Journal of NeuroVirology, 16: 33–40, 2010

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.3109/13550280903555712

informa
healthcare

Quantitation of parvalbumin+ neurons and human immunodeficiency virus type 1 (HIV-1) regulatory gene expression in the HIV-1 transgenic rat: effects of vitamin A deficiency and morphine

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Vitamin A (VA) deficiency in human immunodeficiency virus (HIV) infection has been associated with more progressive HIV disease, which may be enhanced by opioid use. In these studies, we examined the effects of VA deficiency and morphine on frontal cortex neuronal numbers in the HIV-1 transgenic (Tg) rat. These studies showed that total numbers of neurons were similar for rats on the VA-deficient diet as for rats on the normal diet and these numbers were not affected by treatment with morphine. In contrast, numbers of neurons that expressed the calcium-binding protein parvalbumin, which is a marker interneurons that express the inhibitory neurotransmitter γ-amino-butyric acid (GABAergic neurons) were decreased for wild-type (Wt) rats on the VA-deficient diet and for Wt rats treated with morphine. In addition, parvalbumin+ neurons were also decreased for Tg rats on a normal diet but increased to normal levels when these animals were placed on the VA-deficient diet and treated with morphine. Analysis of expression of the genes that code for the HIV regulatory proteins vif, vpr, nef, and tat in frontal cortex and adjacent subcortical white matter showed that tat expression was increased in the morphine-treated Tg rat on the VA-deficient diet as compared to untreated Tg rats on the normal diet and untreated VA-deficient rats. These studies therefore suggest that VA deficiency, opioid exposure, and HIV infection alone and in combination may potentially alter neuronal metabolic activity and induce cellular stress, resulting in the observed changes in levels of parvalbumin expression. The specific mechanisms that underlie these effects require further study. *Journal of NeuroVirology* (2010) 16, 33–40.

Keywords: HIV; morphine; NeuN; parvalbumin; regulatory genes; transgenic rat; vitamin A

Introduction

Neurocognitive impairment is a frequent consequence of human immunodeficiency virus (HIV) infection, which has remained common despite the availability of effective antiretroviral therapy (Cysique *et al*, 2004; Sacktor *et al*, 2002). HIV-infected opioid users have been shown to have an increased risk of developing neurocognitive and motor impairment not only related to the immunosuppression that occurs in the context of HIV infection but also as a consequence of specific effects of opioids (Bell *et al*, 1998; Bell, 2004; Perez-Casanova *et al*, 2007). VA deficiency has been also associated with the development of progressive HIV disease and among individuals with a history of drug use; such deficiency has been associated with lower CD4 counts and a higher mortality (Semba *et al*, 1993). The HIV transgenic (Tg) rat model, which

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Received 24 July 2009; revised 26 November 2009; accepted 2 December 2009.

incorporates a noninfectious viral genome that is under similar regulatory control mechanisms *in vivo* that exist with natural infection (Reid *et al*, 2001), demonstrates many of the clinical characteristic consequences of HIV infection that are seen in humans, including cognitive impairment (Cedeno-Laurent *et al*, 2009; Reid *et al*, 2001, 2004; Vigorito *et al*, 2007). In brains from HIV-infected patients, among the abnormalities that have been observed are cortical thinning with specific loss of neurons in area such as the orbital-frontal cortex, and changes in neuronal morphology that correlates with the severity of HIV encephalitis (Masliah *et al*, 1992; Wiley *et al*, 1991). For individuals with a history of methamphetamine abuse, there has been noted to be a decrease in numbers of neurons that express parvalbumin, a calcium-binding protein that is produced by populations of interneurons that also express the inhibitory neurotransmitter γ-aminobutyric acid (GABA). (Langford *et al*, 2003). Neurons that express parvalbumin are "fast-spiking" neurons that are thought to promote synchronized electrical activity of primary neuronal cortical output through the formation of networks of chemical synapses and electrical gap junctions (Benes and Berretta, 2001; Gibson *et al*, 1999). In the cerebral cortex, inhibitory synapses from these interneurons onto the dendrites, proximal axons and soma of primary neurons modulate the electrical activity of these cells (Benes and Berretta, 2001), and loss of such inputs in specific cortical areas may underlie the development of impaired cognitive performance (Lewis and Moghaddam, 2006).

In this report, we describe studies in which Tg and control rats on a normal or a vitamin A (VA)-deficient diet were examined for effects of morphine on the numbers of neurons that express parvalbumin and possible associations between such effects and expression of HIV regulatory proteins. These studies suggest that vitamin A deficiency and morphine may have specific effects that result in changes in the numbers of these neurons. In addition, these effects may be associated with toxicity induced by HIV tat. The altered numbers of these neurons may potentially result in impaired regional cellular function and global functional abnormalities that, in humans, underlie symptoms that are be associated with HIV-related neurocognitive impairment.

Results

Analysis of NeuN+ and parvalbumin+ neurons

On inspection of the brains from the Tg and wild-type (Wt) rat groups, there was no difference in gross appearance or in measures of cortical thickness (data not shown). Staining for NeuN showed an overall pattern in frontal cortex that was similar for the rats in the various groups (Figure 1). Quantitative analysis of NeuN+ cells in frontal cortex revealed no within- or between-group differences in the mean number of stained cells for Tg and Wt rats (Figure 2).

Analysis of the sections stained for parvalbumin revealed cells with rounded or oval-shaped cell bodies, frequently with the characteristic cell processes (Figure 3). Quantitative analysis of the staining showed that there were similar mean numbers of parvalbumin+ cells for the untreated Wt rats on the normal diet and the morphine-treated Tg rats on the VA-deficient diet (Figure 4). Compared to these groups, there were lower mean numbers of stained cells for the morphine-treated Wt rats on the normal diet, the placebo-treated Wt rats on the VA-deficient diet, and the placebo-treated Tg rats on the normal diet (Figure 4).

HIV-1 regulatory protein gene expression

Tissue blocks from frontal brain cortex from the Tg rats were analyzed for expression of the HIV-1 nef, rev, vif, vpr, and tat protein genes. These studies showed, relative to untreated Tg rats on normal diet,

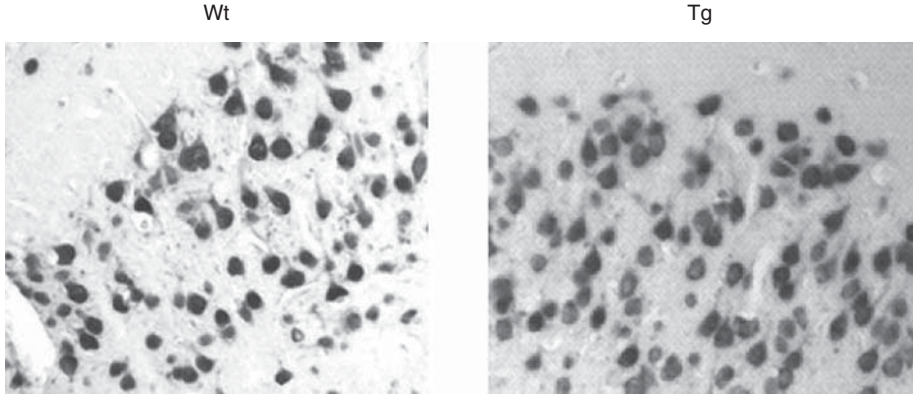

Figure 1 Staining of NeuN+ neurons in frontal cortex from placebo-treated Wt and Tg rats, both on a normal diet, with immunoperoxidase and hematoxylin counterstain (magnification = 10×). The appearance of the cells was similar for these rats and for the Wt and Tg rats in the other treatment and dietary groups (data not shown).

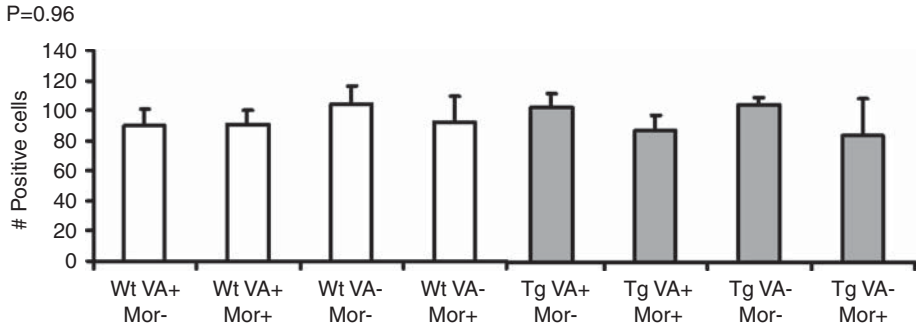

Figure 2 Quantitative analysis of NeuN+ neurons in frontal cortex. NeuN+ neurons in frontal cortex of the Wt and Tg rats on a normal or vitamin A–deficient (VA−) diet were implanted with a placebo (Mor−) or morphine (Mor+) tablet were quantitated and compared as described in Materials and Methods. There was no difference in the mean numbers of neurons for the different groups of rats.

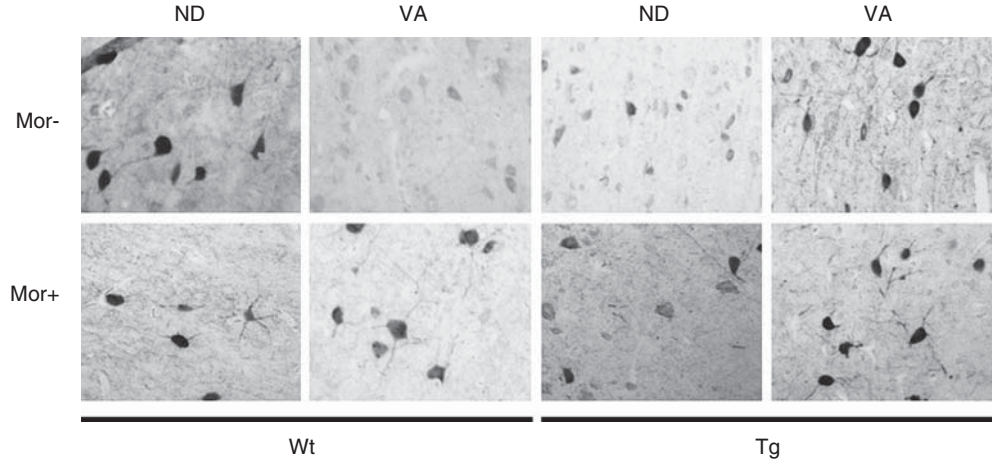

Figure 3 Immunoperoxidase staining of frontal cortex from Wt and Tg rats on a normal (ND) or vitamin A–deficient (VA) diet that were implanted with a placebo (Mor−) or morphine (Mor+) pellet for parvalbumin (magnification = 10×). These representative regions demonstrate the variability in neuronal cell numbers among animal groups.

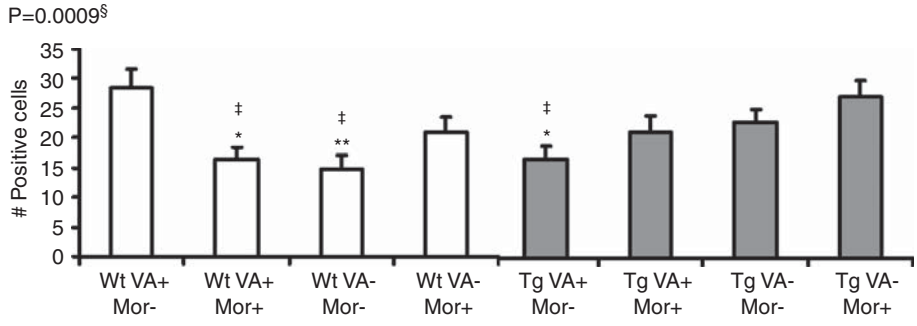

Figure 4 Quantitative analysis of parvalbumin+ neurons in frontal cortex. NeuN+ neurons in frontal cortex of the Wt and Tg rats on a normal or vitamin A–deficient (VA−) diet were implanted with a placebo (Mor−) or morphine (Mor+) tablet were quantitated and compared. Compared to placebo-treated Wt rats on a normal diet and to morphine-treated Tg rats on a VA-deficient diet, mean neuronal numbers were lower for morphine-treated Wt rats on the normal diet, for the placebo-treated Wt rats on the vitamin A–deficient diet, and for the placebo-treated Tg rats on the normal diet. §ANOVA; $*P < .05$, $**P < .01$ (Neumann-Keul test).

no significant difference in nef, rev, vif, and vpr expression for those rats on the VA-deficient diet or in the presence of morphine. In contrast, tat expression was significantly increased for the VA-deficient rats that were treated with morphine relative to placebo-treated rats on either the normal diet or the VA-deficient diet (Figure 5). Also, an increase in relative tat expression was noted for the morphine-treated Tg rats on the normal diet that was not statistically different from that of the

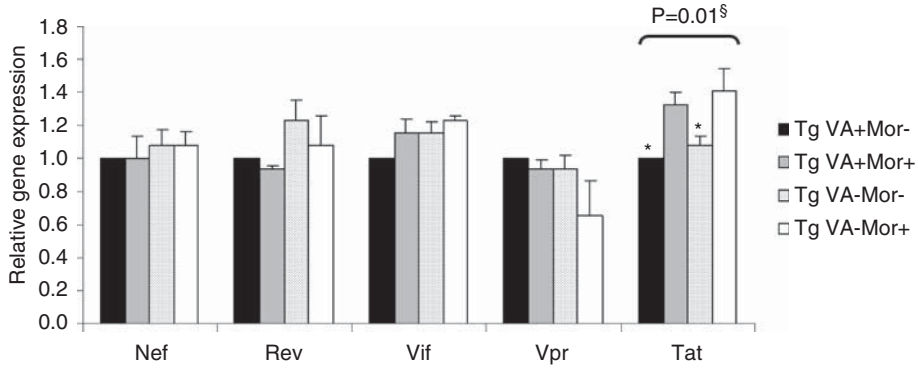

Figure 5 Analysis of HIV-1 regulatory gene expression in frontal cortex of Tg rats on the normal diet or the VA-deficient diet treated with placebo or morphine. Vif, vpr, nef, and tat gene expression was analyzed in frontal cortex from the Tg rats on the normal or deficient (VA−) diet implanted with the placebo (Mor−) or morphine (Mor+) pellet. (N = 2 rats per group with each sample analyzed in triplicate.) §ANOVA; *$P < .05$ versus the Tg VA−Mor+ group (Newmann-Keul test).

placebo-treated Wt rat on the same diet. However, this increase was also were similar to that observed for the morphine-treated, VA-deficient rats.

Discussion

In humans with HIV infection, characteristic neuropathological findings in individuals not on treatment with antiretroviral medications include the presence of marked inflammation, with collections of microglial nodules, multinucleated giant cells, lipid-containing macrophages, and inflammatory cells that stain positive for HIV p24 antigen (Anthony and Bell, 2008). These abnormalities are commonly associated with damage to both neuronal and glial elements (Anthony and Bell, 2008; Wiley *et al*, 1991) and are more prominent for individuals with a history of illicit drug use (Anthony and Bell, 2008). In the HIV transgenic rat, the viral proteins are present from birth and, therefore, do not elicit an immune response. However, gp120 and the regulatory viral proteins tat, vif, vpr and nef have been shown to be capable of exerting direct toxic effects on neurons and glia and such effects, which can occur in the absence of inflammation, can be enhanced by morphine (Bruce-Keller *et al*, 2008; Giunta *et al*, 2009; Gurwell *et al*, 2001; Hauser *et al*, 2009).

The studies reported here demonstrate that changes in parvalbumin+ neuronal cell numbers can occur in this model in the context of the presence of the HIV transgene, exposure to morphine, and the induction of VA deficiency. Parvalbumin is a calcium-binding protein expressed by interneurons, which also produce and secrete GABA, an inhibitory neurotransmitter that regulates neuronal excitability in the central nervous system (Gerfen *et al*, 1985). The cortical interneuron cell types include the so-named large and small basket cells, chandelier cells, double bouquet cells, and tuft cells. Of this group, only the basket cells and the chandelier cells express parvalbumin (a small percentage of basket cells also express calbindin, which is also a calcium-binding protein). The basket cells project axons that terminate on target cell bodies and receive ascending input from the striatum, making these cells candidates for the establishment of center-surround fields. In contrast, the chandelier cell axons project branches that are oriented at right angles to the cell body and terminate on the initial segments of pyramidal cell axons. With immunocytochemical labeling, the stained synapse gives the appearance of a "candle." Both cell types form inhibitory synapses, with the chandelier cell inducing the most prominent inhibitory effect on target cell output (Gerfen *et al*, 1985).

It has been shown that parvalbumin expression is increased as a result of *N*-methyl-D-aspartate (NMDA) receptor activation, which also increases expression of the 67-kDa form of the GABA-synthesizing enzyme, glutamic acid decarboxylase 67 (GAD67) (Cochran *et al*, 2002; Keilhoff *et al*, 2004), in association with activation of the extracelluler signal-regulated kinase 1/2 (Erk1/2) protein kinases and the transcription factor cyclic adenosine monophosphate (cAMP) response element binding (CREB) protein (Kinney *et al*, 2006). This effect on parvalbumin expression is inhibited at increased levels of calcium (Kinney *et al*, 2006), and in studies by Potter *et al*, it was shown that parvalbumin can activate cAMP phosphodiesterase in a calcium-dependent manner (Potter *et al*, 1977), providing feedback mechanisms through which parvalbumin synthesis can be further regulated. Acute morphine exposure has been shown to down-regulate adenyl cyclase activity (Sharma *et al*, 1975; Traber *et al*, 1975), thereby producing decreased intracellular cAMP levels, but with repeated or chronic exposure such activity normalizes and will increase above baseline levels in association with morphine withdrawal (Benalal and Bachrach, 1985; Sharma *et al*, 1975; Traber *et al*, 1975). In mice, symptoms of morphine

withdrawal can be prevented by cAMP analogues and phosphodiesterase inhibitors, which may act by blocking the decrease in cAMP levels that occur with initial morphine exposure (Mamiya *et al*, 2001). In our studies, the rats were exposed to morphine for 5 days, at which time chronic effects from exposure begin to appear.

In the central nervous system, parvalbumin+ interneurons promote the synchronization of cortical neuronal activity (Freund, 2003) and such synchronization is mediated by GABAergic neurons and enhanced by retinoids (Gonzalez-Burgos and Lewis, 2008; Maret *et al*, 2005). In addition to the observed changes with morphine exposure in the Tg rats, levels of parvalbumin increased in these animals in the presence of VA deficiency; this effect was not present for the Wt rats. The mechanisms that are responsible for this effect and the observed discrepancy between the two groups are not clear at this time. The effects of cAMP are mediated by binding of the cyclic nucleotide to protein kinase A (PKA) and retinoids can differentially promote phosphorylation of the type I and type II PKA isoforms (Hohmann and Greene, 1990). Increased levels of cAMP, through binding to CREB and the subsequent phosphorylation of this protein by protein kinase C, can activate expression of GABA receptors (Hu *et al*, 2008). However, this effect can be inhibited by PKA phosphorylation of a repressor of cAMP synthesis (Hu *et al*, 2008). Therefore, in the context of the Tg rat model, it is important to understand the effects of vitamin A on cAMP production, the associated cellular processes, and how they are regulated in the presence or absence of the HIV transgene.

The HIV accessory proteins examined in this report, through a variety of distinct mechanisms, function by either enhancing HIV infectivity (vpr) or replication capacity (nef, vif, and tat) (Trono, 1995). Tat, vpr, and nef have been also shown to be toxic to neurons cultured *in vitro*. In our studies, tat expression was increased in animals on the deficient diet and exposed to morphine, whereas no such associations were noted for the other regulatory proteins. In studies performed *in vitro*, tat-related neurotoxicity has been shown to result from NMDA receptor activation and this effect can be enhanced by morphine (Magnuson *et al*, 1995). Previous studies have demonstrated that morphine and other opioids promote immune cell replication of HIV (Peterson *et al*, 1990, 1993, 1994). Also, the endogenous opioid endomrophin-1 was shown to increase HIV replication by infected human fetal mixed neuronal/glial cells in culture (Peterson *et al*, 1999) and this effect was blocked by specific mu opioid receptor antagonism. However, neither morphine nor the specific mu opioid receptor agonist DAMGO increased HIV expression in the cultures (Peterson *et al*, 1999). Our *in vivo* studies suggest, however, that morphine can indeed have effects that can increase the replication of viral components.

It is possible that the observed changes in the numbers of parvalbumin+ cortical neuronal cells for the Tg and Wt rats in the various dietary and treatment groups were due to alterations in levels of expression of the protein that would render the cells more or less detectible. In studies by Maharajan *et al*, it was shown that morphine administered to female mice for 1 week prior to the mice being mated, during gestation and for 3 weeks postpartum, during which the offspring were allowed to suckle, resulted in increased numbers of parvalbumin+ neurons in layers II to V of parietal cortex I of the offspring (Maharajan *et al*, 2000). These results are similar to the observations made in the Tg rats and opposite what was observed in the Wt rat group. However, parvalbumin, like the other calcium-binding proteins calbindin and calretinin, can regulate levels of free intracellular calcium and therefore cellular functions such as synaptic transmission and apoptosis. These calcium-binding functions of the proteins can have important neuroprotective effects during periods of excessive neuronal excitation, as can occur in experimental models of epilepsy (Schwaller *et al*, 2004), brain ischemia (Gerstein *et al*, 2005), and trauma (Dekkers *et al*, 2002). In the setting of HIV infection, elevated levels of parvalbumin expression may potentially provide protection from toxicity that can be induced by HIV proteins such as tat and by morphine. Conversely, decreased parvalbumin expression may reflect a low metabolic state for the cells, which could potentially occur in association with a number of different scenarios. A better understanding of these potential effects of parvalbumin can be delineated in studies that examine the underlying mechanisms that are involved and their functional consequences.

Materials and methods

Animals

All experiments were performed using 3- to 6-month-old specific pathogen–free Tg and age-matched Wt Fisher 344/NHsd control rats. The details on the construction of the HIV-1 Tg rat have been previously described (Reid *et al*, 2001). The Tg and Wt rats were administered a diet previously used to induce VA deficiency in mice (Carman and Hayes, 1991), except that the rats were fed the Bio-Serv AIN-93M rodent maintenance diet (Bio-Serv; Frenchtown, NJ), which contains 400,000 IU/kg of retinyl palmitate, the major dietary form of VA, or the same diet mix formulated minus retinyl palmitate. Female rats maintained on the normal maintenance diet were mated then randomly divided into two groups at 2 weeks' gestation. One group of pregnant females was subsequently fed a VA-deficient diet and the other was fed the VA-sufficient diet. Weanlings were maintained on the same diets as their dams. For

collection of blood, the rats were anesthetized using a combination of 60 mg/kg ketamine and 7.5 mg/kg xylazine and blood was removed by capillary stick from the cavernous sinus. All studies were approved by the University of Maryland Biotechnology Institute of the University of Maryland, Baltimore Animal Care and Use Committee.

Treatments

Seven-five-milligram morphine tablets and placebo control tablets were obtained through the NIDA Drug Supply Program. Tg and Wt control rats were anesthetized with ketamine then implanted subcutaneously with initially either a whole morphine or placebo tablet. Subsequently, with the occurrence of unanticipated deaths in Tg rats implanted with the morphine 75-mg tablet, the morphine dose was decreased to 37.5 mg (one half tablet). The rats were then observed for 5 days and then sacrificed using ketamine inhalation, and, to obtain brain samples for immunocytochemical analysis, perfused with 1% paraformaldehyde, and the brains then removed, fixed in 4% paraformaldehyde, and embedded with paraffin for subsequent immunocytochemical analysis. Brain tissue for analysis in polymerase chain reaction assays was obtained from rats perfused with saline and then frozen at −80°C until use.

Immunocytochemical studies

Parvalbumin. The tissue sections were deparaffinized and hydrated through xylene and graded alcohol then rinse for 5 min in tap water. The slides were then incubated with 0.3% H_2O_2 in methanol for 30 min then wash in phosphate-buffered saline (PBS) for 5 min. Antigen retrieval was performed by incubating in 10 mM citrate buffer, pH 6.0. The slides were then washed in cold water then the sections incubated with blocking solution (10% horse serum in PBS + 0.1% Triton-X) for 30 min. The slides were then washed and incubated overnight at 4°C with mouse anti-rat parvalbumin, (SWant, catalog no. 235) diluted 1:50,000 in PBS. The following day, the slides were washed three times with PBS, then incubated for 1 h with horse anti-mouse antibody diluted 1:500 with blocking solution (Vectastain ABC kit, catalog no. PK-4002), washed, incubated with AB reagent and diaminobenzadine, and, after processing through graded alcohols and xylene, coverslipped.

NeuN. The sections were deparaffinized and incubated through graded alcohols, xylene, methanol, and then antigen retrieval, incubation in blocking buffer and subsequent washes were performed as described above. NeuN staining was performed by incubating overnight at 4°C with mouse anti-NeuN (US Biological, catalog no. N2173) antibody diluted 1:500 dilution in PBS. The slides were then washed with PBS for 5 min and then incubated for 1 h with anti-mouse immunoglobulin G (IgG) (Vectastain ABC kit peroxidase Mouse IgG, catalog no. 4002) diluted 1:200 in blocking serum. The slides were then washed in PBS for 5 min and incubated for 30 min with AB reagent, washed, then incubated with diaminobenzadine as recommended by the manufacturer (Vector Laboratories). After rinsing, the slides were counterstained with hematoxylin then rinsed, dehydrated, and coverslipped.

Quantitative analysis. Frontal cortex parvalbumin+ cells in four randomly selected, nonoverlapping 10× fields that spanned the cortical region were imaged and manually counted using ImageJ software, National Institutes of Health (Bethesda, MD). Sections were analyzed from three rats per group. For NeuN+ cell quantitation, representative 10× fields were similarly counted.

Polymerase chain reaction (PCR) assays

RNA extracted from thawed blocks of frontal cortical tissue that had been frozen at the time of necropsy was examined in real-time PCR assays for HIV-1 vpr, nef, vif, and tat gene expression. The primers that were utilized were as follows: vpr: forward GCAGGAGTGGAAGCCATAAT, reverse TACTGGCTCCATTTCTTGCTC; nef: forward GCTAACAATGCTGCTTGTGC, reverse TGAATTAGCCCTTCCAGTCC; vif: forward ATTGTGTGGCAAGTAGACAGGATGA, reverse CTAGTGGGATGT GTACTTCTAAACT; tat: forward TCCAGGAAGTCAGCCTAAAACTGCT, reverse TGTCTCCGCTTCTTCCTGCCA; and as an internal control, the ribosomal protein RPL13A (forward GCCTACCAGAAAGTTTGC; reverse CCAAGAGTCCATTGGTCTTG). The thawed tissue was homogenized using Qiagen Qiashredder columns (Qiagen) and the RNA subsequently isolated using RNeasy mini-columns (Qiagen) according to the manufacturer's protocol. Residual contaminating DNA was digested using the RNase-Free DNase set (Qiagen), also as per the manufacturer's protocol. Synthesis of cDNA was performed using the Reverse Transcription system (Promega, Madison, WI). Reactions were carried out in volumes of 20 µl per sample, to which were added 1 µg of template RNA, dNTPs, oligo-dT primer, RNase inhibitor, reverse transcriptase enzyme, and RNase-free water. The cDNA were synthesized at 37°C for 60 min. Real-time PCR of cDNA were performed using an iCycler (BioRad, Hercules, CA) with detection of double-stranded PCR product using SYBR Green (Sigma, St. Louis, MO). The following iCycler settings were utilized for the primer pairs: 3 min at 95°C followed by amplification at 95°C for 30 s, then 30 s at 58°C for 35 cycles. SYBR Green detection was performed during each amplification cycle at 72°C. Following amplification, a melt curve was performed and analyzed to confirm

the absence of primer-dimer formation. Samples were analyzed from two rats per group with each sample analyzed in triplicate. Relative gene expression was expressed using the $\Delta\Delta C_t$ method (Winer *et al*, 1999).

Statistical analyses

Neuronal cell count and HIV gene expression data were analyzed using a between-group analysis of variance (ANOVA) to evaluate the difference between the untreated and morphine-treated TgVA+, TgVA−, WtVA−, and WtVA+ groups, followed by a post hoc between-group comparison using the Neumann-Keul test.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This statement is accurate.

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This paper was first published online on Early Online on 30 January 2010.