and NMDAR expression, time-lapse calcium imaging was performed to determine responsiveness to NMDA/glycine. Briefly, cells were loaded with 10 μ M Fluo-4 AM for 15 to 30 minutes at room temperature. Neurons were imaged in the AECOM Analytical Imaging Facility with a 20 $\times$ objective using an Olympus IX81 inverted microscope run with OpenLab software. Fields were chosen for the maximal number of neurons, and excitotoxic concentrations of 60 μ M NMDA and 10 nmol/L glycine were added to the culture media to stimulate maximal NMDAR activation. Cultures with 25 to 35% of neurons responsive to NMDA/glycine treatment were considered mature.

Treatment of Neuronal Cultures

The exact concentrations of tat *in vivo* are not known. However, it is likely that tat concentrations could be relatively high in small, localized areas of release. We used tat at a range of 10 to 300 ng/ml, which is equivalent to approximately 1.2 to 35.6 nmol/L, based on the concentration at which each preparation induced apoptosis, chemokine production and/or cellular activation.^{25–35} Lyophilized tat protein was resuspended at 50 ng/ μ L in 50 mmol/L Tris buffer containing 100 mmol/L NaCl, 1 mmol/L CaCl₂, and 0.5 mmol/L dithiothreitol. Concentration of tat treatment was dependent on the activity of the recombinant protein as determined by its ability to induce >75% apoptosis in our cultures, and different preparations ranged from 10 to 300 ng/ml (equivalent to approximately 1.2 to 35.6 nmol/L). This is much lower than concentrations used by others^{25,32} to demonstrate neurotoxicity. These preparations were pure recombinant tat protein, as determined by Coomassie staining, and endotoxin was removed before lyophilization. Control cells were treated with the appropriate amount of vehicle. Previous studies using delta tat (tat with amino acids 48–56 deleted) as a control found no induction of chemokine secretion²⁹ or apoptosis (data not shown).³² Src kinase inhibitor I (SrcI) was used at 5 μ M and added 10 minutes before tat treatment. All control cultures were treated with the appropriate diluents (vehicle).

Coimmunoprecipitation

Following tat treatment, cells were washed twice with cold PBS, harvested in lysis buffer (0.5% Triton X-100, 50 mmol/L HEPES (pH 7.4), 40 mmol/L sodium chloride, 2 mmol/L EDTA, 1.5 mmol/L sodium vanadate, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, and 10 mmol/L sodium β -glycerolphosphate) or in radioimmunoprecipitation assay buffer with phosphatase inhibitors (Cell Signaling, Danvers, MA), with protease inhibitors (Roche, Mannheim, Germany) added and sonicated for 10 seconds. The lysate was then centrifuged for 5 minutes to remove particulate matter. The amount of protein obtained was quantified by Bradford assay (Bio-Rad, Hercules, CA). Before the addition of specific antibodies, 150 to 250 μ g of protein were washed and pre-cleared with Immunopure immobilized protein G (Pierce, Rockford, IL). The supernatant was incubated with the appropriate antibody (NR2A, 2.5–3.5 μ g) or control antibody (IgG₁, 3.5 μ g) overnight at 4°C, followed by 10 μ l of protein G for 1 to 3 hours and pelleted. The pellets were washed three times with lysis buffer, resuspended in sample buffer, separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose or PVDF for analysis by Western blotting.

Western Blot Analyses

Lysate was prepared as for immunoprecipitation. Protein concentration was determined by the Bradford method. Protein, 60–100 μ g, was resolved in 10% or 12% SDS-polyacrylamide gel electrophoresis gels (Bio-Rad) or Nu-Page gels (Invitrogen) and then electrotransferred to nitrocellulose or PVDF. Nonspecific protein binding was blocked with 5% nonfat milk in Tween (TBS-T) for 1 hour, followed by overnight incubation at 4°C with primary antibodies at the following dilutions: anti-NR2A (1:250), anti-pTyr-100 (1:2000) anti-pY416 src (Cell Signaling, 1:1000, Biosource, 1:500), anti-src (1:1000), anti-pyk2 (1:1000), anti-fyn (1:500), anti-Y1184 sera (1:100), anti-Y1325 sera (1:100), anti-Y1423 sera (1:100), and anti-pY1325 hybridoma supernatant (1:500–1000). The membrane was then washed with TBS-T and incubated with anti-mouse (1:5000) or anti-rabbit (1:2000) IgG antibody conjugated to horseradish peroxidase. Antigen-antibody complexes were detected on film using ECL reagent (Perkin-Elmer, Waltham, MA) or SuperSignal West Femto Chemiluminescent substrate (Pierce/Thermo Scientific, Rockford, IL).

In Vitro Kinase Phosphorylation Assay

To identify potential tyrosine phosphorylation sites in human NR2A, the amino acid sequence of NR2A (NCBI accession no. NP_000824) was input into NetPhos 2.0.³⁶ Peptides containing those tyrosines in the cytoplasmic tail that scored >0.8 or had been referenced in the literature as potential phosphorylation sites were synthesized and used in our *in vitro* kinase assay. Peptides were synthesized by the Genscript Corporation and had the following sequences: src target consensus se-

quence (EEVYQQWSQ³⁷), Y1151 (FPDPYQDPS), Y1184 (NNDQYKLYS), Y1292 (RQHSYDNIV), Y1325 (EGNFYGSFLF), Y1387 (PSPPYKHSL), Y1400 (VNDSYLRSS), and Y1423 (HNDVYISEH). Src *in vitro* kinase assays were performed by adding 3 active units of recombinant src or distilled, deionized water to a reaction solution containing 20 mmol/L HEPES, pH 7.4, 5 mmol/L MgCl₂, 5 mmol/L MnCl₂, 25 μ mol/L cold ATP, and 1 μ Ci γ -³²P-ATP. After 10 minutes incubation at room temperature, 2 μ g of the consensus sequence peptide or NR2A peptides were added to each reaction and were incubated for 45 minutes at 37°C. To stop the reaction, 2.2 μ l of 5X protein buffer was added and was incubated for 5 minutes at 90°C. Peptides were then run on an Invitrogen 12% Bis-Tris gel with 1X MES buffer, 130 V for 40 to 50 minutes. Gels were imaged using Fuji RX X-ray film.

Generation of Phospho Site-Specific Monoclonal Antibodies

Peptides containing phosphorylated tyrosines were synthesized by Genscript with the following sequences: pY1184 (KNNDQ{pTYR}KLYS), pY1325 (KEGNF{pTYR}GSFLF), and pY1423 (KHNDV{pTYR}ISEH) and coupled to keyhole limpet hemocyanin using the Inject Immunogen EDC kit with mCKLH and OVA (Pierce) as per the manufacturer's instructions. Four 6- to 8-week-old female BALB/c mice were immunized for each antigen with AdjuPrime immune modulator (Pierce) and keyhole limpet hemocyanin-phosphopeptide every two weeks for 6 weeks, and titers were assayed at week 7. Mice were subsequently boosted and sera were redrawn two weeks later and assayed by enzyme-linked immunosorbent assay for reactivity to keyhole limpet hemocyanin, unphosphorylated peptide, and phosphorylated peptide. The sera were pre-absorbed against the unphosphorylated forms of the peptides, and tested and used for Western blotting analysis. Based on data with the anti-sera, the mouse with the best phospho-specific response to Y1325 was used to perform the spleen-myeloma fusion. The fusion and subsequent cloning were performed according to established methods³⁸ with the aid of the AECOM hybridoma facility. Two subclones were found to have strong phospho-Y1325 reactivity with no reactivity to bovine serum albumin, unphosphorylated Y1325, or to phosphorylated Y1423 and Y1184, demonstrating the site- and phospho-specificity of our antibodies. Both subclones, termed clones 11.1 and 11.2, were isotype IgG_{2A}, as determined using the Roche mouse isotyping kit.

Immunofluorescent Labeling and Analysis of Cultured Neurons

For immunofluorescence studies, human primary neurons were plated on glass cover slips in 24-well dishes in Neurobasal media supplemented with N₂, 1% fetal bovine serum and Pen/Strep, and were cultured for up to 28 days. After treatment, cover slips were washed with PBS, fixed in permeabilized ice-cold 70% ethanol and stored

at -20°C until immunostaining. Cover slips were rehydrated with 1X PBS before blocking with blocking solution consisting of 5 mmol/L EDTA, 1% horse serum, 1% Ig free bovine serum albumin, and 1% fish gelatin in PBS. Cover slips were incubated overnight in primary antibody (anti-MAP2, 1:200) diluted in blocking solution or hybridoma supernatant from pY1325 clone 11.1 (used neat), washed with PBS, and then incubated in secondary antibody, anti-mouse Alexa Fluor 488 (1:200), and anti-rabbit Alexa Fluor 594 (1:200) for 1 hour at room temperature. After washing with PBS, cover slips were mounted onto slides using ProLong Gold antifade reagent with DAPI (to identify nuclei) and imaged by confocal microscopy. The number of pixels per cell for pY1325 and MAP-2 staining was determined using ImageJ software by setting the minimum signal threshold and measuring the number of positive pixels in a given frame. Data are reported as pY1325 fluorescence in ratio to MAP-2 to control for the number/size of the neurons per frame.

Immunofluorescent Labeling and Analysis of Human Tissue Samples

Postmortem human tissue sections from cortex and hippocampus of uninfected controls, HIV-infected individuals without encephalitis, and individuals with HIV encephalitis (HIVE) ($n = 4$ cases for each condition, two slides for each anatomical location per individual case) were analyzed by double immunohistochemical staining for MAP-2, a neuronal marker, and NR2A-pY1325 using hybridoma supernatant. Sections of human formalin-fixed, paraffin-embedded tissue 10 μm thick were obtained from individuals with similar postmortem intervals. The ages of the individuals were similar for uninfected and HIVE conditions and gender was equally distributed. In the case of HIVE, low CD4 counts were reported before the time of death, and all HIVE cases were cognitively compromised.

The tissue sections were deparaffinized and then incubated for 12 hours on a UV light box, and subsequently incubated 12 hours on a light box in the visible spectrum to reduce background autofluorescence. After antigen retrieval, the sections were incubated in blocking solution (5 mmol/L EDTA, 1% fish gelatin, 1% essentially Ig-free bovine serum albumin, and 2% horse serum) for 1 hour at room temperature and then incubated with anti-MAP2 antibody (1:300) and anti-pY1325 hybridoma supernatant (1:20) overnight at 4°C . The sections were then washed with PBS, incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma Chemical) or Alexa Fluor 594-conjugated anti-rabbit IgG for 1 hour at room temperature, followed by serial washes in PBS for 1 hour. Samples were then mounted using Prolong Gold antifade reagent (Molecular Probes) and examined by confocal microscopy. Specificity was confirmed by replacing the primary antibody with the appropriate isotype-matched control reagent, anti-IgG2A or the IgG fraction of normal rabbit serum (Santa Cruz Biotechnology). Quantification of the NR2A Y1325 and MAP-2 staining in uninfected, HIV-infected tissue without encephali-

tis, and HIVE tissue sections was performed using NIS Elements Advanced Research software (Nikon, Japan) to determine the total and the mean intensity of fluorescence in each channel. NR2A Y1325 was in the green channel and MAP-2 staining was in the red channel. The background obtained with the respective irrelevant antibodies was subtracted before the analyses.

Results

HIV-tat Treatment of Human Neurons Induces Tyrosine Phosphorylation of the NMDAR Subunit 2A

We previously demonstrated that tat treatment of human cortical neurons induced the formation of a macromolecular complex consisting of LRP, PSD-95, NMDAR, and nNOS at the plasma membrane, and that this complex participated in tat induced neuronal apoptosis, mainly through NMDAR activation and NO production.⁹ The NMDAR found in the complex were composed of NR1 and NR2A subunits. There was little to no NR2B expressed in our culture system (data not shown). As tat induced apoptosis is dependent on NMDAR activation,^{8,9} we examined NMDAR phosphorylation after tat treatment of neuronal cultures as a mechanism of dysregulation of NMDAR activity that can lead to cell death.

Human primary neuronal cultures were treated with tat for 5, 10, 15, 30, and 60 minutes (30 and 60 minutes not shown, no significance compared with control) and tyrosine phosphorylation of the NR2A subunit was examined. Immunoprecipitation analyses indicated that tat treatment induced robust tyrosine phosphorylation of the human NR2A subunit over control levels. Maximal tyrosine phosphorylation was detected between 5 and 15 minutes after tat treatment (Figure 1A, $P < 0.01$, IP: NR2A, WB: P-Tyr). The maximal time point of phosphorylation was somewhat variable, due to the variability inherent in primary cell cultures (Figure 1A, IP: NR2A, WB: P-Tyr, two examples shown). Changes in NR2A phosphorylation were not due to changes in the overall amount of NR2A in the lysates (data not shown), nor to the amount of total NR2A immunoprecipitated (Figure 1A, IP: NR2A, WB: NR2A, loading control). Densitometric analyses of the maximal phosphorylation time point of five independent experiments indicated that tat treatment significantly increased tyrosine phosphorylation of human NR2A as compared with its basal phosphorylation (Figure 1B, $n = 5$, $**P < 0.01$).

src Associates with the NMDAR after tat Treatment

To identify the kinases involved in tat induced tyrosine phosphorylation of the NR2A in human neurons, we analyzed kinases that have been found to regulate NMDAR activity. These kinases include src, pyk2, and fyn.^{13,19,39,40} Tat treated cultures of human neurons (5–15 minutes) were analyzed by co-immunoprecipitation for NR2A and

Figure 1. The human NR2A subunit is tyrosine phosphorylated after tat treatment. **A:** Western blots from two separate, representative experiments demonstrating tat-induced tyrosine phosphorylation of NR2A (IP:NR2A WB: P-Tyr). Human neuronal cultures were treated with HIV-tat protein (10 or 100 ng/ml) for 5–60 minutes; 5–15 minutes shown. After cell lysis, NR2A was immunoprecipitated and electrophoresed. Membranes were probed with a pan phosphotyrosine antibody. The time of maximal phosphorylation varied among experiments, but always occurred between 5 and 15 minutes in patterns similar to those represented. A representative control (IP:NR2A WB:NR2A) demonstrates that changes in tyrosine phosphorylation were not due to changes in the total amount of NR2A immunoprecipitated. **B:** Densitometric analyses of the maximal time point for each individual experiment, normalized to the amount of NR2A immunoprecipitated, indicated a significant increase in the tyrosine phosphorylation of NR2A after tat treatment. (** $P < 0.01$, $n = 5$, 10, or 100 ng/ml of tat).

subsequent Western blotting for total and active src, pyk2, and fyn.

Significant interactions between both active, pY416-Src, and total src and NR2A occurred at 5 minutes after tat treatment of neuronal cultures (Figure 2, A–C, $n = 3$, * $P < 0.05$). This association corresponds to the time frame of enhanced tyrosine phosphorylation of the NR2A subunit after tat treatment (Figure 1). Changes in src association were not due to changes in the total amount of NR2A immunoprecipitated (Figure 2A, loading control) or to nonspecific immunoprecipitation of NR2A or src, as demonstrated by using an irrelevant IgG1 reagent (Figure 2A, IgG1 IPs). Densitometric analyses of those co-immunoprecipitations indicated that src (active and total)–NR2A interaction was increased after tat treatment over basal interaction (Figure 2, B and C, $n = 3$, * $P < 0.05$). As src–NR2A interaction always coincided with phosphorylation of NR2A, we proposed that src was responsible for tat induced phosphorylation of NR2A in human neurons.

In addition, pyk2, another tyrosine kinase shown to be associated with the NMDAR in other experimental models,^{39,41,42} interacted with NR2A in basal conditions, and this interaction was increased by tat treatment (data not shown). However, pyk2–NR2A association occurred 15 to

Figure 2. Src is associated with the NMDAR after tat treatment. **A:** Western blots demonstrating association of active (IP:NR2A WB:Active src) and total src (IP:NR2A WB:Total src) association with NR2A by co-immunoprecipitation and representative control Western blot showing approximately equal amounts of total NR2A immunoprecipitated (IP:NR2A WB:NR2A). Neuronal cultures were treated with tat (10 ng/ml) for 5, 10, and 15 minutes. NR2A was immunoprecipitated from the lysates, and membranes were probed for active src, total src, and NR2A. Changes in association were not due to nonspecific immunoprecipitation of src or NR2A proteins (IP: IgG1 WB: NR2A and WB: src). **B** and **C:** Densitometric analyses of active src (* $P < 0.05$, $n = 3$) (**B**) and of total src (* $P < 0.05$, $n = 3$) (**C**) indicated that maximal association with the NMDAR occurred five minutes after tat treatment.

30 minutes (data not shown) after tat-induced tyrosine phosphorylation of the NR2A. The kinase fyn did not associate with NR2A at baseline or after tat treatment (data not shown).

Tat-Induced Tyrosine Phosphorylation of NR2A in Human Neuronal Cultures Is Dependent on src Activity

As active and total src had increased associations with the NR2A after tat treatment at time points corresponding to tyrosine phosphorylation of NR2A, we hypothesized that src was participating in its phosphorylation. A selective dual-site competitive inhibitor of src, Src kinase inhibitor 1 (SrcI),⁴³ was used in human neuronal cultures in the presence or absence of tat treatment, and tyrosine

Figure 3. Src activity is necessary for tat-induced tyrosine phosphorylation of NR2A. **A:** Western blots demonstrating reduced phosphorylation after tat treatment when src activity is inhibited. Neurons were pretreated with 5 μ M Src inhibitor, SrcI, or vehicle for 10 minutes before tat treatment (10 or 100 ng/ml). NR2A was immunoprecipitated, and Western blotting was performed for phosphotyrosine (IP:NR2A WB:P-Tyr) or NR2A (IP:NR2A WB: NR2A). Tat induced tyrosine phosphorylation of NR2A, which was blocked by pretreatment with src inhibitor. **B:** Densitometric analyses indicated that tat induced tyrosine phosphorylation of NR2A (* P < 0.05, Control versus Tat), and pretreatment with the src inhibitor prevented tat induced phosphorylation (** P < 0.01, Tat versus SrcI + Tat). Although the src inhibitor sometimes induced minimal phosphorylation, it was not significant as compared with control levels (Control versus Src Inhibitor, not significant) (n = 3).

phosphorylation of NR2A was examined by immunoprecipitation. Cells were pretreated with 5 μ M SrcI or vehicle as control for 10 minutes to block src activity before treating with tat for 5, 10, or 15 minutes, the time points at which NR2A tyrosine phosphorylation was found to be maximal (Figure 1). Tat induced tyrosine phosphorylation of NR2A significantly over basal levels (Figure 3, A and B, n = 3, * P < 0.05, tat), and pretreatment with SrcI abolished this increase induced by tat treatment (Figure 3, A and B, n = 3, ## P < 0.01, Src Inhibitor + tat). Although src inhibitor alone caused an occasional induction of tyrosine phosphorylation, it was not significantly different from control conditions (Figure 3, A and B, n = 3, not significant, Src Inhibitor). Changes in tyrosine phosphorylation induced by tat treatment were not due to changes in the amounts of total NR2A in the immunoprecipitations, as Western blotting showed little difference in total NR2A (Figure 3A, IP:NR2A; WB: P-Tyr). These experiments demonstrated that src activity is necessary for tat-induced tyrosine phosphorylation of NR2A.

NR2A Tyrosines 1184, 1325, and 1423 Are Phosphorylated by src in Vitro

To identify the tyrosine residues in the cytoplasmic tail of the human NR2A that could be phosphorylated by src, *in vitro* kinase assays were performed. Using the human amino acid sequence of the NR2A, synthetic peptides were generated containing tyrosines that were likely to be phosphorylated using phosphorylation prediction soft-

Figure 4. Identification of phosphorylation sites on human NR2A. **A:** Schematic depicting the tyrosine residues in the cytoplasmic tail of NR2A that were predicted to have a high likelihood of being phosphorylated according to the computer analysis program NetPhos 2.0. The tyrosines marked with a circled P were phosphorylated in our *in vitro* kinase assay, and Y1325, marked in red, was also found to be phosphorylated after tat treatment in our culture system. **B:** *In vitro* kinase assay. Pure recombinant src was preincubated in reaction buffer with ATP for 5 minutes, allowing for src to trans-autophosphorylate and become maximally active before adding target peptides. CON, src target consensus sequence, our positive control, and Y# indicates the tyrosine residue of the NR2A cytoplasmic tail. Three of seven residues tested were capable of being phosphorylated *in vitro*, Y1184, Y1325, and Y1423 (n = 3). **C:** Y1325 is phosphorylated in our neuronal culture system after tat treatment. Immune sera were generated against the phosphorylated forms of Y1184, Y1325 and Y1423, preabsorbed with the corresponding unphosphorylated peptide, and used for Western blotting after immunoprecipitation for NR2A. Western blotting indicated that Y1325 was a likely target of tat induced phosphorylation. Phosphorylation of Y1184 and Y1423 was present basally but did not change in response to tat treatment (100 ng/ml).

ware, NetPhos 2.0,³⁶ or that were sequences that had been examined previously in other systems as potential src sites⁴⁴ in NR2A (Figure 4A, diagram). A total of seven peptides were synthesized as well as the src target consensus sequence as a positive control: src target consensus (EEVYQQWSQ from³⁷), Y1151 (FPDPYQDPS), Y1184 (NNDQYKLYS), Y1292 (RQHSYDNIV), Y1325 (EGNFYGSFLF), Y1387 (PSPPYKHSL), Y1400 (VNDSYLRS), and Y1423 (HNDVYISEH). *In vitro* kinase assays showed that tyrosines Y1184, Y1325, and Y1423 corresponding to the human sequence of NR2A were phosphorylated by src (Figure 4B, n = 3) and that there was autophosphorylation of src (Figure 4B, n = 3). Interestingly, of the three tyrosines homologous to the mouse NR2A subunit that had been previously identified in the literature as potential src targets, Y1292, Y1325, and Y1387,⁴⁴ only one, Y1325, was phosphorylated by src in our *in vitro* kinase assay (Figure 4B). Tyrosines 1151, 1292, 1387, and 1400 were not phosphorylated by src *in vitro* (Figure 4B, n = 3).

Generation of Phospho Site-Specific Antibodies and Identification of Y1325 as a tat-Sensitive Phosphorylation Site on the Human NR2A

Immune sera were generated in mice to the phosphorylated forms of those peptides found to be phosphorylated

in our *in vitro* kinase assay, specifically Y1184, Y1325, and Y1423, to determine whether the sites phosphorylated *in vitro* were phosphorylated in human primary neuronal cultures after tat treatment. To test for phosphorylation of these three tyrosines, cell cultures were treated with tat for 5, 10, and 15 minutes, lysates were prepared, and immunoprecipitation for NR2A and Western blotting using the immune sera for each phosphorylated peptide was performed. We detected basal tyrosine phosphorylation of the three residues analyzed, Y1184, Y1325 and Y1423. In addition, phosphorylation of Y1325 was significantly increased after tat treatment over control conditions (Figure 4C). Changes in phosphorylation of Y1325 were not due to changes in the total amount of NR2A immunoprecipitated, as determined by Western blot analysis (Figure 4C).

To examine this tat sensitive phosphorylation site of the NR2A, we generated phospho site-specific monoclonal antibodies against pY1325 according to standard procedures³⁸ (AECOM hybridoma facility). Reactivity of the hybridoma supernatants to both the phosphorylated and unphosphorylated Y1325 peptides was determined to confirm phosphorylation specificity. Reactivity to the phosphorylated Y1325 peptide titrated to >1:20,000 dilution. Reactivity to the unphosphorylated form was not above background absorbance (Figure 5A). No nonspecific reactivity was detected to pY1184 (data not shown) and pY1423, demonstrating site specificity, nor to bovine serum albumin (Figure 5A), which was in the screening enzyme-linked immunosorbent assay diluent.

The generated phospho site-specific monoclonal antibody (pY1325 mAb) was then used for Western blotting after immunoprecipitation for NR2A from lysates generated from untreated and tat treated (5, 10, and 15 minutes) primary human neurons. Consistent with the results obtained using immune sera, Western blotting with pY1325 mAb showed some baseline phosphorylation in control conditions (Figure 5B). Tat treatment significantly increased phosphorylation of residue Y1325 of NR2A (Figure 5, B and C, $^{**}P < 0.01$), corresponding to the time in which we detected tyrosine phosphorylation with the pan-phosphotyrosine antibody and with mouse immune sera (Figures 1 and 4). Changes in pY1325 were not due to changes in the total amount of NR2A immunoprecipitated (Figure 5B, IP:NR2A; WB: NR2A).

To determine whether phosphorylation of Y1325 was dependent on src activity, we performed confocal analyses of human neuronal cultures that were treated with tat, src1 + tat, src1 alone and vehicle (Figure 6) and were stained using the pY1325 mAb. Cultures were treated with Src1 (5 μ mol/L) or vehicle for 10 minutes before treating with tat for 5, 10, and 15 minutes. Immunostaining with pY1325 mAb hybridoma supernatant and MAP-2 antibody, a neuronal marker, or the appropriate antibody controls was performed. Tat treatment of neuronal cultures for 5 minutes significantly increased the staining for pY1325 along the cell body and neuronal processes (green, Figure 6, D–F, M, $^{**}P < 0.01$), as compared with cells that received vehicle treatment (Figure 6, A–C). Pretreatment with src1 blocked the tat-induced increase in phosphorylation of Y1325 (Figure 6, G–I) as compared

Figure 5. Monoclonal antibodies to phosphorylated Y1325 were specific, and NR2A was phosphorylated on Y1325 after tat treatment. **A:** Monoclonal antibodies from clone 11.1 were specific for phosphorylated Y1325. Hybridoma supernatant collected from clone 11.1 was diluted up to 1:20,000 in 1% bovine serum albumin in PBS, and enzyme-linked immunosorbent assays were performed. The supernatant titrated to 1:20,000 against phosphorylated Y1325 (pY1325, black square), but did not recognize the unphosphorylated form of the peptide (Y1325, black circle), other phosphorylated peptides (pY1423, gray inverted triangle), or bovine serum albumin (gray triangle), used in the blocking solution. **B:** Y1325 is phosphorylated after tat treatment. Neuronal cultures were treated with tat (100 ng/ml) for 5–30 minutes, and NR2A was immunoprecipitated from the lysates. Western blotting was performed with the hybridoma supernatant, diluted 1:1000. Phosphorylation occurred between 5 and 15 minutes after tat treatment (IP:NR2A WB: pY1325). **C:** Densitometric analyses demonstrated that NR2A was phosphorylated at Y1325 after tat treatment ($^{*}P < 0.01$, $n = 4$, 100 or 300 ng/ml tat).

with tat treatment alone (Figure 6M, $^{###}P < 0.01$). Src kinase inhibitor alone had no effect on the phosphorylation of Y1325 compared with control cells (Figure 6, J–M). No background or nonspecific staining was detected using irrelevant isotype matched antibodies or irrelevant immune hybridoma supernatant (data not shown). Quantification of the pixels positive for NR2A-pY1325 and MAP-2 was performed and the data were expressed as the ratio of NR2A-pY1325/MAP-2, to adjust for numbers of neurons in each frame (Figure 6M). For all conditions, only neurons with some pY1325 staining were examined.

Increased Phosphorylation of NR2A Y1325 in Brain Tissue Sections Obtained from Individuals with HIV Encephalitis (HIVE)

As tat induced phosphorylation of Y1325 in human neuronal cells, we examined phosphorylation of Y1325 of the NMDAR in brain tissue sections obtained from uninfected individuals (Figure 7, Control), HIV-infected individuals

Figure 6. Immunofluorescent staining indicates that tat treatment induces increased phosphorylation of Y1325 and that this is blocked by treatment with src inhibitor. **A–L:** Neuronal cultures were pretreated with SrcI or vehicle for ten minutes before being treated with tat (300 ng/ml) or vehicle for 5, 10, and 15 minutes. The 5-minute treatment is shown. Cover slips were subsequently fixed and stained for MAP2 (red), a neuronal marker, and pY1325 (green). Overlay of pY1325 and MAP2 is shown in the right-hand column (merge). Tat treatment (**D–F**) induced an increase in phosphorylation over controls (**A–C**). Pretreatment for ten minutes with src inhibitor prevented tat induced phosphorylation (**G–I**, **M**). SrcI treatment alone did not affect phosphorylation at the time point examined (**J–L**). **M:** The number of pixels per frame of each color was counted, and the ratio of green pixels to red pixels was calculated. Only those images with at least one spot of pY1325 staining were counted. Tat induced enhanced phosphorylation (* $P < 0.05$). Src inhibitor blocked tat induced phosphorylation (** $P < 0.01$, compared with tat treatment). There was no difference between control and SrcI + tat treated cultures ($n = 3$, with at least three pictures for each cover slip and two cover slips per experiment).

without encephalitis (HIV) and from individuals with HIV encephalitis (HIVE) using immunofluorescent labeling and confocal microscopy. MAP-2 immunostaining was used to identify neurons in the tissue sections and our pY1325 mAb was used to show phosphorylation at this site.

Brain tissue sections obtained from uninfected individuals and HIV-infected individuals without encephalitis showed very low levels of pY1325 mAb immunolabeling in both the cortex (green, Figure 7, B and E, respectively) and hippocampus (data not shown) in neuronal bodies identified by staining with MAP-2 (red, Figure 7, A and D). Tissue sections obtained from individuals with HIVE had highly increased staining with pY1325 mAb in MAP-2 positive cells, especially on the cell bodies of these neurons in both the cortex (Figure 7, G and H) and hippocampus (data not shown) as compared with sections

obtained from uninfected (Figure 7, A–C, Control) and HIV-infected individuals without encephalitis (Figure 7, D–F, HIV). The increased staining with pY1325 mAb in the HIVE tissue was not due to ischemic insult from the postmortem interval before sample collection, as this was similar in all of the cases analyzed.

Quantification of the total fluorescence or the mean fluorescence intensity of all pictures obtained for NR2A Y1325 and MAP-2 using NIS Elements Advance Research software, indicate that control brain sections have a total intensity of Y1325 staining of $440,890 \pm 201,017$ arbitrary units (A.U.) with a mean intensity of 1.71 ± 0.75 A.U. HIV-infected brains without encephalitis have a total intensity of Y1325 staining of $508,661 \pm 202,219$ with a mean intensity of 1.738 ± 0.87 A.U. and HIVE tissue sections have a total intensity of Y1325 staining of $1,004,191 \pm 424,008$ with a mean fluorescence intensity of 3.59 ± 1.26 A.U. These results indicate that control and HIV sections are not significantly different in total or mean intensity of fluorescence ($P = 0.373$ and $P = 0.934$, respectively). In contrast, control or sections from HIV-infected individuals without encephalitis were significantly different from HIVE sections in total and mean fluorescence (control versus HIVE, $P = 2.49 \times 10^{-5}$ and $P = 9.12 \times 10^{-6}$, respectively and HIV versus HIVE, $P = 0.00012$ and $P = 1.79 \times 10^{-5}$, respectively). MAP-2 staining quantification of the human tissue sections showed that control brain sections have a total intensity of MAP-2 staining of $4,410,594 \pm 740,646$ with a mean intensity of 16.54 ± 2.94 A.U. Sections from HIV brains without encephalitis have a total intensity of MAP-2 staining of $4,414,144 \pm 1,004,439$ with a mean intensity of 16.27 ± 3.87 A.U. and HIVE tissue sections have a total intensity of MAP-2 staining of $3,104,139 \pm 1,532,179$ with a mean intensity of 10.86 ± 5.08 A.U. These results indicate that control and HIV without encephalitis brain sections are not statistically significantly different in total and mean intensity of fluorescence ($P = 0.9913$ and $P = 0.828$, respectively). In contrast, control or HIV without encephalitis sections were significantly different from HIVE sections in total and mean fluorescence (control versus HIVE, $P = 0.00313$ and $P = 0.000344$, respectively, and HIV without encephalitis versus HIVE, $P = 0.00529$ and $P = 0.00132$, respectively).

Thus, the total amount of Y1325 was higher in the HIVE sections as compared with control and HIV tissue sections without encephalitis. MAP-2 staining in HIVE was lower than that for the control brains or HIV-infected brains without encephalitis, possibly indicating loss and/or damage of neurons in HIVE as described previously.⁴⁵ Isotype matched control antibodies were negative and were used as background controls for the quantification of fluorescence (data not shown). All cases from the same condition had similar staining patterns as the representative pictures shown in Figure 7.

Discussion

Our results demonstrate that HIV-tat increases tyrosine phosphorylation of Y1325 on the human NMDAR subunit

Figure 7. Phosphorylation of Y1325 of NR2A is increased in HIV encephalitic tissue. Tissue sections from HIV encephalitic brains (HIVE, $n = 4$), HIV-infected without encephalitis (HIV) or from control brains (Control) with a similar interval to autopsy and fixation ($n = 4$) were immunostained for MAP-2 (red, **A**, **D**, and **G**), a neuronal marker and phosphorylated Y1325, labeled pY1325 (green, **B**, **E**, and **H**). Micrographs labeled **C**, **F**, and **I** are the merged images. **A–C**: Control tissue demonstrated robust MAP-2 staining and minimal staining for phosphorylated Y1325. **D–F**: HIV tissue sections without encephalitis (HIV) show a similar minimal staining pattern to control conditions. The merged images show that all Y1325 staining colocalizes with MAP-2 and thus is found only in the neurons (**C** and **F**). HIV encephalitic tissue had greatly increased staining for pY1325 relative to control levels. There was also diminished MAP-2 staining relative to controls cells, perhaps indicative of neuronal damage, as there were also fewer neurons. The MAP-2 staining is so intense for all panels, such that in the merged images it is difficult to see the colocalization of green and red (orange). However, the reactivity with pY1325 is very apparent in **H**, as compared with **B** (Control) and **E** (HIV). The quantification of the fluorescence intensity for Y1325 and MAP-2 staining are described in *Results*.

2A in our primary human neuronal cultures by recruitment and activation of src. Additionally, we found increased phosphorylation of Y1325 in human neuronal cultures and in HIV encephalitic brain sections as compared with uninfected control and HIV-infected without encephalitis brain sections. Thus, our results identify a specific tyrosine residue, Y1325, in the human NR2A whose phosphorylation is dependent on src activity, and increased in response to tat in cultured neurons. Additionally, phosphorylation of the NR2A Y1325 residue was significantly increased in neurons in human brain tissue sections in the context of HIV as compared with sections obtained from uninfected and HIV-infected individuals without encephalitis.

Aberrant activity of the NMDAR is involved in the tat induced apoptotic process of human neuronal cultures, as blocking the NMDA receptor with MK801 or AP-5 inhibited tat induced apoptosis.^{8,9} Additionally, our previous results demonstrated that tat treatment alters protein-protein interactions, channel activity and aggregation of NMDAR channels on the surface of human neurons.^{8,9} Phosphorylation of NMDAR subunits has been shown to affect these processes.^{13–17} The NMDAR can be phosphorylated on serine/threonine residues by protein kinase A, protein kinase C, CaMKII and casein kinase II,⁴⁶ as well as on tyrosine residues by fyn, src, and other kinases.⁴⁶ Aberrant phosphorylation has been observed in several central nervous system pathologies.^{47–49} However, most studies of these diseases were performed in rat and mouse model systems, in which NMDAR differs from the human in subunit composition and amino acid sequence. We are now examining HIV tat induced tyrosine phosphorylation of the human NMDAR subunit 2A in human

neurons as a mechanism that participates in the pathogenesis of neuroAIDS.

Our results demonstrate that tat induces tyrosine phosphorylation of Y1325 on the NR2A subunit in human neurons through the recruitment and activation of src and its interaction with the NMDAR. Src family kinases have been shown to regulate NMDAR activity in a variety of ways, including insertion of new channels onto the surface of neurons⁵⁰ and in the potentiation of NMDAR currents.^{12,19,44,51} Further, others have shown using recombinant expression systems that src increased the glutamate induced current of NR2A containing receptors, but not those receptors containing exclusively NR2B, NR2C or NR2D.¹² We propose that up-regulation of src activity is a mechanism of tat induced activation of NR2A containing NMDAR in our system. The effects of src on tat induced activation are currently being examined.

We found that maximal tyrosine phosphorylation occurred between 5 and 15 minutes after tat treatment. We previously demonstrated the formation of a tat induced membrane complex among LRP, PSD-95, and the NMDAR that participates in tat mediated apoptosis.^{8,9,12} Formation of this complex on the neuronal membrane was maximal at 15 to 30 minutes,⁹ time points after maximal tyrosine phosphorylation. Thus, formation of the complex may also be down-regulating src activity, as PSD-95 can be a negative regulator of src at the NMDAR.^{52–54}

In addition to src, fyn^{20,21} and pyk2^{39,55} kinases have also been implicated in NMDAR activation. However, we did not find fyn associated with the NMDAR in control or tat-treated human cells (data not shown). In contrast, pyk2 was associated with NR2A (data not shown), but the time course of interaction (15 to 30 minutes) did not

correlate with the tyrosine phosphorylation of NR2A, suggesting that pyk2 may have a role in the subsequent events of complex formation and nNOS activation⁹ but not in the initial phosphorylation of Y1325 of NR2A after tat treatment.

Previous reports in rat neurons found that tat induced tyrosine phosphorylation of NR2A and NR2B after 40 minutes of treatment through PKC, IP₃, and G-protein-mediated pathways.^{10,56} The difference in the kinetics of our data may be due to underlying species differences. We observed interspecies differences in the sensitivities of these cultures to tat induced apoptosis. Rat hippocampal neurons have much lower levels of apoptosis in response to tat treatment. Our data and other published reports indicate 5 to 35% apoptosis in rat neurons,^{7,56} as compared with the 65 to 90% apoptosis that we detected in tat treated human neurons.^{8,9} In addition, tat-induced complex formation involves NR2A-containing NMDAR in human neurons,^{8,9} while in rat neurons, we found that the complex is preformed and contains both NR2A and NR2B subunits (data not shown). It has been shown that NMDAR subunits confer different properties that affect receptor function. For example, NR2A receptors have faster deactivation kinetics than NR2B containing receptors,⁵⁷ and they are differentially expressed during development.⁵⁸ It is not yet clear whether the difference in subunit composition between the human and rat, preformation of the complex in rat neurons, and/or alternative signaling contribute to the interspecies differences in apoptosis after tat treatment. It is also important to note that the concentrations of extracellular tat *in vivo* are unknown. It is possible that the concentrations of tat to be used with cultured neurons may differ considerably depending on species, and tat preparations.⁵⁹

We identified three tyrosines in the cytoplasmic tail of NR2A in human primary neurons that are phosphorylated *in vivo* under control conditions: Y1184, Y1325, and Y1423. HIV tat treatment of neuronal cultures resulted in increased phosphorylation of Y1325 of NR2A, but not of Y1184 or Y1423, indicating that the phosphorylation of this particular tyrosine residue is tat sensitive. A previous study had identified 3 sites on NR2A that were phosphorylated when recombinant mouse NMDAR subunits were coexpressed with v-src in HEK293 cells: Y1292, Y1325, and Y1387.⁴⁴ However, of these three residues, only Y1325 was phosphorylated by src in our *in vitro* kinase assay, and thus we did not examine Y1292 or Y1387 phosphorylation in our culture system.

We found that Y1325 is phosphorylated after tat treatment both by Western blot analyses and by immunostaining of tat treated neuronal cultures using a phospho-specific monoclonal antibody that we generated to pY1325. Immunostaining was found after tat treatment of cultured neurons in a similar distribution in the cell body and processes to LRP and NMDAR complexes described previously.⁹ The phosphorylation of NR2A on Y1325 was src dependent. Blocking src activity by pretreating with a src inhibitor completely inhibited tat induced phosphorylation of NR2A.

To demonstrate that the findings in our cultured human neurons could be detected in brain tissue sections ob-

tained from HIV-infected individuals with encephalitis, we examined and quantified the reactivity of our phospho site-specific antibody to pY1325 with human neurons in cortical and hippocampal tissue sections from control and HIV-infected individuals. Basal phosphorylation of Y1325 was low in control tissue sections. Significantly increased staining for pY1325 was observed in the HIV tissue examined as compared with staining of control tissue or HIV-infected tissue without encephalitis, demonstrating a potential role of this phosphorylation in the pathogenesis of neuroAIDS. Phosphorylation of this site in the context of other neuroinflammatory conditions cannot be ruled out. However, the individuals from whom these sections were obtained were well characterized with regard to any existing comorbidities. Thus, in these particular sections, the phosphorylation was solely in the context of HIV encephalitis.

Compelling *in vitro* data suggest the involvement of the NMDAR in HIV pathogenesis³ and other neurodegenerative diseases.⁶⁰ Recent clinical trials have attempted to target this receptor for therapeutic purposes. Memantine, a noncompetitive inhibitor of the NMDAR used to treat other neurodegenerative diseases, is currently being investigated for the treatment of HIV neurocognitive disorders.^{61,62}

In this report, we demonstrate that tat increases tyrosine phosphorylation of the human NR2A subunit of the NMDAR in cultured human neurons, that src associates with the NMDAR after tat treatment, and that NR2A phosphorylation is dependent on the activity of src. We also identify Y1325, Y1184, and Y1423 of the NR2A subunit as phosphorylation targets of src *in vitro*, and Y1325 as a tat-sensitive, src-dependent phosphorylation site *in vivo* in human neurons. Additionally, phosphorylation of Y1325 was enhanced in human tissue sections obtained from individuals with HIV. These findings indicate one of the early mechanisms by which tat may alter NMDAR activation and initiate apoptosis in neurons, contributing to the pathogenesis of neurocognitive impairment observed in HIV-infected individuals. Understanding this disease process is important for the development of therapies to prevent or treat the neurocognitive manifestations seen in HAND (HIV Associated Neurocognitive Disorders).

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