

Original Article

Green Tea-EGCG reduces GFAP associated neuronal loss in HIV-1 Tat transgenic mice

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Abstract: In the current era of antiretroviral treatment, the prevalence of HIV-associated dementia is on the rise. Many past works have associated inflammation and neuronal loss with cognitive deficits inherent to the syndrome. Importantly, HIV-1 induced astrogliosis has been shown to play a central role in this process. Here we examined the effect of green tea derived (-)-epigallocatechin-3-gallate (EGCG) food supplementation for its ability to modulate GFAP expression and neuronal loss in an HIV-1 Tat transgenic mouse model whose expression was controlled by a brain specific doxycycline promoter. By immunohistochemistry we found that EGCG (300mg/kg/day) dramatically reduced astrogliosis as demonstrated by GFAP expression. This was accompanied by a mild reduction in activated microglia by Iba-1 staining and significant reduction in neuronal loss through apoptosis as demonstrated by MAP2 staining and Western blot analysis respectively. Future studies will be required to determine intracellular mechanism involved in EGCG mediated downregulation of GFAP and associated astrocytosis and neuronal loss.

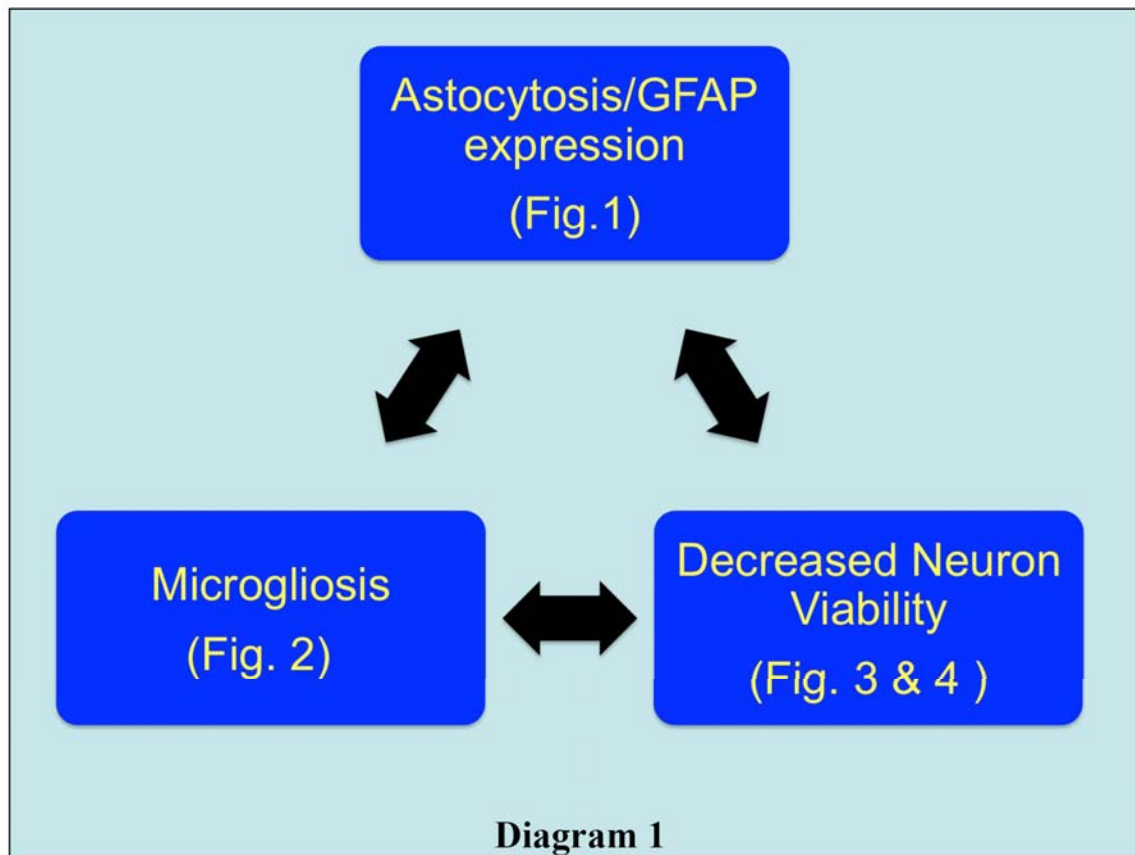
Key Words: HIV, Tat, dementia, astrocytosis, green tea, (-)-epigallocatechin-3-gallate (EGCG)

Introduction

HIV-associated dementia (HAD) is a neurodegenerative disease whose prevalence is on the rise in infected populations. It is believed HIV invades the central nervous system (CNS) through monocyte derived cells, primarily macrophages and microglia. Several studies in HIV patients and HIV-1 Tat transgenic mice have found that HIV resides in astrocytes as well [1-6] which serve as a reservoir for the virus in the brain [7-9]. Indeed, fifty percent of total cells in the brain consist of astrocytes. They have a homeostatic function for neuron support [10-11] as they have regulatory roles in synaptic transmission, and transportation of nutrients and metabolic intermediates to neurons in the brain.

Although there is a controversy whether the virus infects neurons directly, the virus does not replicate in neuron cells [12]. Thus, neuron death in HIV infected patients largely results from the release of viral proteins and neurotoxic factors from microglia, macrophages and astrocytes. Along with extensive reactive astrocytosis, AIDS patients show neuropathological characteristics such as brain atrophy, neuronal death/damage, and formation of multinucleated giant cells from fusion of infected microglia [13].

The main cells in the brain that produce cytokines due to HIV infection [14,15] and express HIV-1 proteins, including the Transactivator of Activation (Tat), are activated microglia [16] and astrocytes [17,18]. It is suggested that Tat, as an important protein that activates HIV-1 transcription, contributes



to HAD pathology and neurotoxicity in two ways. Through the direct pathway, Tat secreted from infected cells, microglia and astrocytes, can be taken up by other neighboring uninfected cells such as neurons [19]. In addition, Tat released from microglia can activate astrocytes which respond rapidly to the neurodegenerative signals resulting in astrocytosis [20] (**Diagram 1**). Importantly, activated astrocytes express glial fibrillary acidic protein (GFAP). The up-regulation of GFAP is considered as a pathological hallmark of brain injury and astrocytosis [21].

Through the indirect pathway, extracellular Tat expressed by astrocytes can contribute to neurodegeneration and impairment of the function of microglia and other astrocytes (**Diagram 1**) by inducing the release of high levels of neurotoxic factors such as IFN- γ from infected cells which are released in the extracellular environment and stimulate degeneration and apoptosis of neurons. For example, HIV-1 Tat protein inhibits the ability of microglia to clear beta amyloid plaques [22] which accumulate with aging and are characteristic pathological findings in many

patients with HAD and all of those with Alzheimer's disease. These plaques are often associated with increased GFAP levels and neuronal death. In addition to brain injury, GFAP expression is up regulated by HIV-1 Tat protein. Different studies in vivo and in vitro have shown that HIV-1 Tat protein induces directly the up-regulation of GFAP expression [18, 23, 24]. The transgenic mouse model utilized in the methods of the following experiment reflect this natural phenomenon as Tat expression is regulated by both the astrocyte-specific GFAP promoter and a doxycycline (Dox)-inducible promoter [23].

Although the highly active antiretroviral therapy (HAART) has reduced the clinical and pathological manifestations of HAD, it has not managed to eliminate and prevent the entry of the HIV-1 virus into the CNS [13]. On the other hand, numerous studies, including many studies in our lab, show that EGCG has multiple neuroprotective properties and it might be a potential therapeutic natural compound in the future to prevent neurodegeneration in HAD patients. This could be important as a vaccine for the

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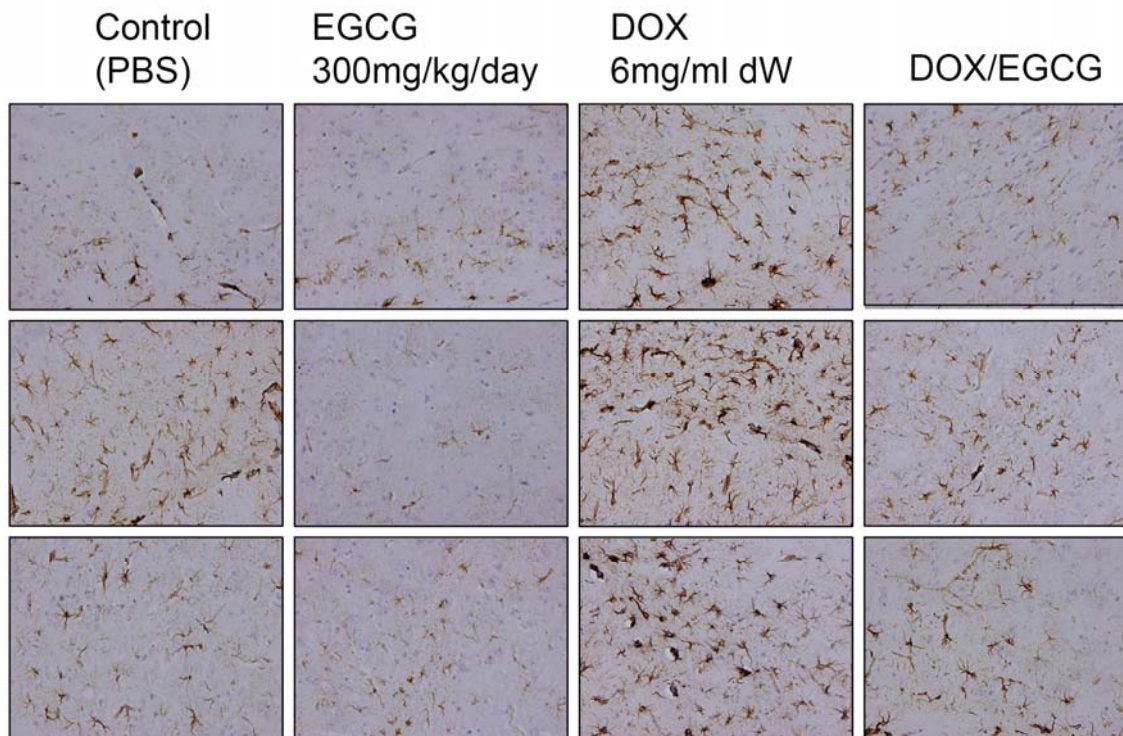


Figure 1. HIV-1 Tat mice treated with EGCG demonstrate marked reductions in astrogliosis after one week of treatment. Brains were harvested from each of the four mouse groups: (1) control (PBS), (2) EGCG treated without Dox induced Tat expression (EGCG), (3) PBS treated with Dox induced Tat expression (DOX), and (4) EGCG treated with Dox induced Tat expression (EGCG/DOX). Coronal, frozen mouse brain sections were stained with GFAP. A marked reduction of GFAP positive cells were observed when EGCG was administered to HIV-1 Tat mice receiving dox compared to those mice receiving dox but not receiving EGCG.

disease is many years away and treatment of HIV-1 symptoms and associated disease are paramount. Previous studies in our lab have shown that one mechanism through which EGCG protects nerve cells in the brain is through the inhibition of JAK/STAT 1 pathway [25]. In the current study, we tested the hypothesis that EGCG protects neuron cells through down-regulation of astrocytosis. After one week of dox-induced HIV-1 Tat expression, Tat transgenic mice were treated with EGCG at a dose of 300mg/kg/day in chow. Immunohistochemical and western blot analysis revealed that EGCG dramatically reduced GFAP expression/astrocytosis, and neuronal apoptosis which was accompanied by a mild reduction in microglia in brain regions examined.

Materials and Methods

Mice

Twenty four HIV-1 Tat transgenic mice whose expression was controlled by a brain specific-GFAP/Doxycycline (Dox) promoter [23] were divided in four groups: (1) control (PBS), (2) EGCG treated without Dox induced Tat expression (EGCG), (3) PBS treated with Dox induced Tat expression (DOX), and (4) EGCG treated with Dox induced Tat expression (EGCG/DOX). Groups were divided evenly between males and females. Transgene expression of GFAP and Tat86 in all 24 mice and Tat expression in brain was confirmed by genotyping and western blot respectively as previously described [23]. The dosing of dox was based on previous methods and administered at a concentration of 6mg/ml in drinking water for one week [23]. Dosing of EGCG in standard mouse chow (LabDiet®) was based on our prior experiments in Tat exposed wild type mice [25] and neuronal cells in vitro [22]. The mice were maintained at the University of South Florida College Of Medicine Animal Facility, and all experiments were in

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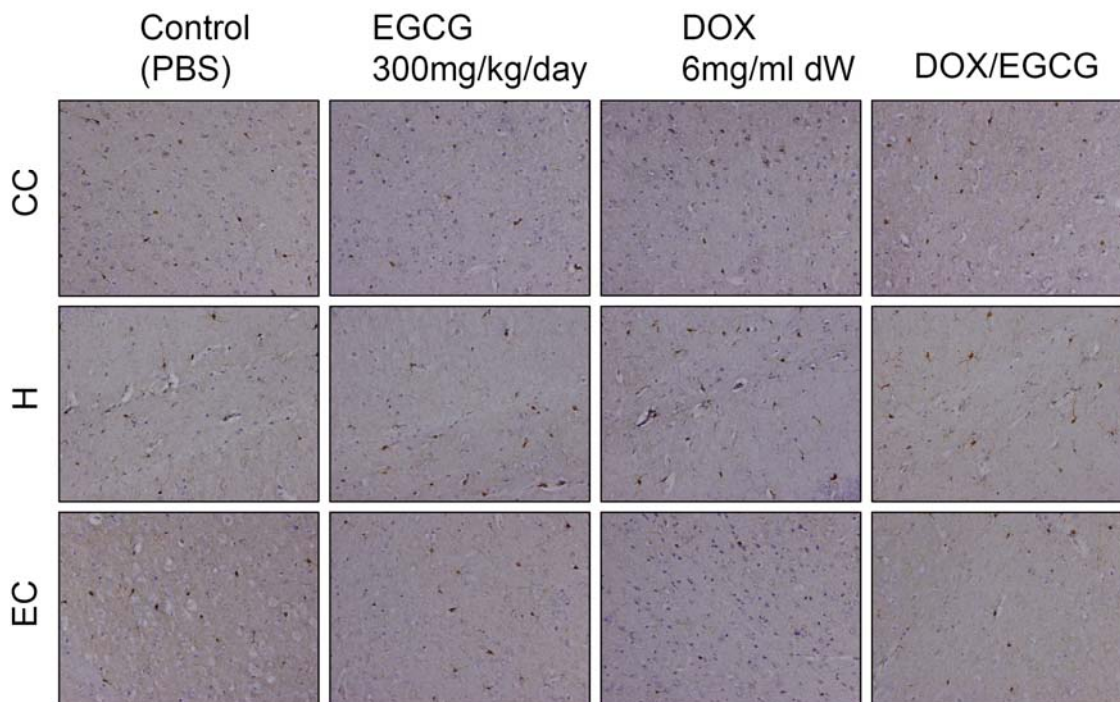


Figure 2. HIV-1 Tat mice demonstrate mild reductions in activated microglia after one week. Coronal, frozen mouse brain sections from the same four mouse groups were stained with Iba-1. A mild reduction of Iba-1 positive cells was observed when EGCG was administered to HIV-1 Tat mice receiving dox compared to those mice receiving dox but not receiving EGCG.

compliance with protocols approved by the University of South Florida Institutional Animal Care and Use Committee.

Immunohistochemical staining

Mice were anesthetized with isoflurane and transcardinally perfused with ice-cold physiological saline containing heparin (10 U/mL). Brains were isolated and quartered using a mouse brain slicer (Muromachi Kikai Co., Tokyo, Japan). The first and second anterior quarters were homogenized for Western blot analysis, and the third and fourth posterior quarters were used for cryostat sectioning. Brains were then fixed in 4% paraformaldehyde in PBS at 4 °C overnight and routinely processed in paraffin. 10 µm coronal brain sections were blocked with 5% horse serum for 1 hour at room temperature. Sections were incubated with primary antibody overnight at 4°C. Antibodies used were as follows: rat polyclonal anti-GFAP antibody (1:1000) (Zymed Laboratories, South San Francisco, CA) mouse polyclonal anti-Map2

antibody (1:500) (Chemicon), and rabbit polyclonal anti-Iba1 antibody (1:1000) (Wako, Osaka, Japan). Then, sections were incubated with the respective biotinylated secondary antibody (1:200) (Pierce Biotechnology, Inc.) for 1 hour at room temperature. After incubation with ABC reagent according to ABC vectastain kit instructions, sections were stained with diaminobenzidine tetrahydrochloride containing 0.01% hydrogen peroxide. To evaluate nonspecific staining, all recommended steps by the manufacturer were followed for the staining of the negative control with omission of the primary antibodies. The sections for GFAP, MAP2 and Iba1 were counterstained with hematoxylin and rinsed in deionized water. After dehydration in graded series of ethanol and xylene, the sections were mounted on microscope slides. Each section was examined under an Olympus IX71/IX51 microscope equipped with a digital camera system.

Western immunoblotting

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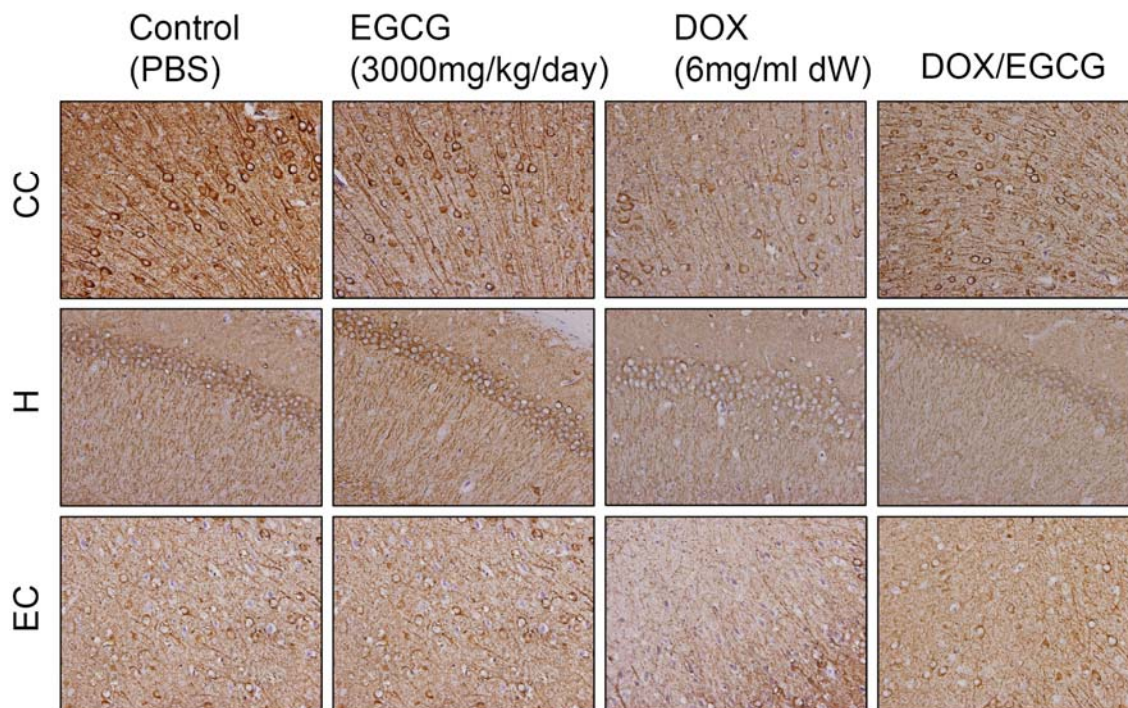


Figure 3. EGCG reduces neuronal loss in HIV-1 Tat mice . Coronal, frozen mouse brain sections from the same four mouse groups were stained with MAP2. A marked increase of MAP2 positive cells were observed when EGCG was administered to HIV-1 Tat mice receiving dox compared to those mice receiving dox but not receiving EGCG.

The first and second anterior quarters of the Tat transgenic mouse brains were homogenized for Western blot analysis and the protein concentration of the supernatant was measured by the BCA Protein Assay System (Pierce). Aliquots containing up to 110 μ g of total protein for HIV-1 Tat western blot and 45 μ g of total protein for Bax and Bcl-xL western blot were electrophoretically separated using 15% and 12 % SDS-PAGE gels respectively. Electrophoresed proteins were then transferred to immunoblotting nitrocellulose membranes (Bio-Rad), washed in Tris-buffered saline (TBS, Bio-Rad), 0.1% Tween-20, and blocked for 1 hour at room temperature in Tris-buffered saline/Tween-20 (0.1%) containing 5% non-fat dry milk. After blocking, membranes were hybridized overnight at 4°C with primary rabbit polyclonal antibody against HIV-1 Tat (Abcam Inc., Cambridge, MA), Bax and Bcl-xL (Cell Signaling Technology Inc., Danvers, MA) diluted 1:5000 for Tat and 1:1000 for Bax and Bcl-xL. Membranes were then washed 3 \times for 5 min each in TBS-T20 and incubated for 1 hour at room temperature

with the appropriate HRP-conjugated secondary antibody (1:10,000 for goat anti-rabbit and 1:2000 for goat anti-mouse, Pierce Biotechnology, Inc.). All antibodies were diluted in TBS/Tween-20 containing 5% non-fat dry milk. The protein bands were detected with a Super Signal west Femto Maximum Sensitivity Substrate (Pierce) and BIOMAX-MR film (Eastman Kodak Co.).

Results

EGCG decreases the number of Tat expressing astrocytes in the brain of Tat transgenic mice

HIV-1 Tat protein expression has been directly associated with up-regulation of GFAP gene in the astrocytes [18], an indicator of astrocyte activation in patients with HAD [26]. Previous works have confirmed that HIV-1 Tat transgenic mice express GFAP in tandem with Tat protein upon dox induction [23]. To determine the effects of EGCG in down-regulation of GFAP in Tat transgenic mice (and thus Tat expression) we performed

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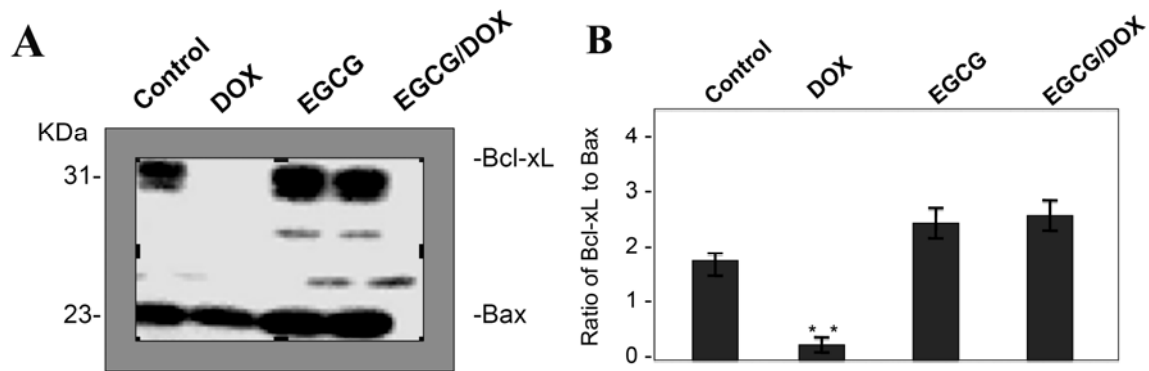


Figure 4. (A) Bcl-xL and Bax protein levels in mouse brain homogenates were analyzed by Western blot. Data are presented as mean \pm SD of Western blot band density ratio of Bcl-xL to Bax (n = 24; 12 female/12 male). (B) One-way ANOVA followed by post hoc comparison revealed significant differences in the band density ratio of Bcl-xL to Bax observed between Dox/EGCG or Dox compared to EGCG or control conditions, respectively (**P < 0.001).

immunohistochemical staining of brain tissue for GFAP. We found that mice which were treated with EGCG had dramatically less GFAP expression in the entorhinal and cerebral cortex as well as hippocampus as compared to the mice treated with dox only. In addition mice that received EGCG in their diet had significantly less GFAP expression in hippocampus, entorhinal and cingulate cortex compared to mice that received regular chow (Figure 1).

EGCG mildly reduces activated microgliosis

To visualize the density of activated microglia in hippocampus (H), Entorhinal cortex (EC), and cingulated cortex (CC), we performed Iba-1 staining of brain tissues. The results revealed a very mild elevation in Dox only treated groups compared to all others however this was considered non-significant (Figure 2).

EGCG enhances neuron survival

To determine the density of neurons in H, CC, and EC, we performed MAP 2 immunohistochemical staining of brain tissues. Staining for MAP 2, a marker for neuron cells, showed that HIV-1 Tat transgenic mice had greater neuronal loss than mice treated with Dox/EGCG (Figure 3). No significant differences were observed between the control group mice and non expressing Tat mice which received EGCG (Figure 3). Also, the western blot of brain homogenates for Bax and Bcl-xL

was performed to observe the degree of apoptosis in neuron cells induced by expression of Tat protein. (Figure 4a & b) One-way ANOVA followed by post-hoc comparison revealed significant differences between the Dox only group compared to all other groups for Western blot band density of Bcl-xL to Bax (**P<0.001).

Discussion

Through quantification of GFAP density, it has been suggested that neuropathogenesis of HAD in patients is most significant and starts in subcortical regions including entorhinal and limbic cortices [26]. Up-regulation of GFAP is associated with proliferation and activation of astrocytes. Since astrocytes are the main reservoir of HIV in the brain [6,8], it is to the virus' advantage to protect proliferation of its host [17,18]. Different studies have shown that Tat prolongs astrocyte survival [9,18], through various mechanisms [27,28]. These results are in accord with the dramatic astrocytosis seen in Dox only treated mice compared to those who received dox followed by EGCG treatment (Figure 1).

Further, astrocytes which have endocytosed Tat or are infected by HIV can no longer maintain brain homeostasis and have a detrimental role in neuron survival as reflected in Figure 3. This dysregulation of astrocyte homeostasis likely plays a central role in the death of neurons in HAD. Indeed, H/E staining

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revealed a disintegration of the granule cell layer in the hippocampus of Tat transgenic mice treated with Dox (Data not shown). Tat expression significantly reduced the ratio of Bcl-xL to Bax protein (**Figure 4b**). On the other hand, administration of EGCG to Tat transgenic mice increased the expression of Bcl-xL protein and reduced the expression of Bax protein; suggesting that EGCG inhibits neuron death by reducing GFAP expression/astrocytosis.

A healthy synaptodendritic network is vital for normal cognitive functioning. HIV-associated progressive impairment of cognitive skills and behavior in individuals infected with AIDS and experimental animals are directly correlated to loss of neurons and synaptodendritic degenerative changes [29,30]. Individuals with AIDS that demonstrate cognitive impairment have neuron loss, low synaptic density, and high synaptodendritic injury which are demonstrated by post-mortem immunostaining with MAP2 [30,31]. Here we show EGCG appears to oppose this pathology in HIV-1 Tat transgenic mice as demonstrated by MAP-2 staining (**Figures 3**) and significantly increased ratio (to near that of control) of Bcl-xL to Bax in EGCG treated Dox expressing mice compared to those not receiving EGCG (**Figure 4**).

Although introduction of (highly active antiretroviral therapy) HAART since 1996 has reduced the incidence rates of HAD [29] new cases of HAD continue due to development of drug resistance. Also many patients experience difficulty in following rigorously the complex HAART medication regimens.

Our results in Tat transgenic mice treated with EGCG suggest that EGCG opposes GFAP associated inflammation and ensuing neuronal apoptosis. In the face of obstacles to produce a vaccine against HIV due to continuous change through mutation, emerges the priority of finding innovative ways to prevent the damage of neuron cells from the HIV. Natural compounds such as the green tea derived flavanoid, EGCG, may provided protection of neurons against HIV in a time when the prevalence of HAD is increasing [22,25]. Studying more in depth the therapeutic and antiviral properties of EGCG in HIV infected humans is essential.

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