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Silencing the PTEN gene is protective against neuronal death induced by human immunodeficiency virus type 1 Tat

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Neurons are targets of toxicity induced by the human immunodeficiency virus (HIV)-1 protein Tat (transactivator of transcription). Exposure to Tat increases $[Ca^{2+}]_i$ in striatal neurons and activates multiple cell death pathways. In earlier studies the authors showed that Tat activated both caspase-3 and endonuclease-G, a caspase-independent effector of apoptosis, and that Tat-induced neurotoxicity was not attenuated by a caspase-3 inhibitor. Because Tat activates multiple, parallel death pathways, the authors attempted to reduce Tat-induced neurotoxicity by manipulating signaling pathways upstream of mitochondrial apoptotic events. PTEN (phosphatase and tensin homolog deleted on chromosome 10), a negative regulator of Akt/PKB (protein kinase B) phosphorylation, was chosen as a target for silencing. Akt/PKB activity directs multiple downstream pathways mediated by GSK3β, BAD, forkhead transcription factors, nuclear factor kappa B (NFκB), and others, in a manner that promotes proliferation and survival. Striatal neurons were nucleofected with short interfering RNA (siRNA) vectors targeting PTEN, or a negative-control siRNA. Although Tat_{1-86} significantly increased the death of neurons transfected with control construct by 72 h, PTEN-silenced neurons were completely protected. These findings indicate that Akt is a critical intermediary in the direct neurotoxicity induced by HIV-1 Tat, and identify Akt regulation as a possible therapeutic strategy for Tat-induced neurotoxicity in HIV encephalitis (HIVE). *Journal of NeuroVirology* (2007) **13,** 97–106.

Keywords: Akt; apoptosis; cell death; endonuclease-G; glial cells; HIVE; neuroAIDS; neuroprotection; phosphoinositide-3-kinase; siRNA

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection can be associated with a decline in neurological function ranging from mild cognitive change to full-blown dementia (Fischer-Smith and Rappaport, 2005; Ghafouri *et al*, 2006; Lipton and Gendelman, 1995; McArthur *et al*, 1993, 2003). The HIV encephalitis (HIVE), which frequently accompanies neurological impairment, consists of microglial activation, loss of neurons, and changes in the architecture of remaining neurons such as dendritic pruning and synaptic loss (Gelbard *et al*, 1995; Lipton,

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[1]*Abbreviations:* Akt/PKB, Akt1/protein kinase B; Bad, bd-2 antagonist of cell death; CMV, cyto-megalovirus; CNS, central nervous system; FACS, fluorescence-activated cell sorting; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; gp120, glycoprotein 120; GSK, Glycogen synthase kinase; HIVE, HIV encephalitis; hDNA, hairpin DNA; Hpt, bacterial hygromycin B phosphotransferase gene; Ikk, I-Kappa-B Kinase; Nef, negative regulatory factor; NeuN, neuronal nuclei; pAkt, phosphorylated Akt; PTEN, phosphatase and tensin homolog deleted on chromosome 10; RNAi, RNA interference; RT-PCR, reverse transcriptase–polymerase chain reaction; siRNA, short interfering RNA; SEC, silencing expression cassette; Tat, transactivator of transcription; Vpr, viral protein R.

1997; Masliah *et al*, 1996; Sa *et al*, 2004). Evidence from a number of studies shows that several HIV-1 viral proteins, including transactivator of transcription (Tat) (Aksenov *et al*, 2003; Bonavia *et al*, 2001; Bruce-Keller *et al*, 2003; Haughey and Mattson, 2002; Kim *et al*, 2003; Kruman *et al*, 1998; Maggirwar *et al*, 1999; Nath *et al*, 1996; Singh *et al*, 2004), glycoprotein 120 (gp120) (Acquas *et al*, 2004; Garden *et al*, 2002; Haughey and Mattson, 2002; Jana and Pahan, 2004; Singh *et al*, 2004), viral protein R (Vpr) (Patel *et al*, 2002; Sabbah and Roques, 2005), and negative regulatory factor (Nef) (Mordelet *et al*, 2004; van Marle *et al*, 2004), have toxicity towards neurons *in vivo* and/or *in vitro*. The apoptotic effector caspase-3 has been shown to be activated in a number of these instances (Acquas *et al*, 2004; Bonavia *et al*, 2001; Garden *et al*, 2002; Kruman *et al*, 1998; Sabbah and Roques, 2005; Singh *et al*, 2004, 2005). Similarly, our previous work has shown that both HIV-1 Tat and gp120 have direct toxicity towards striatal neurons *in vitro*, and that both activate the apoptotic effector caspase-3 (Singh *et al*, 2004). Interestingly, we found that inhibition of caspase-3 was effective in preventing gp120-mediated death, but that Tat-mediated death was completely unaffected (Singh *et al*, 2004). This strongly suggested that although Tat can activate caspase-3–mediated apoptosis, it must also activate caspase-independent effectors of cell death. We subsequently determined that the caspase-independent DNA fragmentation effector endonuclease-G (Li *et al*, 2001; van Loo *et al*, 2001) was activated by Tat, but not by gp120, in striatal neurons (Singh *et al*, 2004). Whether or not endonuclease-G is the only caspase-independent pathway that Tat can induce, the clear implication of these results is that preventing Tat-induced apoptosis may require the simultaneous blockade of multiple cell death pathways.

Akt/protein kinase B (PKB) is recognized as a major determinant of cell survival (Altomare and Testa, 2005; Dudek *et al*, 1997; Kandel and Hay, 1999; Kennedy *et al*, 1997). Phosphorylated Akt (pAkt) regulates the phosphorylation of a number of mediators of survival, including BAD, I$\kappa\kappa$, forkhead transcription factors such as FKHR and FOXO family members, GSK3β, and others. Through phosphorylation of BAD, pAkt can also potentially affect mitochondrial stability and the release of endonuclease-G. In order to increase cellular levels of pAkt and potentially protect striatal neurons from the effects of HIV-1 Tat, we chose to silence a major lipid phosphatase antagonist of Akt phosphorylation, the protein phosphatase and tensin homolog, deleted on chromosome 10 (PTEN). PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate (PIP_3), a product of phosphoinositide 3-kinase (PI3-K) activity. Thus, under normal circumstances, PTEN antagonizes the effect of PI3-K, favoring the accumulation of Akt over pAkt (Figure 1). Dysregulation of PTEN has a potent effect on the balance of cell survival and production, and is well documented to underlie a number of common cancerous conditions, such as glioblastoma multiforme, melanoma, and endometrial and prostate neoplasias (Altomare and Testa, 2005; Bonneau and Longy, 2000; Cantley and Neel, 1999; Rao and James, 2004; Simpson and Parsons, 2001; Stiles *et al*, 2004). As well, PTEN germline mutations underlie disorders associated with increased malignancy, such as Cowden's disease and Bannayan-Riley-Ruvalcaba syndrome (Bonneau and Longy, 2000; Eng, 2003; Zhou *et al*, 2003). Knockouts of PTEN are embryonic lethal, and heterozygous $PTEN^{+/-}$ mice have a decreased life expectancy due to their susceptibility to multiple forms of cancer (Sun *et al*, 1999; Suzuki *et al*, 1998). Because PTEN lies at the core of a crucial cell survival pathway, and because many types of cells tolerate partial loss of PTEN function, silencing PTEN gene expression may offer a novel experimental and therapeutic mechanism for protecting cells from toxic agents, such as Tat, that are able to induce cell death via the activation of multiple cell death pathways.

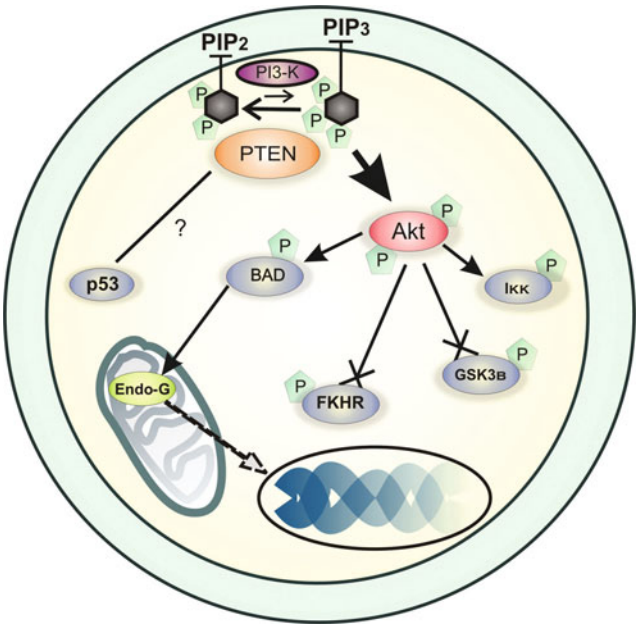

Figure 1 Schematic showing PTEN involvement in multiple signaling pathways related to cell survival and cell death. PTEN normally acts as a tumor suppressor through its ability to limit formation of PIP_3 and phosphorylation of Akt (PKB). By controlling levels of pAkt, PTEN limits cell survival and proliferation via effects on pAkt targets such as GSK3β, I$\kappa\kappa$, forkhead transcription factors (FKHR), BAD, and others not shown on this chart. PTEN also has interactions with the tumor suppressor p53, offering another mechanism for altering cell survival. Tat potentially activates multiple apoptotic pathways that are interrupted by PTEN silencing and accumulation of phosphorylated Akt. PIP_3, phosphatidylinositol 3,4,5-triphosphate; PIP_2, phosphatidylinositol 4,5-biphosphate; PI3-K, phosphoinositide 3-kinase; endo-G, endonuclease-G.

Results

Primary striatal neurons transfected using Amaxa Nucleofector technology showed long-term, high-level expression of GFP. By 48 h after transfection, GFP was detected in approximately 40% of neurons.

Table 1 GFP expression in neurons at 3 to 9 days post transfection

	Day 3	Day 5	Day 7	Day 9
% cells expressing GFP	40.5 ± 2.3	49 ± 3.7	55.4 ± 4.5	43.5 ± 1.5

Notes. Results are mean of $N = 3$ independent experiments ± standard error of the mean.

Even at 9 days after transfection, this percentage had not significantly decreased (Table 1). Although many neurons were lost during the transfection procedure, surviving neurons developed a normal morphology with an extensive network of branching neurites. Transfected, GFP^+ neurons did not appear phenotypically different than neurons in the same culture dish which had not been transfected.

The results show that our PTEN silencing constructs effectively reduce expression of the PTEN gene (Figures 2 and 3). When neurons transfected with either the NCsi-GFP or PTENsi-GFP constructs were separated by GFP expression from untransfected neurons in the same culture dish, real-time RT-PCR analysis showed a significant reduction in the levels of PTEN mRNA in the PTENsi-GFP–transfected cells versus the NCsi-GFP–transfected cells (Figure 3). This is similar to results we obtained

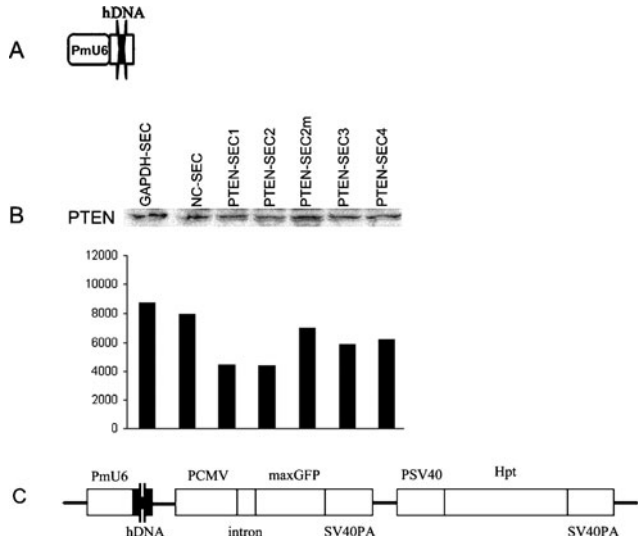

Figure 2 Construction and analyses of siRNA constructs for PTEN. (**A**) Schematic representation of silencing expression cassette used for cell transfection. PmU6: promoter of mouse U6 gene; h-DNA: hairpin DNA. (**B**) Screening the efficacy of PTEN-SECs. The upper panel shows Western blot analyses of PTEN expression in astrocytes after 2 days transfection with GAPDH-SEC, a nonspecific negative control SEC (NC-SEC), four different PTEN-SECs, and a mutated PTEN-SEC (PTEN-SEC-2m). The lower panel shows densitometric analysis of the intensity of the corresponding bands above after normalization to actin. There is efficient silencing of PTEN expression by PTEN-SEC1 and PTEN-SEC2. Results presented are representative of two similar screens. (**C**). Schematic representation of siRNA vectors for PTEN or negative control. PmU6 and h-DNA are as described in **A**; PCMV: promoter of CMV; GFP: green fluorescent protein; PSV40: promoter of SV40; Hpt: bacterial hygromycin B phosphotransferase gene; SV40PA: SV40 polyadenylation signal sequence.

with a similar SEC in an oligodendrocyte-derived cell line (Zhao *et al*, 2006). We also assessed the effects of Tat on untransfected neurons to determine if the transfection procedure itself in any way sensitized the neurons to Tat toxicity. In these cells, Tat induced 34% neuronal death after 72 h. This is 210% of control death, a value that is comparable to that seen in the Ncsi-transfected cells, suggesting that transfected and nontransfected cells were equally sensitive to Tat-induced toxicity.

Although PTEN gene function was not suppressed completely, the level of silencing induced by the PTENsi-GFP construct had significant functional consequences. As has been previously reported by our group and others for a variety of CNS neurons, a 72-h exposure to the HIV-1 Tat protein induced significant cell death in primary striatal neurons. Approximately 15% of neurons transfected with the NCsi-GFP vector died over the course of 72 h in the absence of any treatment. Cell death was significantly increased over this background level to approximately 33% by exposure to 100 nM Tat_{1-86} (Figure 4E). Neurons from the same culture that were transfected with the PTENsi-GFP construct showed the same background rate of cell death in the absence of Tat treatment. Importantly, the PTENsi-GFP–transfected neurons were entirely protected from Tat-induced toxicity over the same 72-h period ($P < 0.001$ versus Tat-treated NCsi neurons). A mutant form of Tat included as a control for specificity was not toxic to either NCsi-GFP–transfected or untransfected neruons (Figure 4F).

Overall, the results suggest that nucleofection is an effective method of promoting relatively long-term silencing expression, at least in postmitotic cells such as CNS neurons. Our finding that PTEN silencing can completely protect striatal neurons against Tat-induced death underscores the potential value of targeting multiple cell death/survival pathways simultaneously in order to achieve therapeutic efficacy.

Discussion

The HIV-1 viral proteins Tat and gp120 are toxic to a variety of neurons both *in vivo* and *in vitro* (Acquas *et al*, 2004; Aksenov *et al*, 2003; Bonavia *et al*, 2001; Bruce-Keller *et al*, 2003; Garden *et al*, 2002; Haughey and Mattson, 2002; Jana and Pahan, 2004; Kim *et al*, 2003; Kruman *et al*, 1998; Maggirwar *et al*, 1999; Nath *et al*, 1996; Singh *et al*, 2004), and we have shown that both of these virotoxins activate caspase-3–dependent pathways in striatal neurons (Singh *et al*, 2004, 2005). Other groups have similarly reported caspase-3 activation in neurons by both Tat and gp120, and identified the involvement of mitochondrial destabilization, and signaling pathways including PI3-K/Akt, GSK3β, and Fas/death receptors (Dou *et al*, 2005; Garden *et al*, 2002; Maggirwar *et al*, 1999). In our hands, striatal neuron death

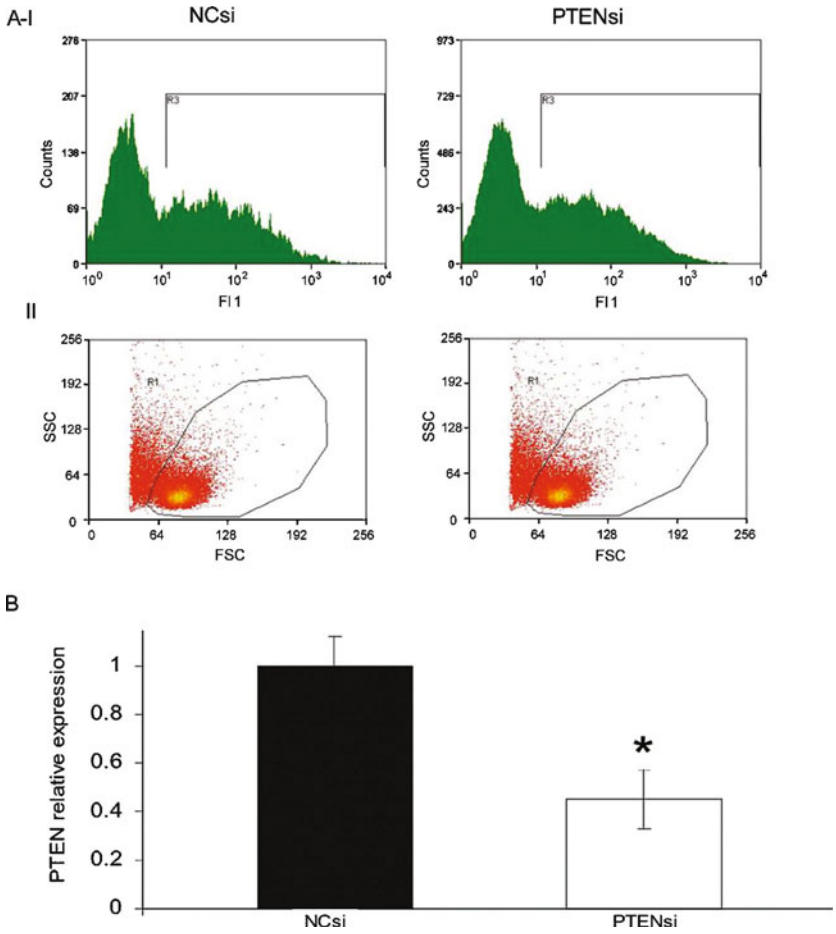

Figure 3 PTEN gene expression is reduced in primary striatal neurons transfected with PTEN a silencing construct. **A,** Results of a representative sort showing FACS intensity and scatter plots of transfected neurons expressing GFP. Primary striatal neurons were transfected prior to plating with either PTENsi-GFP or NCsi-GFP. **A-I,** After 5 days the cells were detached from culture plates and GFP^+ (transfected) cells were gated and analyzed. GFP was identified using a 530/20 bandpass filter and results are shown as fluorescence intensity (FL1) versus cell count. **A-II,** Cells were also gated based on physical parameters of cell size (forward scatter, FSC) versus granularity (side scatter, SSC) to exclude dead cells and debris. Cells within the circled area in **A-II** were chosen for analysis of PTEN gene expression. **B,** Real time RT-PCR analysis shows that PTEN was silenced in sorted, GFP^+ neurons transfected with the PTENsi-GFP vector as compared with GFP^+-expressing neurons transfected with NCsi-GFP vector. NCsi-GFP: nonspecific control silencing vector; PTENsi-GFP: PTEN silencing vector. Data are presented as the mean ± SEM. $N = 3$, $^*P < .01$, as compared to NCsi using Student's *t* test.

was blocked by caspase-3 inhibitors in neurons treated with gp120, although the same inhibitors were unable to block Tat-induced neuron death (Singh *et al*, 2004). This implies that Tat induces activation of multiple caspase-dependent and caspase-independent pathways/effectors leading to cell death. In support of this, we showed activation of endonuclease-G, a caspase-independent DNA fragmentation factor, in Tat-treated, but not gp120-treated, striatal neurons (Singh *et al*, 2004). Whether Tat activates additional caspase-independent proapoptotic targets is not known. Because Tat activates caspase-3 through multiple pathways, and also activates caspase-independent cell death pathways, blocking Tat-induced neuron death requires a strategy designed to include these many downstream effectors. As a method of increasing levels of pAkt, an anti–apoptotic intermediary in this pathway, we developed and tested silencing constructs for PTEN, a negative regulator of Akt phosphorylation. PTEN is also potentially involved in regulation of apoptosis through bidirectional effects on p53 and other pathways (Cully *et al*, 2006; Freeman *et al*, 2003; Stambolic *et al*, 2001; Wee and Aguda, 2006). We initially confirmed the functionality of our silencing constructs in primary astrocytes (Figure 2B). When introduced into striatal neurons, similar suppression was seen at the mRNA level. Importantly, although expression of the PTEN gene was not completely silenced, this suppression resulted in a significant enhancement of survival in the face of HIV-1 Tat exposure (Figure 4). Neurons transfected with the PTENsi-GFP construct were completely protected from Tat_{1-86}-induced toxicity over the 72-h experimental period. Neurons transfected with a nonspecific silencing construct showed a highly

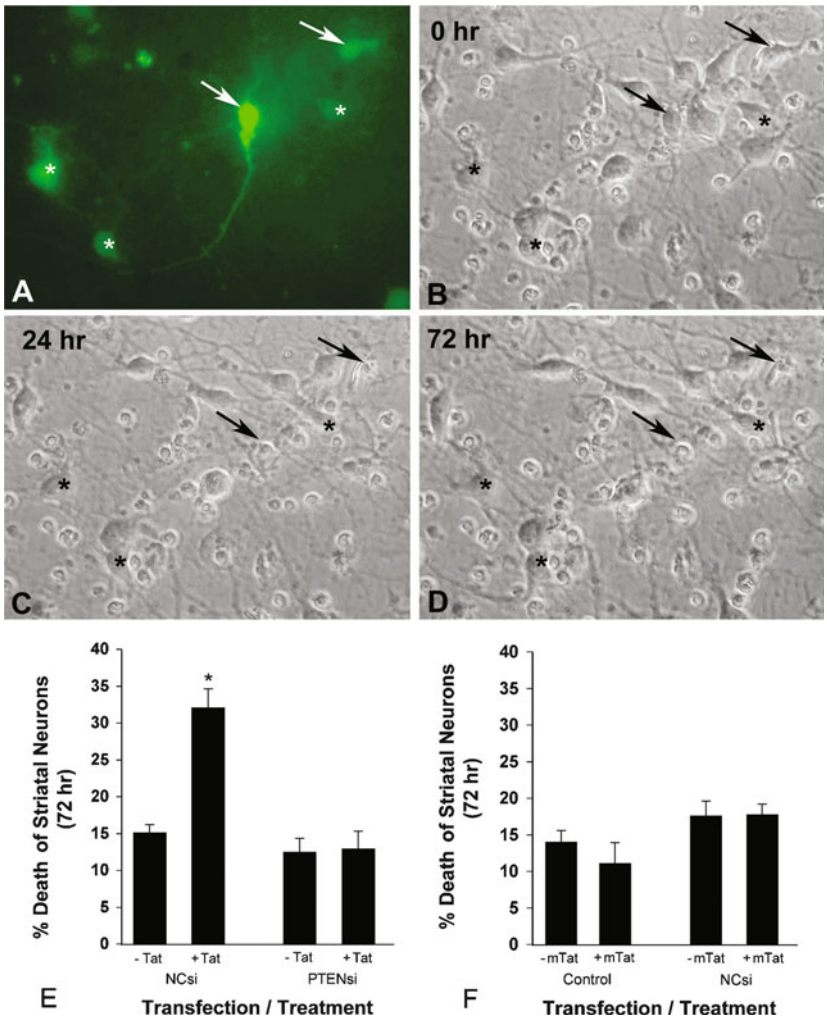

Figure 4 Repeated-measures analysis shows that silencing the PTEN gene reduces HIV-1 Tat–induced death of striatal neurons. Striatal neurons were transfected directly after isolation at embryonic day 15 with NCsi-GFP or PTENsi-GFP constructs, then treated 6 days later with Tat_{1-86} or a mutant form of Tat in which the core and basic domains have been deleted (mTat). **A–D,** Digital phase images of individual fields are recorded starting at day 6 post plating (0 h) and at 24-h intervals (**B–D**). Tat is added at 0 h. A fluorescent image (**A**) is also recorded at 0 h. Individual, transfected (fluorescent) neurons are scored for viability by morphology and/or ethidium homodimer staining. Three transfected neurons that are alive over the 72-h time period are indicated by an asterisk in sequential plates. Two transfected neurons that died between 0 and 72 h are indicated by arrows in sequential plates. **E,** Neurons transfected with the control NCsi-GFP construct show significantly increased death by 72 h when treated with 100 nM Tat_{1-86}. However, neurons transfected with the PTENsi-GFP construct are completely protected from the toxic effects of Tat. $^{*}P < .001$ versus all other groups, $N = 5$. Bars indicate SEM. **F,** The mutant form of Tat (mTat) is not toxic to either control, untransfected neurons or to neurons transfected with the NCsi-GFP construct. There is no difference in neuron death among the four groups. Note that the background percent of neuron death is similar among experiments in **E** and **F**. $N = 3$. Bars indicate SEM.

significant, doubling of cell death over the same time period. Our results also show that functional effects do not require complete silencing.

Our results suggest that neuropathological sequelae in complex disease processes such as HIVE may be more likely to respond to therapeutic strategies that target multiple signaling pathways simultaneously. In the present work we have examined a single HIV virotoxin, Tat, which can invoke cell death through numerous caspase-dependent and -independent effectors. Although Tat appears to play a major role in primary and secondary neuron loss in HIVE, there are several other neurotoxic proteins released during the course of infection, each of which potentially has multiple effectors. PTEN, whose activity can mediate cell survival through the multiple targets of Akt, as well as through interactions with p53, is strategically positioned to act as a buffer against the multiple neurotoxic effects of the several HIV-1 virotoxins. For the same reasons, reducing PTEN activity is also likely to be effective against neurotoxic agents that are not associated with HIV. Regulating PTEN expression may also alter the differentiation and phenotype of multiple cell types through effects on Akt phosphorylation (Costantini *et al*, 2006; Kwon *et al*, 2006; Markus *et al*, 2002; Ogata *et al*, 2004; Yoshimura *et al*, 2006).

Table 2 Primer list

Primer	Sequence (5′ → 3′)
PTEN 1 sense	AACCTACACAAAGTTTCTGCTAACGATCTCTCGGTGTTTCGTCCTTTCCACAAG
PTEN 1 antisense	CGGCGAAGCTTTTTCCAAAAAAAGAGATCGTTAGCAGAAACCTACACAAAGTTT
PTEN 2 sense	TGTCTACACAAAACAAACTGAGGATTGCAAGCCGGTGTTTCGTCCTTTCCACAAG
PTEN 2 antisense	CGGCGAAGCTTTTTCCAAAAAACTTGCAATCCTCAGTTTGTCTACACAAAACAA
PTEN 2m sense	TGTCTACACAAAACATAGTGAAGATAGGAAGCCGGTGTTTCGTCCTTTCCACAAG
PTEN 2m antisense	CGGCGAAGCTTTTTCCAAAAAACTTCCTATCTTCACTATGTCTACACAAAACAT
PTEN 3 sense	ACACTACACAAATGTTTTTGTAAAGTATAGTCGGTGTTTCGTCCTTTCCACAAG
PTEN 3 antisense	CGGCGAAGCTTTTTCCAAAAAAACTATACTTTACAAAAACACTACACAAATGTT
PTEN 4 sense	AAACTACACAAATTTCCAGCTTTACAGTGAACCGGTTTTCGTCCTTTCCACAAG
PTEN-4 antisense	CGGCGAAGCTTTTTCCAAAAAATTCACTGTAAAGCTGGAAACTACACAAATTTC

As a therapeutic target, PTEN has both advantages and disadvantages. Most cells are able to tolerate partial PTEN silencing, because heterozygote PTEN knockout mice develop and reproduce normally. However, the mice have shortened life spans due to increased cancer susceptibility (Sun *et al*, 1999; Suzuki *et al*, 1998). Because of the associated cancer risk, PTEN silencing may be more useful as a therapeutic target in postmitotic cells such as neurons, in which transformation is unlikely. PTEN is ubiquitously expressed, so *in vivo* silencing would require cell specific targeting and control of titer, through genetic or pharmacologic means. Although PTEN itself may be difficult to manipulate therapeutically, our findings imply that proteins whose phosphorylation and activity are altered by PTEN are likely to be important therapeutic objectives in HIV-induced neurodegeneration. In HIVE, or any complex disease process, a single apoptotic factor may induce effects through multiple downstream signaling pathways. Although blocking a single pathway may delay cell dysfunction or death, the most strategic targets will be those that are appropriately positioned to simultaneously intercept multiple deleterious pathways.

Materials and methods

Plasmid construction

Construction of the DNA-based PTEN silencing (PTENsi) and nonspecific, negative-control silencing (NCsi) vectors and silencing expression cassettes (SECs) has been previously described (Zhao *et al*, 2006). In brief, plasmids pSEC hygro and pMaxGFP were purchased from Becton Dickinson (Palo Alto, CA) and Amaxa (Gaithersburg, MD), respectively. siRNA expression cassettes (SECs) for PTEN and NCsi genes were prepared using the mouse U6 gene promoter to drive a gene specific or non-specific hairpin DNA using the Silencer Express Kit (Ambion, Austin, TX) protocol (Zhao *et al*, 2006) (Figure 2A). siRNA targets for the PTEN gene were selected according cDNA sequence (GenBank number 1916329) using online Ambion software (siRNA Target Finder), and were determined to be nonhomologous to other genes by BLAST research. Sense and antisense primers for PTEN were designed based on target sequences using the same software. Sense and antisense primers for PTEN-2–mutated (PTEN2m) SEC were designed based on the mutated PTEN-2 SEC target sequence, in which two base pairs were changed (Table 2). Sense and antisense primers for the nonspecific negative-control gene were provided in the Silencer Express Kit. The SECs were cloned into the pSEC hygro vector to form siRNA vectors for PTEN (PTENsi) or a negative-control sequence (NCsi) (Figure 2A).

The green fluorescence protein (GFP) expression cassette, containing the human cytomegalovirus (CMV) promoter driving the GFP coding region, was amplified by PCR from the pMaxGFP plasmid using forward (5′-CCCAAGCTTAGTTATTAATAGTAATC-3′) and reverse (5′-CCCAAGCTTACGCGTTAAGATAC ATTGA-3′) primers. A restriction site for Hind III was included in each primer to facilitate cloning. PCR products were purified using a PCR purification kit (Qiagen, Valencia, CA), digested with Hind III, and then gel purified. The purified fragments were then ligated into a dephosphorylated Hind III–digested siRNA vector to form two new vectors, PTENsi-GFP and NCsi-GFP (Figure 2C). The orientation of GFP was determined by restriction enzyme analysis and verified by sequencing.

Culture and transfection

For neuron cultures, embryonic day 15 (E15), timed-pregnant female ICR mouse were euthanized by cervical dislocation. Brains were removed from embryos and transferred to 35-mm dishes containing Neurobasal medium after carefully removing the skin, skull, and meninges. The cerebellum was removed and the brain was split down the midline. The striatum was dissected out under a microscope and placed in a 60-mm Petri dish containing trypsin (0.25%) and DNase (0.015 mg/ml) and incubated for 15 min at 37°C. The digested tissue was filtered twice through 135-μm nylon filter to remove debris. The neurons were counted and resuspended in 2.0 ml Neurobasal medium with 25 μM glutamate, B27 additive (StemCell Technologies, Vancouver, BC, Canada) and antibiotics, then transfected as described below prior to plating at a density of 4.5×10^5 cells per 35-mm Petri dish. Both transfected and nontransfected cells were cultured for 6 days at 37°C, 5% CO_2 before

experimental use. Viability of the cells is routinely assessed after attachment in sister culture dishes using a fluorescent live-dead cell assay kit (Molecular Probes, Eugene, OR). Growth in Neurobasal medium favors the survival of neurons versus glial cells, and at the time of experimental use the cultures are routinely 85% to 95% neurons by morphological and immunohistochemical assessment. Astrocytes were cultured from E15 cerebral cortex as previously published (Hostettler *et al*, 2002). Briefly, the meninges were carefully removed, dissociated enzymatically in trypsin and DNase (15 min at 37°C) and dissociated to a fine suspension by triturating through increasingly fine bore glass pipettes. After filtering as described above to remove debris, cells were plated at low density to favor astrocyte growth (5×10^5 per 60-mm dish) in Dulbecco's modified Eagle's medium supplemented with 0.5% glucose, 100 units/ml penicillin-streptomycin, and 10% characterized fetal bovine serum (HyClone, Logan, UT) at 37°C, 5% CO_2. Astrocyte cultures are routinely >95% pure as judged by expression of glial fibrillary acidic protein (GFAP).

Experiments involving neuronal transfection use the Amaxa Nucleofection system. Striatal neurons were transfected prior to their initial plating, at embryonic day 15, using the Amaxa Mouse Nucleofector kit (Amaxa Technology, catalog numberVPG-1001). A total of 3×10^6 pelleted cells were suspended in 75 μl of transfection buffer, to which was added 35 μl of Ca^{2+}-free Dulbecco's modified Eagle's medium containing 10 to 20 μg DNA. The cuvette containing the cells was placed in the Amaxa Nucleofector and set for program O-05, which was empirically determined to be favorable for striatal neuron transfection. Immediately afterwards, 900 μl of Neurobasal medium with 25 μM glutamate, B27, and antibiotics was added, and the cells were plated. Regular medium was replaced after 20 min. Cells that survive the nucleofection procedure have transfection rates of 40% as judged by GFP expression, and are generally quite healthy, exhibiting strong neurite outgrowth and survival rates indistinguishable from nontransfected neurons. All experimental treatments with Tat_{1-86} (100 nM; Immunodiagnostics, Woburn, MA) were begun at day 6 post plating, a time when neurons appear morphologically mature, with an extensive process network typical of medium spiny neurons. At this *in vitro* age, we and others have shown that striatal neurons exhibit many typical *in vivo* characteristics, including opiate, dopamine, glutamate, and cannabinoid receptors, typical voltage-gated K^+ channel subunits, calbindin, substance P, and enkephalins (Falk *et al*, 2006; Goody *et al*, 2003; Haberstock-Debic *et al*, 2005; Mao and Wang, 2001).

For experiments involving transfected astrocytes, confluent primary cells were passed and replated into 6-well culture plates, then transfected 24 h later using the Ambion siPORT XP-1 transfection reagent (Ambion). All transfection procedures followed the manufacturer's recommended protocol. The amounts of siPORT XP-1 (4.8 μl) and DNA (2 μg) were optimized for transfection efficiency based on GFP expression in one well of cells (5×10^5 cells).

Functional testing of silencing constructs

The functionality of the PTEN SEC was initially tested in astrocytes instead of primary neurons because of their abundance and relative ease of transfection. Cells were transfected with PTEN silencing, PTEN mutated (PTENm), GAPDH, or nonspecific negative-control SECs using the Ambion siPORT XP-1 reagent, as described above. Expression of PTEN was assessed by Western blotting after 48 h. Total protein was extracted using RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS]) with one tablet of mixed protease inhibitors (Roche Diagnostics, Indianapolis, IN) per 10 ml. Protein concentration was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL). For Western blots, 20 μg of total protein was separated on 15% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA) and blotted to Hybond-P membrane (Amersham Biosciences, Piscataway, NJ). Blots were probed with a polyclonal antibody to PTEN (Upstate Biotechnology, Lake Placid, NY) and then reprobed for actin (Chemicon, Temecula, CA). Blots were detected using the ECL-Plus (Amersham Biosciences, Piscataway, NJ) or Supersignal (Pierce) detection systems and visualized on a Kodak Image Station 440. Densitometric evaluations were performed using Kodak 1D Software. Signal for PTEN was normalized to the actin signal.

Repeated-measures analysis

Phase-contrast digital images of individual fields in the culture dish were recorded prior to the start of treatment with Tat proteins at day 6 post plating. Immediately after this, either Tat_{1-86} (100 nM) or a control, Tat deletion mutant (mTat; 100 nM), in which the the core and basic domains (amino acids 31 to 61) have been removed, were added to experimental dishes. Phase images were taken of the same fields at 24-h intervals up to 72 h. A single fluorescent image was also taken, either at 0 h or 72 h. Cells that were transfected with either the nonspecific control silencing (NCsi-GFP) or PTEN silencing (PTENsi-GFP) vectors were identified on the basis of their GFP expression. These cells were tracked forward and/or backward in time through the digital images to assess survival. Neurons that were alive at 0 h, but died between 0 and 72 h, were identified on the basis of morphology and ethidium monoazide nuclear staining as previously described (Hostettler *et al*, 2002). In all, an average of 65 transfected cells were examined per culture dish and treatment regimen in five different primary cultures ($N = 5$). Cultures that exhibited more than 25% loss of neurons in untreated, control dishes over the 72-h experimental period were not included in the quantitation. Statistical differences

were assessed using one-way analysis of variance (ANOVA). Post hoc comparisons were performed using Duncan's test (Jmp 5.0; SAS Institute, Cary, NC).

Real-time RT-PCR analysis of gene expression

Real-time RT-PCR was used to document silencing of the PTEN gene in transfected neurons. Because nucleofection efficiency is only 40% in primary striatal neurons, the cells were sorted by FACS prior to analysis of gene expression. At 5 days after transfection, neurons were detached from culture plates with cell dissociation buffer (Sigma) for 20 min, collected by centrifugation, and suspended in sterile sort buffer (1 × phosphate-buffered saline [PBS], Ca/Mg-free; 1 mM EDTA; 25 mM HEPES, pH 7; 1% fetal bovine serum) at a density of 10^7 cells/ml. Cell suspensions were filtered through a 70-μm nylon filter, transferred to 5 ml polypropylene tubes and sorted into GFP^+ (transfected) and GFP^- (untransfected) groups using a 530/20 bandpass fluorescent filter. After sorting, an aliquot of GFP^+ cells was plated, and cultured for 24 h. Several hundred random cells analyzed for purity under fluorescent optics were all GFP positive (data not shown), suggesting that FACS was highly efficient and that GFP^+ cells were not contaminated with GFP^- cells.

For real time RT-PCR analysis of PTEN expression in sorted NCsi-GFP and PTENsi-GFP neurons, total RNA was isolated using GenElute Mammalian Total RNA kit (Sigma). cDNA was synthesized from 2 μg of total RNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Warrington, UK). Standards containing up to 16 × dilution of cDNA template were prepared to generate a standard curve. SYBR green mixture was purchased from Applied Biosystems. Primers for PTEN (primer forward: 5′-CTTTTGAAGACCATAACCA-3′; primer reverse: 5′-TGACTCCCTTTTTGTCTCTG-3′) and primers for 18S (primer forward: 5′-CACTTGTCCCTCTAAGAAGTTG-3′; primer reverse: 5′-GACAGGATTGACAGATTGATAG-3′) were used for quantitative gene expression assay. Levels of PTEN mRNA determined by real time RT-PCR (Applied Biosystems Prism 7500 system) were normalized against 18S mRNA and analyzed using a Student's *t* test (Jmp 5.0; SAS Institute).

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