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RESEARCH PAPER

The cannabinoid CB_2 receptor agonist AM1241 enhances neurogenesis in GFAP/Gp120 transgenic mice displaying deficits in neurogenesis

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BACKGROUND AND PURPOSE

HIV-1 glycoprotein Gp120 induces apoptosis in rodent and human neurons *in vitro* and *in vivo*. HIV-1/Gp120 is involved in the pathogenesis of HIV-associated dementia (HAD) and inhibits proliferation of adult neural progenitor cells (NPCs) in glial fibrillary acidic protein (GFAP)/Gp120 transgenic (Tg) mice. As cannabinoids exert neuroprotective effects in several model systems, we examined the protective effects of the CB_2 receptor agonist AM1241 on Gp120-mediated insults on neurogenesis.

EXPERIMENTAL APPROACH

We assessed the effects of AM1241 on survival and apoptosis in cultures of human and murine NPCs with immunohistochemical and TUNEL techniques. Neurogenesis in the hippocampus of GFAP/Gp120 transgenic mice *in vivo* was also assessed by immunohistochemistry.

KEY RESULTS

AM1241 inhibited *in vitro* Gp120-mediated neurotoxicity and apoptosis of primary human and murine NPCs and increased their survival. AM1241 also promoted differentiation of NPCs to neuronal cells. While GFAP/Gp120 Tg mice exhibited impaired neurogenesis, as indicated by reduction in $BrdU^+$ cells and doublecortin$^+$ (DCX^+) cells, and a decrease in cells with proliferating cell nuclear antigen (PCNA), administration of AM1241 to GFAP/Gp120 Tg mice resulted in enhanced *in vivo* neurogenesis in the hippocampus as indicated by increase in neuroblasts, neuronal cells, $BrdU^+$ cells and $PCNA^+$ cells. Astrogliosis and gliogenesis were decreased in GFAP/Gp120 Tg mice treated with AM1241, compared with those treated with vehicle.

CONCLUSIONS AND IMPLICATIONS

The CB_2 receptor agonist rescued impaired neurogenesis caused by HIV-1/Gp120 insult. Thus, CB_2 receptor agonists may act as neuroprotective agents, restoring impaired neurogenesis in patients with HAD.

Abbreviations

ACEA, arachidonyl-2′-chloroethylamide; AEA, anandamide; BrdU, bromodeoxyuridine; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; DAGL, diacylglycerol lipase; DCX^+, doublecortin; EB, embryoid body; ES, embryonic stem cells; FAAH, fatty acid amide hydrolase; GFAP, glial fibrillary acidic protein; HAART, highly active antiretroviral therapy; HAD, HIV-associated dementia; HAND, HIV-associated neurocognitive disorder; NPC, neural progenitor cell; PCNA, proliferating cell nuclear antigen; SGZ, subgranular zone; SVZ, subventricular zone; Tg, transgenic

Introduction

Neurological impairment affects approximately 40% of HIV-infected patients. HIV-1 virus enters the CNS at the early phase of infection and induces motor and cognitive dysfunction and behavioural changes (Wiley *et al.*, 1999; Shah and Kumar, 2010). Despite the use of highly active antiretroviral therapy (HAART), the prevalence of HIV-associated neurocognitive disorder (HAND) is increasing and remains a significant risk factor for AIDS mortality (Cherner *et al.*, 2004; Price and Spudich, 2008). The HIV-1 envelope protein Gp120 is a mediator of HAND (Mattson *et al.*, 2005; McArthur *et al.*, 2010; Scott *et al.*, 2011) and decreases adult hippocampal neural precursor cell proliferation. Gp120 immunoreactivity has been demonstrated throughout the brain, including the hippocampus, in individuals infected with HIV-1 (Mattson *et al.*, 2005; Scott *et al.*, 2011). Gp120 is a potent neurotoxin that triggers inflammatory response by increasing production and secretion of proinflammatory cytokines such as TNF-α, IL-1β and IL-6 in HIV-1 encephalitis (Fauci, 1988; Shah and Kumar, 2010; Louboutin and Strayer, 2012). Gp120 also increases the number of activated microglia in the brain (Albright *et al.*, 2001).

The glial fibrillary acidic protein (GFAP)/Gp120 transgenic (Tg) mouse model reproduces some of the neurological, learning and memory deficits seen in patients with HIV-1 (Toggas *et al.*, 1994; 1996; Mucke *et al.*, 1995; Krucker *et al.*, 1998). Astrogliosis appears around 5–6 months and degeneration of neurons appears at 7–9 months in brains of these mice (Toggas *et al.*, 1994; 1996; Mucke *et al.*, 1995). These mice display deficits in neurogenesis in the hippocampus where adult neural progenitor cells (NPCs) have decreased proliferation via checkpoint kinase-mediated cell-cycle withdrawal and G_1 arrest (Okamoto *et al.*, 2007). The severity of damage in brains of GFAP/Gp120 Tg mice is correlated positively with the level of Gp120 expression in various brain regions (Toggas *et al.*, 1994).

The endogenous cannabinoid (endocannabinoid) system, an important lipid signalling system in modulating physiological responses in CNS and immune system, plays a role in modulating neurotoxic and inflammatory processes in the brain and exhibits neuroprotective properties (Ramírez *et al.*, 2005; Sarne and Mechoulam, 2005). This system comprises the endogenous ligands (endocannabinoids) chiefly anandamide (AEA) and 2-arachidonylglycerol, agonists at the cannabinoid receptor type 1 (CB_1) and cannabinoid receptor type 2 (CB_2) receptors respectively (receptor nomenclature follows Alexander *et al.*, 2013)

In vivo, cannabinoids decrease hippocampal neuronal loss and infarct volume after cerebral ischaemia (Nagayama *et al.*, 1999), sacute brain trauma (Panikashvili *et al.*, 2006) and ouabain-induced excitotoxicity (van der Stelt *et al.*, 2001). Recently, it has been reported that activation of CB_2 receptors promoted NPC proliferation via mTORC1 signalling, and inhibited HIV-1/Gp120-induced synapse loss between hippocampal neurons (Kim *et al.*, 2011).

Here we have examined whether selective agonists of the CB_2 receptor exerted neuroprotective effects on Gp120-mediated insults on NPCs *in vitro* and *in vivo*. We found that, *in vitro,* the CB_2 receptor agonist AM1241 inhibited HIV-1/Gp120-mediated apoptosis and enhanced the survival of both human and murine NPCs as well as their differentiation to neuronal cells. Further, administration of AM1241 to GFAP/Gp120 Tg mice *in vivo* enhanced neurogenesis in the hipppocampus.

Methods

Culture of murine embryonic stem (ES) cells and their differentiation to NPCs

NeuroCult proliferation medium was prepared with 1:9 ratio of NeuroCult Proliferation Supplements (#05701; StemCell Technologies Vancouver, Canada) to NeuroCult basal Medium (StemCell Technology #05700), supplemented with 20 $ng{\cdot}mL^{-1}$ of rhEGF (StemCell Technology #02633), 20 $ng{\cdot}mL^{-1}$ of rhFGF-b (StemCell Technology #02634), 20 $ng{\cdot}mL^{-1}$ of rmLIF (StemCell Technology #02740).

Formation of embryoid bodies (EBs) from murine ES cells was carried out using established protocols (www.lifetechnologies.com). Murine ES cells, E14/GFP cells, were obtained commercially from StemCell Technologies and were cultured following the protocol provided by StemCell Technologies. At day 4 of culture, EBs were harvested and placed into sterile polypropylene tubes. EBs were allowed to settle for about 5 min, the supernatant were aspirated and NeuroCult Proliferation medium was added. About 30 EBs per well or about 300 EBs per plate were seeded, supplemented with 100 nM of cannabinoid agonist. The cell cultures were incubated at 37°C with 5% CO_2 for 10 to 12 days, in a medium that was changed every 3 days. Cells were analysed by immunostaining, RT-PCR and Western blot as indicated.

Primary human and murine neural progenitor cells

We used primary normal human NPCs (hNPCs), from Chemicon (Catalogue Number SCC007; Upstate Chemicon, Billerica, MA). The hNPCs were characterized regularly by the human neural stem cell characterization kit (Catalogue Number SCR060; EMD Millipore, Billerica, MA), which contained the molecular markers Nestin, Sox 2 and Musashi.

These cells were maintained and cultured using the manufacturer's protocol (Chemicon, Inc.). In addition, we employed primary normal murine NPCs (mNPCs), commercially available from Chemicon, which were characterized by molecular markers for neural stem cells based on the manufacturer's protocol.

CB_1 and CB_2 receptor expression in human NPCs

RNA from human NPC was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. cDNA and PCR amplification were performed with the TITANIUM One-Step RT-PCR Kit (BD Biosciences, San Jose, CA). CB_1 was amplified using primers: 5′-CGT GGG CAG CCT GTT CCT CA-3′ and 5′-CAT GCG GGC TTG GTC TGG-3′, which yielded a product of 403 bp. CB_2 was amplified using: 5′-CCA TGG AGG AAT GCT GGG TG-3′ and 5-TCA GCA ATC AGA GAG GTC TAG-3′, which yielded a product of 1100 bp. GAPDH was used as a positive control with primers: 5′-CTC ACT GGC ATG GCC TTC CG-3′ and 5′-ACC ACC CTG TTG CTG TAG CC-3′, which yielded a product of 292 bp.

Western blots were used to analysis CB_1 and CB_2 receptor expression. NPCs were lysed in RIPA lysis buffer and the samples were separated by SDS-PAGE. The antibodies for human CB_1 and CB_2 receptors were from Cayman. SH-SY5Y human neuroblastoma cells were used as a negative control for CB_2 receptor expression and as a positive control for CB_1 receptors.

RT-PCR analysis of CB_1 and CB_2 receptor expression

RNA from total mES cells was extracted using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. A QIAshredder spin column and DNase digestion were included in the isolation procedure to limit the possibility of PCR amplification of CB_1 and CB_2 receptors from genomic DNA. cDNA and PCR amplification were performed with the TITANIUM One-Step RT-PCR Kit using 200 ng of RNA as a template for first-strand synthesis. CB_1 was amplified using primers: 5′-CGT GGG CAG CCT GTT CCT CA-3′ and 5′-CAT GCG GGC TTG GTC TGG-3′, which yielded a product of 403 bp. CB_2 was amplified using: 5′-CCG GAA AAG AGG ATG GCA ATG AAT-3′ and 5-CTG CTG AGC GCC CTG GAG AAC-3′, which yielded a product of 479 bp. GAPDH was used as a positive internal control with primers: 5′-CTC ACT GGC ATG GCC TTC CG-3′ and 5′-ACC ACC CTG TTG CTG TAG CC-3′, which yielded a product of 292 bp. The template was first denatured at 94°C for 2 min followed by 35 cycles (94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min), then by 68°C for 2 min in a myCycler Personal Thermal Cycler (Bio-Rad Laboratories, Inc). Aliquots (20 μL) of the PCR products were run on a 1.2% agarose gel containing 0.5 mg/mL ethidium bromide.

Animals

All animal care and experimental protocols complied with the guidelines from the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care & Use Committee of Beth Israel Deaconess Medical Center, affiliated with Harvard Medical School (protocol number #047-2-1226). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath et al., 2010). A total of 35 animals were used in the experiments described here.

C57BL16 CB1-I knockout mice and WT littermate controls were bred from founders that were generously provided by Dr. Andreas Zimmer (Rhemische Evedeurich Wilhelms University, Bonn, Germany). Female mice at 5-8 weeks were used, as indicated. GFAP/Gp120 Tg mice and their control littermates (C57BL16X 129s strain) were kindly obtained from Dr. Eliezer Masliah's lab at the University of California, San Diego. Female mice at the age of 7 months were used, as indicated.

Treatment of GFAP/Gp120 Tg mice and control littermates with CB_2 receptor agonist AM1241

Control littermates and GFAP/Gp120 Tg mice (eight mice per group per treatment) were either treated with vehicle control or with the CB_2 receptor agonist, AM1241 at 10 $mg{\cdot}kg^{-1}$. Mice received i.p. injection once a day continuously for 10 days, followed by 10 days of no treatment. After 10 days, these mice received again daily i.p. injection of AM1241 or vehicle control for another 10 days. Mice were then treated with bromodeoxyuridine (BrdU) for 5 days at 50 $mg{\cdot}kg^{-1}$ and after 30 days, neurogenesis in the brain of these mice was analysed as described by Rockenstein *et al.*, 2007. A diagram of the dosing regimen is shown in Fig 5A.

Analysis of neurogenesis and apoptosis in vivo

Neurogenesis in the hippocampus of GFAP/Gp120 Tg mice and control mice was assessed as described previously (Rockenstein *et al.*, 2007). Briefly, mice were anesthetized with chloral hydrate and perfused transcardially with 0.9% saline. Brains were removed and divided sagitally. One hemibrain was snap frozen and stored at -70°C for protein analysis and the other fixed in 4% paraformaldehyde (pH 7.4) at 4°C for 48 h and sectioned with a Vibratome 2000 (Leica, Germany).

For detection of markers of neurogenesis, sagittal sections (see above) of mouse brain were incubated with antibodies against BrdU (marker of dividing cells; rat monoclonal, 1:100, Oxford Biotechnology, Oxford, UK), proliferating cell nuclear antigen (PCNA, marker of proliferation; mouse monoclonal, 1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or doublecortin (DCX, marker of migrating neuroblasts; goat polyclonal, 1:500, Santa Cruz) overnight at 4°C. Sections were then incubated with biotinylated secondary antibodies directed against rat, mouse, or goat. After rinsing in Tris-buffered saline (TBS), avidin– biotin–peroxidase complex was applied (ABC Elite kit, Vector) followed by peroxidase detection with diaminobenzidine. For analysis of the proportion of $BrdU^+$ cells converting into neurons or astroglial cells, double immmunofluorescence labeling was performed with antibodies against BrdU and NeuN, and BrdU and GFAP. All sections

were processed under the same standardized conditions. The immunolabeled blind-coded sections were imaged with the LSCM (MRC1024, BioRad).

For detection of apoptosis in NPCs, the terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) detection method using the ApopTag *in situ* Apoptosis Detection Kit (Chemicon) was used with slight modifications. Detection was performed with Avidin-FITC and sections were mounted under glass coverslips with anti-fading media (Vector) for confocal microscopy analysis. To confirm that NPC undergo apoptosis, sections were double-labeled with a monoclonal antibody against activated caspase-3 (1:200, Stressgen Bioreagents, Ann Arbor, MI) and the polyclonal antibody against DCX (1:500, Santa Cruz), followed by incubation with Fluorochrome-labeled secondary antibodies and imaging on the LSCM.

Data analysis

All values are expressed as mean ± SEM. Analyses were carried out with the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Differences among group means were assessed by one-way ANOVA with *post hoc* Dunnett's test (when comparing to the non-Tg control group) or the Tukey–Kramer test (when comparing between treatment groups). Means of two groups were compared with the two-tailed unpaired Student's *t*-test. Correlation studies were carried out by simple regression analysis and the null hypothesis was rejected at the 0.05 level.

Materials

HIV-1/Gp120 protein from HIV-1 strain III B recombinant protein, originally isolated from a CXCR4-preferring strain of HIV-1 (ImmunoDiagnostics Inc. Woburn, MA, USA), was used at an optimal concentration of 1 nM as reported earlier (Iskander *et al.*, 2004, Walsh *et al.*, 2004; Bunnik *et al.*, 2010). This is based on dose responses and kinetics studies performed in our lab (data not shown). In some experiments, we used Gp120 R5 strain (Ba-L) of HIV-1 (ImmunoDiagnostics, Inc).

Anti-CB_1 and anti-CB_2 receptor antibodies (ABR-Affinity BioReagents, Golden, CO, USA) were used for immunostaining. Immunophenotyping of CB_2 receptors was confirmed by using another anti-CB_2 receptor antibody obtained from Sigma (St. Louis, MO, USA).

Arachidonyl-2′-chloroethylamide (ACEA), a selective agonist at CB_1 receptors, and the cannabinoid receptor antagonists AM251 (for CB_1) and AM630 (for CB_2) were purchased from Tocris Cookson (Ellisville, MO, USA). AM1241 is a CB_2 receptor agonist with a K_i volume of 2 nM and greater than 100-fold selectivity over CB_1 receptors *in vitro*. The antinociceptive actions of AM1241 are blocked by the selective CB_2 receptor antagonist AM630 (Ibrahim *et al.*, 2003; 2005). AM630 with $K_i = 31.2$ nM displays 165-fold selectivity for CB_2 over CB_1 receptors (Tocris). URB597, an inhibitor of fatty acid amide hydrolase (FAAH) was obtained from Cayman Chemical (Cat #10046; Ann Arbor, Michigan). Another CB_2 receptor agonist JWH-015 was used at 10 μM (Tocris Cookson). The CB_2 receptor agonist AM1241 was synthesized by Dr. A. Makriyannis.

Results

Expression of CB_1 and CB_2 receptors in human NPCs

First, we examined the expression of cannabinoid receptors in hNPCs. RT-PCR analysis (Figure 1A) and Western blot analysis (Figure 1B) showed hNPCs express both CB_1 and CB_2 receptors. The specificity of CB_1 and CB_2 antibodies was tested by Western blot and immunostaining analysis using cells positive for CB_1 receptors only (human SH-SY5Y; Figure 1C1) and cells positive for CB_2 receptors only (Jurkat T-cells; Figures 1 and 2). These results suggest that human NPCs express CB_1 and CB_2 receptors similar to murine NPCs. Therefore, we proposed that hNPCs might be responsive to the protective effects of cannabinoid receptor agonists in response to challenge with HIV-1/Gp120, as detailed below.

Effects of cannabinoid receptor agonists and HIV-1/Gp120 on human NPC proliferation

The CB_2 receptor agonist HU-308 is reported to stimulate NPCs (Palazuelos *et al.*, 2006). Thus, we examined the effects of another CB_2 receptor agonist AM1241 on hNPC proliferation, at various concentrations with or without HIV-1/Gp120. hNPCs were either untreated or treated with AM1241, with or without HIV-1/Gp120. After 7 days, the cells were fixed and immunostained with mouse anti-human Ki-67 PCNA expressed in the active stages of the cell cycle. The coverslips were counterstained with Hoechst 33342 to determine total cell numbers. As shown in Figure 2A, Gp120 inhibited hNPC proliferation and AM1241 prevented Gp120 from inhibiting hNPC proliferation. The optimal concentration was 1nM for Gp120 based on our preliminary experiments and earlier

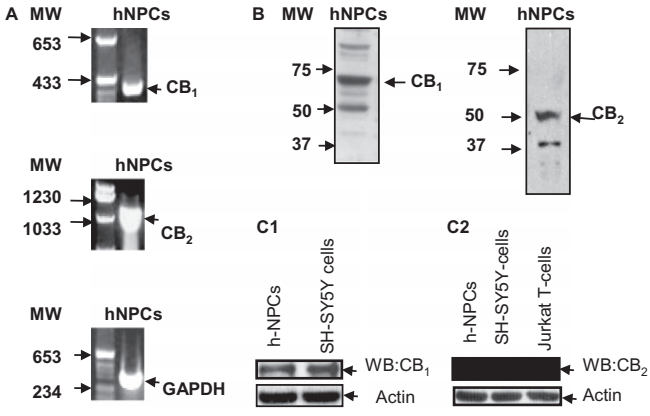

Figure 1

CB_1 and CB_2 receptor expression in human NPCs. (A) RNA from human NPC cells was extracted and then cDNA and PCR amplification were performed CB_1 was amplified and yielded a product of 403 bp. CB_2 was amplified and yielded a product of 1100 bp. GAPDH was used as a positive control yielding a product of 292 bp. (B, C) Western blot analysis of CB_1 and CB_2 receptor expression. NPC cells were lysed and the lysates were separated by SDS-PAGE. Lysates of the SH-SY5Y human neuroblastoma cell line were used as a negative control for CB_2 receptor expression and as a positive control for CB_1 receptors.

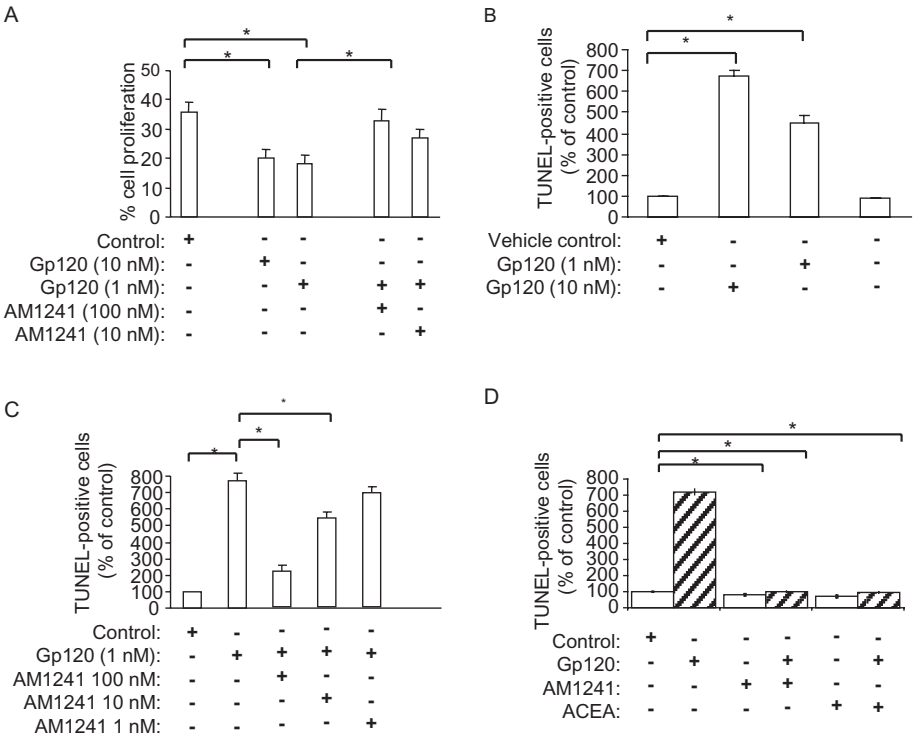

Figure 2

Functional analysis of the effects of CB receptor agonists on human NPCs following exposure to HIV-1/Gp120. (A) Gp120 and AM1241 were used as indicated. hNPCs were grown in the presence of control vehicle; varying concentrations of Gp120 (as indicated); or Gp120 (1 nM) and varying concentrations of AM1241 (as indicated) for 48 h. The cells were monitored and analysed by microscopy after 48 h, using MTT assay. hNPC survival was increased on addition of AM1241 to the Gp120-treated cells. *$P < 0.05$, significant differences as indicated; $n = 3$. (B) Effects of Gp120 and CB receptor agonists on apoptosis of hNPC cells. Human NPCs ($3x10^4$) were cultured on eight-well chamber slides coated with laminin and maintained in ReNcell NSC Medium with freshly added FGF and EGF. Cells were treated as follows: untreated, with Gp120, with CB receptor ligands and/or Gp120 for 48 h as indicated. (C) Effects of AM1241 on Gp120-induced apoptosis. Apoptosis was detected using TUNEL assay after 48 h. Nuclei were stained with DAPI. Negative control included cells treated with proteinase K and positive control included cells digested with DNase I. For quantification of cell death by the TUNEL assay, approximately 50 hNPCs were analysed in each experiment. The proportion of TUNEL-positive cells was increased significantly on addition of AM1241 to the Gp120-treated cells. (D) Effects of AM1241 (100 nM) and the CB_1 receptor agonist ACEA (1 μM) on apopotosis induced by Gp120 (1nM). *$P < 0.05$, significant differences as indicated; $n = 3$.

reports Iskander *et al.*, 2004 and Bunnik *et al.*, 2010). The optimal concentration was 100 nM for AM1241 (Figure 2A). Similar effects on hNPCs were obtained with the the CB_2 receptor agonist JWH-015 (data not shown). Thus, Gp120 (1 nM) decreased hNPC proliferation, and AM1241 inhibited Gp120-induced decrease proliferation of hNPCs. These studies (Figure 2A) are in agreement with reports that cannabinoids increased NPC proliferation *in vivo* (Aguado *et al.*, 2005).

Effects of HIV-1/Gp120 and cannabinoid receptor agonists on apoptosis of human NPCs

We then examined the effects of the CB_2 receptor agonist AM1241 on hNPC apoptosis, with or without treatment with HIV-1/Gp120. Detection of hNPC apoptosis was performed by TUNEL assay. Gp120 at 1 nM caused cell death (Figure 2B), which was inhibited by AM1241 optimally at 100 nM (Figure 2B, C). In addition, quantitative analysis of cell death by TUNEL assay revealed that AM1241 or the selective CB_1 receptor agonist ACEA significantly reduced cell death mediated by Gp120 (Figure 2D), indicating the importance of endocannabinoids in protecting hNPCs (Figure 2D).

Effects of Gp120 and cannabinoid receptor agonists on human NPC differentiation

To examine the effects of cannabinoid receptor agonists on hNPC differentiation to neuronal cells, we employed the known marker MAP-2 (Maurin *et al.*, 2009). Gp120 induced significant cell death of hNPCs and few MAP-2-positive cells were detected in samples treated by Gp120 alone (Figure 3A,B). However, differentiation of hNPCs to neuronal cells in the presence of AM1241 (data not shown) or URB597 was observed (Figure 3A). Further, increase in MAP-2 staining was observed in hNPCs treated with Gp120+AM1241 or Gp120+URB597, compared with the staining in control hNPCs (Figure 3A). Quantitative analysis of the effects of AM1241 and URB597 on differentiation of hNPCs to neuronal cells is shown in Figure 3B.

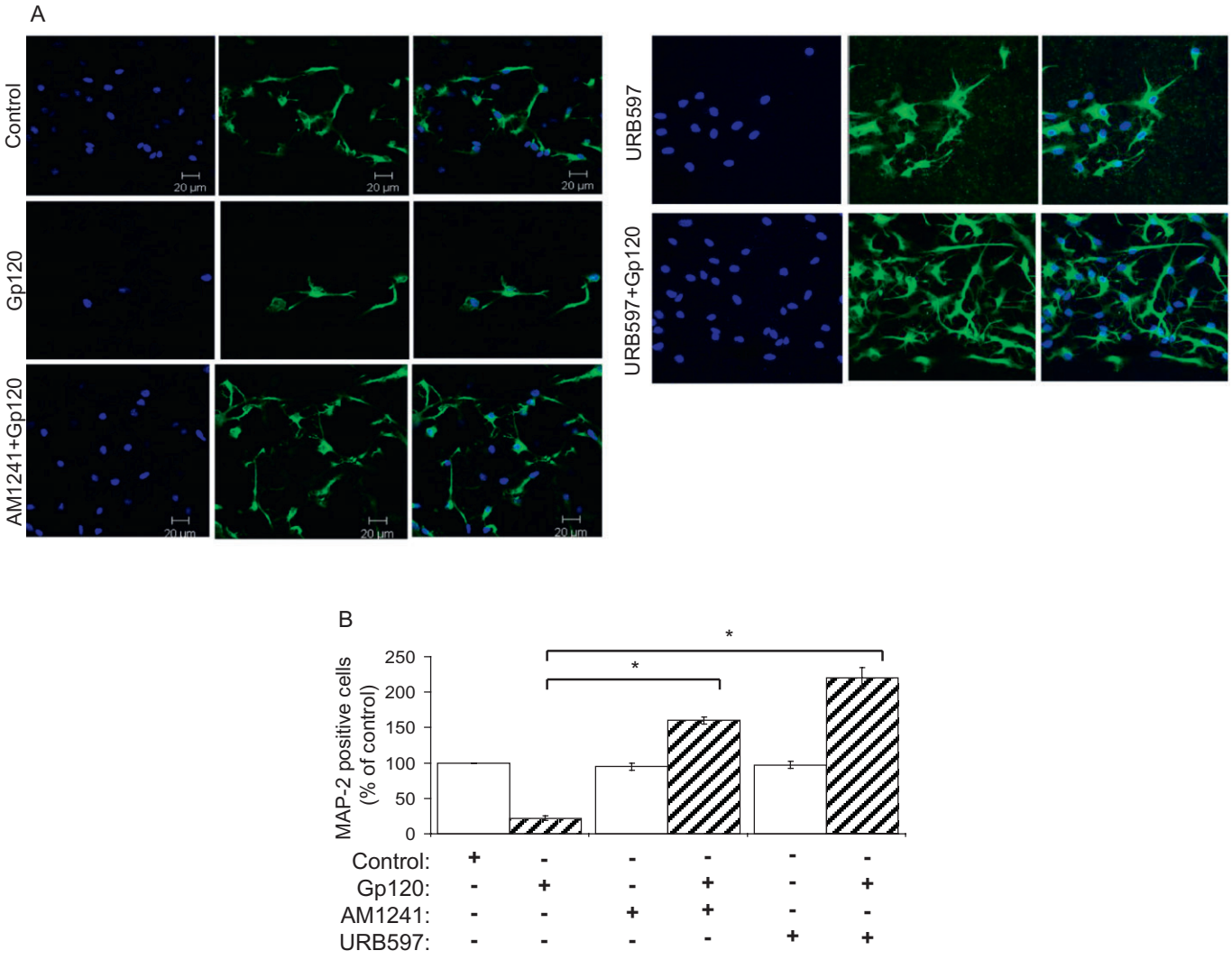

Figure 3

Effects of Gp120 and CB receptor agonists on hNPC differentiation. (A) Immunostaining analysis of positive neuronal cells derived from hNPC. hNPCs ($3x10^4$) were cultured on an eight-well chamber slide, as described above The cells were either treated or untreated with AM1241 (100 nM) or the FAAH inhibitor URB597, in the presence or absence of 1 nM of Gp120. The medium was replaced every 2 days and the cultures were kept for 2 weeks. Differentiation was detected by immunofluorescence staining using MAP-2 antibody, a marker for neuronal cells. Confocal microscopy analysis was performed (using a Zeiss LAM 510Meta). This is a representative experiment out of three experiments. (B) Quantification of neuronal cells derived from hNPCs, staining positive for MAP-2 antibody in the presence or absence of AM1241 or URB597. hNPCs were treated as indicated in Figure 3A. MAP-2-positive cells were determined with an optical fluorescence microscope analysing approximately 100 hNPCs from each experiment.* $P < 0.05$, significantly different from cells treated with Gp120 alone; $n = 3$.

The in vivo *effect of cannabinoid receptor agonists on murine NPCs*

To examine the effects of CB_2 receptor agonists on murine and hNPCs, we established conditions where mES cells can differentiate into NPCs. For this purpose, we first used the mES cell line E14/GFP cells, where GFP was tagged to mES cells (Figure 4A). These cells did not express CB_1 or CB_2 receptors (Figure 4B). However, NPCs derived from these mES cells showed CB_1 and CB_2 receptor expression by RT-PCR analysis and Western blot analysis (Figure 4C). The specificity of the CB_1 receptor antibody is shown by its reactivity to CB_1 receptors expressed in brain of WT mice but not in brain from $CB_1^{-/-}$ mice (see Figure 4D–E). NPCs isolated from adult mice showed similar pattern of CB_1 and CB_2 receptor expression as in NPCs derived from mES cells (data not shown). Functional assays showed that the CB_2 receptor agonist AM1241 inhibited Gp120-induced toxicity on murine NPCs. Specifically, AM1241 inhibited Gp120-induced apoptosis (Figure 4H). Further, AM1241 enhanced survival (Figure 4H) and increased differentiation of murine NPCs to neuronal cells (Figure 4I). These effects were mediated by CB_2 receptors as pretreatment of NPCs with the CB_2 receptor antagonist AM630 abolished these AM1241-mediated effects on survival and differentiation of NPCs (Figure 4H–I).

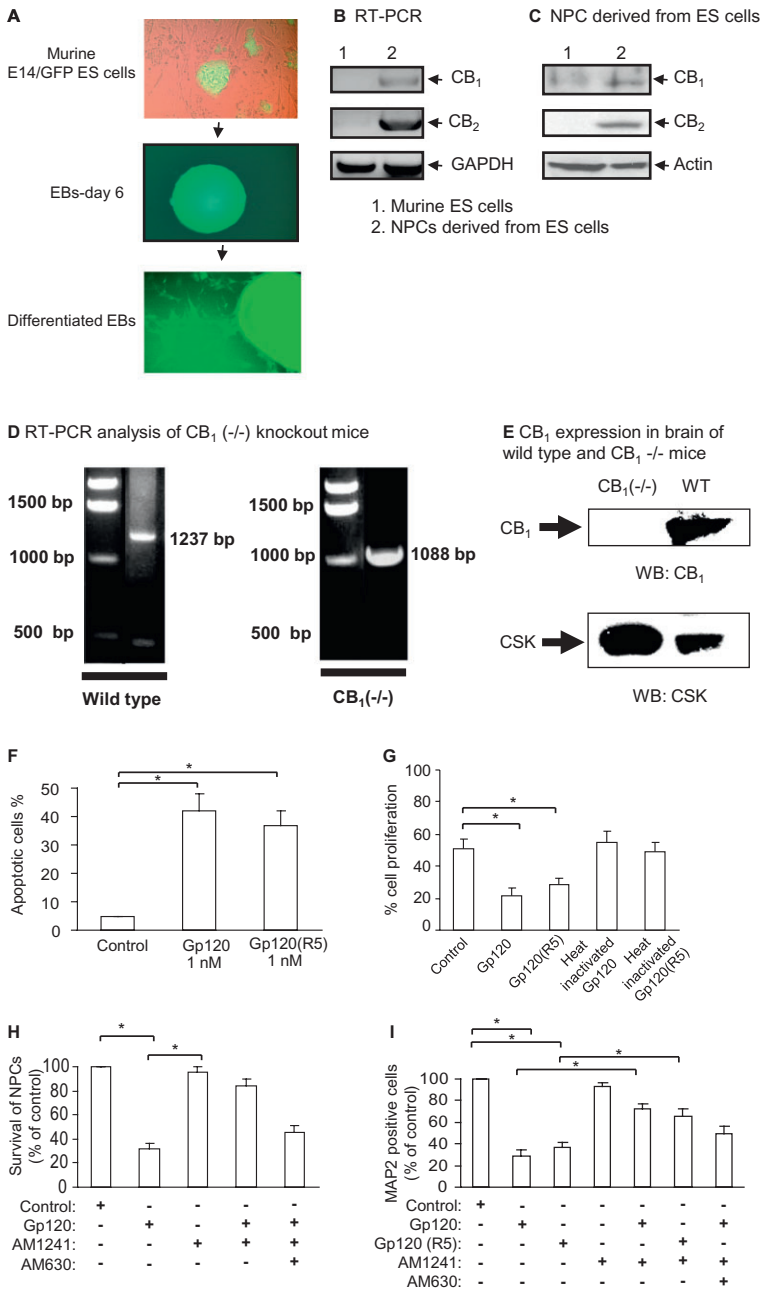

Figure 4

Expression of CB_1 and CB_2 receptors and functional analysis of neurogenesis in murine NPCs, derived from murine ES cells. (A) Differentiation of murine E14/GFP ES cells to EBs. (B) RT-PCR analysis of mES cells and neural progenitor cells derived from mES cells. Lane 1: murine ES cells; lane 2: neural progenitor cells derived from ES cells. (C) Western blot analysis of CB_1 and CB_2 in mES cells (lane 1) and neural progenitor cells derived from ES cells (lane 2). (D) Genotype screening and protein expression in CB_1 knockout and WT mice. The size of the cDNA fragments obtained by PCR for the WT mice was 1237 bp and 1088 bp for the CB_1 knockout mice. (E) Protein expression analysis by Western blot assay in brain total lysates derived from CB_1 knockout mice and WT mice. Brain tissues were obtained, lysed and analysed by Western blotting with specific antibodies against CB_1 receptors and tyrosine kinase C-terminal SRC kinase (CSK), as loading control. (F) Apoptosis: Gp120 was added to murine NPCs at 1nM for 48 h. Apoptotic cell death was determined by TUNEL assay. (G) Proliferation: murine NPCs were exposed to Gp120 (1 nM) or Gp120 (R5) (1 nM) or Gp120-denatured at 100°C for 15 min (1 nM) or control solution. Cells were grown in media containing low concentration of EGF and FGF (1 ng·mL^{-1}). After 7 days, the cells were fixed and immunostained with ki67. In addition, coverslips were counterstained with Hoechst 33342 (2 mg·mL^{-1}) to determine total cell numbers. To assess the percentage of cell proliferation, the number of ki67-positive cells as well as the Hoechst-stained cells were counted for five fields each per coverslip. (H) Survival: quantitation of murine NPCs following exposure to Gp120 for 48 h, untreated or treated with AM1241 (100 nM) in the presence or absence of CB_2 receptor antagonist AM630 (1 mM). Percentages of survival of NPCs were analysed using MTT assay. (I) Quantification of positive neuronal cells derived from murine NPCs stained with MAP-2 antibody in the presence or absence of CB_2 receptor agonist AM1241 and CB_2 receptor antagonist AM630, as indicated. In all panels (A–I), the data are presented as mean ± SEM (n = 3). *P < 0.05, significant differences as indicated.

The in vivo *effect of AM1241 on neurogenesis in brains of GFAP/Gp120 transgenic mice*

GFAP/Gp120 Tg mice display deficits in neurogenesis in the hippocampus (Okamoto *et al.*, 2007). To investigate if the alterations in neurogenesis in GFAP/Gp120 Tg can be reversed by CB_2 receptor agonists, 7-month old GFAP/Gp120 Tg mice and age-matched WT littermate control mice received AM1241 or vehicle control daily for 10 days, followed by 10 days of no treatment and again administered with vehicle control or AM1241 daily for 10 days (see protocol in Figure 5A). Next, a series of five BrdU injections were administered daily for 5 days and after 30 days (Figure 5A), the levels of markers of neurogenesis were analysed in the hippocampal subgranular zone (SGZ). As predicted, compared with the WT vehicle group, the GFAP/Gp120 Tg vehicle group displayed a reduction in the numbers of $BrdU^+$ (Figure 5B, a–b), doublecortin positive (DCX^+; Figure 5B, c–d) and $PCNA^+$ (Figure 5B, e–f) cells in the SGZ. Moreover, double labelling studies with antibodies against BrdU, NeuN and GFAP showed a decrease in neuroblasts converting to neurons (Figure 6A,B) and an increase in neural precursors converting to astroglial cells (Figure 6C,D) in the brain of GFAP/Gp120 Tg vehicle group, compared with control mice treated with vehicle. In contrast, analysis of the SGZ of the GFAP/Gp120 Tg mice treated with AM1241, compared with GFAP/Gp120 Tg mice treated with vehicle, showed an increase in $BrdU^+$ cells (Figure 5B, panels a,b); DCX^+ cells (Figure 5B, panels c,d); and $PCNA^+$ cells (Figure 5B, panels e,f). In addition, decreased numbers of GFAP-positive cells (Figure 6C,D) were observed in GFAP/Gp120 Tg mice treated with AM1241, compared with those in GFAP/Gp120 Tg mice treated with vehicle only. Thus, treatment with the CB_2 receptor agonist AM1241 prevented deficits in neurogenesis observed in GFAP/Gp120 Tg mice.

Discussion

This study focused on understanding the inhibitory effects of Gp120 on neurogenesis both *in vitro* and *in vivo* and the protective effects of CB_2 receptor agonists on this process. Using *in vitro* cultures of human and murine NPCs, we showed that two agonists for CB_2 receptors, JWH-015 and AM1241, inhibited Gp120-mediated toxicity in both murine (Figure 4) and human NPCs (Figures 1–3). AM1241 also promoted the differentiation of human and murine NPCs into neuronal cells (Figures 3 and 4I). Further, our *in vivo* data showed that administration of AM1241 significantly improved neurogenesis in GFAP/Gp120 Tg mice and decreased astrogliosis and gliogenesis (Figures 5 and 6; anti-inflammatory effects).

Several studies have confirmed the toxic effects of Gp120 on the neuronal population (Bari *et al.*, 2010). Neonatal rats treated systemically with Gp120 showed retardation in behavioral development (Corasaniti *et al.*, 2001). Gp120 induced apoptosis in the brain neocortex of adult rats treated with a dose of 100 ng of viral protein for 7 consecutive days (Corasaniti *et al.*, 2001). Bilateral injection of Gp120 (10–100 nM) into the intermediate medial mesopallium of chicken forebrain caused amnesia (Corasaniti *et al.*, 2001). Some studies show that Gp120 impairs memory retention in rodents (Corasaniti *et al.*, 2001). The ability of macrophages to produce pro-inflammatory cytokines like TNF-α and IL-1 may further increase neuronal cell death (Wyss-Coray *et al.*, 1996; Corasaniti *et al.*, 2001). In addition, HIV-1/Gp120, by activating FAAH and inducing neuronal apoptosis, enhanced AEA degradation (Maccarrone *et al.*, 2004).

The ability to isolate and differentiate CNS progenitor cells in culture allows studies to focus on functional and neurodevelopmental consequences of progenitor cell infection in the brain following exposure to HIV-1-derived toxins (such as Gp120). HIV-1/Gp120 is a potent neurotoxin in NPCs in which its effects are mediated through its interaction with the chemokine receptor CXCR4 expressed on hNPCs (Tran *et al.*, 2005). Using human NPCs, we were able to show that HIV-1/Gp120 inhibits proliferation optimally at concentration of 1 nM (Figure 2A) and induces apoptosis of in these cells (Figure 2B). The endocannabinoid system exerts an important neuromodulatory role in different types of synapses (Galve-Roperh *et al.*, 2009) and is involved in the regulating the fate of of neural cells. There is an orchestrated pattern of CB_1 receptor expression, endocannabinoid production and appearance of endocannabinoid-metabolizing enzymes during cortical development (Galve-Roperh *et al.*, 2009). In this regard, it is important to note that the selective CB_1 receptor agonist ACEA was also neuroprotective and inhibited Gp120-induced damage of NPCs *in vitro* (Figure 2D). Further, we demonstrated neuroprotective effect of the CB_2 receptor agonist AM1241 on neurogenesis in GFAP/Gp120 Tg mice *in vivo* (Figure 5). These mice display deficits in neurogenesis in the hippocampus. However, after administration of AM1241 to these mice, enhanced *in vivo* neurogenesis was observed as indicated by a significant increase in the number of neuroblasts and neuronal cells, increase in the number of $BrdU^+$ cells and DCX^+ cells as well as an increase in the number of $PCNA^+$ cells (Figure 5B), and a significant decrease in astrogliosis and gliogenesis (Figure 6). These results demonstrate that AM1241 reversed the impaired neurogenesis caused by HIV-1/Gp120. The one possible mechanism underlying the protective effects of AM1241 on neurogenesis might be via modulating inflammatory pathways, as demonstrated by decrease in astrogliosis and gliogenesis (Figure 6). Indeed, endocannabinoids protect neurons during CNS inflammation induced by MKP-1 microglial cells (Eljaschewitsch *et al.*, 2006).

The proliferating cells in the adult mouse subventricular zone (SVZ) express DAG lipases (DAGLs), enzymes that synthesize the endocannabinoid agonist ligands (Goncalves *et al.*, 2008). While both DAGL and CB_2 receptor antagonists inhibited the proliferation of cultured neural stem cells and the proliferation of progenitor cells in young animals, CB_2 receptor agonists stimulated progenitor cell proliferation *in vivo* (Goncalves *et al.*, 2008). Earlier studies showed that CB_2 receptor agonists stimulated NPC proliferation as analysed in NPC cultures and in $CB_2^{-/-}$ mice using positive $BrdU^+$ cells (Palazuelos *et al.*, 2006). Agonists at CB_2 receptors promoted NPC proliferation via mTORC1 signalling (Palazuelos *et al.*, 2012). However, these studies lack the detailed analysis of the *in vivo* neurogenesis in these mice, and therefore, it is not

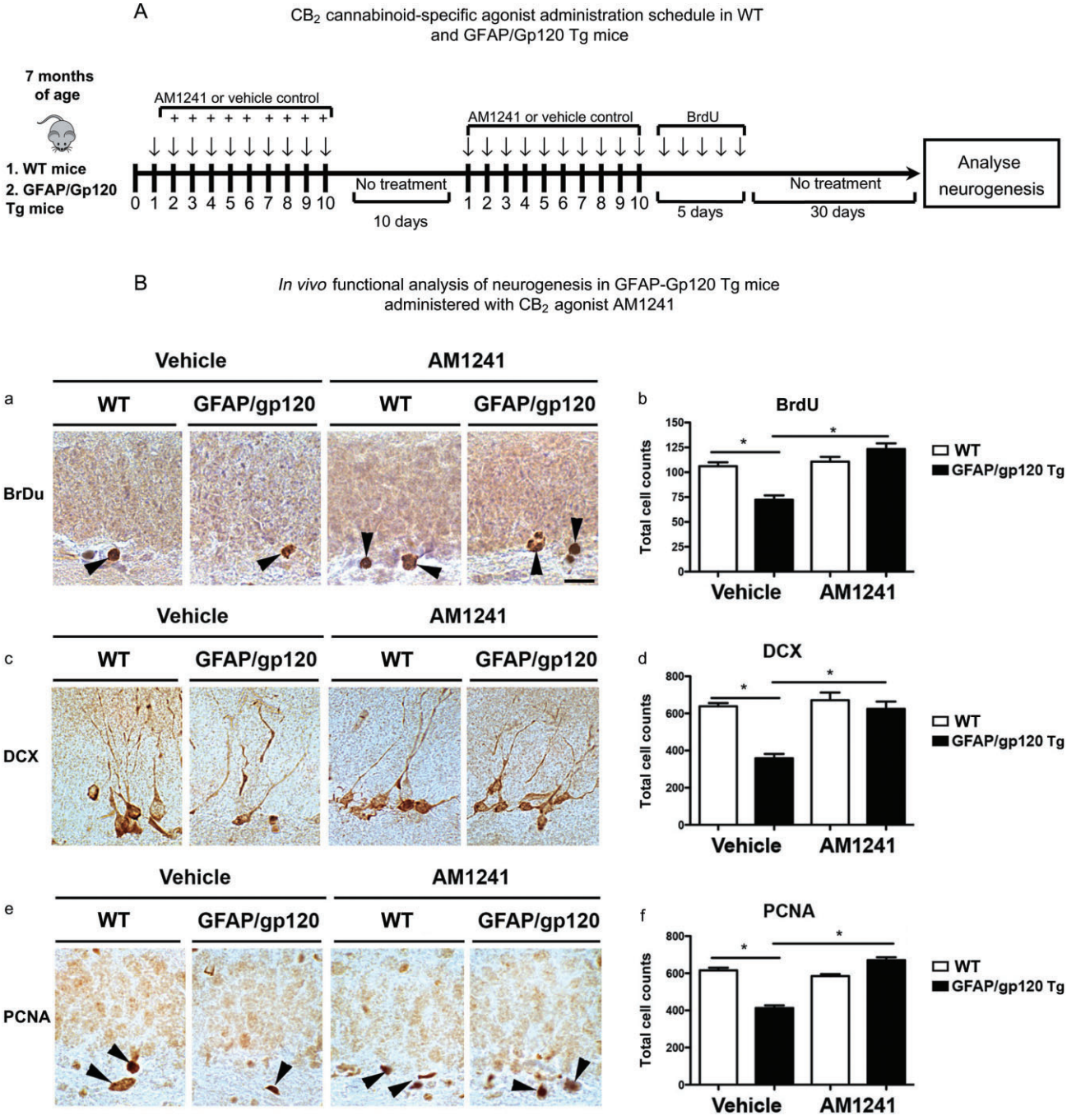

Figure 5

Effects of AM1241 on neurogenesis *in vivo*. (A) GFAP/Gp120 Tg mice and their WT littermate control were either treated with vehicle control or with AM1241 (10 mg·kg^{-1}) for the first 10 days. No treatment was given for the following 10 days. Then, AM1241 or vehicle control was administered daily for the following 10 days. All mice then received BrdU (50 mg·kg^{-1}) by injection for 5 days, and the experiments were terminated after 30 days and brains were analysed for neurogenesis. (B) Analysis of neurogenesis in GFAP/GP120 transgenic mice and WT mice, treated with AM1241. (a–b) Immunohistological analysis of BrdU$^+$ cells and quantitative analysis in the SGZ of BrdU$^+$ cells in GFAP/Gp120 Tg mice treated with AM1241. *P < 0.001, significantly different from WT mice treated with AM1241; Student's t-test; n = 8 per group. (c–d) Immunocytochemical analysis of DCX$^+$ and quantitative analysis of DCX$^+$ cells using the disector method in the SGZ shows increased numbers of DCX$^+$ neurons in GFAP/Gp120 Tg mice after AM1241 treatment, compared with vehicle control treatment. *P < 0.001. GFAP/Gp120 Tg with vehicle significantly different from WT mice with vehicle control; **P < 0.001, GFAP/Gp120 Tg with AM1241 significantly different from GFAP/Gp120 treated with vehicle; one-way ANOVA with *post hoc* Dunnett's test; n = 8. (e–f) Immunocytochemical analysis of PCNA$^+$ cells in GFAP/Gp120 Tg and WT mice treated with vehicle or AM1241. Quantitative analysis using the disector method in the SGZ showing the numbers of PCNA$^+$ NPC cells. *P < 0.001, GFAP/Gp120 Tg treated with AM1241 significantly different from vehicle; one-way ANOVA with *post hoc* Dunnett's test; (n = 8).

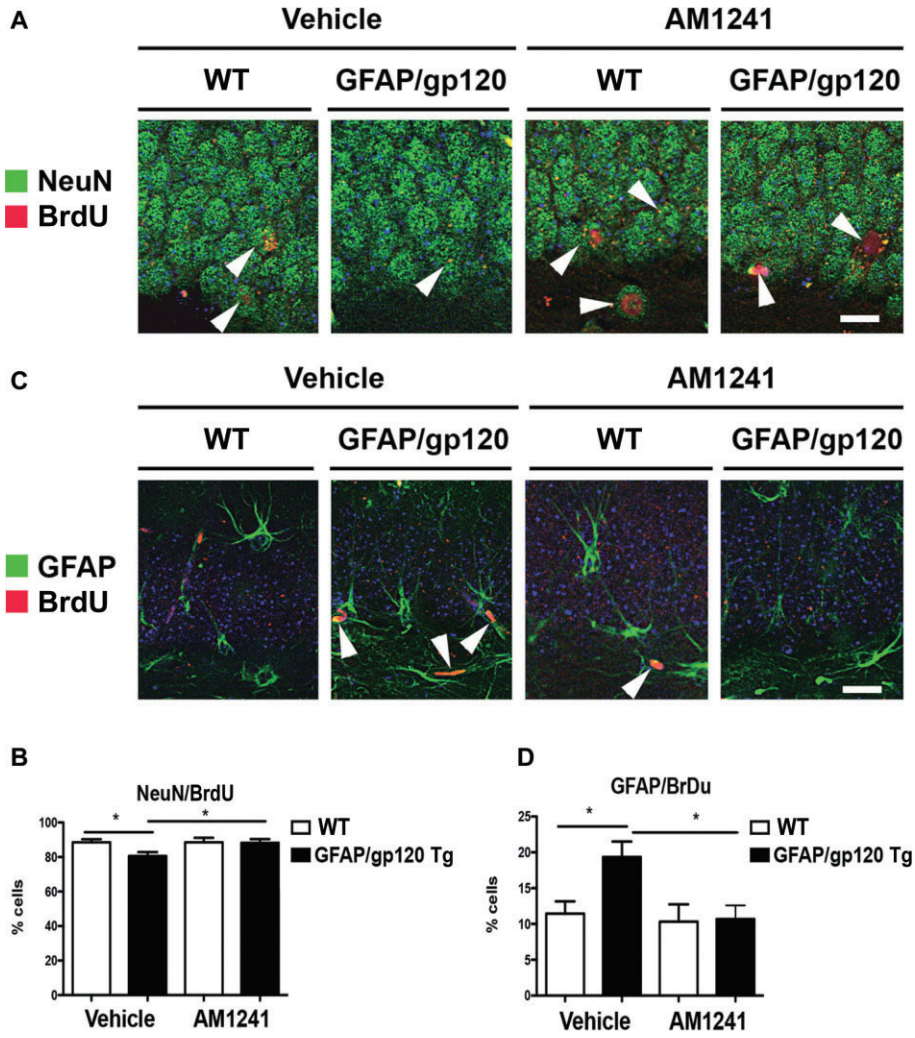

Figure 6

Effects of *in vivo* administration of AM1241 on $NeuN^+$ cells and astrogliosis. (A–B) Immunohistological analysis of $NeuN^+$ cells within the BrdU+ cell population and estimates of the numbers of $NeuN^+$ neurons within the BrdU+ cells in the hippocampal dentate gyrus using the disector method. *$P < 0.005$, significant differences as indicated; one-way ANOVA with *post hoc* Tukey Kramer test; $n = 8$. (C–D) Immunostaining and analysis of NPCs converting to astroglial cells. Conversion of NPCs to astroglial cells (GFAP immunoreactivity) within the BrdU+ cells in the hippocampal dentate gyrus. *$P < 0.001$, significant differences as indicated; one-way ANOVA with *post hoc* Tukey Kramer test; $n = 8$.

clear whether CB_2 receptor-mediated cell proliferation is absent in basal conditions *in vivo*, as indicated by our studies.

CB_2 receptor agonists decreased chronic neuroinflammation and restored hippocampal neurogenesis and improved memory in aged rats (Marchalant *et al.*, 2008; 2009a,b,c; 2012). The endocannabinoids may regulate many aspects of the brain's inflammatory response, including the release of pro-inflammatory cytokines and modulation of astrocytic and microglial activation. In addition to the CB_2 receptor-mediated effects on neurogenesis, it is important to note that we also observed significantly decreased astrogliosis and gliogenesis in GFAP/Gp120 Tg mice following the administration of AM1241. Thus, AM1241 enhanced neurogenesis and attenuated the neuroinflammation induced by Gp120 in GFAP/Gp120 Tg mice.

Based on the results presented in this study focusing on CB_2 receptor-mediated effects, we propose that selective agonists at the CB_2 receptor may protect NPCs from the neurotoxic effects of HIV-1 proteins such as Gp120 and may therefore represent a potentially promising neuroprotective treatment for patients with HIV-1-associated neurocognitive disorders. A new opportunity for the development of cannabinoid-based analgesics emerged from data showing that selective CB_2 receptor agonists are anti-nociceptive in animal pain models, suggesting that neural cells are involved in pain perception and/or modulation through CB_2 receptors (Marsicano *et al.*, 2003; Ramírez *et al.*, 2005; Sarne and Mechoulam, 2005; Galve-Roperh *et al.*, 2006; 2008; 2009; Panikashvili *et al.*, 2006; Katona and Freund, 2008; Heifets and Castillo, 2009). We have shown here that NPCs expressed CB_2 receptors abundantly and that the CB_2 receptor agonist

AM1241 exerted neuroprotective effects on NPCs after insult by Gp120.

In summary, these findings support the hypothesis that CB_2 receptor agonists have neuroprotective effects on impaired neurogenesis induced by HIV-1/Gp120 in GFAP/Gp120 Tg mice.

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Conflicts of interest

There exist no conflicts of interest.

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