Citation: Cell Death and Disease (2012) 3, e381; doi:10.1038/cddis.2012.114

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Exosome-mediated shuttling of microRNA-29 regulates HIV Tat and morphine-mediated Neuronal dysfunction

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Neuronal damage is a hallmark feature of HIV-associated neurological disorders (HANDs). Opiate drug abuse accelerates the incidence and progression of HAND; however, the mechanisms underlying the potentiation of neuropathogenesis by these drugs remain elusive. Opiates such as morphine have been shown to enhance HIV transactivation protein Tat-mediated toxicity in both human neurons and neuroblastoma cells. In the present study, we demonstrate reduced expression of the tropic factor platelet-derived growth factor (PDGF)-B with a concomitant increase in miR-29b in the basal ganglia region of the brains of morphine-dependent simian immunodeficiency virus (SIV)-infected macaques compared with the SIV-infected controls. *In vitro* relevance of these findings was corroborated in cultures of astrocytes exposed to morphine and HIV Tat that led to increased release of miR-29b in exosomes. Subsequent treatment of neuronal SH-SY5Y cell line with exosomes from treated astrocytes resulted in decreased expression of PDGF-B, with a concomitant decrease in viability of neurons. Furthermore, it was shown that PDGF-B was a target for miR-29b as evidenced by the fact that binding of miR-29 to the 3′-untranslated region of PDGF-B mRNA resulted in its translational repression in SH-SY5Y cells. Understanding the regulation of PDGF-B expression may provide insights into the development of potential therapeutic targets for neuronal loss in HIV-1-infected opiate abusers.
Cell Death and Disease (2012) **3,** e381; doi:10.1038/cddis.2012.114; published online 30 August 2012
Subject Category: Neuroscience

Though the majority of HIV-infected individuals acquired infection through sexual contact, a growing number acquire the virus via intravenous drug use. The most commonly injected illicit drug is heroin. Combined HIV-1 infection and opiate dependence is becoming an emerging problem in the post-combination anti-retroviral treatment era, as infected individuals continue to live longer. It is estimated that drug abuse may be the leading cause of HIV transmission in the United States. The interplay of these two thus raises concerns regarding their effects on disease severity and rate of progression.[1] Opiates, such as morphine and heroin, are drugs of choice in a large number of injecting drugs users. There exists a strong connection between opiate usage and HIV-1 neuropathogenesis, and opiate use has been reported to correlate with increased severity of central nervous system (CNS) disease through a number of potential mechanisms.[2] Morphine is an opioid analgesic that is used extensively in the clinical setting. Heroin is a semi-synthetic ester of morphine and exerts its effect following its conversion to morphine in the brain.[3] Opiate abuse has the potential to destabilize neuronal functions, thereby leading to acceleration of neurological complications. Studies on effects of drugs of abuse on HIV infection have been problematic to conduct, primarily because of high variability in drug-usage patterns, length of use and multidrug use, as well as differences in nutritional status and access to medical care.[4] Animal models therefore provide a valuable approach to study the effects of opiates on lentivirus pathogenesis in a more controlled environment. Several studies have utilized the simian immunodeficiency virus (SIV)/AIDS macaque model to better understand the influence of opiate dependency on HIV progression to AIDS. The questions on morphine effects regarding worsening or prevention of disease are relevant in this model system. Previous study reported that morphine dependence in SIV (R71/17E)-infected rhesus macaques, a SIVmac239 derivative, resulted in potentiation of virus replication and concomitant neuronal apoptosis in the CNS compared with macaques infected with the virus alone.[5,6]

Neuronal homeostasis is controlled by neurotropic factors, such as brain-derived neurotrophic factor and platelet-derived growth factor (PDGF).[7] Interestingly, HIV proteins such as Tat and gp120 can downregulate expression of tropic factors such as PDGF, leading to increased neuronal apoptosis. PDGF belongs to a family of five dimeric ligands assembled from four gene products (PDGF-A–D) that act via two classical receptor tyrosine kinases, PDGF-αR and PDGF-βR. It must be noted

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Keywords: HAND; morphine; miRNA; PDGF-B; neuronal dysfunction
Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; 3′-UTR, 3′-untranslated region; CM, conditioned media; CACM, control astrocyte CM; DMA, dimethyl amiloride; HAND, HIV-associated neurological disorder; PDGF, platelet-derived growth factor; SIV, simian immunodeficiency virus; TACM, Tat-treated astrocyte CM; MTACM, morphine plus TACM; MβCD, methyl-β-cyclodex; MTT, 3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
Received 13.12.11; revised 12.4.12; accepted 18.4.12; Edited by D Bano

that nomenclature of PDGF-B refers to RNA expression, whereas that of PDGF-BB refers to the protein expression of PDGF-B chain genes. Members of the PDGF family have multiple functions during embryogenesis and in a variety of pathological situations in the adult.[8] Among these members, PDGF has been demonstrated to protect primary hippocampal neurons against glutamate-induced neuronal damage.[9] PDGF has been shown to promote long-term survival in cultured brain neurons and cultured mesencephalic dopaminergic neurons.[10] In addition, PDGF has also been shown to exert protection against the HIV envelope protein-mediated toxicity in neurons.[11] In our recent study on SIV-infected morphine-dependent macaques, we observed high viral load in cerebrospinal fluid (CSF) that was accompanied with increased neuronal apoptosis.[5] We thus hypothesized that morphine potentiated SIV-mediated neuronal apoptosis likely via downregulation of the tropic factor PDGF-B chain in neurons, and this in turn, could be regulated by microRNA(s) specific for PDGF-B.

MicroRNAs are small RNA regulators that have essential roles in various biological processes.[12] MiRNA-mediated regulation is gaining popularity in neuronal development, neuronal plasticity and neuronal differentiation.[12] Elegant studies have revealed that miRNAs have pivotal roles in neuronal development as well as in the control of neuronal functional elements. Various miRNAs are being recognized with links to HIVE/SIVE,[13] such as Parkinson's, Alzheimer's and Huntington's diseases.[14]

In this study, we demonstrate that miR-29b is increased in the basal ganglia in brains of SIV-infected rhesus macaques that were morphine-dependent. We further demonstrate that this effect was indirect as exosomes released from astrocytes treated with morphine and HIV Tat can shuttle miR-29b, which can be taken up by neurons leading to neuronal death. Moreover, conditioned media (CM) from astroctyes treated with both Tat and morphine decreased PDGF-B expression and enhanced neuronal apoptosis in neurons.

Results

MiRNA profiling in the basal ganglia of SIV-infected macaques with and without morphine dependence. As miRNAs have pivotal roles in neuronal function, we first examined the expression profile of miRNAs in the basal ganglia of uninfected ($n=5$) and SIV-infected macaques with ($n=5$) and without ($n=5$) morphine dependence. For details of morphine dose and treatment, and the virus used, please refer to the study by Bokhari *et al.*[5] Figure 1a is a heat map of the miRNA profile observed in the basal ganglia of SIV-infected macaques with and without morphine dependence compared with the uninfected controls. Supplementary Table 1 demonstrates some of the other up- and down-regulated miRNAs in the basal ganglia of SIV-infected macaques with and without morphine dependence.

Of these altered miRNA profiles, miR-29b was of particular interest, as it was upregulated 4.3-fold in the brains of SIV-infected macaques that are morphine-dependent, and more so, as one of its predicted target genes, *PDGF-B* chain, has been demonstrated to regulate neuronal survival.[15] To validate upregulation of miR-29b, we sought to evaluate its expression in the basal ganglia isolated from the various macaque groups using the mature miRNA-specific quantitative PCR. As shown in Figure 1b, and in keeping with the miRNA array data, there was increased expression of miR-29b in the basal ganglia of SIV-infected macaques that were morphine-dependent compared with either the SIV-infected or -uninfected control groups. These findings were further validated by *in situ* hybridization, demonstrating increased expression of miR-29b both in the neurons and astrocytes in the brains of SIV-infected macaques with morphine dependence (Figure 1c) compared with SIV-infected macaques. Upregulation of miR-29b was not specific to basal ganglia, as other brain regions such as cortex also demonstrated increased expression of miR-29b in both SIV and morphine-dependent macaques (Supplementary Figure 1).

Downregulation of PDGF-B in basal ganglia of SIV-infected macaques with opiate abuse. Our previous studies have demonstrated decreased expression of PDGF-B in neurons exposed to HIV proteins as well as in the brains of SIV-infected macaques.[15] Intriguingly, PDGF-B is a neurotropic factor whose downregulation has been shown to correlate with neuronal damage.[15] To understand the impact of opiate dependence on neuronal damage and, in turn, expression of PDGF-B, we examined by western blot and real-time PCR the levels of PDGF-B in the basal ganglia of untreated or SIV-infected macaques with or without drug dependence. As shown in Figure 2a, basal ganglia from SIV-infected, morphine-dependent macaques demonstrated significant decrease in the expression of PDGF-B compared with the untreated or SIV-infected groups. However, contrary to the decrease in protein levels, PDGF-B mRNA levels in the basal ganglia of SIV-infected, morphine-dependent macaques were upregulated, thereby suggesting posttranscriptional regulation of PDGF-B protein (Figure 2b).

CM from HIV-1 Tat and morphine-treated astrocytes downregulates PDGF-B expression in neurons. Having determined the effect of SIV-infection and morphine-dependence on downregulation of PDGF-B, the next step was to confirm these findings in purified cultures of SH-SY5Y neurons. We therefore sought to determine whether treatment of either a neuron cell line or primary rat neurons to exogenous morphine and/or HIV protein Tat (neurotoxin used here as a substitute for SIV/HIV infection in the CNS, as neurons are not directly infectable by the virus, but are affected by viral proteins) could downregulate expression of PDGF-B. Interestingly, treatment of SH-SY5Y cells or rat primary neurons with morphine (10^{-7} M; concentration based on previous findings[16]) and/or Tat protein (200 ng/ml) failed to decrease PDGF-B expression (data not shown). These finding are consistent with previous reports that neurons are more sensitive to Tat and/or morphine in the presence of astrocytes,[17] based on the fact that in the CNS microenvironment, neuronal homeostasis depends on continuous communication between the astrocytes and neurons.[18]

On the basis of these reports and the fact that astrocytes have pivotal roles in neuron survival via transport of nutrients and other substances to the neurons,[19] we sought to examine the effect of morphine and/or Tat on the ability of astrocytes to

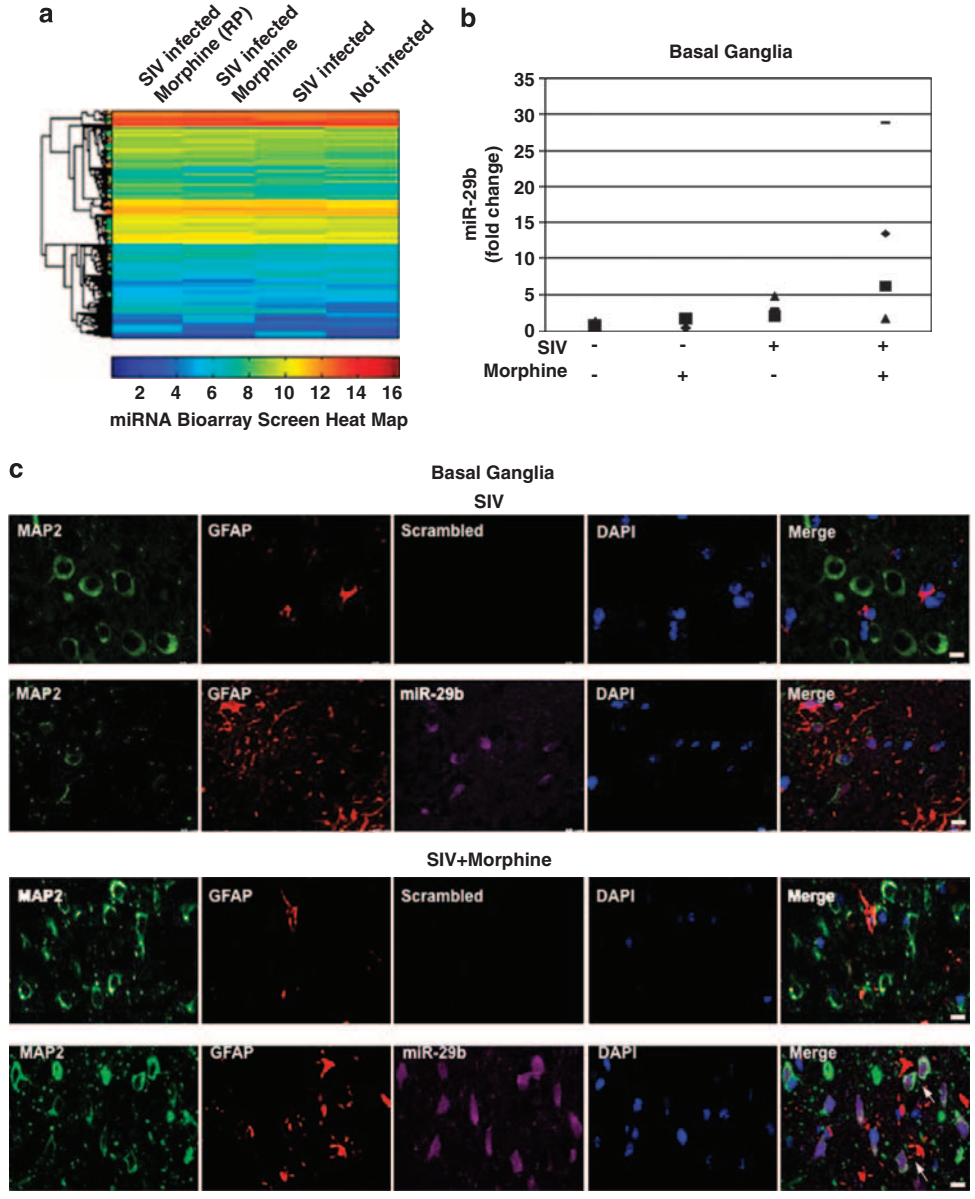

Figure 1 SIV and morphine alter miRNA profiling in basal ganglia of macaques. (**a**) Unbiased heat map correlating the expression of various miRNAs in the basal ganglia of SIV-infected macaques with and without morphine dependence, with colors indicating relative expression levels. (**b**) Real-time PCR demonstrating increased miR-29b expression in basal ganglia of SIV-infected and morphine-dependent macaques. (**c**) *In situ* hybridization demonstrating increased miR-29b expression in neurons adjacent to astrocytes (arrows) in the basal ganglia of morphine-dependent SIV-infected macaques. Scale bar = 10 μm

provide tropic support to neurons. The next series of experiments were thus done using CM collected from either rat primary astrocytes or human astrocytoma A172 cells treated with morphine and/or Tat for 24 h. The nomenclature used for various CM were as follows – untreated, control astrocyte CM (CACM); morphine-treated astrocyte CM; Tat-treated astrocyte CM (TACM); and morphine plus TACM (MTACM). As shown in Figure 3, exposure of either the rat primary neurons (Figure 3a), the SH-SY5Y cell line (Figure 3c) or differentiated (retinioic acid-treated) SH-SY5Y cells (Figure 3e) to MTACM resulted in decreased expression of PDGF-B compared with the exposure of same cells to CACM. However, exposure of neurons to CM from all the treatments failed to decrease PDGF-B mRNA (Figures 3b, d and f), thereby suggesting a posttranscriptional regulatory mechanism.

Exosomes released from astrocytes treated with morphine and Tat contain miR-29b that can be taken up by neurons. Exosomes are extracellular vesicles derived from cells that function as 'bioactive vesicles' to promote cell–cell communication and immunoregulation.[20] They have been isolated from bronchoalveolar lavage, CSF, urine, serum, bile and breast milk. Exosomes can shuttle various molecules, including miRNAs that can be transported to the recipient cells, and impair their functioning. Intriguingly, the

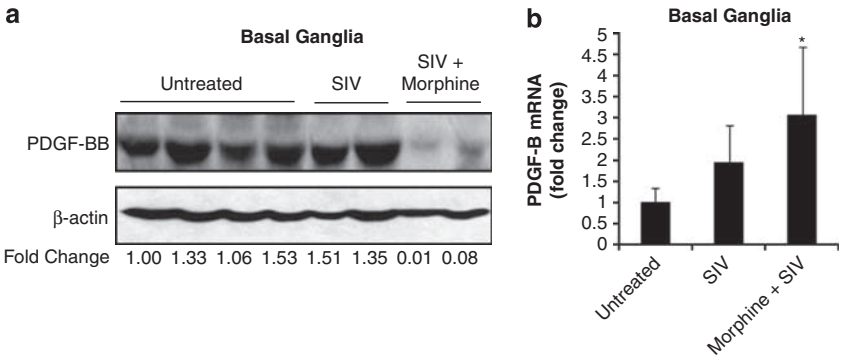

Figure 2 Downregulation of PDGF-BB protein in basal ganglia of SIV-infected macaques with opiate abuse. (**a**) Western blot analysis of PDGF-BB expression in basal ganglia of untreated or SIV-infected macaques with and without morphine dependence. (**b**) Real-time PCR analysis of PDGF-B mRNA expression in basal ganglia of SIV-infected macaques with and without morphine dependence. Bars represent the mean ± S.D. from three independent experiments. $^*P<0.05$ *versus* untreated group

number of exosomes as well as their miRNA content has been found to be elevated in various diseased states in humans.[21] For example, it has been reported that during inflammation, glial cells can release transmit signals and soluble mediators through exosomes, which, in turn, have critical roles in vascular and neuronal regeneration.[22,23] On the basis of these findings, we hypothesized that SIV/SIV protein Tat and/or morphine could alter the expression of astrocyte-released miR-29b, which, in turn, could be delivered via the exosomes to the neighboring neurons, resulting in targeted downregulation of the neurotrophic factor PDGF-B. In our efforts to test this hypothesis, we first monitored the release of exosomes in CM of astrocytes. Exosomes were readily detectable in A172 CM as assessed by electron micrography (Figure 4a) and western blotting (Figure 4b), using the antibody specific for exosome marker-Tsg101. Intriguingly, elevated miR-29b expression was detected in exosomes purified from CM of astrocytes treated with morphine and Tat, and this effect was inhibited by pre-treating the cells with either the exosome inhibitor (dimethyl amiloride, DMA) or the lipid-raft disruption drug (methyl-β-cyclodex, MβCD), both of which are known to inhibit exosome release (Figure 4c). Presence of miR-29 in the released exosomes was further confirmed by virtual northern blots (Figure 4d). In addition to miR-29b, there was also decreased expression of other irrelevant miRs, such as miR-221 and miR-222, in the exosomes purified from CM of astrocytes treated with morphine and Tat (Supplementary Figure 2). The next step was to examine the transfer of miR-29b from the astrocytes to the neurons. For this, neurons were cultured in the presence of exosomes released from astrocytes transfected with fluorescently labeled reporter miR-29. As observed in Figure 4f, neurons cultured with the isolated exosomes for 4 h demonstrated a fine granular fluorescent pattern within their cytoplasm, suggesting the incorporation of the fluorescently labeled reporter FAM-miR-29 within the neurons. Having determined the transfer of miR-29b from the astrocyte released exosomes to the neurons, the next step was to examine the functionality of the transferred miR-29b in the neurons by assessing the expression of its target gene, *PDGF-B*. Neuronal SH-SY5Y cells cultured in the presence of exosomes purified from CM obtained from either untreated or Tat- and morphine-treated

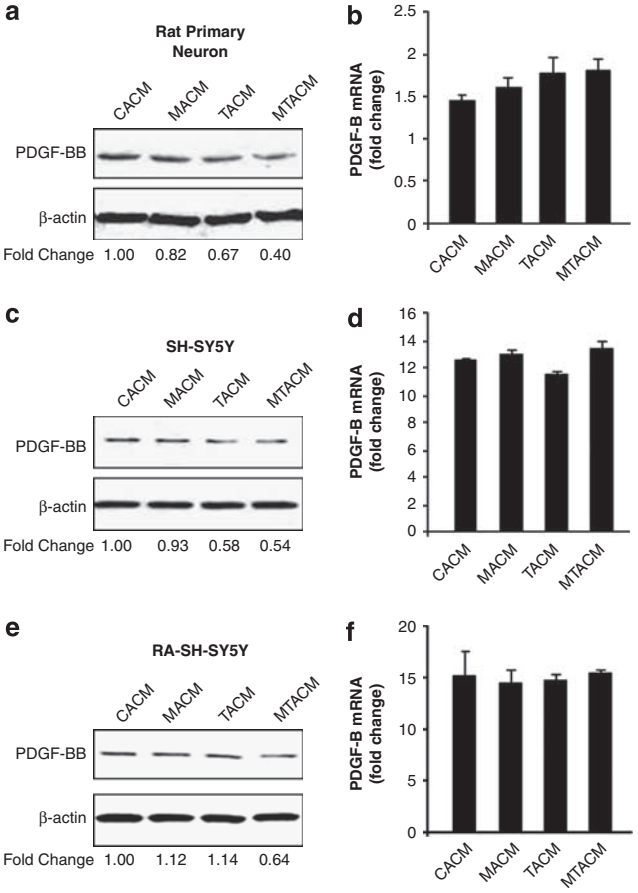

Figure 3 Conditioned media from HIV-1 Tat-and morphine-treated astrocytes downregulated PDGF-BB expression in neurons. (**a**, **c** and **e**) Western blot analysis of PDGF-BB protein expression in neurons treated with astrocyte conditioned medium. (**b**, **d** and **f**) Real-time PCR analysis of PDGF-B chain expression in neurons treated with astrocyte conditioned medium. Bars represent the mean ± S.D. from three independent experiments. NCM, nonculture medium; CACM, control astrocyte CM; MACM, morphine-treated astrocyte CM; TACM, Tat-treated astrocyte CM; MTACM, morphine and TACM

A172 cells were assessed for expression of the PDGF-B chain protein. As shown in Figure 4e, neuronal cells cultured with miR-29b containing exosomes purified from stimulated A172 cells demonstrated reduced expression of the survival

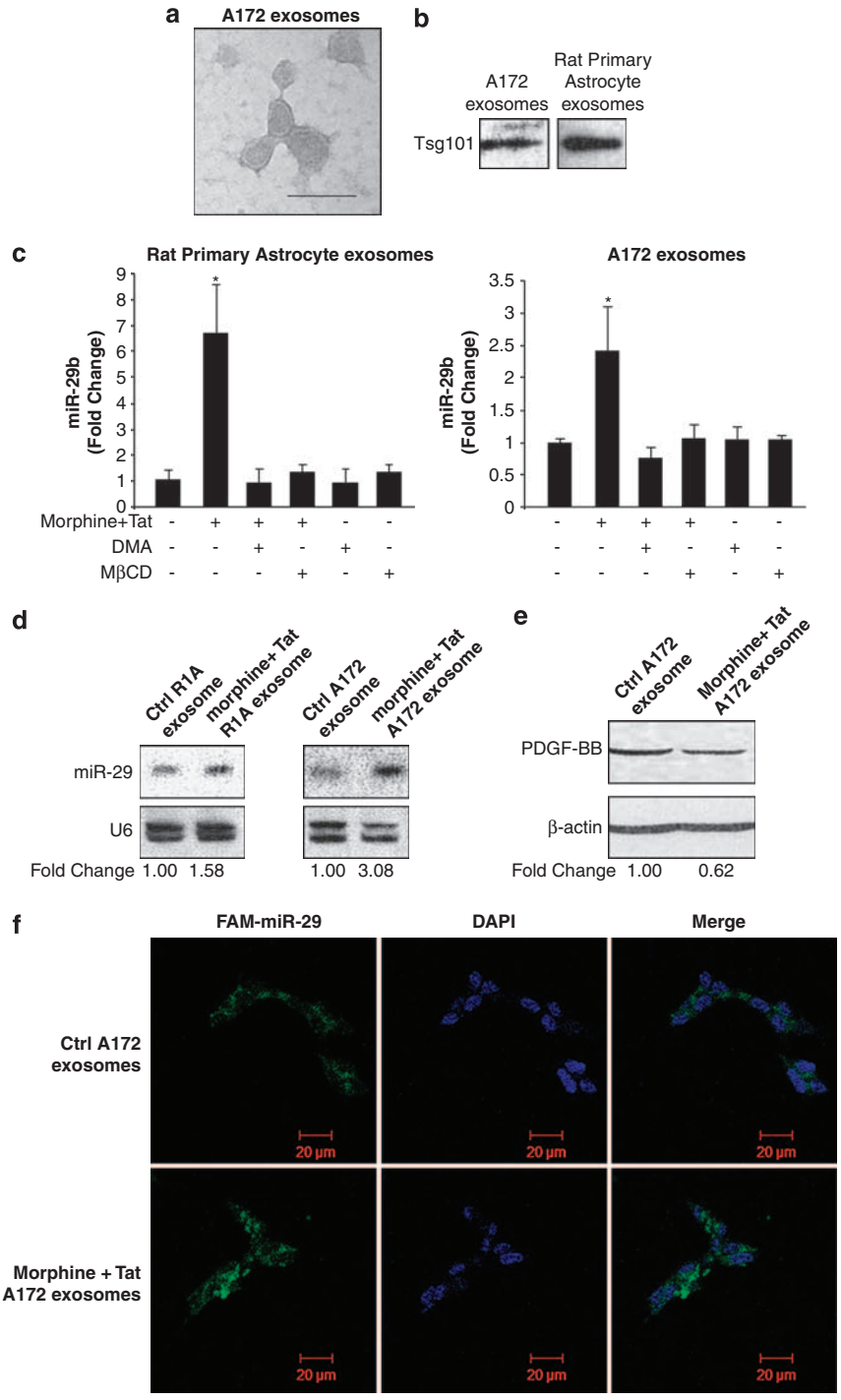

Figure 4 Exosomes released from astrocytes treated with morphine and Tat contain miR-29b that can be taken up by neurons. (**a**) Electron micrograph of A172 exosomes. The image shows small vesicles of approximately 50–80 nm in diameter. Scale bar = 100 nm. (**b**) Western blot characterization of astrocyte exosomes. Exosome protein released from astrocytes was separated on SDS-PAGE and electroblotted to nitrocellulose membrane. Blots were probed with exosome marker antibody against Tsg101. (**c**) Rat primary astrocytes and A172 cells were pre-treated with exosome inhibitor (DMA) or lipid-raft pathway inhibitor (MβCD) for 1 h (to inhibit exosome release), followed by morphine and Tat treatment exposure for 24 h. Total RNA from exosomes was then analyzed by real-time PCR for miR-29b. Bars represent the mean ± S.D. from three independent experiments. *$P < 0.05$ *versus* control. (**d**) MiR-29 was increased in morphine and Tat-treated A172 released exosomes, as assessed by virtual northern blot. Cells were exposed to morphine and Tat for 24 h. Exosomes were isolated and followed by virtual northern blot for miR-29. Small nuclear RNA RNU6B (U6) was blotted to ensure equal loading. (**e**) SH-SY5Y cells were incubated with exosomes released from control or morphine- and Tat-treated A172 cells for 24 h, followed by western blot for PDGF-BB. (**f**) Detection of miR-29 transferred into SH-SY5Y cells using FAM-labeled microRNA mimic (miR-29). SH-SY5Y cells were incubated for 3 h at 37 °C with exosomes isolated from A172 cells transfected with FAM-labeled miR-29 mimic (exosome FAM miR-29). The uptake of exosomes was evaluated by confocal microscopy. MiRNAs incorporated within SH-SY5Y cells were detected as green fluorescence signal of FAM fluorophores (original magnification × 400). Scale bar = 20 μm

gene – *PDGF-B*. This result was further confirmed by detection of miR-29b in SH-SY5Y cells using FAM-labeled microRNA mimics (miR-29), as shown in Figure 4f. Taken together, these findings implicate that Tat- and morphine-stimulated astrocytes release exosomes containing miR-29b, which can be taken up by neighboring neurons, resulting in downregulation of the expression of the target gene – *PDGF-B*.

MiR-29b targets PDGF-B 3′-UTR resulting in translational suppression. Having determined the uptake of exosomes containing miR-29b by SH-SY5Y cells, it was subsequently important to determine whether the transferred miR-29b, once inside the cells, could bind directly to the 3′-untranslated region (3′-UTR) of the target mRNA and inhibit its translation. SH-SY5Y cells were therefore transfected with a PDGF-B Luc reporter construct, wherein Luc expression was regulated by the PDGF-B 3′-UTR, an miR-29b potential binding element (Figure 5a). Cotransfection of SH-SY5Y cells with both miR-29b with PDGF-B-Luc constructs resulted in a significant decrease in Luc activity, suggesting thereby the preferential binding of miR-29b with the 3′-UTR of PDGF-B. As a negative control, neuronal cells were also cotransfected with a construct containing mutations in the miR-29-binding region of the 3′-UTR–PDGF-B. As expected, transfection of cells with the PDGF-B 3′-UTR Mut did not downregulate Luc activity (Figure 5b). Reciprocally, these findings were further validated by knocking down the expression of endogenous miR-29b by cotransfecting cells with anti-miR-29b. As expected, cotransfection with anti-miR-29b resulted in significant enhancement of Luc activity (Figure 5b). Further confirmation of the role of miR-29b in regulating the PDGF-B chain translation was carried out by overexpressing miR-29b in the neuronal SH-SY5Y cells, which were transfected with the precursor miR-29b and subsequently examined for the expression of PDGF-B protein. As shown in Figure 5c, cells transfected with the precursor miR-29b demonstrated reduced expression of PDGF-B protein compared with cells transfected with an unrelated precursor miRNA control. Reciprocally, and in contrast, transfection of anti-miR-29b (to knock down endogenous miR-29b) resulted in enhanced expression of target protein. These results thus underpin the role of miR-29b in regulating PDGF-B protein expression via a post-transcriptional mechanism.

MiR-29b shuttled through exosomes released from stimulated astrocytes regulates neuronal survival. To determine whether miR-29b transferred from the exosomes released in the CM of astrocytes stimulated with Tat- and morphine-controlled neuronal survival by regulating the survival factor PDGF-B, both rat primary neurons as well as SH-SY5Y cells (undifferentiated and differentiated) were treated with the respective CM (untreated and treated with Tat and morphine) and monitored for cell viability. As shown in Figure 6a, exposure of both primary as well as neuronal cell line with MTACM resulted in decreased cell viability compared with the cells treated with CACM. These findings were also confirmed using MAP-2 staining on cultured rat primary neurons. As shown in Figure 6b, MTACM exposure of neurons resulted in increased neuronal damage compared

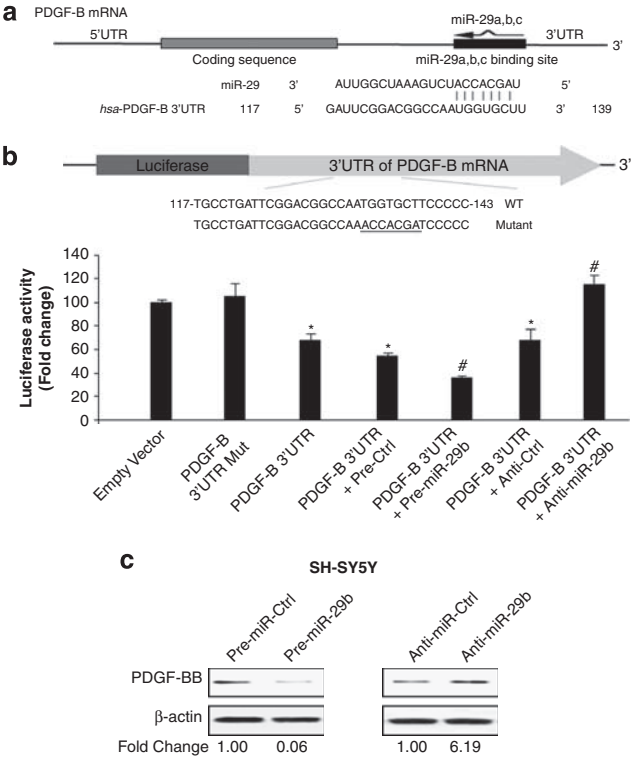

Figure 5 MiR-29b targets PDGF-B 3′-UTR resulting in translational suppression. (**a**) PDGF-B mRNA shows one potential binding site in the 3′-UTR for miR-29. (**b**) Binding of miR-29b to the potential binding site in the PDGF-B 3′-UTR resulted in translational suppression. SH-SY5Y cells were cotransfected with the reporter construct and miR-29b precursors anti-miR-29b for 24 h. Luc activity was then measured and normalized to the control β-gal levels. (**c**) SH-SY5Y cells were transfected with either miR-29b precursor or anti-miR-29b for 24 h followed by western blotting for PDGF-BB. Bars represent the mean ± S.D. from three independent experiments. $^{*}P<0.05$ *versus* control; $^{\#}P<0.05$ *versus* treated control

with cells exposed to CACM. To investigate whether miR-29b present in the Tat- and morphine-treated astrocyte CM regulated cell viability, SH-SY5Y cells were transfected with either the pre-miR-29b or anti-miR-29b for 48 h and subsequently assessed for cell viability using the MTT (3-(4,5-dmethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. As shown in Figure 6c, cells transfected with pre-miR-29b demonstrated decreased cell viability, and cells transfected with the anti-miR-29b, in contrast, exhibited increased cell viability compared with the controls. To further confirm the specificity of miR-29b, SH-SY5Y cells were transfected with miR-29 precursor in the presence or absence of PDGF-BB protein and assessed for cell viability. As shown in Figure 6c, cells transfected with miR-29b precursor exhibited decreased cell viability, and in contrast, transfected cells treated with PDGF-BB demonstrated a reversal of cell viability. To further examine whether exosomes, the potential carriers of miR-29b, in the stimulated astrocyte CM were the key factors involved in regulating cell viability, neurons exposed to purified exosomes isolated from CM of either untreated or HIV-1 Tat- and morphine-treated astrocytes were monitored 48 h later for cell viability using the MTT assay. As shown in Figure 6d, neurons cultured with purified exosomes isolated from

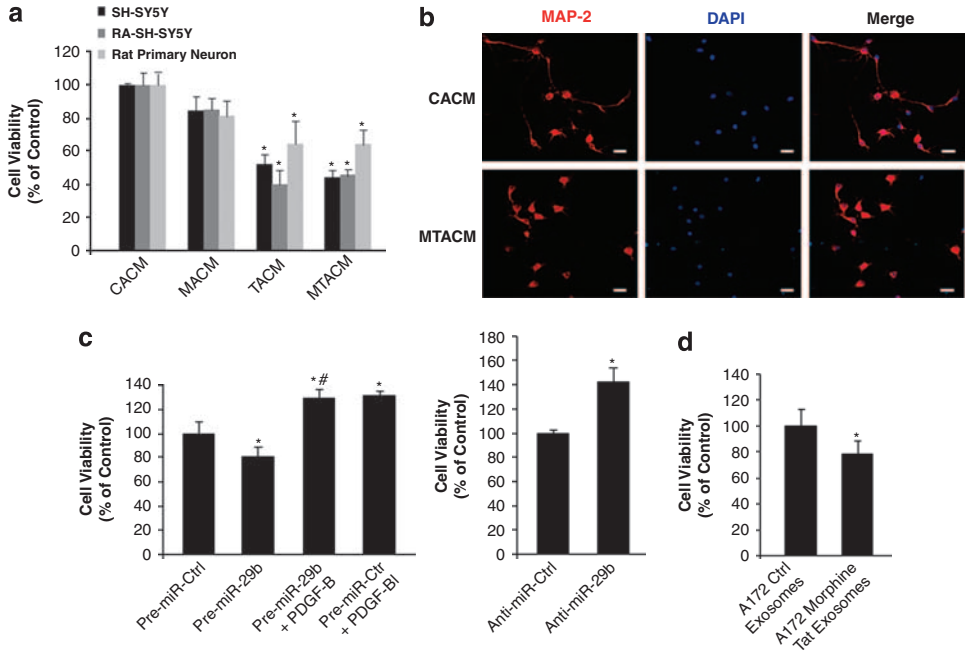

Figure 6 MiR-29b is involved in neuronal death induced by morphine and Tat astrocyte conditioned media. (**a**) Cell viability of rat primary neurons and SH-SY5Y cells (± retinoic acid) treated with the indicated conditioned media using the MTT assay after 48 h. (**b**) MAP-2 staining in rat primary neurons treated with indicated conditioned media. Scale bar = 10 μm. (**c**) Cell viability of SH-SY5Y cells transfected with miR-29b precursors with or without PDGF-B protein (100 ng/ml) or anti-miRs for 48 h using MTT assay. (**d**) Cell viability of SH-SY5Y cells treated with indicated exosomes for 48 h followed using MTT assay. Data are mean ± S.D. of three independent experiments performed in triplicate. $^{*}P<0.05$ *versus* control group; $^{\#}P<0.05$ *versus* treated control

stimulated astrocytes exhibited decreased viability likely via downregulation of PDGF-B expression in these cells.

Discussion

Despite the advent of anti-retroviral therapy, prevalence of HIV-1-associated complications is on the rise, affecting almost 60% of the infected individuals and thereby impairing their quality of life. Use of illicit drugs, a commonly observed feature among HIV-infected individuals, further compounds this problem. Combined HIV-1 infection and opiate dependence is becoming an emerging problem in the post-HAART era, as infected people continue to live longer. In fact, it is estimated that drug abuse may be the leading cause of HIV transmission in the United States. Brain tissues express a high number/density of μ-opioid receptors, making the CNS uniquely susceptible to the combined effects of opiate substance abuse and HIV-1 infection. The interplay of the two thus raises concerns regarding their effects on disease severity and rate of progression.[24]

Given the difficulties of studies on drug abusers, controlled studies on the effects of drugs are best done in animal models. Several studies have utilized the SIV/AIDS macaque model to better understand the influence of opiate dependency on HIV progression to AIDS. SIV infection in rhesus macaques is one of the best models of HIV-1 infection in humans, as the virus has a CCR5 phenotype similar to that of HIV.[4] Although SIV infection in macaques has proved to be an excellent model of HIV encephalitis, studies on the impact of morphine on SIVE, as to whether it protects or mediates increased pathogenesis, have been controversial in the field.[6,24] More recently, findings from our lab have demonstrated that morphine-dependence in SIV-infected macaques resulted in increased mortality in a subset of animals, and this was accompanied by exacerbated neuropathology.[5] We thus hypothesized that some of the adverse effects of opiates on HIV/SIV-infected CNS could be modulated by miRNA-mediated downmodulation of neuroprotective factors. We performed miRNA arrays on the RNA isolated from archival brain tissue (basal ganglia region – critical for HIV predilection) of uninfected and SIV-infected macaques with and without morphine dependence. Although significant numbers of miRNAs were differentially regulated by SIV and morphine, we focused our studies on miRNA-29b, which has potential binding sites on the PDGF-B 3′-UTR.

In the present study, we identified miR-29b targeting the 3′-UTR region of the neurotropic gene *PDGF-B* chain as a highly expressed miRNA in the basal ganglia region of SIV-infected, morphine-dependent macaques. To validate these findings in cell culture, we first treated neuronal cell line SH-SY5Y and rat primary neurons with morphine and HIV Tat to mimic the *in vivo* scenario. Intriguingly, treatment of neurons with the two agents did not result in upregulation of miR-29b. A possible explanation could be that neurons need signal from the astrocytes to respond effectively. Astrocytes have been shown to communicate to neighboring cells via gap junctions. For example, Eugenin *et al.*[25] have elegantly demonstrated the role of astrocyte gap junctions in mediating blood–brain barrier damage, possibly by triggering bystander cellular dysfunction and endothelial apoptosis. In the present study, we demonstrate another mechanism of cellular communication involving indirect intercellular transfer of miRNAs by exosomes during neuronal degeneration.

Neurons were treated with CM from astrocytes exposed to morphine and Tat, followed by the assessment for the expression of miR-29b. Our findings demonstrated that there was increased expression of miR-29b in exosomes released from astrocytes treated with morphine and Tat, and these exosomes could be taken up by the neurons, leading to neuronal death. A recent study demonstrated an increase in a different miRNA, miR-21, in SIV and HIV encephalitis in the absence of drugs of abuse.[13] Although expression was found in neurons in the infected brains, it is not known whether miR-21 can be induced in neurons or, similar to the current findings, in exosomes, or whether other mechanisms can lead to their transfer to neurons

There is ample evidence in literature that many cells either constitutively or following stimulation release vesicles or microparticles that can function as vehicles for intercellular communication. Basically, two modes of microvesicle release have been characterized: exocytosis of multivesicular bodies (MVBs) and the direct budding from the plasma membrane small vesicles. Meldolesi and colleagues[26] coined the term 'exosomes' for the MVB-derived microvesicles, whereas vesicles directly budding from the plasma membrane are referred to as 'shedding vesicles'. Previous studies have reported that exosomes express certain specific marker proteins such as ALix, Tsg101 and CD9.[22] In the brain, one of the functions of astrocytes is their bidirectional communication with other cells specifically neurons. Interestingly, Guescini *et al.*[22] have reported that astrocytes release exosomes carrying protein and mitochondrial DNA, which can be transferred between cells. Our *in vitro* data demonstrates that following exposure of astrocytes to HIV Tat and morphine, there is increased expression of miR-29b that is released via the exosomes and can be taken up by the neurons, resulting in concomitant decrease in PDGF-B expression in the latter cells. These studies highlight the significance of communication between astrocyte and neuron through exosome modulating miRNAs and their target gene *PDGF-B* in neurons.

It is intriguing to speculate that such a cell-to-cell communication could have advantages for transmission of signals from one cell to the other. As one of the functions of astrocytes is to maintain neuronal homeostasis, the exosomes/microvesicles mode of shuttling mRNAs and miRNAs could be an additional mechanism by the cells for regulating the genetic makeup of the recipient cells. Furthermore, the release of exosomes/microvesicles could be a means to allow a cell to communicate to another cell that is physically apart. Exosomes have been identified in body fluids such as cerebrospinal fluid, urine, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk, saliva and blood, which indicate organs might exchange information via exosomes.[27]

MiR-29a and b are highly expressed in the CNS. According to deep-sequencing studies by different groups, miR-29 is one of the most abundant miRNAs in the adult hippocampus and cortex.[28–30] In rodents, the expression of miR-29 increases with neuronal development and reaches highest levels in adulthood.[31] Moreover, miR-29 contributes to increased BACE1 and $\alpha\beta$ levels in sporadic Alzheimer's disease.[31] Although there is increased expression of miR-29b in the CNS, its expression in the basal ganglia is lower compared with other brain regions (Supplementary Figure 3). Furthermore, reports that miR-29 is more strongly expressed in astrocytes than in neurons[32] lends credence to our findings that miR-29b is shuttled via exosomes from stimulated astrocytes to neurons. Although our findings readily suggest that shuttling of exosomal miR-29 from astrocyte affects neuronal viability by targeting PDGF-B expression, we cannot directly evaluate the stoichiometric relationship between the amount of exosomal miR-29 and PDGF-B expression *in vivo*. In keeping with the findings on the role of dysregulated miR-29 in cardiac fibrosis, we also observed that even a minimal decrease in miRNA expression was accompanied by a robust increase in its target gene expression.[33] Intriguingly, in our studies we demonstrate that inhibiting miR-29b expression in neurons resulted in enhanced neuronal survival *in vitro*, whereas overexpressing miR-29b mimic suppressed neuronal viability. Tat and morphine could thus modulate the expression of miR-29b, resulting in downregulation of the neurotrophic factor PDGF-B in neurons. This is the first report to demonstrate that exosomes released from astrocytes can shuttle the miRNAs to neurons, thereby functionally regulating gene expression in neurons.

In addition to regulating the neurotropic factors, studies have also indicated that miR-29b can also function as an inhibitor of tumor growth and a stimulator of cell differentiation.[34,35] Furthermore, Nathans *et al.*[36] also identified that miR-29a can specifically target HIV-1 mRNA and represses viral replication by accumulating viral mRNA in P-bodies in human T lymphocytes. Our studies have revealed that PDGF-B is an important target for miR-29b in neurons. Upregulated expression of miR-29b in HIV/SIV and drug abusers, through repression of PDGF-B, and potentially other target genes such as *MCP-1*, could lead to deficits in neurocognitive functions as seen in HIV-infected drug abusers.

In conclusion, our data suggest that the exosomes released from astrocytes exerted neurotoxic effects on neurons. The mechanism of this effect is, at least in part, related to the increased miR-29b, which was taken up by neurons, leading to decreased PDGF-B expression. Regulation of miR-29b expression in exosomes released from astrocytes might be a putative therapeutic strategy against the pathogenesis of HIV-associated neurological disorder.

Materials and Methods

Macaques and viruses. Sixteen, 2- to 3-year-old, Indian strain rhesus macaques (*Macaca mulatta*) were purchased from the Caribbean Research Primate Center and individually housed in steel-holding cages in two dedicated rooms within the AAALAC-approved animal facility at the University of Kansas Medical Center. The monkeys were exposed to 12-h light–dark cycles daily and given laboratory chow and water *ad libitum* along with daily snacks. All cages were equipped with environmental enrichments. The animals were tested for tuberculosis, herpes B virus and simian retrovirus, and were found to be negative in all these tests. All animal protocols were approved by the local animal care committee in accordance with the Guide for the Care and Use of Laboratory Animals. The animals were randomly divided into two groups: SIV only (V, $n=4$) and morphine + SIV (M + V, $n=12$). Morphine was administered intramuscularly (IM) to M + V macaques 4 times daily at 6-h intervals, at a dose of 3 mg/kg with a 1-ml syringe (27.5 G needle). The macaques were gradually acclimated to morphine by starting with 1 mg/kg for 1 week and escalating to a final dose of 3 mg/kg in 1 mg/kg increments per week. The V-group animals were injected with saline IM at the same time intervals. Morphine administration was maintained

throughout the study to avoid withdrawal effects. For all animal studies, clinical-grade morphine was purchased from the University of Kansas Pharmacy and was used at a final dose of 3 mg/kg.[5]

Reagents. Morphine was purchased from R&D Systems (Minneapolis, MN, USA) and Tat1-72 was obtained from the University of Kentucky. Chemical inhibitors, including MβCD and DMA were purchased from Sigma (St. Louis, MO, USA). PDGF-B antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tsg101 and β-actin antibodies were purchased from Abcam (Cambridge, MA, USA) and Sigma, respectively.

Isolation of rat primary neurons and astrocytes. Primary cultures of rat neurons and astrocytes were prepared as described previously, with slight modifications.[37] Briefly, cortex tissues were dissected from embryonic day 18–19 Sprague-Dawley rats and dissociated with mild mechanical trituration. Dissociated cells were seeded firstly at 5–10^5 cells/well on 24-well culture plates, and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 10% fetal bovine serum and 1% antibiotic. After 1 day, the cultures were supplemented with serum-free neurobasal medium containing B27 (50 : 1), 2 mM glutamax and 1% antibiotic. Seven days following the initial plating, cultures consisted of 98% neuron-specific nuclear protein-immunoreactive (IR) neurons.

Rat primary astrocytes were prepared from whole brains of postnatal (1- to 2-day-old) Sprague-Dawley rats and plated on poly-D-lysine pre-coated cell culture flasks containing DMEM (10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin). The cells were grown in a humidified atmosphere of 5% CO_2/95% air at 37 °C. When the astrocytes reached confluence, they were passaged by trypsinization and plated at a density of 10^6 cells/well on 24-well culture plates in a final volume of 1 ml of DMEM and grown in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Two days later, the astrocytes were used for experimentation. Immunocytochemical analyses demonstrated that the cultures comprised of $>$95% glial fibrillary acid protein-positive astrocytes.

Cell culture and treatments. Neuroblastoma SH-SY5Y cells were plated at a density of 1×10^5 per ml and cultured in a 1 : 1 mixture of Eagle's minimum essential medium containing nonessential amino acids (Gibco, Gaithersburg, MD, USA) and F12 medium (Gibco) supplemented with heat-inactivated fetal bovine serum (10% v/v) and 2 mM glutamine at 37 °C in 5% CO_2. For some experiments, confluent cells were replated at 1–5 $\times$ 10^5 cells/ml and differentiated by treatment with 10 μM retinoic acid (Sigma) for 7 days with medium changes every 2 days. For the experiments with differentiated cells, the cells were serum-starved for 24 h in the presence of 10 μM retinoic acid (Sigma). Human astrocytoma cell line A172 (ATCC CRL-1620) were grown in DMEM supplemented with 10% fetal calf serum plus antibiotics.

Western blots. Whole-cell lysates were obtained from cells or tissues with the Mammalian Cell Lysis kit (Sigma). Equal amounts of the proteins were electrophoresed in a SDS-polyacrylamide gel (12%) under reducing conditions, followed by transfer to PVDF membranes. The blots were blocked with 5% nonfat dry milk in TBST. Western blots were then probed with antibodies recognizing the PDGF-B (1 : 1000, Santa Cruz Biotechnology), Tsg101 (1 : 1000, Abcam) and β-actin (1 : 5000, Sigma). The secondary antibodies were alkaline phosphatase conjugated to goat anti-mouse/rabbit IgG (1 : 5000) as described previously.[37] Densitometric levels of PDGF-B signals were quantified and expressed as their ratio to β-actin.

MiRNA microarray studies. Total cellular RNA was isolated from frozen (-80 °C) brain specimens by using TRIzol (Invitrogen, Carlsbad, CA, USA) for the monkey samples, followed by column purification (miRNeasy; Qiagen, Valencia, CA, USA) as per the manufacturer's instructions. Affymetrix microarray was performed by Asuragen, Inc. (Austin, TX, USA).

Real-time PCR. For quantitative analysis of mRNA expression, comparative real-time PCR was performed with the use of the SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). The sequences for the amplification of human PDGF-B were: 5′-TGTGGGCAGGGTTATTTAATATGG-3′ (forward) and 5′-CCATTGGCCGTCCGAATC-3′ (reverse). The primer sequences for the amplification of *GAPDH* were as follows: 5′-GCCAAAAGGGTCATCATCTC-3′ (forward); 5′-GGCATGGACTGTGGTCATGAG-3′ (reverse).

For analysis of miR-29b, total RNA was isolated from cells as described above. Comparative real-time PCR was performed by using the Taqman Universal PCR Master Mix (Applied Biosystems). Specific primers and probes for mature miR-29b and snRNA RNU6B were obtained from Applied Biosystems. All reactions were run in triplicate. The amount of miR-29b was obtained by normalizing to snRNA RNU6B and relative to control as previously reported.[38]

Virtual northern blots. MiR-29 and snRNA U6 real-time PCR products were run on a 15% Tris/Borate/EDTA (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA (pH8.3)) urea gel (Invitrogen) and transferred to a Nytran nylon transfer membrane (Ambion, Austin, TX, USA). An LNA DIG-probe for miRNA-29b (Exiqon, Vedbaek, Denmark) was hybridized using UltraHyb reagents (Ambion) according to the manufacturer's instructions, with blotted snRNA RNU6B as a control.

MiRNA *in situ* hybridization. *In situ* hybridization for miRNA was performed as described previously. Following deparaffinization and antigen retrieval with citrate, sections were pre-hybridized in hybridization buffer (50% formamide, 10 mM Tris-HCl, pH 8.0, 200 μg/ml yeast tRNA, 1 $\times$ Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, 10% dextran sulfate) for 1 h at 37 °C in a humidified chamber. LNA-modified miR-29b, labeled at both the 5′- and 3′-ends with digoxigenin (Exiqon), were diluted to a final concentration of 2 pM in hybridization buffer, heated to 65 °C for 5 min and separately hybridized to the sections at 37 °C overnight. The slides were then washed twice in hybridization buffer (without probe) at 37 °C, followed by washing three times in 2 $\times$ SSC and twice in 0.2 $\times$ SSC at 42 °C. They were then blocked with 1% BSA and 3% normal goat serum in 1 $\times$ PBS for 1 h at room temperature, and incubated with anti-digoxigenin conjugated with horseradish peroxidase (1 : 100, Roche Diagnostics GmbH, Mannheim, Germany) and anti-MAP2 (1 : 1500, Sternberger Monoclonals Inc., Baltimore, MD, USA) antibodies overnight at 4 °C. The slides were washed twice with PBS and incubated with Alexa Fluor 568 goat anti-mouse IgG (1 : 400, Invitrogen) antibody for 1 h at room temperature. This was followed by two PBS washes and signal amplification (for the *in situ*, now labeled with horseradish peroxidae) using TSA Cy5 kit (Perkin Elmer, Waltham, MA, USA) according to the manufacturer's protocol. The slides were mounted in Prolong gold anti-fade reagent with DAPI (Invitrogen).

Transient transfection assays. Transfections of miRNAs to A172 cells were performed using the Lipofectamine 2000 Transfection Reagent (Invitrogen) according to the manufacturer's protocol. FAM-conjugated MISSIONH microRNA Mimics (Sigma; miR-29 20 nM) were used in each experiment.

Exosome purification. Exosomes were prepared from the supernatant of rat primary astrocytes and A172 cells by differential centrifugations as previously described,[20] with some modifications. Briefly, cells were harvested, centrifuged at 1000 r.p.m. for 10 min to eliminate cells, and again spun at 10 000 r.p.m. for 30 min, followed by filtration through 0.22 μm filter to remove cell debris. Exosomes were pelleted by ultracentrifugation (Beckman Ti70 rotor, Brea, CA, USA) at 44 000 r.p.m. for 70 min. Exosomes were assessed for their protein content using BCA Protein Assay Kit (Pierce, Rockford, IL, USA). For real-time PCR, the RNA from the cell pellets was extracted by Trizol. Tsg101 was detected by western blot as an exosome marker.

Electron microscopy. Exosome pellets were prepared for negative staining and immunogold double-labeling employing a slightly modified procedure. Using wide-bore tips, 3 μl of exosome pellet was gently placed on 200-mesh formvar-coated copper grids, allowed to adsorb for 4–5 min, and processed for standard uranyl acetate staining. In the last step, the grid was washed with three changes of PBS and allowed to semi-dry at room temperature before observation in TEM (Hitachi H7500 TEM, Tokyo, Japan).

Luciferase activity assays. Complementary 37 bp DNA oligonucleotides containing the putative miR-29 target site within the 3′-UTR of human PDGF-B were synthesized and cloned into the multiple cloning sites of the pMIR-REPORT Luc vector (Ambion) as previously reported.[11] In this vector, the posttranscriptional regulation of Luc was potentially regulated by miRNA interactions with the PDGF-B 3′-UTR. Another pMIR-REPORT Luc construct containing mutant 3′-UTR (5′-TGGTGCT-3′ to 5′-ACCACGA-3′) was applied as a control. SH-SY5Y cells were transfected with each reporter construct, as well as anti-miR-29b or miR-29b precursor, followed by assessment of Luc activity 24 h after transfection. Luc activity was measured and normalized to control β-gal.

Immunohistochemistry. Immunohistochemical analysis of PDGF-B was carried out on archival paraffin-fixed tissue sections of brain from uninfected or

SIV-infected macaques with or without morphine dependence, as described earlier.[5] Sections from basal ganglia region of macaques were treated with rabbit polyclonal PDGF-B antibody (Santa Cruz Biotechnology), followed by treatment with Alexa Fluor 594-conjugated anti-rabbit secondary antibody (1 : 500; Invitrogen) for 30 min at room temperature and mounted in Slow Fade anti-fade reagent with 4,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR, USA). Images were captured using inverted fluorescence microscope TE2000-E (Nikon, Tokyo, Japan).

MTT assay. Cell viability was measured by the MTT method. Briefly, cells were collected and seeded in 96-well plates. Different seeding densities were optimized at the beginning of the experiments. Cells were exposed to nonculture medium or astrocyte-CM. After incubation for up to 48 h, 20 μl MTT tetrazolium salt dissolved in Hank's balanced salt solution at a final concentration of 5 mg/ml was added to each well and incubated in CO_2 incubator for 1–4 h. Finally, the medium was aspirated from each well and 200 μl of dimethyl sulfoxide was added to dissolve the formazan crystals, and the absorbance of each well was obtained using a plate counter at test and reference wavelengths of 570 and 630 nm, respectively. All experiments were repeated at least three times.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance with a *post hoc* Student's *t*-test. Results were judged statistically significant if $P<0.05$ by analysis of variance.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We thank Shannon Callen and Yaman Lu for their outstanding technical assistance. This work was supported by grants DA020392, DA023397, DA024442 (SB) from the US National Institutes of Health.

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Supplementary Information accompanies the paper on Cell Death and Disease website (http://www.nature.com/cddis)