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A synthetic cannabinoid agonist promotes oligodendroglialogenesis during viral encephalitis in rats

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Abstract

Chronic CNS infection by several families of viruses can produce deficits in prefrontal cortex (PFC) and striatal function. Cannabinoid drugs have been long known for their anti-inflammatory properties and their ability to modulate adult neuro and gliogenesis. Therefore, we explored the effects of systemic administration of the cannabinoid agonist WIN55,212-2(WIN) on prefrontal cortex(PFC) and striatal cytogenesis in a viral model of CNS injury and inflammation based on Borna Disease (BD) virus encephalitis. Active BrdU⁺ progenitor populations were significantly decreased 1 week after BrdU labeling in BD rats [$p < 0.001$ compared to uninfected (NL)controls] while less than 5% of BrdU⁺ cells colabeled for BDV protein. Systemic WIN (1mg/kg i.p. twice daily x7 days) increased the survival of BrdU⁺ cells in striatum ($p < 0.001$) and PFC of BD rats, with differential regulation of labeled oligodendroglia precursors versus microglia/macrophages. WIN increased the percentage of BrdU⁺ oligodendrocyte precursor cells and decreased BrdU⁺ ED-1-labeled phagocytic cells, without producing pro- or antiviral effects. BDV infection decreased the levels of the endocannabinoid anandamide(AEA) in striatum ($p < 0.05$ compared to NL rats), whereas 2-AG levels were unchanged. Our findings indicate that: 1)viral infection is accompanied by alterations of AEA transmission in the striatum, but new cell protection by WIN appears independent of its effect on endocannabinoid levels; and 2)chronic WIN treatment alters the gliogenic cascades associated with CNS injury, promoting oligodendrocyte survival. Limiting reactive gliogenesis and macrophage activity in favor of oligodendroglia development has significance for demyelinating diseases. Moreover, the ability of cannabinoids to promote the development of biologically supportive or symbiotic oligodendroglia may generalize to other microglia-driven neurodegenerative syndromes including NeuroAIDS and diseases of aging.

Keywords

Borna Disease Virus; endocannabinoid; anandamide; neurogenesis; BrdU; WIN 55,212-2

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Introduction

The adult mammalian CNS is highly vulnerable to various insults and injuries, due in part to its limited capacity to replace dying or dead cells. Recent advances in developmental and stem cell biology show that regeneration-based therapies via activation of endogenous neuronal progenitor (NPC) and stem cells may be a promising approach to CNS repair. The discovery of neurogenesis in the adult subventricular (SVZ) and subgranular zone (SGZ), and the continuous generation of astrocytes and oligodendrocytes throughout the adult CNS has led to early experimental applications of stem-cell-based therapies in trauma, stroke and degenerative diseases (Gage, 2000; Alvarez-Buylla and Lim, 2004; Duan et al., 2008). Although viruses are implicated in a wide spectrum of inflammatory/degenerative CNS disorders, studies on the plasticity of adult neural stem cells in viral brain injury have been limited (Krathwohl and Kaiser, 2004; van Marle et al., 2005; Schwartz et al., 2007; Peng et al., 2008). In this regard, animal models can be helpful as they allow the study of NPC *in vivo* during progressive viral, immune and inflammatory processes.

Striatum and prefrontal cortex (PFC) structural and physiologic injury are implicated in the pathological behaviors and many of neurobiological phenomena associated with the Borna Disease (BD) syndrome in rats. Adolescent rats experimentally infected with Borna Disease virus (BDV) develop a chronic microglial and inflammatory encephalitis (Hatalski et al., 1998; Solbrig et al., 2002; Stitz et al., 2002; Ovanesov et al., 2006) with frontostriatal injury producing stereotypical, perseverative and disinhibited dyskinetic behaviors (Solbrig et al., 1994). In rats, the adult rat medial PFC is equivalent to the human dorsolateral PFC (Gabbott et al., 2005) and, just as in humans, these cortices are functionally coupled to the striatum for the execution of planned behaviors and problem solving tasks (Dias-Ferreira et al., 2009). Indeed, when human PFC and striatal regions are targeted by viruses such as HIV-1 (Aylard et al., 1993; Stout et al., 1998; Chang et al., 2001; Archibald et al., 2004; Thompson et al., 2005), the net result is disruption of problem solving, planning, and set-shifting tasks (Melrose et al., 2008).

Given the importance of the corticostriatal circuitry in syndromes emerging during chronic viral infections and the proximity of the neuroproliferative SVZ to these sites, we examined changes in cell genesis as mechanisms underlying neurotoxic effects of virus. In this study, we focused on gliogenesis because medial PFC precursors are mostly gliogenic, and until recently, their neurogenic capacity in rat has been uncertain or controversial (Dayer et al., 2005; Mandyam et al., 2007). Controversial also are types of cells generated in the injured striatum, with different models having different outcomes. We focused on gliogenesis in striatum because of the BD rat similarities to several rat models in which cytogenesis is mainly gliogenic. Specifically, BD rats have viral-induced partial dopamine lesions (Solbrig et al., 1994), similar to partial, progressive 6-OHDA lesioned rats in which cytogenesis in the corpus striatum is reported as gliogenic (Aponso et al., 2008). Also, BD rats resemble d-amphetamine-treated rats (Solbrig et al., 2000). Rats, after d-amphetamine injection, show striatal cytogenesis that is gliogenic, not neurogenic (Mao and Wang, 2001).

Because of the importance of corticostriatal injury in viral behavioral syndromes, we also sought a pharmacologic strategy to support and manipulate the differentiation of NPC *in vivo* in these structures. Cannabinoids were selected because of their role in: (1) early neuronal differentiation and cell survival (Jiang et al., 2005; Galve-Roperh et al., 2007; Galve-Roperh et al., 2008; Marchalant et al., 2009; Nones et al., 2009) (2) inflammatory and degenerative processes including immunosuppression (Klein et al., 2003; Wolf et al., 2008) migration, invasion and angiogenesis (Velasco et al., 2007; Miller and Stella, 2008; Cencioni et al., 2010). In previous work carried out in BD rats, we showed that cannabinoid treatment increased

BrdU⁺ cells in the SVZ (Solbrig and Hermanowicz, 2008), although the identity and functional significance of the surviving cells was not known.

In this study, we tested the hypothesis that adult striatal and PFC cell genesis is vulnerable to viral injury due to a dysregulation of the endogenous cannabinoid system and that cell genesis can be rescued by exogenous cannabinoid treatment. We found that BDV infection affected the levels of the endocannabinoid anandamide (AEA), and that systemic administration of the non-selective cannabinoid agonist WIN55,212-2 (WIN) promoted oligodendroglia progenitor cell (OPC) survival and differentiation through mechanisms that were independent of its ability to elevate brain anandamide. The mechanisms involved reductions in inflammation and neovascularization, and alterations in the gliogenic cascades associated with CNS viral injury.

Methods

Animals

Male Lewis rats (Charles River Labs, Wilmington, MA USA) were group-housed on a 12 hour light-dark cycle with *ad libitum* access to food and water. All experimental procedures were performed in compliance with institutional (University of California-Irvine Institutional Animal Care and Use Committee; Animal Welfare Assurance no. A3416-01, University of Manitoba Protocol Management and Review Committee) and National Institutes of Health guidelines. Under methoxyflurane anesthesia, 4 week old males were infected with BDV by intracerebral injection of 1.6×10^4 tissue culture infectious dose units (strain He/80-1) into the right lateral ventricle (BD rats), or sham-infected with sterile phosphate buffered saline (NL rats) (Solbrig et al., 1994).

BrdU Incorporation

The S phase marker 5-bromo-2'-deoxyuridine (BrdU) (Sigma St Louis MO USA) was dissolved in saline and administered to rats at 5 weeks of age (1 week after BDV infection) at the dose of 50 mg/kg i.p. (3 times on day 0) to label dividing cells (Kuhn et al., 1996). As previously shown (Solbrig et al., 2006), this dose does not promote neuronal apoptosis.

Drugs

The non-selective cannabinoid receptor agonist R(+)- WIN 55,212-2 (WIN) (Sigma, St Louis, MO USA) (1 mg/kg i.p. $\times$ 7 days, starting 1 week after BDV or sham infection), or an equal volume of vehicle (saline), was administered after the last BrdU injection.

To evaluate effects of BDV and WIN on the survival of newly born cells, rats were sacrificed 1 week after the last BrdU injection (n=4–5 per experimental group). WIN treatment groups were injected with BrdU, treated for 7 days with WIN and sacrificed 1 week after the last BrdU injection (n=4–5 per experimental group).

BrdU Quantification, Imaging and Analysis

Brains from experimental groups were processed as described (Solbrig et al., 2006) and examined for changes in precursor cell survival, BrdU-immunoreactive (BrdU⁺) cells, in PFC and striatum. These areas and their subregions were chosen for morphological studies because of their known neuropathology (Gosztonyi and Ludwig, 1995) and their role in behavioral dysfunction (Solbrig et al., 1994; Solbrig et al., 1996).

Fifty micrometer sections were collected on a freezing microtome and the left and right hemisphere of every sixth section slide-mounted. Single labeling for BrdU was performed with biotinylated secondary antibodies (1:200 Vector, Burlingame, CA USA) processed by ABC histochemical method and developed with 3,3'-diaminobenzidine. All BrdU⁺ cells from

orbitofrontal, anterior cingulate, prelimbic and infralimbic cortex (Bregma +4.20 to Bregma +2.70 mm, Paxinos and Watson, 1998), and the entire rostral-caudal extent of the striatum and SVZ (Bregma +2.50 mm to Bregma -0.80 mm) were counted under x400 magnification with the aid of Image-Pro Plus software (n=4–5 per group). To avoid misidentification of repairing or dying cells as newborn cells, histologic markers of apoptosis were also examined. To control for differences in bioavailability or cell uptake of BrdU between NL and BD rats, BrdU⁺ cells were also counted in the habenula, a non-neurogenic region. In adjacent sections Nissl substance was stained by Cresyl violet. Brightfield images were examined with an Olympus AX70 microscope equipped with Image-Pro Plus software (Media Cybernetics, Bethesda, MD USA).

For analysis of cell phenotype, a one-in-six series of sections from NL, BD, and WIN-treated BD rats were processed for BrdU immunostaining (1:100, Accurate, Westbury, NY USA; DAB, 1:400, Chemicon, Billerica, MA USA) for the following cell type markers 1 week after the BrdU injections: NG2 (AB5320 1:250, Chemicon, Billerica, MA USA) anti-NG2 chondroitin sulfate proteoglycan for polydendrocytes; GFAP (REF20334 1:2000, Dako, Mississauga, ON Canada) glial fibrillary acidic protein for astroglia and primary neurogenic SVZ stem cells; and NeuN (MAB377 1:1000, Chemicon) anti-neuronal nuclei for neurons. Although NeuN is a mature neuronal marker, it can be found in 7–10 day old cells in adult rodent cortex (Shapiro et al., 2007) and it was chosen because it is unlikely to show overlap with NG2 and GFAP.

Other primary antibodies were ED-1 (1:100 AbD Serotec, Raleigh, NC USA) for tissue macrophages and activated microglia, O4 (MAB345 1:250, Chemicon) for oligodendroglia precursors, O1 (MAB344 1:250, Chemicon) for more differentiated oligodendroglia. While O4 recognizes a sulfated glycolipid antigen of late oligodendrocyte progenitors, the protoligodendroblast antigen (POA) and a sulfated galatocerebroside of oligodendrocytes that have entered terminal differentiation, O1 reacts with a galatocerebroside that is not synthesized by oligodendrocytes until they enter terminal differentiation. The ED-1 monoclonal antibody recognizes an antigen in the lysosomal membranes of phagocytes that is expressed by the majority of tissue macrophages and activated microglia. Control sections were processed with omission of primary antisera.

Antigens were visualized with Alexa-488 or Alexa-546 secondary antibodies (1:1000, Molecular Probes, Carlsbad, CA USA). Colocalization of antibodies was assessed with an Olympus FluoView Laser Scanning Confocal Microscope at 600x using multitrack scanning and an optical section thickness of 0.50 μ m in the Z-plane. Reconstructions of stacked images using the Visualize function confirmed colocalization. At least 50 BrdU⁺ cells in the striatum and 25 cells in PFC were used to examine colocalization in randomly selected view frames in a minimum of 4 sections per animal (striatum, +2.16 to +1.70; PFC, +3.20 to +2.70, from Bregma)(n=4 animals per group). Data were expressed as percentage of double-labeled cells for BrdU. In selected sections, cell morphology was also examined in wide fields by fluorescence signals detected with an Olympus AX70 microscope.

Virus Effects

BDV effects were assessed in sections processed and examined for new cell infection and changes in viral burden, apoptotic cell death, myelin staining and neovascularization. New cell infection was examined in sections processed for BrdU and BDV-nucleoprotein, the most abundantly expressed BDV protein, 1 day and 1 week after BrdU administration (1 and 2 weeks after infection). BDV p38/40 nucleoprotein was recognized using a mouse monoclonal antibody (38/15 H76, gift of L. Stitz, 1:250). Percentage of cells colabeled for BDV-p38/40 and BrdU was determined by counting at least 50 BrdU⁺ cells in striatum and SVZ, and 20 BrdU⁺ cells in PFC. Total viral burden was estimated from sections in a one-in-six series

immunostained with the BDV-p38/40 monoclonal antibody and processed for DAB. Cells immunoreactive for BDV-nucleoprotein were counted in the entire rostral-caudal extent of the striatum and SVZ and in the anterior cingulate cortex in a 1mm width field at 200x magnification. BrdU⁺ cells undergoing apoptosis 1 day or 1 week after birth, were examined by confocal microscopy in PFC and striatal sections labeled with BrdU and anti-activated caspase-3 (AB3640 AC-3, 1:500, Chemicon) rabbit polyclonal antibody. Data were expressed as percent of BrdU⁺ cells (n=3–4 per group).

Viral-induced angiogenesis was assessed by morphometric values (area) obtained from the examination of Nissl-stained, coronal PFC and striatum sections using an Olympus AX70 microscope equipped with Image-Pro Plus software (Media Cybernetics, Bethesda, MD, USA). Data were expressed as percent of total section area (n=3,4 per group). Luxol Fast Blue-Periodic acid-Schiff (LFB-PAS) staining was used for myelin (Bancroft and Stevens, 1996).

Endocannabinoid Measurements

A separate group of animals (n=8–11 per experimental group) were anesthetized by inhaled halothane and sacrificed by decapitation 1 hr after the last injection of the cannabinoid agonist WIN55,212-2 or vehicle. Brains were rapidly removed, snap-frozen in cold 2-methylbutane (–50°C), and cut into 2 mm coronal slices with razor blades using an ice-cold aluminum alloy mold. Tissue sections (ranging from 5 to 20 mg) were excised from PFC and striatum sections and thawed in 1 ml of methanol containing 50 pmol of [²H₄]-anandamide (AEA) and [²H₅]-2-arachidonyl glycerol (2-AG) (internal standards). Samples were homogenized with a PowerGen 125 homogenizer (Fisher Scientific; Pittsburgh, PA, USA) and lipids were extracted by adding chloroform and water to yield a methanol/chloroform/water ratio of 1:2:1 (v/v/v), and centrifuged at 800 × g for 5 min at room temperature to allow for phase separation. The lower organic layer (2 ml) was further purified by solid phase extraction using C₁₈ Bond Elut cartridges (100 mg, Varian, Harbor City, CA USA) as previously described (Hardison et al., 2006). Endocannabinoid-containing fractions were derivitized with 30 ml of BSTFA at room temperature for 30 min, dried under nitrogen, resuspended in 5 ml of hexane and analyzed by gas chromatography/chemical ionization mass spectrometry (GC/MS) with positive ion detection (PCI) using a TraceDSQ (Thermo Electron; San Jose, CA USA) equipped with an Rtx-5MS column (15 m × 0.25 mm; Restek; Bellefonte, PA USA). Endocannabinoid quantification was carried out using an isotope dilution GC/MS procedure as previously described (Hardison et al., 2006).

Protein Determination

Isolated brain regions were homogenized in (1:5 v/v) Tris-EDTA lysis buffer (1% Triton X-100, 10% glycerol, 20mM Tris, pH 7.5, 1 mM EDTA) with freshly added 1% protease inhibitor cocktail (Sigma P8340, St Louis MO USA). Ten microgram protein samples were size fractionated by 15% SDS-PAGE. MBP was quantified by Western blotting using mouse anti-Myelin Basic Protein 119–131 monoclonal antibody (MAB381 1:500, Chemicon), an HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove PA USA), ECL detection kit (Amersham Biosciences, Buckinghamshire, UK) and image analysis (Gel Doc 2000 Biorad Laboratories, Mississauga Ontario CA). Actin, detected by mouse anti-actin monoclonal antibody (A5316 1:1000 Sigma) served as a control for protein loaded (n=4 per group).

Data analysis

Numeric data were presented as mean ± SEM and considered significant if p < 0.05. Statistical analysis was performed using a two-tailed Student's *t* tests (for comparisons of two groups) or one-way ANOVA followed by Tukey's *post hoc* test (for comparisons of more than 2 groups) if data or scores did not violate the assumption of homogeneity of variance. To compare co-

expression of various markers in BrdU⁺ cells, percentage or proportional data for differences in treatment groups, set in 2 X 2 tables, were analyzed by Chi-square test with Yate's correction. All analyses were carried out using the GraphPad Software (San Diego CA USA).

Results

WIN55,212-2 increases survival of striatal and PFC progenitor cells

BrdU immunohistochemistry was performed to quantify proliferating cells 7 day after BrdU injection, as an indicator of precursor cell survival in PFC (orbitofrontal cortex, anterior cingulate, prelimbic cortex and infralimbic cortex, from Bregma +4.20 to Bregma +2.70 mm), striatum (including the SVZ, from Bregma +2.50 mm to Bregma -0.80 mm) (Fig 1). In PFC, we observed differences in the number of BrdU⁺ cells across groups [$F(3,12)=182.1$, $p<0.0001$] with significant decreases in BD compared to NL rats ($p<0.001$, Tukey's *post hoc* following significant ANOVA; $n=4$ per group) (Fig 1B,C). Systemic administration of the cannabinoid agonist WIN, which significantly increased BrdU⁺ cells in NL rats (NL-WIN vs NL, $p<0.01$, Tukey's) (Fig 1C), also increased the number of surviving BrdU⁺ cells in BD rats, (BD-WIN: 1754 ± 313.3 vs BD: 672.0 ± 221.7) (Fig 1B,C) but the difference between BD and BD-WIN groups was not statistically significant ($p>0.05$, Tukey's; $n=4$ per group).

In the striatum, the number of BrdU⁺ cells was significantly different across groups [$F(3,14)=279.3$, $p<0.0001$], with a decrease in BD compared to NL rats ($p<0.001$, Tukey's *post hoc* following significant ANOVA; $n=4-5$ per group). (Fig 1D,E). WIN treatment significantly increased the number of BrdU⁺ cells (BD-WIN vs BD, $p<0.001$, Tukey's; $n=4-5$ per group) (Fig 1D,E), as already shown in the SVZ alone after WIN injection (Solbrig et al., 2008). Cell counts in the habenula, a control region for bioavailability of BrdU, were similar across experimental groups (35–40 BrdU⁺ cells per section).

To investigate the occurrence of apoptosis in BrdU⁺ cells, newly generated PFC and striatal BrdU⁺ cells were colabeled with antibodies against activated caspase 3 (AC-3). One day after BrdU injections, BD rats showed significant increases in the percent of colabeled cells compared to NL rats in both PFC (38% BD vs 0% NL; X^2 with Yate's correction=44.48 $p<0.0001$) and in striatum (52% BD vs 1% NL; X^2 with Yate's correction=64.18 $p<0.0001$). AC-3 data comparing BD rats 1 week after BrdU injections and BD-WIN treated rats 1 week after BrdU injections showed that WIN inhibited activation of the mitochondrial intrinsic apoptotic pathway, as indicated by a reduction of the percent of BrdU⁺ cells in both striatum (16% in BD-WIN rats versus 32% in BD group, X^2 with Yate's correction=6.168, $p=0.013$) and PFC (15% in BD-WIN versus 30% in BD group, X^2 with Yate's correction=5.62, $p=0.017$) ($n=3$ per group).

WIN had no apparent pro- or anti-viral effects, as the numbers of infected cells were similar in BD and BD WIN-treated groups. Total counts of cells immunoreactive for the BDV-p38/40 nucleoprotein were: in the striatum, 6955 ± 563 for BD-WIN; 6820 ± 320 for BD ($t=0.208$ $p>0.05$; $n=3$ per group); in the PFC (cells counts from a 1 mm width section of the anterior cingulate cortex) 99 ± 4.7 for BD-WIN; 102 ± 6.2 for BD ($t=0.383$ $p>0.05$; $n=3$ per group). In addition, BDV-infected BrdU⁺ cells accounted for a small percentage of total cells in both BD and BD-WIN groups at the time point studied (striatum, 5% for BD and BD-WIN; PFC, 3% for BD and 4% for BD-WIN). As in previous reports (Gosztonyi and Ludwig, 1995; Solbrig et al., 2002), BDV nucleoprotein was detected in large neurons, such as striatal medium spiny neurons or PFC pyramidal neurons.

WIN 55,212-2 increases oligodendroglia precursors and reduces phagocytic macrophages/microglia

Newly generated cells in the PFC and striatum were examined using cell-type specific markers for polydendrocytes (chondroitin sulfate proteoglycan NG2) astrocytes (GFAP) and neurons (NeuN) (Fig 2A). Phenotypic analysis of BrdU⁺ cells in both PFC and striatum from NL, BD and BD-WIN-treated rats (7 days after BrdU injection) showed similar ratios of BrdU/NG2, BrdU/GFAP and BrdU/NeuN colabeling across groups (Fig 2B). In both structures, the majority of cells were glial, labeled with NG2 or GFAP, whereas a small proportion was NeuN⁺ (1–6%). These cells were round, had small processes or NeuN staining restricted to the nucleus and no apparent polarity or migratory phenotype. The approximately 1% NeuN⁺ cells detected in NL rats suggests that neurons could be produced in the same proportions as reported for adult rat neocortex (Dayer et al., 2005). A proportion (approximately 5–10%) of BrdU⁺ cells from these groups that did not colocalize with NeuN, GFAP or NG2 were designated as “other”.

When BrdU/NG2, BrdU/GFAP and BrdU/NeuN cells were quantified to assess changes in cell phenotype in WIN-treated NL rats, we found WIN significantly decreased the percentage of BrdU/GFAP colabeled cells in striatum, compared with controls (12% NL-WIN vs 38% NL; X^2 with Yates's correction=16.67, $p<0.0001$), while modestly increasing BrdU/NeuN cells (5% NL-WIN vs 1% NL; X^2 with Yates's correction=1.546, $p>0.05$) and maintaining percentages of BrdU/NG2 (Fig 2B).

Although NG2⁺ cells are widely accepted as oligodendroglia precursor cells (OPC), NG2 immunoreactivity is also present in blood-borne macrophages (Nishiyama et al. 2009), and some reactive NG2⁺ cells express microglia/macrophage antigens such as ED-1. Therefore, to assign the BrdU⁺/NG2⁺ cells observed in our study to the oligodendrocyte or macrophage/microglia lineage, cells in adjacent sections were colabeled for BrdU and OPC markers (O4 and O1) and the ED-1 antigen, which is used to detect myeloid cells such as tissue macrophages and activated microglia.

In BD rats, WIN treatment altered the phenotypic ratio of clinically important subsets of NG2⁺ cells in favor of the oligodendrocyte lineage. Analysis of BrdU⁺ cells from NL, NL-WIN, BD and BD WIN-treated groups showed that WIN significantly increased the percentage of BrdU⁺ cells that survived and matured into O4⁺ OPC in both PFC (10% in BD vs 31% in BD-WIN rats; X^2 with Yates correction = 12.27, $p<0.001$) (Fig 3A,C) and striatum (14% in BD vs 45% in BD-WIN rats; X^2 with Yates's correction = 21.64, $p<0.0001$) (Fig 3B,D). In PFC, WIN also increased the percentage of more differentiated O1⁺ OPC [7% in BD group vs 20% in BD-WIN rats (X^2 with Yates correction = 6.166, $p<0.05$; $n=4$ per group)] (Fig 3A,C).

WIN treatment significantly reduced the percentage of BrdU/ED-1 colabeled cells in both PFC (32% in BD vs 8% in BD-WIN rats; X^2 with Yates's correction = 16.53, $p<0.0001$) and striatum (29% in BD versus 9% in BD-WIN rats; X^2 with Yates's correction = 11.73, $p=0.001$) ($n=3$ per group) (Fig 4A,B). No ED-1⁺ cells were observed in NL and NL-WIN rats (Fig. 4B), whereas NG2⁺ cells were detected in the brain parenchyma of all animals studied (Fig. 4C). Populations of process-bearing cells were different across groups. The thickened processes and phagocytic morphology of reactive NG2⁺/BrdU⁺ cells found in BD rats were not seen in NG2⁺ cells of NL, NL-WIN and BD-WIN rats. In these groups, NG2⁺ cells were stellate with round or oval cell bodies and had multiple long or fibrillar processes, more consistent with OPC morphology (Fig 4C).

WIN reduces angiogenesis

One-week after BDV infection, there were reductions in Luxol Fast Blue staining for myelin in the perivascular areas of PFC and striatum of BD rats (Fig 5A) and significantly lower levels of myelin basic protein in PFC ($p < 0.05$, $t = 3.420$, $df = 5$, Student's t test) (Fig 5A). Morphometric analysis of brain tissue vascularization using the Image-Pro Plus software for detection and measurements of section area of medium or large lumen vessels, revealed limited changes in the PFC of infected rats compared to NL controls ($p > 0.05$ n.s.), but significant increases in the striatum 1 week after BDV infection ($p < 0.05$, $t = 0.4652$, $df = 4$, Student's t test) (Fig 5B). Systemic administration of WIN reduced vascular areas in PFC ($p < 0.01$, Tukey's *post hoc* following significant ANOVA, $F(3,11) = 25.75$ $p < 0.001$) and striatum ($p < 0.05$, Tukey's *post hoc* following significant ANOVA, $F(3,14) = 7.918$ $p < 0.01$; $n = 3-4$ per group) (Fig 5C,D).

Regional endocannabinoid levels: BDV infection reduces striatal AEA

Since the endocannabinoid system has been shown to promote adult neurogenesis and gliogenesis by providing instructive precursor cell signals (Molina-Hogado et al., 2002; Aguado et al., 2005; Aguado et al., 2006; Arevalo-Martin et al., 2007; Harkany et al., 2007), we tested the hypothesis that the viral-induced decrease of neuro/gliogenesis observed in BD rats was associated with reduced endocannabinoid transmission.

Quantification of the endocannabinoids anandamide (AEA) and 2-AG by GC/MS isotope dilution showed significant differences in striatal AEA across groups [$F(2,27) = 41.76$, $p < 0.0001$]. There was a decrease of AEA levels in the striatum of BD rats compared to NL controls ($p < 0.05$, Tukey's *post hoc* following significant ANOVA; $n = 9-11$ per group), and a further decrease of striatal AEA in BD rats following WIN administration ($p < 0.001$, Tukey's *post hoc* following significant ANOVA) (Fig 6A). However, the changes in AEA levels were not equivalent in every region of BD rat, as there were no differences in PFC AEA levels between NL, BD, and BD-WIN treated groups (Fig 6A). Additionally, there were no group differences in striatal or PFC levels of 2-AG (Fig 6B). Thus, the protection of cytogenesis appears to be independent of its ability to affect endocannabinoid levels.

Discussion

Except for antivirals, there are few specific therapies available for neurological syndromes associated with chronic CNS viral infections. Using the BD adolescent rat model of persistent CNS infection, we found that BDV encephalitis was associated with a dysregulated endocannabinoid system in the striatum and decreased new cell survival in the striatum and PFC. Early treatment with the general cannabinoid agonist WIN stimulated or preserved oligodendroglialogenesis while having a regulatory role on several stages of BDV-induced inflammatory pathology. Therefore, these data suggest that lipid imbalance is an aspect of the altered microenvironment of encephalitic brains that may be remedied or overcome by cannabinoid-based treatments. Such treatments have the potential to improve the frontostriatal neurological syndromes associated with chronic viral encephalitis.

Although BD pathology of adolescent- or adult-infected mammals has been characterized by neuronal tropism, transynaptic spread, inflammatory-mediated neuronal loss and reactive astrogliosis (Gosztonyi and Ludwig, 1995), in some hosts or under certain experimental conditions, BD can also present demyelinating features. For example, BDV proteins and antibodies have been reported in the cerebrospinal fluid of Multiple Sclerosis (MS) patients (Deuschle et al., 1998). In addition, oligodendrocyte cell lines can be infected by BDV *in vitro* (Jordan et al., 1999), antigen and viral nucleic acid have been detected in Schwann cells after *in vivo* infections (Carbone et al., 1989; Carbone et al., 1991) and oligodendrocytes would be vulnerable to the oxidative stress and the inflammatory injury triggered by BDV and

antiviral CD8⁺ T cells (Planz and Stitz, 1999; Stitz et al., 2002). In this study, we observed neural progenitor cell loss, some perivascular white matter pallor, limited infection of BrdU⁺ cells, and, in agreement with previous investigations, microglia and/or macrophage infiltration (Carbone et al., 1989; Deschl et al., 1990; Stitz et al., 2002).

Microglia are activated by BDV infection of neurons (Ovanesov et al., 2006) and mixed inflammatory cells, mainly macrophages/microglia and CD8⁺ T cells, which produce CNS inflammation in adolescent or adult-infected rodents (Carbone et al., 1989; Deschl et al., 1990; Stitz et al., 2002; Solbrig et al., 2002; Solbrig et al., 2006). The CNS inflammation observed in BD differs, in this respect, from immune-mediated models of demyelinating diseases or MS, such as experimental allergic encephalomyelitis (EAE), a T cell-mediated autoimmune disease, and Theiler's murine encephalomyelitis (TME), which is triggered by the mouse virus TMEV. In EAE, infiltrating T cells sensitized to myelin protein(s) drive pathology (Leung et al., 2009) and in the Theiler's viral model, CD4⁺ Th1 cells produce a delayed-type hypersensitivity reaction with epitope spreading to viral and endogenous myelin epitopes (Miller et al., 1997; Tompkins et al., 2002); in addition, encephalitogenic T cells can be primed by TMEV antibodies that cross react with galactocerebroside (Yamada et al., 1990). Instead, BD leukoencephalitis evolves from an oligodendroglial, inflammatory, non-autoimmune mechanism of white matter injury. The increased percentage of BrdU⁺ OPC and reduced proportion of BrdU⁺ ED-1 cells found in BD-WIN rats, indicates that the general cannabinoid agonist WIN55,212-2 can treat the viral inflammatory disease that spills over into myelin.

Previous studies have shown that application of WIN promotes OPC survival *in vitro* (Molina-Holgado et al., 2002) and limits neurodegeneration in EAE (Pryce et al., 2003; Ni et al., 2004; Sanchez et al., 2006) and Theiler's viral model of MS (Arevalo-Martin et al., 2003; Croxford and Miller, 2003; Mestre et al., 2009). In addition, several groups have reported that pharmacological enhancement of endocannabinoid signaling can improve the pathological and functional outcome of Theiler's virus in infected mice (Mestre et al., 2005; Ortega-Gutierrez et al., 2005; Loria et al., 2008; Loria et al., 2010). In agreement with these studies, the increase in OPC and the reduction of new microglia/macrophages in BD rats chronically treated with WIN supports a role for this cannabinoid agonist in limiting the progression of viral-induced inflammatory neurodegeneration. Although the molecular targets mediating WIN effects have not been determined in this study, numerous reports indicate that cannabinoid agonists reduce inflammatory neurodegeneration and MS-like pathology by targeting both CB₁ and CB₂ cannabinoid receptors. There is experimental evidence of (a) CB₁-mediated protection of neurons and oligodendroglia in EAE (Pryce et al., 2003; Cabranes et al., 2005; Maresz et al., 2007), (b) CB₁ receptor stimulation of neural progenitor proliferation and differentiation into astroglia (Aguado et al., 2005; Aguado et al., 2006), (c) CB₂-mediated support of SVZ development (Arevalo-Martin et al., 2007) and oligodendrocytes differentiation (Molina-Holgado et al., 2002), and (d) CB₂-mediated inhibition of (1) bone-marrow-derived myeloid recruitment (Palazuelos et al., 2008), (2) T cells (Maresz et al., 2007), (3) leukocyte/endothelial interactions (Ni et al., 2004), (4) macrophage/microglia activation in animal models of neurodegenerative disorders, (Stella, 2009; Price et al., 2009) and (5) angiogenesis (Schley et al., 2009).

The cellular alterations produced by WIN in NL animals suggest WIN could have some beneficial direct effects on neurogenesis and oligodendroglialogenesis. There was an overall increase in BrdU labelled cells, compared to drug-naïve NL animals, along with increase in percentages of BrdU/NeuN colabeled cells (from 1 to 5% in both PFC and striatum) and BrdU/NG2 cells (from 55 to 61% in PFC) in WIN-treated NL animals. However, considering the overall results from BD animals at this dose, the main effect of WIN in BD rats is more likely indirect stimulation of neurogenesis and gliogenesis via inhibition of microglia activation and

inflammation. First, WIN significantly increased survival of progenitor cells in the BD rat striatum, a structure with marked inflammation due to the intraventricular source of virus. This was in contrast to the modest effect of WIN in NL rat striatum. Second, WIN increased progenitor cell survival, an effect that was accompanied by reductions in BrdU labeled macrophages, activated microglia, and inflammatory angiogenesis. Third, WIN treatment of BD rats preserved basal BrdU/GFAP, BrdU/NG2 and BrdU/NeuN cell proportions observed in untreated groups, and did not reproduce the reductions in the GFAP⁺ phenotype seen in WIN-treated NL rats. This suggests WIN's effect was on an extrinsic rather than intrinsic factor in neural or glial genesis in infected rats. Since WIN is a non-selective cannabinoid receptor agonists, our results suggest that either or both cannabinoid receptor subtypes might be implicated in the therapeutic effects of WIN and draw attention to the importance of targeting neural, glial, inflammatory and immune systems to achieve optimal clinical benefit. Further studies are in progress to assess whether the beneficial effects of WIN might be ascribed to CB₁/CB₂ or, alternatively, to non-cannabinoid targets.

In agreement with previous observations in the adult rat neocortex (Dayer et al., 2005), IHC phenotype analysis revealed that a small percentage of BrdU⁺ cells were NeuN⁺ (1-6%), a finding that further supports the neurogenic capacity of the postnatal PFC (Dayer et al., 2005). Finding a few new neurons in both cortex and striatum would be in agreement with general ideas that brain regions showing adult neurogenesis are functionally linked, or the specific hypothesis that some medial ganglionic eminence-derived precursor cells in different regions of the adult brain may retain their ability to divide and generate new neurons such as GABAergic interneurons (Dayer et al., 2005) and be resistant to infection and injury. Therefore, we have initiated new investigations to fully characterize these cells with neuronal phenotypes.

New cell protection by WIN appears independent of its effect on endocannabinoid levels in the structures studied. For example, WIN had stronger effects on oligodendrogenesis in PFC vs. striatum of BD rats (Fig 3), with all other effects being comparable. One possibility for the differential effect of WIN in the two structures is that the phenotype, in this case, compensatory cellular responses, is determined by the most prominent break with homeostasis, which for this example, is myelin loss. Measurements of myelin basic protein revealed greater demyelination in the PFC than striatum.

Interestingly, BDV infection caused a significant reduction of AEA levels in the striatum of BD rats, possibly reflecting an infection-induced decrease of synthesis and/or increase of catabolism induced by the infection, whereas it had no effect in PFC. Although the specific effects of BDV on endocannabinoid anabolic or catabolic pathways are not known, the further reduction of striatal AEA observed after WIN treatment suggests that WIN can effectively penetrate the CNS and inhibit AEA signaling either indirectly (i.e. by reducing the release of neurotransmitters driving the on-demand synthesis of AEA) or by direct inhibition and/or activation of the respective AEA biosynthetic (NAPE-PLD) or catabolic (fatty acid amide hydrolase or FAAH) enzymes. In keeping with this hypothesis, prenatal administration of WIN has been shown to dysregulate the activity of NAPE-PLD and to inactivate FAAH in rat brain in a region-specific fashion (Castelli et al., 2007). In addition, prolonged activation of cannabinoid receptors has been shown to decrease AEA content in rat striatum (DiMarzo et al., 2000). If AEA synthesis is regulated through CB₁ receptors, which desensitize upon repeated WIN administration (DiMarzo et al., 2000), the lower expression of CB₁ receptors in the PFC versus striatum (Herkenham et al., 1990), or differences in viral penetration in the two structures, may explain why the WIN-induced decline of AEA levels was limited to the striatum. Also of interest, neither BDV infection nor WIN affected 2-AG levels. The lack of effect may result from virus- or inflammatory-induced disruption of biological membrane/lipid raft integrity, and the consequent uncoupling of the interactions between metabotropic glutamate 5 (mGLUR5) and CB₁ receptors, which is known to affect 2-AG production

(Uchimagashima et al. 2007, Maccarrone et al., 2008; Maccarrone et al., 2009). Consistent with this hypothesis, lipid rafts are involved in BDV cell entry (Clemente et al., 2009; Clemente et al., 2010), and synaptic pathology and disruption of striatal synapses have been reported in more advanced BD (Gonzalez-Dunia et al., 2000; Solbrig et al., 2000). Nevertheless, our data indicate that the beneficial effects of WIN do not require the reversal of AEA deficit and suggest that the decreased endocannabinoid levels observed in the striatum of BD rats may be a correlate rather than a cause of BDV-induced inflammatory response. For example, astrocytes have been shown to overexpress the cannabinoid-degrading enzyme FAAH in response to SIV-induced neuroinflammation (Benito et al., 2005).

Additional studies of the *in vivo* effects of WIN are needed to establish whether this drug acts at the cellular and/or circuit levels in a way that specifically protects progenitor cells of diseased animals. For example, WIN might rebalance the equilibrium between excitation and inhibition in the basal ganglia circuits, and generate neurochemical or neurotrophin signals to promote a supportive environment for progenitor cells.

Further studies of the *in vivo* effects of WIN are also needed to establish its effect on cytogenesis in NL healthy animals, particularly the meaning of the reductions in percent BrdU/GFAP cells (from 38% to 12% in striatum and 30% to 18% in PFC). Similar, selective reductions of BrdU/GFAP cells in medial PFC have also been shown after daily methamphetamine administration to rats (Mandyam et al., 2007). As a result, daily drug administration or dependance models may be used to examine a role of adult-generated glia in drug-related behaviors dependent on these structures (Mandyam et al., 2007).

In summary, administration of cannabinoid agonists has been shown to be neuroprotective in various models of brain injury, but *in vivo* studies in viral-induced degenerative conditions have been limited. Our results indicate that cannabinoids can stimulate survival of adult-born OPC during viral encephalitis and underlie the possibility of cannabinoid-based therapies to promote oligodendroglialogenesis in diseases with direct or indirect viral-induced oligodendroglia loss and demyelination. Support of early phases of oligodendroglialogenesis in viral encephalitis is a previously unrecognized action of cannabinoids, and underscores the importance of studying lipid systems in disease processes. Future advances in neuroprotection during CNS viral infection will require a systematic approach that includes: using longer survival periods, determining optimal conditions of cannabinoid stimulation or utilization, identifying factors that confer sensitivity or resistance to cannabinoid treatment, and ascertaining specific requirements for cannabinoid receptors through the use of more selective agonists and antagonists. It remains to be seen whether screening compounds for their neurogenic and gliogenic potential can be a meaningful approach in antiviral drug discovery.

Beyond demyelinating diseases, the ability of cannabinoids to replace activated microglia and macrophages with oligodendroglia may broadly apply to microglia-driven neurodegenerative diseases. Since many forms of neural injury share the common neuropathologic trait of microglia activation, the BDV model is relevant to other neurodegenerative syndromes, such as NeuroAIDS, recently described NeuroIRIS (Neuro immune-reconstitution inflammatory syndromes) (McCombe et al., 2009) and diseases of aging.

Research Highlights

1. Borna Disease viral infection reduces active BrdU⁺ progenitor populations
2. A synthetic cannabinoid (WIN) increases BrdU⁺ cell survival
3. New cell protection by WIN is independent of its effect on endocannabinoid levels

4. WIN indirectly stimulates neurogenesis and oligodendroglialogenesis via inhibition of inflammation

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Abbreviations

BD	Borna Disease
WIN	WIN 55,212-2

References

- Aguado T, Monory K, Palazuelos J, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I. The endocannabinoid system drives neural progenitor proliferation. *FASEB J* 2005;19:1704–1706. [PubMed: 16037095]
- Aguado T, Palazuelos J, Monory K, Stella N, Cravatt B, Lutz B, Marsicano G, Kokaia Z, Guzman M, Galve-Roperh I. The endocannabinoid system promotes astroglial differentiation by acting on neural progenitor cells. *J Neurosci* 2006;26:551–561.
- Alvarez-Buylla A, Lim DA. For the long run: maintaining germinal niches in the adult brain. *Neuron* 2004;41:683–686. [PubMed: 15003168]
- Aponso PM, Faull RLM, Connor B. Increased progenitor cell proliferation and astrogenesis in the partial progressive 6-hydroxydopamine model of Parkinson's Disease. *Neuroscience* 2008;151:1142–1153. [PubMed: 18201835]
- Archbald SL, Masliah E, Fennema-Notestine C, Marcotte TD, Ellis RJ, McCutchan JA, Heaton RK, Grant I, Mallory M, Miller A, Jernigan TL. Correlation of in vivo neuroimaging abnormalities with postmortem human immunodeficiency virus encephalitis and dendritic loss. *Arch Neurol* 2004;61:369–376. [PubMed: 15023814]
- Arevalo-Martin A, Vela JM, Molina-Holgado E, Borrell J, Guaza C. Therapeutic actions of cannabinoids in a murine model of Multiple Sclerosis. *J Neurosci* 2003;23:2511–2516. [PubMed: 12684434]
- Arevalo-Martin A, Garcia-Ovejero D, Rubio-Araiz A, Gomez O, Molina-Holgado F, Molina-Holgado E. Cannabinoids modulate Olig2 and polysialylated neural cell adhesion molecule expression in the subventricular zone of post-natal rats through cannabinoid receptor 1 and cannabinoid receptor 2. *Eur J Neurosci* 2007;26:1548–1559. [PubMed: 17880390]
- Aylward EH, Henderer JD, McArthur JC, Brettschneider PD, Harris GJ, Barta PE, Pearlson GD. Reduced basal ganglia volume in HIV-1-associated dementia: results from quantitative neuroimaging. *Neurology* 1993;43:2099–2104. [PubMed: 8413973]
- Bancroft, JD.; Stevens, A. Theory and practice of histological techniques. Edinburgh: Churchill Livingstone; 1996.
- Benito C, Kim WK, Chavarria I, Hillard CJ, Mackie K, Tolon RM, Williams K, Romero J. A glial endogenous cannabinoid system is upregulated in the brains of macaques with simian immunodeficiency virus-induced encephalitis. *J Neurosci* 2005;25:2530–2536. [PubMed: 15758162]
- Cabranes A, Venderova K, de Lago E, Fezza F, Sanchez A, Mestre L, Valenti M, Garcia-Merino A, Ramos JA, Di Marzo V, Fernandez-Ruiz J. Decreased endocannabinoid levels in the brain and beneficial effects of agents activating cannabinoid and/or vanilloid receptors in a rat model of multiple sclerosis. *Neurobiol Dis* 2005;20:207–217. [PubMed: 16242629]

- Carbone KM, Trapp BD, Griffin JW, Duchala CS, Narayan O. Astrocytes and Schwann cells are virus-host cells in the nervous system of rats with Borna disease. *J Neuropathol Exp Neurol* 1989;48:631–644. [PubMed: 2507750]
- Carbone KM, Moench TR, Lipkin WI. Borna disease virus replicates in astrocytes, Schwann cells and ependymal cells in persistently infected rats: location of viral genomic and messenger RNAs by in situ hybridization. *J Neuropathol Exp Neurol* 1991;50:205–214. [PubMed: 2022964]
- Castelli MP, Paola Piras A, D'Agostino A, Pibiri F, Perra S, Gessa GL, Maccarrone M, Psitis M. Dysregulation of the endogenous cannabinoid system in adult rats prenatally treated with the cannabinoid agonist WIN 55,212-2. *Eur J Pharmacol* 2007;573:11–19. [PubMed: 17644084]
- Cencioni MT, Chiurciu V, Catanzaro G, Borsellino G, Bernardi G, Battistini L, Maccarrone M. Anandamide suppresses proliferation and cytokine release from primary human T-lymphocytes mainly via CB2 receptors. *PLoS One* 2010;5:e8688. [PubMed: 20098669]
- Chang L, Speck O, Miller EN, Braun J, Jovicich J, Koch C, Itti L, Ernst T. Neural correlates of attention and working memory deficits in HIV patients. *Neurology* 2001;57:1001–1007. [PubMed: 11571324]
- Clemente R, de Parseval A, Perez M, de al Torre JC. Borna disease virus requires cholesterol in both cellular membrane and viral envelope for efficient cell entry. *J Virol* 2009;83:2655–2662. [PubMed: 19129439]
- Clemente R, Sisman E, Aza-Blanc P, de al Torre JC. Identification of host factors involved in Borna disease virus cell entry through a siRNA functional genetic screen. *J Virol*. 2010 Jan 13;
- Croxford JL, Miller SD. Immunoregulation of a viral model of multiple sclerosis using the synthetic cannabinoid R+WIN55,212. *J Clin Invest* 2003;111:1231–1240. [PubMed: 12697742]
- Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J Cell Biol* 2005;168:415–427. [PubMed: 15684031]
- Deschl U, Stitz L, Herzog S, Frese K, Rott R. Determination of immune cells and expression of major histocompatibility complex class II antigen in encephalitic lesions of experimental Borna disease. *Acta Neuropathol* 1990;81:41–50. [PubMed: 2085093]
- Deuschle M, Bode L, Heuser I, Schmider J, Ludwig H. Borna disease virus proteins in cerebrospinal fluid of patients with recurrent depression and multiple sclerosis. *Lancet* 1998;352:1828–1829. [PubMed: 9851390]
- Dias-Ferreira E, Sousa JC, Melo I, Morgado P, Mesquita AR, Cerqueira JJ, Costa RM, Sousa N. Chronic stress causes frontostriatal reorganization and affects decision-making. *Science* 2009;325:621–625. [PubMed: 19644122]
- Di Marzo V, Berrendero F, Bisogno T, Gonzalez S, Cavaliere P, Romero J, Cebeira M, Ramos JA, Fernandez-Ruiz JJ. Enhancement of anandamide formation in the limbic forebrain and reduction of endocannabinoid contents in the striatum of delta9-tetrahydrocannabinol-tolerant rats. *J Neurochem* 2000;74:1627–1635. [PubMed: 10737621]
- Duan X, Kang E, Liu C, Ming GI, Song H. Development of neural stem cell in the adult brain. *Curr Opin Neurobiol* 2008;18:108–115. [PubMed: 18514504]
- Gabbott PL, Warner TA, Jays PR, Salway P, Busby SJ. Prefrontal cortex in the rat: projections to subcortical autonomic, motor and limbic centers. *J Comp Neurol* 2005;492:145–177. [PubMed: 16196030]
- Gage F. Mammalian neural stem cells. *Science* 2000;287:1433–1438. [PubMed: 10688783]
- Galve-Roperh I, Aquado T, Palazuelos J, Guzman M. The endocannabinoid system and neurogenesis in health and disease. *Neuroscientist* 2007;13:109–114. [PubMed: 17404371]
- Galve-Roperh I, Aquado T, Palazuelos J, Guzman M. Mechanisms of control of neuron survival by the endocannabinoid system. *Curr Pharm Des* 2008;14:2279–2288. [PubMed: 18781978]
- Gonzalez-Dunia D, Watanabe M, Syan S, Mallory M, Masliah E, de la Torre JC. Synaptic pathology in Borna disease virus persistent infection. *J Virol* 2000;74:3441–3448. [PubMed: 10729116]
- Gosztonyi G, Ludwig H. Borna disease-neuropathology and pathogenesis. *Curr Top Microbiol Immunol* 1995;190:39–73. [PubMed: 7789150]
- Hardison S, Weintraub ST, Giuffrida A. Quantification of endocannabinoids in rat biological samples by GC/MS: technical and theoretical considerations. *Prostaglandins Other Lipid Mediat* 2006;81:106–112. [PubMed: 17085319]

- Harkany T, Guzman M, Galve-Roperh I, Berguis P, Devi LA, Mackie K. The emerging functions of endocannabinoid signaling during CNS development. *Trends Pharmacol Sci* 2007;28:83–92. [PubMed: 17222464]
- Hatalski CG, Hickey WF, Lipkin WI. Evolution of the immune response in the central nervous system following infection with Borna disease virus. *J Neuroimmunol* 1998;90:137–142. [PubMed: 9817441]
- Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC. Cannabinoid receptor localization in brain. *Proc Natl Acad Sci USA* 1990;87:1932–1936. [PubMed: 2308954]
- Jiang W, Zhang Y, Xiao L, Van Cleemput J, Ji SP, Bai G, Zhang X. Cannabinoids promote embryonic and adult hippocampus neurogenesis and produce anxiolytic- and antidepressant-like effects. *J Clin Invest* 2005;115:3104–3116. [PubMed: 16224541]
- Jordan I, Briesse T, Averatt DR, Lipkin WI. Inhibition of Borna disease virus replication by ribavirin. *J Virol* 1999;73:7903–7906. [PubMed: 10438889]
- Klein TW, Newton C, Larsen K, Lu L, Perkins I, Nong L, Friedman H. The cannabinoid system and immune modulation. *J Leukoc Biol* 2003;74:486–496. [PubMed: 12960289]
- Krathwohl MD, Kaiser JL. HIV-1 promotes quiescence in human neural progenitor cells. *J Infect Dis* 2004;190:216–226. [PubMed: 15216454]
- Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 1996;16:2027–2033. [PubMed: 8604047]
- Leung PS, Shu SA, Kenny TP, Wu PY, Tao MH. Development and validation of gene therapies in autoimmune diseases: Epidemiology to animal models. *Autoimmun Rev*. 2009
- Loria F, Petrosino S, Mestre L, Spagnolo A, Correa F, Hernangomez M, Guaza C, DiMarzo V, Docagne F. Study of the regulation of the endocannabinoid system in a virus model of multiple sclerosis reveals a therapeutic effect of palmitoylethanolamide. *Eur J Neurosci* 2008;28:633–641. [PubMed: 18657182]
- Loria F, Petrosino S, Hernangomez M, Mestre L, Spagnolo A, Correa F, Di Marzo V, Docagne F, Guaza C. An endocannabinoid tone limits excitotoxicity in vitro and in a model of multiple sclerosis. *Neurobiol Dis* 2010;37:166–176. [PubMed: 19815071]
- Maccarrone M, Rossi S, Bari M, De Chiara V, Fezza F, Musella A, Gasperi V, Prosperetti C, Bernardi G, Finazzi-Agro A, Cravatt BF, Centonze D. Anandamide inhibits metabolism and physiological actions of 2-arachidonoylglycerol in the striatum. *Nat Neurosci* 2008;11:152–159. [PubMed: 18204441]
- Maccarrone M, De Chiara V, Gasperi V, Viscomi MT, Rossi S, Oddi S, Molinari M, Musella A, Finazzi-Agro A, Centonze D. Lipid rafts regulate 2-arachidonoylglycerol metabolism and physiological activity in the striatum. *J Neurochem* 2009;109:371–381. [PubMed: 19187444]
- Mandyam CD, Wee S, Eisch AJ, Richardson HN, Koob GF. Methamphetamine self-administration and voluntary exercise have opposing effects on medial prefrontal cortex gliogenesis. *J Neurosci* 2007;27:11442–11450. [PubMed: 17942739]
- Mao L, Wang JQ. Gliogenesis in the striatum of the adult rat: alteration in neural progenitor population after psychostimulant exposure. *Brain Res Dev Brain Res* 2001;130:41–51.
- Marchalant Y, Brothers HM, Norman GJ, Karelina K, DeVries AC, Wenk GL. Cannabinoids attenuate the effects of aging upon neuroinflammation and neurogenesis. *Neurobiol Dis* 2009;34:300–307. [PubMed: 19385063]
- Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, Ledent C, Cheng X, Carrier EJ, Mann MK, Giovannoni G, Pertwee RG, Yamamura T, Buckley NE, Hillard CJ, Lutz B, Baker D, Dittel BN. Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB1 on neurons and CB2 on autoreactive T cells. *Nat Med* 2007;13:492–497. [PubMed: 17401376]
- McCombe JA, Auer RN, Maingat FG, Houston S, Gill MJ, Power C. Neurologic immune reconstitution inflammatory syndrome in HIV/AIDS. *Neurology* 2009;73:835–841. [PubMed: 19255411]
- Melrose RJ, Tinaz S, Castelo JM, Coutney MG, Stern CE. Compromised fronto-striatal functioning in HIV: an fMRI investigation of semantic event sequencing. *Behav Brain Res* 2008;188:337–347. [PubMed: 18242723]

- Mestre L, Correa F, Arevalo-Martin A, Molina-Holgado E, Valenti M, Ortaz G, Di Marzo V, Guaza C. Pharmacological modulation of the endocannabinoid system in a viral model of multiple sclerosis. *J Neurochem* 2005;92:1327–1339. [PubMed: 15748152]
- Mestre L, Docagne F, Correa F, Loria F, Hernangomez M, Borrell J, Guaza C. A cannabinoid agonist interferes with the progression of a chronic model of multiple sclerosis by downregulating adhesion molecules. *Mol Cell Neurosci* 2009;40:258–266. [PubMed: 19059482]
- Miller SD, Vanderlugt CL, Begolka WS, Pao W, Yauch RL, Neville KL, Katz-Levy Y, Carrizosa A, Kim BS. Persistent infection with Theiler's virus leads to CNS autoimmunity via epitope spreading. *Nat Med* 1997;3:1133–1136. [PubMed: 9334726]
- Miller AM, Stella N. CB2 receptor-mediated migration of immune cells: it can go either way. *Br J Pharmacol* 2008;153:299–308. [PubMed: 17982478]
- Molina-Holgado E, Vela JM, Arevalo-Martin A, Almazan G, Molina-Holgado F, Borrell J, Guaza C. Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. *J Neurosci* 2002;23:2511–2516. [PubMed: 12684434]
- Ni X, Geller EB, Eppihimer MJ, Eisenstein TK, Adler MW, Tuma RF. WIN 55,212-2, a cannabinoid receptor agonist, attenuates leukocyte/endothelial interactions in an experimental autoimmune encephalomyelitis model. *Mult Scler* 2004;10:158–164. [PubMed: 15124761]
- Nishiyama A, Komitova M, Suzuki R, Zhu X. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nat Rev Neurosci* 2009;10:9–22. [PubMed: 19096367]
- Nones J, Spohr TC, Furtado DR, Sartore RC, Paulsen BS, Guimaraes MZ, Rehen SK. Cannabinoids modulate cell survival in embryoid bodies. *Cell Biol Int*. 2009
- Ortega-Gutierrez S, Molina-Holgado E, Arevalo-Martin A, Correa F, Viso A, Lopez-Rodriguez ML, DeMarzo V, Guaza C. Activation of the endocannabinoid system as therapeutic approach in a murine model of multiple sclerosis. *FASEB J* 2005;19:1338–1340. [PubMed: 15941768]
- Ovanesov MV, Sauder C, Rubin SA, Richt J, Nath AM, Carbone KM, Pletnikov MV. Activation of microglia by borna disease virus infection: in vitro study. *J Virol* 2006;80:12141–12148. [PubMed: 17020949]
- Palazuelos J, Davoust N, Julien B, Hatterer E, Aguado T, Mechoulam R, Benito C, Romero J, Silva A, Guzman M, Nataf S, Galve-Roperh I. The CB(2) cannabinoid receptor controls myeloid progenitor trafficking: involvement in the pathogenesis of an animal model of multiple sclerosis. *J Biol Chem* 2008;283:13320–13329. [PubMed: 18334483]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. 4. Academic Press; San Diego: 1998.
- Peng H, Whitney N, Wu Y, Tian C, Dou H, Zhou Y, Zheng J. HIV-1-infected and/or immune-activated macrophage-secreted TNF-alpha affects human fetal cortical neural progenitor cell proliferation and differentiation. *Glia* 2008;56:903–916. [PubMed: 18383342]
- Planz O, Stitz L. Borna disease virus nucleoprotein (p40) is a major target for CD8(+)-T-cell-mediated immune response. *J Virol* 1999;73:1715–1718. [PubMed: 9882386]
- Price DA, Martinez AA, Seillier A, Koek W, Acosta Y, Fernandez E, Strong R, Lutz B, Marsicano G, Roberts JL, Giuffrida A. WIN55,212-2, a cannabinoid receptor agonist, protects against nigrostriatal cell loss in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Eur J Neurosci* 2009;29:2177–2186. [PubMed: 19490092]
- Pryce G, Ahmed Z, Hankey DJ, Jackson SJ, Croxford JL, Pocock JM, Ledent C, Petzold A, Thompson AJ, Giovannoni G, Cuzner ML, Baker D. Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. *Brain* 2003;126:2191–2202. [PubMed: 12876144]
- Sanchez AJ, Gonzalez-Perez P, Galve-Roperh I, Garcia-Merino A. R-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN-2) ameliorates experimental autoimmune encephalomyelitis and induces encephalitogenic T cell apoptosis: partial involvement of the CB(2) receptor. *Biochem Pharmacol* 2006;72:1697–1706. [PubMed: 17007821]
- Schley M, Stander S, Kerner J, Vajkoczy P, Schupfer G, Dusch M, Schmelz M, Konrad C. Predominant CB2 receptor expression in endothelial cells of glioblastoma in humans. *Brain Res Bull* 2009;79:333–337. [PubMed: 19480992]

- Schwartz L, Civitello L, Dunn-Pirio A, Ryschkewitsch S, Berry E, Cavert W, Kinzel N, Lawrence DM, Hazra R, Major EO. Evidence of human immunodeficiency virus type 1 infection of nestin-positive neural progenitors in archival pediatric brain tissue. *J Neurovirol* 2007;13:274–283. [PubMed: 17613718]
- Shapiro LA, Ng KL, Kinyamu R, Whitaker-Azmitia P, Geisert EE, Blurton-Jones M, Zhou QY, Ribak CE. Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. *Brain Struct Funct* 2007;212:133–148. [PubMed: 17764016]
- Solbrig MV, Koob GF, Fallon JH, Lipkin WI. Tardive dyskinetic syndrome in rats infected with Borna disease virus. *Neurobiol Dis* 1994;1:111–119. [PubMed: 9173990]
- Solbrig MV, Koob GF, Fallon JH, Reid S, Lipkin WI. Prefrontal cortex dysfunction in Borna disease virus (BDV)-infected rats. *Biol Psychiatry* 1996;40:629–636. [PubMed: 8886296]
- Solbrig MV, Koob GF, Lipkin WI. Key role for enkephalinergic tone in cortico-striatal-thalamic function. *Eur J Neurosci* 2002;18:1819–1822. [PubMed: 12431235]
- Solbrig MV, Schlager R, Briese T, Horscroft N, Lipkin WI. Neuroprotection and reduced proliferation of microglia in ribavirin-treated bornavirus-infected rats. *Antimicrob Agents Chemother* 2002;46:2287–2291. [PubMed: 12069992]
- Solbrig MV, Adrian R, Baratta J, Piomelli D, Giuffrida A. A role for endocannabinoids in viral-induced dyskinetic and convulsive phenomena. *Exp Neurol* 2005;194:355–362. [PubMed: 16022863]
- Solbrig MV, Adrian R, Baratta J, Lauterborn JC, Koob GF. Kappa opioid control of seizures produced by a virus in an animal model. *Brain* 2006;129:642–654. [PubMed: 16399805]
- Solbrig MV, Hermanowicz N. Cannabinoid rescue of striatal progenitor cells in chronic Borna disease viral encephalitis in rats. *J Neurovirol* 2008;14:5191–5194.
- Solbrig MV, Koob GF, Parsons LH, Kadota T, Horscroft N, Briese T, Lipkin WI. Neurotrophic factor expression after CNS viral injury produces enhanced sensitivity to psychostimulants: potential mechanism for addiction vulnerability. *J Neurosci* 2000;20:RC104. [PubMed: 11050146]
- Stella N. Endocannabinoid signaling in microglial cells. *Neuropharmacology* 2009;56(Suppl 1):244–253. [PubMed: 18722389]
- Stitz L, Biltzer T, Planz O. The immunopathogenesis of Borna disease virus infection. *Front Biosci* 2002;7:d541–555. [PubMed: 11815301]
- Stout JC, Ellis RJ, Jernigan TL, Archbald SL, Abramson I, Worlfson T, McCutchan JA, Wallace MR, Atkinson JH, Grant I. Progressive cerebral volume loss in human immunodeficiency virus infection: a longitudinal volumetric magnetic resonance imaging study. *HIV Neurobehavioral Research Center Group Arch Neurol* 1998;55:161–168.
- Thompson PM, Dutton RA, Hayashi KM, Toga AW, Lopez OL, Aizenstein HJ, Becker JT. Thinning of the cerebral cortex visualized in HIV/AIDS reflects CD4+ T lymphocyte decline. *Proc Natl Acad Sci USA* 2005;102:15647–15652. [PubMed: 16227428]
- Tompkins SM, Fuller KG, Miller SD. Theiler's virus-mediated autoimmunity: local presentation of CNS antigens and epitope spreading. *Ann NY Acad Sci* 2002;958:26–38. [PubMed: 12021081]
- Uchigashima M, Nanushima M, Fukaya M, Katona I, Kano M, Watanabe M. Subcellular arrangement of molecules for 2-arachidonoyl-glycerol-mediated retrograde signaling and its physiological contribution to synaptic modulation in the striatum. *J Neurosci* 2007;27:3663–3676. [PubMed: 17409230]
- Van Marle G, Anthony JM, Silva C, Sullivan A, Power C. Aberrant cortical neurogenesis in a pediatric neuroAIDS model: neurotrophic effects of growth hormone. *AIDS* 2005;19:1781–1791. [PubMed: 16227785]
- Velasco G, Carracedo A, Blazquez C, Lorente M, Aguado T, Haro A, Sanchez C, Galve-Roperh I, Guzman M. Cannabinoids and gliomas. *Mol Neurobiol* 2007;36:60–67. [PubMed: 17952650]
- Wolf SA, Tauber S, Ullrich O. CNS immune surveillance and neuroinflammation: endocannabinoids keep control. *Curr Pharm Des* 2008;14:2266–2278. [PubMed: 18781977]
- Yamada M, Zurbruggen A, Fujinami RS. Monoclonal antibody to Theiler's murine encephalomyelitis virus defines a determinant on myelin and oligodendrocytes, and augments demyelination in experimental allergic encephalomyelitis. *J Exp Med* 1990;171:1893–1907. [PubMed: 1693653]

Figure 1. Cannabinoid effects on cell survival in PFC and striatum

(A) Schematic representation of PFC subregions showing the areas examined for cell quantification. Subregions included anterior cingulate, prelimbic, infralimbic cortices (shaded in light blue) and orbitofrontal cortex (shaded in blue-gray) (adopted from Paxinos and Watson 1998).

(B) Representative images of DAB-stained sections showing BrdU⁺ cells in the anterior cingulate cortex from uninfected normal (NL), BDV-infected (BD), and WIN-treated BD rats (BD-WIN) examined 1 week after BrdU administration. The arrowhead indicates the medial edge of the cortex. Clusters of BrdU⁺ cells distributed along linear diagonals from the forceps minor of the corpus callosum to the medial edge of the anterior cingulate cortex (arrowhead) in NL rats, but were absent from BD and BD-WIN groups. Scale bar = 100 μ m.

(C) Quantitative data demonstrating the effect of BD (BD) and WIN (NL-WIN, BD-WIN) on number of BrdU⁺ cells. BDV-infected animals had significantly fewer BrdU⁺ cells in PFC (***) $p < 0.001$ vs NL, Tukey's *post hoc* test following significant ANOVA, $n = 4$ per group). Although WIN treatment of BD rats increased the number of BrdU⁺ cells, the difference

between BD and BD-WIN groups was not significant. WIN treatment of NL rats increased BrdU⁺ cells (**p<0.01 vs NL, Tukey's).

(D) BrdU⁺ cells were present in the striatum and subventricular zone (SVZ) of NL, BD, BD-WIN animals near Bregma 2.16 (Paxinos and Watson 1998) 1 week after BrdU administration. Scale bar = 200 um. The insets are magnifications of dorsomedial striatum and SVZ.

(E) Quantitative data demonstrating the effect of BD (BD) and WIN (NL-WIN, BD-WIN) on number of BrdU⁺ cells. BrdU⁺ cells were reduced in the striatum and SVZ of BD rats (**p<0.001 vs NL) and significantly increased by WIN treatment (###p<0.001 vs untreated BD rats). (Tukey's *post hoc* test following significant ANOVA) (n=4-5 per group) Data are expressed as mean ± SEM.

Figure 2. Phenotypic analysis of surviving BrdU⁺ cells in the PFC and striatum

(A) Single Z-plane (0.50 μm thick) confocal images of striatal BrdU⁺ cells showing immunoreactive cells positive for BrdU/NG2, BrdU/GFAP, BrdU/NeuN (red, BrdU; green, cell marker; scale bar = 10 μm).

(B) Analysis of BrdU⁺ cells from PFC and striatum of NL, NL-WIN, BD and BD-WIN rats (n=4 per group) showed cells immunoreactive to glial cell markers, but rarely with neuronal antigens. While there were no differences in the percentages of BrdU/NeuN, BrdU/GFAP and BrdU/NG2 colabeling across NL, BD and BD-WIN groups (expressed as mean ± SEM), WIN treatment significantly decreased the percentage of BrdU/GFAP colabeled cells in striatum of NL rats (NL-WIN vs NL ***p<0.001 X²; n=4 per group). A proportion of cells in which BrdU that did not colocalize with NeuN, GFAP, NG2 was designated as “other”.

Figure 3. Cannabinoid effects on oligodendroglia precursors

(A) WIN treatment increases oligodendroglia lineage cells. In PFC, the percentages of BrdU⁺/O4⁺ and BrdU⁺/O1⁺ cells were significantly higher in BD-WIN vs BD rats (for O4, ***p<0.001 X²; for O1 *p<0.05 X²; n=4 per group).

(B) The percentage of BrdU⁺/O4⁺ oligodendroglia precursors in the striatum was significantly higher in BD-WIN vs BD rats (***p<0.001 X²; n= 4 per group).

(C) and (D) Single Z-plane (0.50 um thick) images of and PFC (C) and striatal (D) BrdU⁺ cells show colabeling for BrdU/O4 and BrdU/O1 (red, BrdU; green, cell marker; scale bar = 10 um).

Figure 4. Cannabinoid effects on ED-1⁺ cells and cell morphology

(A) Single Z-plane (0.50 μ m thick) confocal images of striatal BrdU⁺/ED-1⁺ cells from BD rats showing cells of various morphology. Process-bearing cells as well as cells with only BrdU⁺ nuclei and surrounding cytoplasm labeled for ED-1 (red, BrdU; green, cell marker; scale bar = 10 μ m).

(B) Cannabinoid treatment reduced ED-1⁺ cells. The percentage of BrdU⁺/ED-1⁺ cells was significantly lower in BD-WIN vs BD rats in PFC and striatum (** p <0.001 vs. BD rats, X^2 ; n =3 per group).

(C) NG2⁺ cells from adjacent striatal section in BD rats have thickened processes and phagocytic morphology, and differ in appearance from populations of fibrillar NG2⁺ cells from BD-WIN and NL rats. Scale bar = 20 μ m

Figure 5. Effect of cannabinoids on viral angiogenesis

(A) Quantification of regional differences in myelin basic protein expression, normalized to B-actin (* $p < 0.05$, Student's t test) and qualitative representative images of coronal sections stained for myelin with LFB-PAS showing reduction in LFB staining in perivascular areas of orbitofrontal subcortical white matter and striatum in BD rats 1 week after infection. The arrowhead points to a perivascular demyelinated area in a striatal myelin bundle of a BD rat. Values represent mean optical density in arbitrary units ($n = 4-5$ per group). Scale bar = 20 μm .

(B) Morphometric analysis of Nissl-stained sections 1 week after BDV infection, showing significantly increased vascularity (* $p < 0.05$, Student's t test) in the striatum of BD rats compared to NL uninfected animals.

(C) One week treatment with WIN 1 mg/kg BID, significantly reduced vasculature in BD-WIN rats compared to vehicle-treated BD rats (## $p < 0.01$ # $p < 0.05$, Tukey's following significant ANOVA). Values represent percentage of endovascular space per unit tissue area ($n = 3-4$ per group).

(D) Representative PFC and striatal sections showing blood vessels in WIN-treated BD rats (right) and networks of dilated vessels and perivascular cells in untreated BD rats (left). Scale bar = 500 μm .

Figure 6. Regional changes in endocannabinoid levels

(A) Anandamide (AEA) levels were significantly decreased in the striatum of BD (* $p < 0.05$ vs NL) and BD-WIN rats (** $p < 0.001$ vs NL), (### $p < 0.001$ compared to untreated BD) (Tukey's following significant ANOVA). There were no group differences in tissue levels of AEA in PFC.

(B) There were no group differences in tissue levels of 2-arachidonyl glycerol (2-AG) in PFC and striatum. Values represent the mean $\pm$ SEM of $n = 8-11$ independent measurements per group.