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Short Communication

Cannabinoid rescue of striatal progenitor cells in chronic Borna Disease viral encephalitis in rats

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A growing number of environmental and pharmacologic manipulations have been shown to influence adult neurogenesis. Borna disease virus (BDV) in rats causes cortical and subcortical infection with extrapyramidal motor symptoms, and hippocampal infection suppresses neurogenesis. Given the known effects of cannabinoids in promoting neural progenitor cell survival, the authors examined *in vivo* effects of chronic BDV infection in rats on BrdU-positive progenitor cells in striatum, together with neuroprotective actions of cannabinoids. Birth and survival of BrdU-positive progenitor cells in striatum of BDV-infected rats treated with a general cannabinoid agonist (WIN 55,212 1 mg/kg i.p. b.i.d. × 7 days) were examined, as well as anti-inflammatory, antiviral, and nutritional effects of cannabinoids. Cannabinoid treatment protected BrdU-positive progenitor cells in striatum that were susceptible to virus-induced injury ($p < .01$) through suppression of microglia activation ($p < .001$). As a consequence of their anti-inflammatory actions and support of neural progenitor cell survival, cannabinoids may be adjunctive treatment for encephalitides with microglial inflammation and neurodegeneration. *Journal of NeuroVirology* (2008) **14,** 252–260.

Keywords: inflammation; microglia; neurogenesis; Parkinson's disease

There has been increasing interest in the role of drugs in expression of central nervous system (CNS) viral disease, with drugs of abuse receiving a large amount of attention. Drug-virus interactions can occur through immune suppression, or dysregulation, or site- or circuit-specific neurotoxicity. Opiates, amphetamines, cocaine, alcohol, cannabinoids, and their endogenous transmitter systems have been implicated in manifestations and acceleration of CNS viral disease elicited by retroviruses, i.e., human immunodeficiency virus (HIV) (Anthony *et al*, 2004 2005; Arango et al, 2004; Cherner *et al*, 2005; El-Hage *et al*, 2006; Hauser *et al*, 2005; Langford *et al*, 2003; Rippeth *et al*, 2004; Venkatesan *et al*, 2007); simian immunodeficiency virus (SIV) (Benito *et al*, 2005; Czub *et al*, 2004; Molina *et al*, 2006; Perez-Casanova *et al*, 2007); feline immunodeficiency virus (FIV) (Gavrilin *et al*, 2002)]; and also by Borna disease virus (BDV) (Fu *et al*, 1993; Solbrig *et al*, 1994, 1998, 2000, 2002a, 2005, 2006; Solbrig and Koob, 2003).

Endocannabinoids (the endogenous lipid messengers that bind cannabinoid receptors) serve broad homeostatic and regulatory functions. Stimulation of cannabinoid (CB) receptors is neuroprotective in models of brain injury with striatal dysfunction, including Parkinson's disease (Garcia-Arencibia *et al*, 2007; Morgese *et al*, 2007; Sagredo *et al*, 2007), and cannabinoid receptor expression is altered in striatal neurodegenerative diseases such as Huntington's disease (Curtis *et al*, 2006). Some synthetic cannabinoids, like opiates, are anti-inflammatory and have been proposed as suppressors of inflammatory-mediated neurologic disease (Centonze *et al*, 2007; Maresz *et al*, 2007; Sanchez *et al*, 2006; Witting *et al*, 2006). Cannabinoids, however, are unique among drugs of abuse because of their in ability to support neurogenesis (Aguado *et al*, 2005, 2007; Crews and

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Nixon, 2003; Dominguez-Escriba *et al*, 2006; Eisch *et al*, 2000; Eisch and Harburg, 2006; Harkany *et al*, 2007; Jiang *et al*, 2005; Venkatesan *et al*, 2007; Yamaguchi *et al*, 2004). Recent studies have demonstrated the presence of a functional endocannabinoid system in neural progenitor cells that participates in the regulation of cell proliferation, differentiation, and survival (Harkany *et al*, 2007). Accordingly, cannabinoids could have a role in treatment of neurodegenerative illnesses. The presence of ongoing neurogenesis in the adult mammalian brain in striatum and hippocampus raises the possibility that endogenous neural progenitor cells may be able to generate new neurons to replace cells lost to brain injury or neurodegenerative disease, including virus-induced neurodegenerative diseases.

In immunocompetent animals, such as rats experimentally infected as adolescents, BDV produces an immune-mediated meningoencephalitis. There are time-based neurological sequelae, including movement, behavioral, convulsive, and dementing disorders (Narayan *et al*, 1983; Solbrig *et al*, 1994), driven by inflammatory-mediated neural cell loss (Bilzer and Stitz, 1994; Planz and Stitz, 1999; Stitz *et al*, 2002). In neonatal rats, infection with BDV on day 1 of life severely disrupts neural development and maturation, but confers a degree of tolerance that generally limits inflammatory reaction to activation of microglia and astrocytes, resident CNS cells.

Persistent CNS infections in rats occur with both neonatal and adolescent introduction of virus. BDV productively infects neurons, young neurons, and glia, as shown in neonatal infection models (Bautista *et al*, 1994; Carbone *et al*, 1989, 1991; Gosztonyi and Ludwig, 1995; Hornig *et al*, 1999), in adolescent/adult models (Gosztonyi and Ludwig, 1995) and *in vitro* (Billaud *et al*, 2000). BDV also interferes with synaptic connectivity or plasticity of differentiated neurons in neonatal models (Dietz *et al*, 2004; Gonzalez-Dunia *et al*, 2000; Gosztonyi and Ludwig 2001; Pletnikov *et al*, 2002; Williams *et al*, 2006, 2007), in adolescent models (Gosztonyi and Ludwig 2001; Solbrig *et al*, 2000), and *in vitro* (Hans *et al*, 2004; Kamitani *et al*, 2001), to provide informative paradigms of inflammatory and virus-based motor, psychomotor, and cognitive syndromes and neural injury.

Neural progenitor cells may be an additional population of cells to consider in BD pathogenesis and disease expression. Adult neurogenic zones: the subventricular zone (SVZ) of striatum, subgranular zone of hippocampus, and olfactory areas, are viral targets (Solbrig *et al*, 1994, 1998), and immature neurons of different subtypes support BDV infection to variable degrees, including self-renewing, Mash1 + cells (mammalian achaete-scute homologue 1) of hippocampus (Mayer *et al*, 2005; Solbrig *et al*, 2006). Furthermore, the association of striatal and hippocampal injury with the pathologic behaviors of adolescent-infected rats: dyskinesias, stereotypic behaviors, seizures, and impaired procedural learning (Solbrig *et al*, 1994, 2005, 2006), also supports the possibility of neurogenesis as a functional target of infection. Should defects in neurogenesis contribute to the behavior phenotype, structures that maintain adult neurogenesis, the striatum (ventriculo-olfactory system) and hippocampus, would be the regions to give rise to aberrant behaviors.

Given the supportive roles of cannabinoids in neural development, survival and repair, the hypothesis that cannabinoids rescue subventricular zone (SVZ) progenitor cells during encephalitis was tested by examining the ability of cannabinoid treatments to protect cell birth and survival in the "neurogenic" region of striatal SVZ in BDV-infected rats. To determine whether the protective actions of cannabinoids were through a mechanism that reduces brain inflammation, microglia numbers and activation were examined in striatum of cannabinoid-treated and untreated Borna groups.

Under methoxyflurane anesthesia, 4-week-old male Lewis rats were infected intracerebrally (i.c.) with BDV (BD rats) by injection of 1.6×10^4 tissue culture infectious dose units, strain He/80-1, into the right lateral ventricle, or sham-infected with sterile phosphate-buffered saline (PBS) (NL rats) (Solbrig *et al*, 1994). 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) and National Institutes of Health guidelines. Cell birth and survival were examined in the neurogenic region of the subventricular zone with morphologic and histologic analyses 2 weeks after infection.

To track proliferating cells, rats were given BrdU to label dividing cells and sacrificed at selected time points. BrdU (Sigma, St Louis MO, USA) dissolved in saline was administered to rats at a dose of 50 mg/kg i.p. × 3 (3 times on day 0) to label S phase dividing cells (Kuhn *et al*, 1996). A one-in-six series of sections from uninfected control and BD animals sacrificed 1 day or 1 week after the injection of BrdU were processed for BrdU (1:400 for DAB; Chemicon/Millipore, Billerica, MA, USA) as described (Solbrig *et al*, 2006). Immunoreactive (IR) cells were quantified along the longitudinal extent of striatum from Bregma +3.00 mm to Bregma −0.80 mm (Paxinos and Watson, 1998).

Cell counts were performed with a Nikon E800 microscope as described (Solbrig *et al*, 2006). Raw data for cell counts were statistically analyzed using analysis of variance (ANOVA) and *post hoc* Tukey test ($p < .05$) or Student's t test when comparing two groups. To control for differences in bioavailability or cell uptake of BrdU between uninfected and BD rats, BrdU-labeled cells in a non-neurogenic region, the habenula, were counted in selected sections of age-matched NL and BD rats.

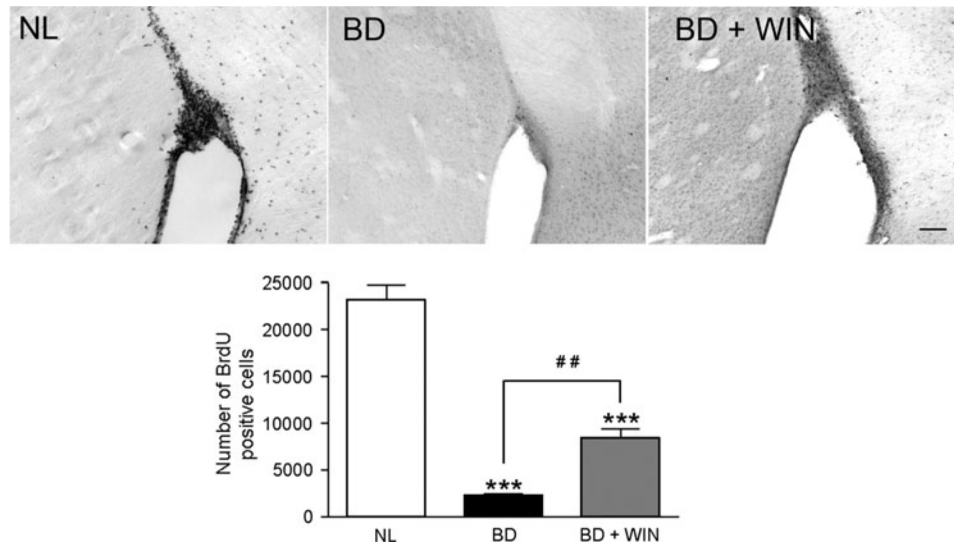

Figure 1 Cannabinoid therapy partially restores neural progenitor cells to BDV-infected rats. (*Upper panels*) Photomicrographs of BrdU-labeled cells in striatal subventricular zone of NL (uninfected), BD (Infected), and BD-WIN (BD WIN 55,212–treated) animals 7 days after BrdU administration and 2 weeks after infection (scale bar = 100 μm). (*Lower panel*) The number of BrdU-labeled cells in the SVZ of striatum was examined 1 week after BrdU injection. Infected animals had significantly fewer BrdU cells in SVZ than uninfected NL animals (*** $p < .001$) and cannabinoid treatment significantly increased total number of SVZ BrdU-positive cells compared to BD untreated animals (## $p < .01$) (Tukey's *post hoc* test following significant ANOVA). Values are mean ± SEM cell counts per animal for entire striatal SVZ ($n = 4$ per group).

The distinct morphology of proliferating cells—small, irregularly shaped, and often in clusters of contiguous or overlapping immunoreactive cells—was similar in normal uninfected and BD rats of the 1-day groups. BrdU-labeled cells were seen throughout the SVZ of striatum. Adjacent to the ventricle, BrdU cells were dense and overlapping, estimated to be greater than 42,000 per animal in NL rats, but accurate counts above that number were not obtained due to degree of overlap.

In the groups 1 week after BrdU injections, there were significantly fewer numbers of newly generated cells (BrdU-IR cells) in SVZ of BD rats relative to uninfected control rats (2% of control) (Figure 1). The habenula, counted as a control for bioavailability of BrdU, showed no difference in the average number of BrdU-IR cells per section (NL, 38.33±1.67; BD, 40.75±3.39; $t(1,4) = .649$, $p > .05$; $n = 4$ per group). These data show that BD rats (of the 1 week group) have significantly fewer BrdU-IR cells in the SVZ relative to NL and that this decrease is independent of bioavailability of BrdU.

Cannabinoids, specifically CB_1, stimulation, has been shown to confer neuroprotection and promote cell survival in various experimental paradigms (Guzman and Galve-Roperh, 2002, Mechoulam *et al*, 2002). During CNS development, endocannabinoid signaling regulates the proliferation, migration, specification, and survival of neural progenitors, directs the phenotypic differentiation of neurons and controls establishment of synaptic communication (Harkany *et al*, 2007). Neural progenitors have a functional endocannabinoid loop: the capacity to synthesize endocannabinoids, functional CB_1 receptors, and their catabolic enzyme. Because cannabinoid receptors are on neurons and glial cells and populations of progenitor cells (Curtis *et al*, 2006), cannabinoid signaling systems potentially influence neural development and survival in various physiological and pathophysiological conditions.

To determine whether exogenous cannabinoid administration had an effect on SVZ progenitor cells of BD rats, the general CB agonist $R(+)$-WIN 55,212-2 (Sigma) at doses of 1 mg/kg intaperitoneal (i.p.) twice per day (b.i.d.) × 7 days, or vehicle saline control, was administered to rats beginning at 5 weeks of age (1 week after BDV infection). To evaluate the effect of WIN 55,212 on cell proliferation, rats received 7 days of WIN treatment, and then were injected with BrdU and sacrificed the following day. To evaluate effects of WIN 55,212 on survival of newly born cells, rats were injected with BrdU, received 7 days of WIN treatment, and were sacrificed 1 week after the last BrdU injection ($n = 4$ per experimental group).

Progenitor cell survival, and not proliferation, was affected by WIN 55,212. There were no differences in SVZ cell proliferation, measured by BrdU + cells 1 day after BrdU injection in vehicle treated BD rats and WIN 55,212 treated BD rats [BD 39376.25 ± 2171.96 versus BD + WIN 40278.00 ± 2000.51; $t(1,6) = -.305$, $p = .7704$; $n = 4$ per group). However, there were group differences in SVZ progenitor cell survival, measured as numbers of SVZ BrdU-positive cells counted 1 week after BrdU injection in NL, BD, and BD-WIN animals ($F(2,9) = 102.27$, $p < .001$) and BrdU cells were significantly elevated

in BD-WIN compared to BD-vehicle group (BD-WIN 8460.00 ± 973.46 versus BD 2277.00 ± 174.49; $p <$ .01) (Figure 1). Thus, cannabinoid treatment rescued depressed SVZ cytogenesis in the setting of BDV infection by increasing progenitor cell survival.

Although cannabinoids stimulate feeding, there were no differences in body weights of the two 7-day BrdU groups (BD 120.75 ± 4.35 g versus BD + WIN 125.75 ± 2.84 g; $t(1,6) = .963$, $P = .37$; $n = 4$ per group], rendering differences in BrdU cell survival independent of nutrition status.

Any antiviral effect of cannabinoids was estimated by immunohistochemical detection and inspection of BDV-infected cells using a monoclonal BDV antibody to p38/40 nucleoprotein (38/15 H76, gift of L Stitz; 1:200). Abundant BDV-IR cells were found in striatal sections at comparable levels in untreated and cannabinoid-treated BD rats, with *in vivo* infection of progenitor cells appearing greater in untreated rats. Fewer small, round cells and more immunoreactive mature neurons were seen in cannabinoid-treated BD rats (Figure 2).

This preliminary result, that different (more juvenile) cell types could be more permissive for infection in untreated BD groups, raises the possibility that

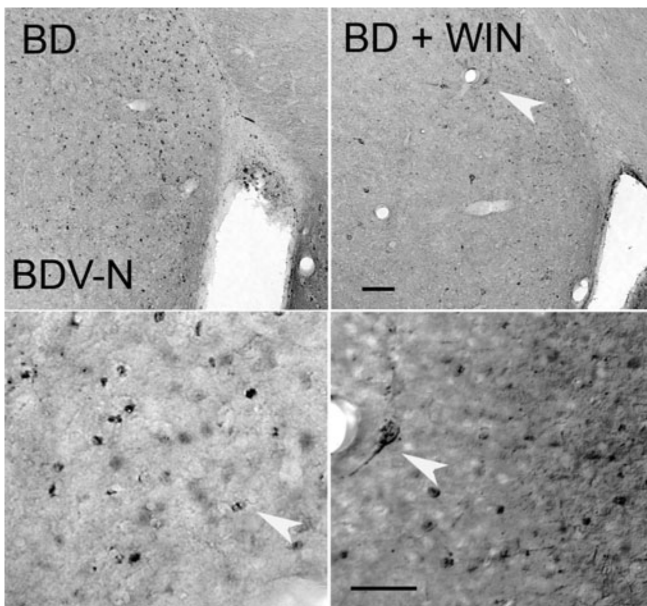

Figure 2 Cannabinoid treatment alters populations of infected cells in striatum. Immunohistochemical detection of infected cells 2 weeks after infection using monoclonal antibody to Borna p38/40 nucleoprotein. Vehicle-treated BD rats (*left*) have a homogeneous population of small cells recognized by BDV antibody (BDV-N). Rare cells appear to have an orientation or polarity, and possess a flagellum (*arrow*). In contrast, WIN 55,212–treated BD rats (*right*) have heterogeneous immunoreactive (IR) populations with fewer small, round cells and more mature neurons. Nucleus, perikaryon, and processes of a differentiated neuron stain with p38/40 mAb (*arrow*). The IR granules are Joest-Degen inclusion bodies, characteristic of BD (Haas *et al*, 1986; Sasaki and Ludwig, 1993). (Scale bar = 100 μm [upper], 50 μm [lower]. Top panels photographed at 100×; bottom panels at 400×.)

cannabinoids alter susceptibility of neural progenitor cells to infection. There may be cannabinoid interference with surface binding or internalization of virus in particular cells. The host cellular receptor for BDV is not known. Also unknown are consequences of cannabinoid treatment for baseline cell populations of striatum, the context for considering cell-type sensitivity to infection. Whether WIN 55,212 treatment biases toward mature populations of neural and glial cells would influence distribution of infected cells.

The existence and importance of *trans*-signaling between cannabinoid and other G-protein–coupled receptors (GPCRs) has been shown for growth factors in many cellular and tissue contexts (Harkany *et al*, 2007). Similar heterologous signaling between cannabinoids and other soluble GPCR ligands, cytokines, or chemokines may occur. Should cannabinoids modulate the chemokine signals recognized by migratory neural forms (Bagri *et al*, 2002; Lu *et al*, 2002, Stumm *et al*, 2003), neural cell orientation and development could be affected. Cannabinoid treatment appeared to eliminate BDV-IR polarized cells from BD animals, whereas polarized cells containing tails were present in sections from untreated BD rats (Figure 2).

CNS inflammation has been shown harmful to adult neurogenesis (Ekdahl *et al*, 2003; Monje *et al*, 2003). Yet cannabinoids, acting through CB_2 receptors or a palmitoylethanolamide (PEA)-sensitive non-CB_1/CB_2 cannabinoid receptor (Mackie and Stella, 2006), can regulate aspects of brain and peripheral inflammatory responses to mitigate deleterious effects of inflammation. Protective actions of CB_2 stimulation include modulation of microglia activation and release of proinflammatory cytokines (Walter and Stella, 2004; Miller and Stella, 2007).

After experimental infection of adolescent rats, BDV causes immune-mediated meningoencephalitis or encephalomyelitis with microglial activation and infiltrating immune cells, which have been characterized as CD4-positive, CD8-positive T cells, macrophages, and B cells. CD8-positive T cells represent the effector cell population showing antigen specificity for the nucleoprotein (Stitz *et al*, 2002).

To examine the influence of cannabinoids on inflammatory aspects of BD disease, immunohistochemical (IHC) staining for ED1 (AbD Serotec, Raleigh, NC, USA; 1:100) was performed. The ED1 monoclonal antibody recognizes an antigen in lysosomal membranes of phagocytes. It is expressed on lysosomal membranes of myeloid cells, weakly on their cell surfaces. It is also expressed by activated microglia, by the majority of tissue macrophages, and weakly by peripheral granulocytes. Immunoreactivity is high in cells with morphologic features of activated microglia (Bauer *et al*, 1994), and the ED1-positive cells in BD rat brains had small cell bodies and processes characteristic of activated microglia. Previous results with OX42 staining of microglia in comparable BD rat brains (Solbrig

et al, 2002b) would support the designation of ED1-positive cells as activated microglia, based on distribution and morphology. Because we could not exclude some ED1-positive cells as bone marrow derived macrophages, ED1-positive cells were denoted as microglia/macrophage cells. ED1-positive cell numbers and activity were susceptible to suppression via cannabinoid administration in comparisons of sections from cannabinoid-treated and vehicle-treated BD rats (Figure 3).

To describe further the relation between microglia inflammation and new cells, BrdU-positive cells were plotted against numbers of ED1-positive cells for each animal using the groups sacrificed 1 week after BrdU injection. There was a significant negative correlation between numbers of BrdU-positive cells and numbers of ED1-positive cells ($R = -.7021$, $R^2 = .4930$, slope $= -.3960 \pm .07589$; with significance for deviation from 0: $F(1,28) = 27.22$, $p < .0001$). Thus, the extent of inflammation had a direct titrating effect on new cells in SVZ, with microglia, and possibly macrophage, infiltrates lowering BrdU-positive cell survival.

Taken together, the results of our studies indicate that survival of adult neural precursor cells of the striatum of BD rat declines in infection, but can be rescued by cannabinoid administration. One mechanism of this restorative effect is prevention of microglia activation and reduction in microglia/macrophage numbers by a general cannabinoid agonist. As sensors of tissue health and disease, microglia have important roles in numerous neurologic conditions. Therefore, cannabinoid effects on microglia may have broad application to cases of pathological activation due to infection, toxins, or genetics, inclucing frontal and striatal syndromes or Parkinson's disease (Wahner *et al*, 2007).

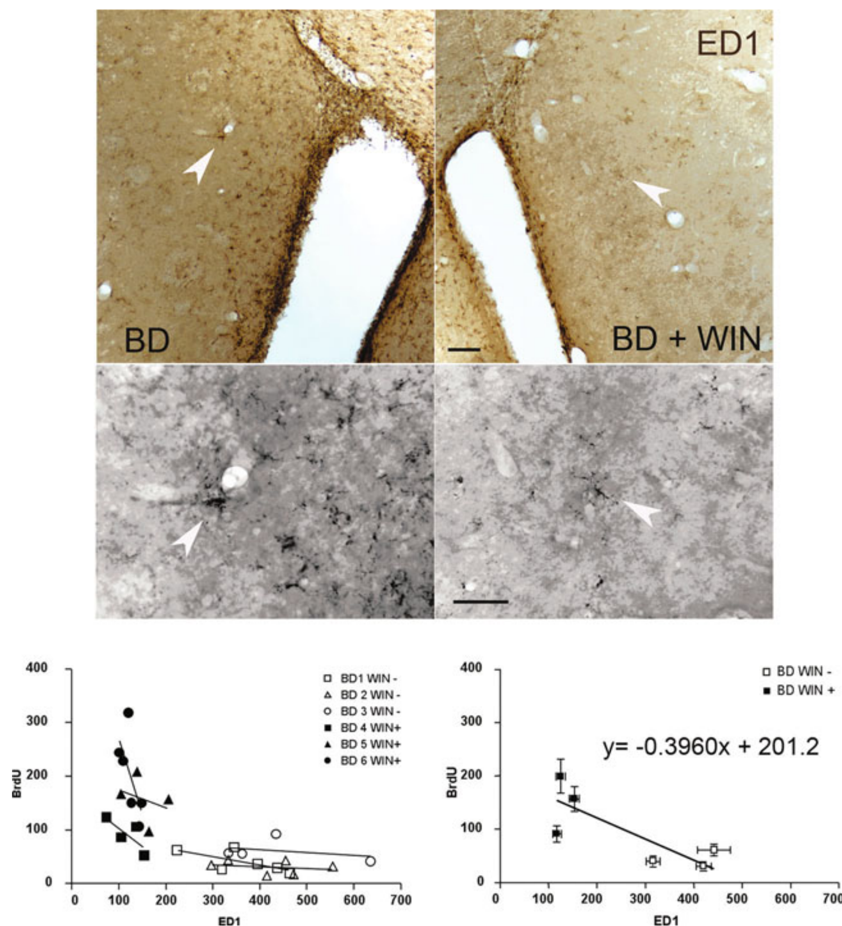

Figure 3 Cannabinoid treatment suppresses SVZ microglial inflammation during infection. Low- and high-power photomicrographs of ED1 + cells in BD (*left*) and BD-WIN 55,212–treated animals (*right*) 2 weeks after infection. WIN treatment for 7 days reduced numbers of ED1 IR cells. Arrows indicate ED1-immunoreactive cells and higher magnifications (*lower panels*) show process-bearing morphology, amoeboid, and ramified forms (Scale bar = 100 μm [upper], and 50 μm [lower]. Top panels photographed at 100×; bottom panels at 400×.) Inflammation negatively correlates with accumulation of BrdU + cells (*bottom panels*). Plots of numbers of ED1-positive and BrdU-labeled cells per hemisection of individual animals (BD: white symbols; BD-WIN: black symbols) show numbers of BrdU cells were inversely related to total numbers of activated microglia/macrophages in individual animals (*left*). Regression analysis showed a negative correlation between number of ED1 and BrdU labeled cells (*right*). Data points represent average (mean ± SEM) number of cells per section per animal ($n = 3$ per group).

Our studies support the premise that cannabinoid receptor agonists would be beneficial for treatment of chronic viral encephalitis with mixed degenerative and inflammatory features. CB_1 receptor stimulation could directly support neurons and neural progenitors, restoring neurogenesis and neural plasticity described in development, injury, and repair (Pryce *et al*, 2003, Jin *et al*, 2004, Harkany *et al*, 2007), whereas CB_2 receptor activation could reduce inflammation (Miller and Stella, 2007). Cannabinoid ligands acting on CB_2 receptors expressed by immune cells inhibit cytokine production (Ehrhart *et al*, 2005), decrease antigen presentation (McCoy *et al*, 1995, 1999), modulate inflammatory cell migration (Miller and Stella, 2008), and are protective in inflammatory neuropathies (Guindon and Hohmann, 2008, Jhaveri *et al*, 2007) and experimental allergic encephalomyelitis (Maresz *et al*, 2007). Suppression of inflammation indirectly supports neurogenesis, releasing neural progenitor cells from suppressive inflammation (Ekdahl *et al*, 2003 Monje *et al*, 2003). The relative contribution of neurotropic and anti-inflammatory actions to precursor cell rescue can be investigated with CB receptor–specific agonists and antagonists. The investigation should extend to ligands of the "independent" endocannabinoid signaling system that involves PEA. There is experimental evidence of non-CB_1/CB_2 receptors on neurons, with involvement in synaptic transmission and spinal analgesia (Hajos and Freund, 2002, Welch *et al*, 1998), and on inflammatory cells, with roles in peripheral inflammatory pain responses (LoVerme *et al*, 2006) microglia and neutrophil migration (Franklin and Stella, 2003, Walter et al, 2003, Nilsson *et al*, 2006).

This current work expands on the protective role for cannabinoids and opens a novel therapeutic window for manipulating neural progenitor cell fate. The apparent action of cannabinoids on cell survival merits future studies to define at cellular and network levels, the spatial and temporal characteristics of cannabinoid signaling important to neuroprotection and neurogenesis. Further work will be required to unravel the receptor (CB_1, CB_2, non-CB_1/CB_2) and signaling mechanisms that subserve cannabinoid protective effects and to establish phenotypes of rescued cells. Whether cannabinoids have antiviral effects and whether protective effects of cannabinoids generalize to other viral encephalitides can then be addressed.

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