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Role of the cannabinoid system in the transit of beta-amyloid across the blood-brain barrier

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Running title
Cannabinoids promote Aβ BBB clearance
Abstract

Emerging evidence suggests beta-amyloid (Aβ) deposition in the Alzheimer’s disease (AD) brain is the result of impaired clearance, due in part to diminished Aβ transport across the blood-brain barrier (BBB). Recently, modulation of the cannabinoid system was shown to reduce Aβ brain levels and improve cognitive behavior in AD animal models. The purpose of the current studies was to investigate the role of the cannabinoid system in the clearance of Aβ across the BBB. Using *in vitro* and *in vivo* models of BBB clearance, Aβ transit across the BBB was examined in the presence of cannabinoid receptor agonists and inhibitors. In addition, expression levels of the Aβ transport protein, lipoprotein receptor-related protein1 (LRP1), were determined in the brain and plasma of mice following cannabinoid treatment. Cannabinoid receptor agonism or inhibition of endocannabinoid-degrading enzymes significantly enhanced Aβ clearance across the BBB (2-fold). Moreover, cannabinoid receptor inhibition negated the stimulatory influence of cannabinoid treatment on Aβ BBB clearance. Additionally, LRP1 levels in the brain and plasma were elevated following cannabinoid treatment (1.5-fold), providing rationale for the observed increase in Aβ transit from the brain to the periphery. The current studies demonstrate, for the first time, a role for the cannabinoid system in the transit of Aβ across the BBB. These findings provide insight into the mechanism by which cannabinoid treatment reduces Aβ burden in the AD brain and offer additional evidence on the utility of this pathway as a treatment for AD.

**Keywords:** Alzheimer’s disease, amyloid, cannabinoid, blood-brain barrier, clearance, low density lipoprotein receptor-related protein 1
Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative process characterized by neuronal cell loss (Donev et al., 2009) and a global decline in cognitive function (Citron, 2010). The major pathological hallmarks of AD include the formation of neurofibrillary tangles and the deposition of beta-amyloid proteins (Aβ) in the brain and cerebrovasculature (Mehta, 2007). Moreover, AD pathophysiology is associated with increased neuroinflammation and oxidative stress (Galasko and Montine, 2010) in addition to excitotoxicity and dysregulated calcium homeostasis (LaFerla, 2002). While the etiology and pathogenesis of AD are poorly understood, Aβ accumulation in the brain appears to be a key factor in the development of AD as it precedes the neuroinflammatory and neurotoxic aspects of this disease (LaFerla et al., 2007). Furthermore, soluble Aβ levels in the brain correlate with the severity of neurodegeneration (McLean et al., 1999; Weller et al., 2009) and are a predictor of cognitive impairment in AD (Nordberg, 2008).

An increasing body of evidence suggests a neuroprotective role for the cannabinoid system in the brain that may have applications in the treatment of neurodegenerative disorders, including AD (Campbell and Gowran, 2007; Sarne and Mechoulam, 2005). The cannabinoid system consists of two G protein-coupled membrane receptors (CB1 and CB2) and a number of endogenous agonists (endocannabinoids), one of which is 2-arachidonoyl-glycerol (2-AG) (Kirilly et al., 2012). 2-AG activity is predominantly regulated by monoacylglycerol lipase (MAGL) (Dinh et al., 2002), and to a lesser extent, fatty acid amide hydrolase (FAAH) (Bisogno et al., 2002) and α/β-hydrolase domain 6 (ABHD6) (Marrs et al., 2010). These enzymes hydrolyze and inactivate 2-AG and, as a consequence, mitigate signaling through the CB receptors (Savinainen et al., 2012). In addition, hydrolysis of 2-AG in the brain generates arachidonic acid and the production of neuroinflammatory prostaglandins. Disruption of MAGL activity suppresses these inflammatory processes, resulting in neuroprotection.
The neuroprotective function of the cannabinoid system is thought to occur through a variety of mechanisms. Activation of CB1, which controls the release of excitatory neurotransmitters from the pre-synaptic neuron, can protect against excitotoxicity (Marsicano et al., 2003; Shen and Thayer, 1998) and promote neurogenesis (Aguado et al., 2007; Jiang et al., 2005; Jin et al., 2004). Meanwhile, stimulation of the CB2 receptor attenuates oxidative stress (Horvath et al., 2012) and reduces neuroinflammation by suppressing microglial activation (Ehrhart et al., 2005) and controlling the production of inflammatory mediators (Campbell and Gowran, 2007).

With respect to the cannabinoid system and AD, a number of recent studies explored the influence of the cannabinoid system on Aβ accumulation in the brain. CB receptor stimulation reduced Aβ brain deposition (Martin-Moreno et al., 2012; Wu et al., 2013), restored synaptic plasticity, and improved cognitive behavior in mouse models of AD (Aso et al., 2012; Haghani et al., 2012). Similarly, inactivation of the MAGL enzyme lowered Aβ brain burden (Chen et al., 2012; Piro et al., 2012) and improved long-term synaptic plasticity, spatial learning, and memory (Chen et al., 2012; Pan et al., 2011) in transgenic AD animals. Recent work has suggested the impact of the cannabinoid system in ameliorating Aβ brain burden is the result of an increased transport of Aβ out of the brain (Martin-Moreno et al., 2012). As Aβ transit across the blood-brain barrier (BBB) is an important determinate of Aβ accumulation in the AD brain, we investigated the role of the cannabinoid system in the clearance of Aβ across the BBB. We found that endocannabinoid treatment or MAGL inhibition facilitated Aβ transit across the BBB in vitro and in vivo. This effect appears to be the result of increased expression of the low density lipoprotein receptor-related protein 1 (LRP1), which is known to participate in the brain-to-blood transport of Aβ (Shibata et al., 2000). These studies suggest a role for the cannabinoid system in the elimination of Aβ from the brain to the periphery and may explain the impact of this system on Aβ brain burden and AD pathophysiology.
Material and methods

Primary human brain microvascular endothelial cells (HBMEC) and associated culture reagents were purchased from Sciencell Research Laboratories (Carlsbad, CA, USA). Fibronectin solution was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fluorescein-labeled Aβ(1-42) was purchased from rPeptide (Bogart, GA, USA). JZL195 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). All other small drug molecules used for these studies were purchased from Tocris Bioscience (Ellisville, MO, USA). Unlabeled human Aβ(1-42) and the human Aβ(1-42) enzyme linked immunosorbent assay (ELISA) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). The ELISA kit for mouse LRP1 was purchased from Cedarlane Labs (Burlington, NC, USA). Halt enzyme inhibitor cocktails and the BCA protein assay were purchased from Thermo Scientific (Waltham, MA, USA). The 24-well membrane inserts (translucent, 0.4µm pore) and 24-well companion plates were purchased from Fisher Scientific (St. Louis, MO, USA). The stereotaxic apparatus and large probe holder were purchased from Stoelting Co. (Wood Dale, IL, USA). Wild-type (C57BL/6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and allowed to adapt to the vivarium for 2 weeks prior to any experimental procedures. Mice were singly housed under standard laboratory conditions (23±1°C, 50±5% humidity, and a 12-hour light/dark cycle) with free access to food and water throughout the study. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

Aβ transcytosis across an in vitro BBB model

The in vitro model of the BBB used for these studies was characterized previously by our group (Bachmeier et al., 2010). In brief, HBMEC were seeded at 50,000 cells cm⁻² onto fibronectin-coated, 24-well, membrane inserts to establish a polarized, confluent monolayer representative of the BBB. The layer of endothelial cells separates this system into apical (“blood” side) and basolateral (“brain” side)
compartments (for a schematic see (Bachmeier et al., 2011)) and provides a mechanism for administering or sampling compounds on either side of the BBB model. The effect of cannabinoid treatment on Aβ transcytosis across the BBB model was examined using a fluorometric Aβ assay previously reported by our group (Bachmeier et al., 2010). Additionally, using the same cannabinoid modulators and concentrations tested in the Aβ assay, we evaluated the effect of each treatment on BBB monolayer integrity in the same manner described previously (Bachmeier et al., 2010). No difference was observed in the movement of a paracellular marker (fluorescent 10kDa dextran) across the in vitro BBB model between treated and control conditions, indicating the barrier properties of the BBB model are maintained in the presence of drug exposure (data not shown). A schematic of the cannabinoid pathway and the modulators used in these studies is depicted in Figure 1.

Cannabinoid receptor agonism

To examine the basolateral-to-apical transcytosis of Aβ, medium consisting of 2µM fluorescein-Aβ(1-42) was placed in the basolateral compartment of the BBB model while the apical side of the membrane was exposed to various concentrations (1, 2, 5, 10, and 20µM) of: 1) cannabinoid agonists (2-AG, CB13), 2) monoacylglycerol lipase (MAGL) inhibitors (JZL184, JZL195), 3) fatty acid amide hydrolase (FAAH) inhibitors (PF750, JNJ 166), or 4) an α/β-hydrolase domain 6 (ABHD6) inhibitor (WWL70). For a summary of the drug treatment groups and the concentrations used in the in vitro studies, see Table I. Samples were collected from the apical compartment at 0, 30, and 60 minutes to assess the rate of fluorescein-Aβ(1-42) transcytosis across the cell monolayer (basolateral-to-apical). For all of the in vitro BBB studies, the apparent permeability of fluorescein-Aβ(1-42) for each treatment condition was determined as previously indicated (Bachmeier et al., 2010) and expressed as a percentage of control. Moreover, using a standard process to limit aggregation, the Aβ peptides used in each of the studies
were solubilized in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to acquire a monomeric/dimeric sample and minimize the formation of β-sheet structures as we previously described (Bachmeier et al., 2013).

Cannabinoid receptor modulation

In the same manner as the agonism studies above, the basolateral-to-apical transit of Aβ was examined in the presence of a combination of cannabinoid pathway modulators. Medium consisting of 2µM fluorescein-Aβ(1-42) was placed in the basolateral compartment of the BBB model while the apical side of the membrane was exposed to: 1) a cannabinoid receptor agonist, 2) cannabinoid receptor type 1 or 2 (CB1, CB2) inhibitor, or 3) a combination of both agonists and inhibitors. As above, samples were collected from the apical compartment at 0, 30, and 60 minutes to assess fluorescein-Aβ(1-42) transcytosis across the cell monolayer (basolateral-to-apical). In a separate study, we examined Aβ transit across the BBB in the opposing direction (apical-to-basolateral). Here, 2µM fluorescein-Aβ(1-42) was placed in the apical compartment of the BBB model while the basolateral side of the membrane was exposed to the same combination of agonists and inhibitors mentioned above. This time, samples were collected from the basolateral compartment at 0, 30, and 60 minutes to assess the apical-to-basolateral penetration (i.e., brain entry) of fluorescein-Aβ(1-42) across the BBB model.

Intracerebral Aβ(1-42) injections

These studies were performed using a mouse Aβ BBB clearance model described by our group previously (Paris et al., 2011). Briefly, male wild-type (C57BL/6) mice (8-10 months of age) were anesthetized via inhalation using a 4% isoflurane / oxygen mix. While under anesthesia, the mice were administered vehicle (1:1 DMSO:PBS), JZL195 (10 mg/kg) alone or JZL195 in combination with PF514 (10 mg/kg) or AM630 (10 mg/kg) via intraperitoneal (i.p.) injection. One hour after the i.p. injection, the mice were stereotaxically injected unilaterally with 3µl of 1mM human Aβ(1-42) into the caudate putamen of the right hemisphere of the brain (0.5mm anterior to the bregma, 2 mm lateral to the
midline, and 3 mm below the surface of the skull). Ten minutes after intracerebral administration of human Aβ(1-42), the mice were euthanatized and plasma samples were collected via cardiac puncture. Additionally, the brain (minus the cerebellum) was extracted and all tissue samples were immediately snap frozen in liquid nitrogen.

**Tissue processing and analysis**

Plasma samples were analyzed by ELISA for human Aβ(1-42) (Invitrogen Corp., Carlsbad, CA, USA) or mouse LRP1 (Cedarlane Labs, Burlington, NC, USA) as recommended by the manufacturers and expressed as pg per ml or µg per ml, respectively. To determine LRP1 levels in the brain, the contralateral hemisphere (i.e., non Aβ-injected) was homogenized at 4°C in 700µL of ice-cold M-PER reagent (Pierce Biotechnology, Rockford, IL, USA) containing phenylmethanesulfonyl fluoride (1mM) and Halt protease and phosphatase inhibitor cocktail (1X) (Thermo Scientific, Waltham, MA, USA). Homogenates were centrifuged at 14,000g for 20 minutes and the resulting supernatant was analyzed for mouse LRP1 using an ELISA and normalized to total protein content as determined by the bicinchoninic acid (BCA) method. Expression levels in brain tissue were expressed as ng of LRP1 per mg protein.

**Statistics**

Statistical analyses were performed using an ANOVA and Bonferroni post-hoc test.

**Results**

**Cannabinoid receptor agonism**

The role of the cannabinoid pathway in fluorescein-Aβ(1-42) transcytosis was examined using an established *in vitro* model of the BBB (Bachmeier *et al.*, 2010). Cannabinoid receptor stimulation with an endocannabinoid agonist (2-AG) dose-dependently enhanced the basolateral-to-apical transit of Aβ across the BBB model, increasing Aβ transcytosis to nearly twice that of control (Figure 2). The
synthetic CB receptor agonist, CB13, also facilitated Aβ transcytosis across the BBB model, though the impact of this compound was not as potent or as pronounced as 2-AG (Figure 2). Aβ BBB transcytosis was also significantly altered upon treatment with the MAGL inhibitors JZL184 and JZL195. The profile with these drugs was similar to that observed for 2-AG, resulting in an approximately 2-fold increase in Aβ BBB transit compared to control (Figure 3A). It should be noted that JZL195 is a dual inhibitor of MAGL and FAAH (Long et al., 2009b), whereas JZL184 is selective for MAGL only (Long et al., 2009a). We also examined the impact of other 2-AG hydrolyzing enzymes (FAAH and ABHD6) in the BBB model. Treatment with the FAAH inhibitors JNJ166 and PF570 had no effect on Aβ transit at concentrations up to 10\(\mu\)M (Figure 3B). Similar results were obtained for the ABHD6 enzyme inhibitor, WWL70 (Figure 3C). While these drugs did demonstrate an effect at 20\(\mu\)M, the magnitude of this effect was less than that observed with MAGL inhibition or direct 2-AG agonism.

**Cannabinoid receptor modulation**

To determine whether the effects we observed with endocannabinoid agonism and MAGL inhibition were mediated through the CB receptors, we examined Aβ BBB transcytosis upon direct CB receptor inhibition. Aβ BBB transcytosis was unaffected by CB receptor modulation as treatment with inhibitors of the CB1 (PF514) and/or CB2 (AM630) receptors did not alter the basal rate of Aβ transit across the BBB model (Figures 4 and 5). However, when the CB receptors were stimulated with 2-AG or the MAGL inhibitor, JZL195, we observed a reduction in Aβ BBB transcytosis in the presence of CB receptor antagonism. For CB1 inhibition, we observed approximately a 50% reduction in 2-AG or JZL195 stimulated Aβ BBB transit for each concentration of PF514 tested (Figure 4). CB2 inhibition by AM630 resulted in a dose-dependent decrease in Aβ BBB transit following receptor stimulation by 2-AG or JZL195 (Figure 5). At 10\(\mu\)M, AM630 completely negated the stimulatory effects of 2-AG and JZL195, reducing Aβ BBB transytosis to control levels (Figure 5). Interestingly, when both CB1 and
CB2 were inhibited simultaneously, not only were the stimulatory effects of 2-AG and JZL195 fully
negated, but Aβ BBB transcytosis was diminished below baseline to approximately half that observed
under control conditions (Figure 6). Alternatively, we also examined the effect of cannabinoid receptor
modulation on the brain entry of Aβ (i.e., “blood”-to-“brain” direction). Once again, CB receptor
inhibition alone did not influence Aβ BBB transit, however, CB receptor stimulation with 2-AG
significantly reduced (>40%) the BBB penetration of Aβ in the *in vitro* model (Figure 7). When the
treatments were combined, CB receptor blockade slightly abrogated the stimulatory effects of 2-AG on
Aβ BBB entry, nevertheless Aβ transit remained well below that observed for control (Figure 7).

**Aβ BBB clearance *in vivo***

Using an *in vivo* model of Aβ clearance across the BBB (Paris *et al.*., 2011), we intracranially
administered human Aβ(1-42) to wild-type mice and examined the appearance of Aβ(1-42) in the
plasma following treatment with various modulators of the cannabinoid pathway. Upon stimulation of
the cannabinoid pathway with the MAGL inhibitor JZL195, plasma Aβ(1-42) was significantly elevated
(*P* < 0.05) compared to vehicle (2.4-fold), demonstrating enhanced Aβ(1-42) clearance from the brain
parenchyma to the periphery (Figure 8). Alternatively, when mice were administered a CB1 inhibitor
(PF514) or CB2 inhibitor (AM630), the effect of JZL195 on Aβ BBB clearance was fully negated, as no
difference in plasma Aβ(1-42) levels was observed between mice receiving vehicle and mice treated
with the combination of JZL195 and PF514 or AM630 (Figure 8).

**LRP1 expression**

As LRP1 levels in the brain and periphery are known to influence Aβ removal from the brain
(Deane *et al.*., 2008), we examined LRP1 expression following treatment with various modulators of the
cannabinoid pathway. In the brain, we observed a 50% increase in LRP1 levels (*P* < 0.05) following
JZL195 treatment in comparison to vehicle (Figure 9A). When mice were treated with the combination
of JZL195 and a CB1 inhibitor (PF514) or CB2 inhibitor (AM630), LRP1 levels were reduced in comparison to JZL195 alone, however, LRP1 levels in these groups were still above that observed for vehicle, though statistical significance from vehicle was not achieved for either inhibitor (Figure 9A). Similarly, plasma LRP1 levels in the JZL195-treated mice were 50% greater ($P < 0.05$) than in vehicle-treated mice (Figure 9B). Additionally, as in the brain, CB receptor inhibition attenuated the effect of JZL195 on LRP1 levels in the periphery, with the CB1 inhibitor PF514 having the greatest impact, fully reducing LRP1 levels to that observed in vehicle-treated mice (Figure 9B).

**Discussion**

Several studies have established a role for the central cannabinoid system in regulating age-related processes like neuroinflammation, neurogenesis, and memory, which has implicated this pathway as potential target for the treatment of AD (Marchalant *et al.*, 2012). In particular, prior work has indicated endocannabinoid exposure can prevent (Milton, 2002) or mitigate (Harvey *et al.*, 2012) Aβ-related neurotoxicity. More recently, administration of a natural cannabimimetic was shown to inhibit neuroinflammation, prevent amyloidogenesis, and attenuate disease progression in AD animal models (Gertsch and Anavi-Goffer, 2012). Recent work has suggested the Aβ-lowering effect of CB receptor stimulation may be due to enhanced Aβ transport across the blood-cerebrospinal fluid (CSF) barrier (Martin-Moreno *et al.*, 2012). Aβ elimination is an important means of reducing Aβ brain burden as evidence now suggests Aβ accumulation in sporadic AD is not due to aberrant Aβ production, but the result of impaired Aβ clearance from the brain (Castellano *et al.*, 2011; Mawuenyega *et al.*, 2010). More specifically, Aβ clearance via the BBB is reduced by approximately 30% in AD patients (Krohn *et al.*, 2011). In response to this, the purpose of the current studies was to investigate the role of the cannabinoid system in the clearance of Aβ across the BBB, an avenue which has yet to be explored.
The two best characterized endocannabinoids are N-arachidonoyl ethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG), which is present in endothelial cells (Gauthier et al., 2005). While these agonists bind to and activate both the CB1 and CB2 receptors, 2-AG is more potent than AEA (Sugiura et al., 2006) and is found in the brain at a concentration 150 times that of AEA (Scotter et al., 2010). In the present studies, treatment with 2-AG or the synthetic CB receptor agonist, CB13, resulted in a dose-dependent increase in the basolateral-to-apical transcytosis of Aβ across an in vitro model of the BBB. The impact of 2-AG on Aβ BBB transcytosis appears to be mediated through the cannabinoid receptor as co-treatment with a CB1 or CB2 inhibitor mitigated the effects of 2-AG. It should be noted that for all studies, inhibition of CB1 or CB2 in the absence of receptor stimulation did not significantly alter Aβ transytosis in the in vitro BBB model. Interestingly, dual inhibition of both CB1 and CB2 not only mitigated the stimulatory effect of 2-AG on Aβ BBB transcytosis, but reduced Aβ transit below that observed for control. Seemingly, inhibition of both CB receptors results in an overcompensation in response to 2-AG stimulation, an understanding of which requires further exploration. Additionally, we investigated the effect of the cannabinoid system on the brain entry of Aβ in the BBB model and observed a decrease in the apical-to-basolateral transcytosis of Aβ upon 2-AG stimulation. Thus, CB receptor activation appears to facilitate brain Aβ elimination across the BBB while at the same time preventing brain re-entry. Both CB1 and CB2 receptors are expressed in the brain endothelium (Golech et al., 2004) and appear to be localized on both the luminal and abluminal membranes of the BBB (Maccarrone et al., 2006), which may describe the bidirectional impact of CB receptor activation on Aβ BBB transcytosis, though further investigation is warranted.

In addition to testing the effect of direct cannabinoid receptor stimulation on Aβ BBB transcytosis, we also examined the impact of inhibiting the enzymes responsible for endocannabinoid hydrolysis and inactivation. MAGL is the enzyme primarily responsible for 2-AG inactivation (Dinh et
accounting for approximately 85% of 2-AG hydrolysis (Savinainen et al., 2012). MAGL is present in endothelial cells (Ho and Randall, 2007) and its activity and expression is elevated in the brains of AD patients (Farooqui et al., 1988) and Aβ-exposed rodents (van der Stelt et al., 2006). In vitro, we used two MAGL inhibitors (JZL184 and JZL195) to evaluate the role of MAGL on Aβ BBB transcytosis. Both inhibitors significantly enhanced Aβ transit across the BBB model in a manner comparable to that observed for 2-AG. It should be noted that JZL195 is an inhibitor of both MAGL and another 2-AG degrading enzyme, FAAH (Long et al., 2009b), while JZL184 selectively inhibits MAGL (Long et al., 2009a). The similarity of the effect of JZL184 and JZL195 on Aβ BBB transcytosis suggests that FAAH has minimal impact on Aβ transit at the BBB level. We evaluated this aspect more directly by testing specific inhibitors of FAAH and ABHD6 in the BBB model. While we observe an effect on Aβ BBB transcytosis at the highest concentration tested for each inhibitor (20µM), the potency of FAAH and ABHD6 on Aβ transit is more modest than that observed for MAGL. Like with 2-AG, CB1 or CB2 receptor inhibition mitigated the stimulatory effect of MAGL inhibition on Aβ transcytosis in the in vitro BBB model, once again indicating this process is CB receptor-dependent.

Using a mouse model of Aβ BBB clearance previously described by our group (Paris et al., 2011), we continued our evaluation of MAGL inhibition in an in vivo paradigm. Consistent with our in vitro findings, MAGL inhibition by JZL195 significantly enhanced the clearance of intracerebrally-administered Aβ from the brain to the periphery in wild-type mice. Moreover, administration of a CB1 or CB2 inhibitor completely reversed the stimulatory effect of JZL195 on Aβ BBB clearance, lowering plasma Aβ levels to the baseline established in control mice. In line with our observations, a recent study demonstrated a role for the cannabinoid system in the cellular transport of Aβ. Prolonged administration of two different CB receptor agonists reduced neuroinflammation, lowered brain Aβ levels, and improved cognitive performance in transgenic AD animals (Martin-Moreno et al., 2012). It
was determined that the decrease in Aβ brain burden following cannabinoid treatment was not due to changes in Aβ synthesis or release, but likely the result of an increased Aβ transport from the brain to the periphery by way of the blood-brain or blood-cerebrospinal fluid (CSF) barriers. Further analysis in choroid plexus cells, which constitute the blood-CSF barrier, revealed that treatment with the cannabinoid agonists significantly enhanced Aβ transport across the cell monolayer in as little as 1 hour (Martin-Moreno et al., 2012). The results of the present studies are consistent with this line of investigation as Aβ transit across the BBB model was enhanced upon CB receptor stimulation in the same 1 hour time frame. Thus, modulation of the cannabinoid system may provide an effective means of lowering Aβ burden in the AD brain by promoting Aβ clearance across both the blood-CSF and blood-brain barriers.

Accumulating data have implicated cannabinoids as acting members of peroxisome proliferator-activated nuclear receptors (PPAR) (O'Sullivan, 2007), which function as ligand-activated transcription factors. In fact, the anti-inflammatory properties of some endocannabinoids, including 2-AG, are mediated by one of the PPAR subtypes, PPAR-gamma (PPARg) (Du et al., 2011). Specifically, 2-AG binds to the PPAR-ligand binding domain (Bouaboula et al., 2005) and activates PPARg transcriptional activity (Rockwell et al., 2006). Furthermore, activation of PPARg has been shown to stimulate the expression of the low density lipoprotein receptor-related protein 1 (LRP1) (Gauthier et al., 2003), which is a major transporter in the brain-to-blood clearance of Aβ across the BBB (Shibata et al., 2000). Moreover, it has been demonstrated that circulating soluble LRP (sLRP) in the plasma binds Aβ and prevents brain re-entry, exerting a peripheral sink that promotes Aβ efflux from the brain (Sagare et al., 2007). Based on these reports, we evaluated LRP1 levels in the brain and plasma of mice treated with the MAGL inhibitor, JZL195. MAGL inhibition elevated LRP1 levels to a similar extent in brain and plasma, an effect that was attenuated by CB1 or CB2 blockade, consistent with our observations of Aβ
BBB clearance. The combination of increased brain LRP1 and plasma sLRP could provide explanation for the increased Aβ BBB transit we observe in the presence of CB receptor stimulation and may offer an effective strategy in lowering Aβ burden in the AD brain (Deane et al., 2008).

Recently, several studies have demonstrated a role for the cannabinoid system in ameliorating the AD phenotype. As mentioned earlier, direct CB receptor stimulation or modulation of the MAGL enzyme in AD mouse models was shown to reduce Aβ levels and plaque burden (Chen et al., 2012; Piro et al., 2012), enhance synaptic plasticity (Haghani et al., 2012; Wu et al., 2013), and improve spatial learning (Aso et al., 2012; Wu et al., 2013) and memory (Chen et al., 2012; Haghani et al., 2012). In the course of these studies, several mechanisms were investigated to describe the impact of the cannabinoid system on AD pathophysiology which included: 1) reduced neuroinflammatory prostaglandin production and cytokine signaling (Piro et al., 2012), 2) altered ion channel activation protecting against Aβ-induced neurotoxicity (Haghani et al., 2012), 3) decreased Aβ production due to reduced β-site amyloid precursor protein cleaving enzyme 1 (BACE1) expression (Chen et al., 2012), 4) increased phagocytic clearance of brain Aβ (Wu et al., 2013), 5) enhanced Aβ transport from the brain to the periphery (Martin-Moreno et al., 2012). The current studies expand upon the latter mechanism by demonstrating cannabinoid system modulation can promote Aβ clearance across the BBB by increasing LRP1 expression in the brain and plasma. It is unlikely that a single mechanism is responsible for the observed impact of cannabinoid treatment on the AD phenotype, but more plausibly a combination of mechanisms acting through several pathways. If so, a multimodal therapy such as this may be particularly effective in ameliorating the complex etiology inherent in AD.
Conclusions

The current studies demonstrate a novel role for the cannabinoid system in the transit of Aβ across the BBB. Direct stimulation of the CB receptors (2-AG) or inhibition of endocannabinoid-inactivating enzymes (MAGL) facilitated Aβ BBB clearance *in vitro* and *in vivo*. These effects appear to be mediated through the CB receptors as modulation of CB1 or CB2 mitigated the stimulatory impact on Aβ BBB clearance. Furthermore, we observed an increase in LRP1 levels in the brain and plasma upon cannabinoid treatment, providing rationale for the increase in Aβ transit from the brain to the periphery. Our findings are consistent with recent work describing an effect of cannabinoid treatment on Aβ transport out of the brain (Martin-Moreno *et al.*, 2012) and provide further insight into the mechanism by which cannabinoid system modulation has been shown to reduce Aβ brain burden and abrogate AD pathophysiology and cognitive decline. More work is necessary to fully understand the nature of the cannabinoid system in neurodegenerative disease and its utility as a treatment for AD.
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References


dihydropyridines lower abeta accumulation by targeting both the production and the clearance of abeta across the blood-brain barrier. Mol Med. 17, 149-162.


Figure 1
Figure 2
Figure 3A
Figure 3B
Figure 3C

A bar graph showing the effect of different concentrations of WWL 70 on % Control. The x-axis represents the apical treatment concentration in μM (control, 1, 2, 5, 10, 20), and the y-axis represents the % Control. The graph shows a significant increase in % Control at 20 μM compared to the control.
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9A
Figure 9B
Figure 1. Schematic of the cannabinoid pathway and the modulators used in the current studies. The cannabinoid receptors, CB1 and CB2, are both stimulated by 2-AG (endogenous agonist) and CB13 (synthetic agonist) and selectively inhibited by PF514 (CB1) and AM630 (CB2). 2-AG is hydrolyzed by the enzymes MAGL, FAAH, and ABHD6, which inactivates 2-AG and mitigates CB receptor stimulation. Alternatively, inhibitors of MAGL (JZL184, JZL195), FAAH (PF750, JNJ 166), and ABHD6 (WWL70) attenuate 2-AG conversion, which promotes stimulation of the CB receptors. Note: JZL195 is an inhibitor of both MAGL and FAAH. Straight arrows indicate stimulation, blunt lines indicate inhibition, curved arrows indicate metabolism, and the dotted line indicates conversion to an inactive metabolite. For illustrative purposes, not all of the molecules that influence the cannabinoid pathway are depicted.

Figure 2. Basolateral-to-apical transcytosis of fluorescein-Aβ(1-42) across an in vitro model of the BBB in the presence of an endogenous (2-AG) or synthetic (CB13) cannabinoid agonist. Fluorescein-Aβ (1-42) (2 µM) was exposed to the basolateral (“brain”) compartment while various concentrations of each agonist (1, 2, 5, 10, and 20 µM) were exposed to the apical (“blood”) compartment of the BBB model. Samples were collected from the apical compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 3). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

Figure 3. Basolateral-to-apical transcytosis of fluorescein-Aβ(1-42) across an in vitro model of the BBB in the presence of various cannabinoid enzyme inhibitors. Fluorescein-Aβ (1-42) (2 µM) was exposed to the basolateral (“brain”) compartment while various concentrations (1, 2, 5, 10, and 20 µM) of a (A) MAGL inhibitor (JZL184, JZL195), (B) FAAH inhibitor (PF750, JNJ166), or (C) ABHD6 inhibitor (WWL70) were exposed to the apical (“blood”) compartment of the BBB model. Samples
were collected from the apical compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 3). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

**Figure 4.** Basolateral-to-apical transcytosis of fluorescein-Aβ(1-42) across an *in vitro* model of the BBB in the presence of CB1 receptor inhibition. Fluorescein-Aβ (1-42) (2µM) was exposed to the basolateral (“brain”) compartment while the apical (“blood”) compartment of the BBB model was exposed to 2-AG (20µM), JZL195 (20µM), PF514 (1, 5, and 10µM), or combinations thereof. Samples were collected from the apical compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 4). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

**Figure 5.** Basolateral-to-apical transcytosis of fluorescein-Aβ(1-42) across an *in vitro* model of the BBB in the presence of CB2 receptor inhibition. Fluorescein-Aβ (1-42) (2µM) was exposed to the basolateral (“brain”) compartment while the apical (“blood”) compartment of the BBB model was exposed to 2-AG (20µM), JZL195 (20µM), AM630 (1, 5, and 10µM), or combinations thereof. Samples were collected from the apical compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 4). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

**Figure 6.** Basolateral-to-apical transcytosis of fluorescein-Aβ(1-42) across an *in vitro* model of the BBB in the presence of CB1 and CB2 receptor inhibition. Fluorescein-Aβ (1-42) (2µM) was exposed to the basolateral (“brain”) compartment while the apical (“blood”) compartment of the BBB model was
exposed to 2-AG (20µM), JZL195 (20µM), PF514 (1, 5, and 10µM), AM630 (1, 5, and 10µM), or combinations thereof. Samples were collected from the apical compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 3). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

**Figure 7.** Apical-to-basolateral transcytosis of fluorescein-Aβ(1-42) across an *in vitro* model of the BBB in the presence of CB1 and CB2 receptor inhibition. The apical (“blood”) compartment of the BBB model was exposed to fluorescein-Aβ (1-42) (2µM) and 2-AG (20µM), PF514 (1, 5, and 10µM), AM630 (1, 5, and 10µM), or combinations thereof. Samples were collected from the basolateral (“brain”) compartment at 60 minutes to determine fluorescein-Aβ(1-42) transcytosis across the BBB model and the values expressed as a percentage of control. Values represent mean ± SEM (n = 3). *P < 0.05 compared to control as determined by ANOVA and Bonferroni post-hoc test.

**Figure 8.** Appearance of human Aβ(1-42) in the plasma following intracerebral stereotaxic Aβ(1-42) administration. Vehicle (1:1 DMSO:PBS, n=10), JZL195 (10 mg/kg, n=10) alone, or JZL195 in combination with PF514 (10 mg/kg, n=6) or AM630 (10 mg/kg, n=6) was administered intraperitoneally (i.p.) to 8-10 month-old male wild-type (C57BL/6) mice 1 hour prior to intracerebral injection of Aβ(1-42). Plasma was collected 10 minutes after the intracerebral injection and evaluated for human Aβ(1-42) by ELISA. Values represent mean ± SEM and are expressed as pg per ml. *P < 0.05 compared to vehicle as determined by ANOVA and Bonferroni post-hoc test.

**Figure 9.** LRP1 levels in mouse (A) brain and (B) plasma following cannabinoid pathway modulation. Vehicle (1:1 DMSO:PBS, n=7), JZL195 (10 mg/kg, n=7) alone, or JZL195 in combination with PF514 (10 mg/kg, n=5) or AM630 (10 mg/kg, n=5) was administered intraperitoneally (i.p.) to 8-10 month-old male wild-type (C57BL/6) mice. Brain and plasma were collected 1 hour after the i.p. injection and
evaluated for mouse LRP1 by ELISA. Values represent mean ± SEM and are expressed as µg of LRP1 per ml plasma or ng of LRP1 per mg protein for brain. *P < 0.05 compared to vehicle as determined by ANOVA and Bonferroni post-hoc test.
Table I. Cannabinoid system modulators used to examine Aβ transcytosis across an *in vitro* model of the BBB.

<table>
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<th>Category</th>
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<th>Concentration in BBB model</th>
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<td>CB13</td>
<td>synthetic CB1 and CB2 receptor agonist</td>
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