Role of microRNA in CB1 antagonist-mediated regulation of adipose tissue macrophage polarization and chemotaxis during diet-induced obesity

Pegah Mehrpouya-Bahrami¹, Kathryn Miranda¹, Narendra P. Singh¹, Elizabeth E. Zumbrun¹, Mitzi Nagarkatti¹, and Prakash S. Nagarkatti¹,²

From the ¹Department of Pathology, Microbiology and Immunology, University of South Carolina School of Medicine, Columbia, SC 29208, USA

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² Address correspondence and reprint requests to: Prakash S. Nagarkatti: Vice President for Research, Carolina Distinguished Professor, University of South Carolina, 202 Osborne Administration Building, Columbia, SC 29208; prakash@mailbox.sc.edu; Tel. (803) 777-5458; Fax (803) 777-5457

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ABSTRACT

While cannabinoid receptor 1 (CB1) antagonists have been shown to attenuate diet-induced obesity (DIO) and associated inflammation, the precise molecular mechanisms involved are not clear. In the current study, we investigated the role of microRNA (miR) in the regulation of adipose tissue macrophage (ATM) phenotype following treatment of DIO mice with the CB1 antagonist SR141716A. DIO mice were fed high-fat diet (HFD) for 12 weeks then treated daily with SR141716A (10mg/kg) for four weeks while continuing HFD. Treated mice experienced weight loss, persistent reduction in fat mass, improvements in metabolic profile, and decreased adipose inflammation. CB1 blockade resulted in down-regulation of several miRs in ATMs including the miR-466 family and miR-762. Reduced expression of the miR-466 family led to induction of anti-inflammatory M2 transcription factors KLF4 and STAT6, while downregulation of miR-762 promoted induction of AGAP-2, a negative regulator of the neuroimmune retention cues, Netrin-1, and its co-receptor UNC5b. Furthermore, treatment of primary macrophages with SR141716A upregulated KLF4 and STAT6, reduced secretion of Netrin-1, and increased migration towards the lymph node chemo attractant, CCL19. These studies demonstrate for the first time that CB1 receptor blockade attenuates DIO-associated inflammation through alterations in ATM miR expression that promote M2 ATM polarization and macrophage egress from adipose tissue. The current study also identifies additional novel therapeutic targets for diet-induced obesity and metabolic disorder.

The incidence of obesity has grown significantly in the last 25 years leading to upwards of 1.45 billion overweight adults in the world, of which approximately 500 million are obese (1). Chronic low-grade, systemic inflammation associated with obesity plays a major role in the development of various chronic disease states, including type 2 diabetes, metabolic syndrome and cardiovascular disease, which together contribute to high rates of mortality and morbidity (2). The chronic inflammation observed is primarily driven by the intense migration and accumulation of adipose tissue macrophages (ATMs), which drive insulin resistance (3–6). The recruitment of ATMs to adipose tissue positively correlates with the production of pro-inflammatory molecules, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 that potentiate insulin resistance (7–9).

Macrophages show significant heterogeneity in phenotype and function, as factors in the local milieu determine their activation state and subsequent properties.
Macrophages can be activated under various conditions that lead to polarization between two main states, routinely classified as “M1” or “M2”. M1 macrophages are induced during obesity and are said to be ‘classically activated’ cells triggered by LPS and IFNγ that secrete pro-inflammatory cytokines (TNF-α, IL-6, IL-12) and generate nitric oxide (NO), a reactive oxygen species via iNOS activation (10, 11). However, M2 macrophages are considered “alternatively activated” and populate lean adipose tissue. They can be activated by IL-4 and IL-13, secrete anti-inflammatory cytokines, and upregulate arginase, which opposes NO production (12). Additionally, studies have shown that the M1 and M2 macrophage phenotypes are not clearly defined because multiple subtypes exist, which are activated by various stimuli, and it is possible to dynamically switch their polarization (13). The key signaling molecules such as DNA methyltransferase 3b (DNMT3b) and peroxisome proliferator activated receptor-γ (PPAR-γ) deregulate ATM polarization, inflammation, and insulin insensitivity (14, 15). In addition, other studies have shown that besides the molecules that trigger macrophage recruitment in adipose tissue, other signaling molecules such as neuronal guidance cues of the Semaphorin, Ephrin and Netrin families modulate macrophage retention in adipose tissue and regulate immunometabolism (16, 17). Indeed recent studies have shown Semaphorin 3E and Netrin-1 are involved in chemo attraction and retention of macrophages in the visceral adipose tissue of DIO mice (17, 18). Collectively, studies on neuroimmune guidance cues in macrophages define them as key regulators for ATM accumulation and active participants in the induction of chronic inflammation.

Weight loss is associated with beneficial effects of reducing the underlying inflammation in adipose tissue and subsequent amelioration of insulin resistance (19, 20). The endocannabinoid system plays a major role in food intake and energy balance. Over-activity of the endocannabinoid system in human obesity and in animal models of genetic and diet-induced obesity has been reported (21). Clinical studies on treatment of obesity and metabolic syndrome with the cannabinoid CB1 receptor antagonist SR141716A (“Rimonabant”) have shown greater weight loss in obese patients compared with placebo (22). However, this drug was removed from the market due to adverse psychiatric effects (23). Nevertheless, the mechanistic effect of CB1 receptor antagonist on inflammation and ATMs has not been well studied. Previous studies have shown that blockade of CB1 receptors suppressed inflammation in adipose tissue of DIO mice (24, 25). However, the signals controlling the beneficial effect of the CB1 receptor antagonists on inflammation in adipose tissue remain poorly understood.

MicroRNAs (miR) are short non-coding RNAs that can inhibit translation of their mRNA targets (26). Regulatory roles of miRs in many biological processes associated with obesity have been defined. They can synchronize obesity-related pathways such as immune-mediated inflammation, insulin action, fat metabolism, and energy homeostasis (27–29). Recently, we identified miRs involved in regulation of ATM phenotype during DIO (30). However, associations between aberrant miR expression and over-activation of the endocannabinoid system in obesity have not been previously reported to our knowledge. In the present study, we tested the role of miRs in SR141716A-mediated amelioration of obesity-induced inflammation, specifically by focusing on macrophage polarization and retention in visceral adipose tissue. Our study unveiled specific miRs that promote a switch from M1 to M2 phenotype as well as miRs that target neuroimmune guidance cues. Taken together, our findings suggest CB1 blockade in DIO mice promotes miR dysregulation, which suppresses adipose tissue inflammation and contributes to improved metabolism.

Results
SR141716A attenuates HFD-induced obesity

To study the effects of CB1 antagonist on obesity parameters, we treated DIO mice that were fed a 60% HFD for 12 weeks with SR141716A (SR) by daily oral gavage for 4 weeks (Figure 1A). All other experimental groups were treated with the vehicle (Veh) 0.1% Tween 80. In addition to the HFD+SR group, other experimental groups included mice fed a
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10% low-fat control diet (LFD) (LFD+Veh), ad-libitum HFD-fed mice (HFD+Veh), and HFD-fed mice that were pair-fed to the HFD+SR group (HFD-PFSR) (Figure 1B). Blockade of CB1 receptors with SR141716A resulted in acute and persistent weight loss, as well as transient reduction of calorie intake in DIO mice (Figure 1C & 1D). At the baseline of the study, the mice were stratified into groups balanced by mean fat mass. After 4 weeks of treatment, reduction in the fat mass and fat percentage was significant in HFD+SR group when compared to both HFD+Veh and HFD-PFSR groups, while the changes in lean mass were not significant (Supplementary Table 1). Fasting parameters including blood glucose, insulin, insulin resistance index (HOMA-IR), total cholesterol and triglycerides were examined pre- and post-the 4-week intervention with SR compound. Our data demonstrated improvement in metabolic parameters in the HFD+SR group when compared to HFD+Veh and HFD-PFSR groups, while SR141716A reduces adipose tissue inflammation and alters ATM miR profile

In a previous report, we showed that treatment of DIO mice with SR reduces both ATM infiltration and M1 polarization in epididymal adipose tissue (31). To further confirm the overall inflammatory status in adipose tissue, we quantitated the expression of macrophage polarization genes Nos2 (M1) and Arg1 (M2) in epididymal fat. SR treatment lowered Nos2 expression and increased expression of Arg1, when compared to HFD+Veh and HFD-PFSR, which validated an anti-inflammatory state in the adipose tissue of DIO mice results in amelioration of obesity, independent of its effect on calorie intake.

SR141716A reduces adipose tissue inflammation and alters ATM miR profile

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In order to investigate the effect of SR141716A on miR profile independent of its effect on calorie restriction, we identified miRs that were similarly dysregulated the HFD+SR group vs. both the HFD+Veh and the HFD-PFSR groups (Fig 3D). A heatmap of mean normalized expression of these altered miRs demonstrated that SR treatment alters miR expression independent of food restriction (Figure 3E).

We next used in silico analyses to identify potential pathways targeted by the dysregulated miRs. First, using Cytoscape analysis modules, we identified the targeted gene ontologies of dysregulated miRs. The main affected pathways following SR treatment included regulation of various components of the immune system (Supplementary Figure 1). Next, we performed analysis using Ingenuity Pathway Analysis (IPA, Qiagen). Predicted interactions between miRs and their targeted genes following SR treatment revealed that the altered miR profile might skew the ATM balance to a more anti-inflammatory macrophage phenotype (M2, Arginase+) through various cytokine, chemokine, transcription factor, and signaling networks (Supplementary Figure 2).

SR141716A-altered miRs promote a shift towards M2 macrophage phenotype

Interestingly, pathway analysis with IPA uncovered SR141716A-mediated alterations in miRs that may induce anti-inflammatory M2 macrophages by targeting the M2 related transcription factors (STAT3, STAT6, LCN2, KLF4, PPARg, SIRT1) (Figure 4A). The list of corresponding targets and miRs, along with each algorithm is summarized in Supplementary Table 2. More interestingly, for the first time we
uncovered that most of the members of the miR-466 family were down-regulated following SR141716A treatment regardless of calorie intake (Figures 3D and 4A). qRT-PCR of the miR-466 family from the ATMs validated that these were down-regulated following SR141716A treatment (Figure 4B). Furthermore, expression of the M2-transcription factor genes Klf4 and Stat6 were upregulated in HFD+SR ATMs (Figure 4C and 4D).

In order to validate the impact of the miR-466 family in induction of M2 phenotype in ATMs, peritoneal macrophages were isolated and cultured in conditioned medium from differentiated 3T3-L1 adipocytes, following transfection with miR-466* and miR-466f LNA™ power inhibitors. The data showed that Klf4 and Stat6 were over-expressed following miR-466 inhibition (Figures 4E and 4F), thereby supporting that miR-466 targeted these transcription factors. Moreover, the inflammatory cytokines such as TNF-α, IL-6 and IFN-γ were suppressed following miR-466 inhibition (Figures 4G-4I). Taken together these data suggested that blockade of CB1 in DIO mice results in down-regulation of the miR-466 family in ATMs, which promotes M2 macrophage polarization and thereby induces a switch towards anti-inflammatory state in adipose tissue.

**SR141716A ameliorates ATM retention in adipose tissue**

In addition to the effect of SR141716A in promoting M2 macrophages, we previously demonstrated significant decrease in the number of pro-inflammatory M1 macrophages in adipose tissue of the HFD+SR treated group when compared to the HFD+Veh group (31). In the current study, we investigated if this resulted from altered migration of ATM due to miR-mediated regulation of neuronal guidance cues. Filtering the IPA modules to the neuroimmune guidance cue canonical pathways demonstrated a novel series of miRs that targeted AGAP2, which is an upstream negative regulator of the Netrin-1 receptor UNC-5 Homology B (UNC5B) (Figure 5A). This suggested that downregulation of miR-762 may increase AGAP-2, and thus inhibit Netrin-1-mediated ATM detainment (Figure 5A). qRT-PCR validated expression of miR-762 was reduced while Agap2 was increased following SR treatment (Figures 5B and 5C). Interestingly, we noted that the level of expression of Netrin-1 at both protein and transcription level in ATM was significantly reduced in HFD+SR mice when compared to HFD+Veh mice (Figures 5D and 5E). Unc5b was also down-regulated in the HFD+SR vs. HFD+Veh (Figure 5F).

Additionally, we quantified gene expression of other Netrin family receptors (Supplementary Table 3). To validate the direct interaction of miR-762 and AGAP-2, we used BMDM that were treated with conditioned medium from 3T3-L1 adipocytes. Such BMDMs were transfected with a Renilla luciferase plasmid (transfection efficiency control) containing the 3’UTR of Agap2 and a Firefly luciferase reporter gene. Luciferase expression was suppressed by endogenous miR-762. Subsequently, upon transfection with Anti-miR-762 LNA microRNA inhibitor (5 nM), increased luciferase activity/luminescence was detected thereby demonstrating that the Agap2 3’UTR is a direct target of miR-762 (Figure 5G).

**Macrophage-dependent CB1 blockade promotes M2 polarization and decreased retention**

Next, to confirm that the miR-mediated M2 polarization and decreased ATM retention observed in HFD+SR treated mice was a direct effect of CB1 blockade and not a secondary effect due to weight loss or other factors, we treated primary macrophages in vitro with 3T3-L1 adipocyte conditioned media and SR141716A or DMSO vehicle control. Treatment of (3%) thioglycollate-elicited peritoneal macrophages with adipocyte conditioned medium and SR141716A or DMSO vehicle control. Treatment of (3%) thioglycollate-elicited peritoneal macrophages with adipocyte conditioned medium and SR141716A or DMSO vehicle control. Consistent with decreased miR-466, SR141716A treatment increased expression of the miR-466 targets Klf4 and Stat6 indicating a shift towards M2-phenotype (Figures 6B and 6C). Additionally, expression of the miR-762 target Agap2 was increased following SR141716A treatment (Figure 6D). Concomitantly, Netrin-1 secretion was reduced.
following SR141716A treatment (Figure 6E). Macrophage migration rate towards CCL19 was also measured because CCL19 is the primary chemokine implicated in emigration of tissue macrophages towards draining lymph nodes. SR141716A-treated macrophages exhibited higher migration towards CCL19 than DMSO-treated macrophages (Figure 6F), though the level expression of CCL19 receptor, CCR7, was similar in both groups (Figure 6G). These data suggested that SR141716A attenuates the over-secretion of Netrin-1 from ATMs in obese phenotype, and potentially promotes their emigration from adipose tissue through miR-762-mediated regulation of AGAP-2. Together these findings confirmed that blockade of macrophage CB1 signaling has anti-inflammatory and anti-retention effects in macrophages due to altered miR expression.

**Overexpression of miR-466 and miR-762 prevents the effects of CB1 antagonism in macrophages**

To verify that miRs mediate regulation of macrophage polarization and migration following CB1 antagonist treatment, we performed rescue experiments by overexpressing miR-466 and miR-762 in macrophages treated with SR141716A. Thioglycollate-elicited peritoneal macrophages were harvested from naïve mice then transfected with miR mimics followed by 24h incubation with conditioned medium from 3T3-L1 adipocytes and 10µM SR141716A or DMSO vehicle. Transfection of miR-466f and miR-466j mimics led to upregulation of the respective miRs, and likewise SR treatment reduced their expressions (Fig. 7a). Consistent with our claims that downregulation of miR-466 promotes M2 polarization, gene expression of *Klf4* and *Stat6* showed negative correlation with expression of miR-466f and miR-466j, albeit miR-466f appeared to better regulate *Stat6*, and miR-466j better regulated *Klf4* (Fig. 7a).

In order to confirm that miR-466 directly targets the 3’UTR of *Klf4* we performed a luciferase reporter assay in the RAW264.7 macrophage cell line. RAW264.7 cells were transfected with empty vector, vector containing the 3’UTR binding site for miR-466, or a scrambled 3’UTR negative control. The cells were then treated with SR141716A to induced downregulation of miR-466. Cells transfected with the 3’UTR of *Klf4* + SR had significantly elevated luciferase activity that was not observed in negative controls (Fig. 7b). It should be noted that the luciferase activity was slightly increased in pmiR-Glo-KLF4-UTR + DMSO vs. PmiR-Glo + DMSO, which could have resulted from experimental variation during transfection of various constructs or differential endogenous expression levels of miR-466 following transfection. Nonetheless, the significant increase in luciferase activity in pmiR-Glo-KLF4-UTR + SR vs. pmiR-Glo-KLF4-UTR + DMSO confirmed that miR-466 likely directly targets the 3’UTR of *Klf4*.

Next, to validate that miR-762 mediates regulation of macrophage chemotaxis following CB1 blockade, we plated peritoneal macrophages in transwell inserts and transfected them with miR-762 mimic followed by culture in conditioned medium from 3T3-L1 adipocytes + SR141716A. Half-way through the 24h incubation, medium containing CCL19 was added to the bottom well and the migrated cells were collected for gene expression and migration analysis at the 24h timepoint. As expected miR-762 expression was elevated in macrophages transfected with miR-762 mimic, but reduced following SR treatment (Fig. 7c). Expression of the miR-762 target *Agap2* inversely correlated with miR-762 expression (Fig. 7c). In accordance with *Agap2* being a negative regulator of Netrin-1, *Ntn1* expression was negatively correlated with that of *Agap2* (Fig. 7c). Overexpression of miR-762 reduced migration towards CCL19 (Fig. 7d). Correspondingly, SR treatment rescued migration towards CCL19 in cells transfected with miR-762 (Fig. 7d). We also noted that expression of *Ccr7*, which encodes the CCL19 receptor CCR7, was mostly similar between the treatment groups (less than 25% difference from control) (Fig. 7c).

**Discussion**

The phytocannabinoid Δ⁹-tetrahydrocannabinol, a CB1 agonist, has been well characterized to stimulate appetite thereby prompting studies to test if CB1 antagonists such as SR141716A would exhibit anti-obesity
CB1 blockade reduces obesity-induced inflammation by miRNA properties (32, 33). SR141716A, also known as Rimonabant, was tested in clinical trials and while it showed great potential as an anti-obesity agent, psychiatric side effects prevented it from being approved for use by the Food and Drug Administration (23). Nonetheless, understanding the mechanisms through which SR141716A mediates anti-obesity and anti-inflammatory activity may help develop alternative targets against obesity. Consistent with previous studies, the current study demonstrated the beneficial effects of a CB1 receptor antagonist, specifically SR141716A, as an interventional treatment of obese phenotype (24, 34–36). Our data demonstrated that SR141716A suppressed food intake transiently, however weight loss and reduction in fat mass was persistent.

Interestingly, in the current study, blockade of CB1 receptors in DIO phenotype resulted in altered expression of several miRs in ATMs; specifically, down-regulation of the miR-466 family in ATMs, which targeted M2 transcription factors KLF4 and STAT6, thereby promoting M2 macrophage phenotype and accordingly inducing an anti-inflammatory state in adipose tissue. We confirmed miR-466 directly targets the 3’UTR of Klf4. SR141716A treatment reduced macrophage miR-466 expression both in vivo and in vitro. Overexpression of miR-466 in macrophages treated with SR141716A reversed SR-mediated induction of Klf4 and Stat6. Together these data confirm that SR141716A treatment promotes M2 macrophage polarization through regulation of miR-466.

Additionally, we noted that treatment with SR141716A caused down-regulation of miR-762, which was found to target the 3’UTR of the AGAP-2 gene, a negative regulator of UNC5B and Netrin-1 signaling. Previous studies have shown that Netrin-1, a neuroimmune guidance cue, promotes ATM retention, inflammation, and insulin resistance in obesity (17). Consistent with this observation, our studies demonstrated that treatment with SR141716A led to a decrease in the expression of UNC5B and Netrin-1 and increased macrophage migration towards lymph nodes. Thus, together, our studies demonstrated that treatment with SR141716A leads to alterations in the expression of miRs that promote macrophage differentiation into M2 as well as promote the emigration of ATMs from the adipose tissue thereby supporting anti-inflammatory microenvironment, which contributes to attenuation of insulin resistance and obesity.

Various studies have identified miRs involved in the regulation of obesity and macrophage polarization (37–39). More recently, we demonstrated that ATMs from lean and DIO mice display distinct transcript and miR expression profiles, which indicates that miRs are involved in regulation of ATM phenotype (30). Together these findings suggest miR-based therapies may be investigated as potential therapeutic interventions for metabolic diseases (40). Our results uncovered a key role for dysregulated miR in ATMs following SR141716A treatment in promoting an anti-inflammatory state in the visceral adipose tissue of DIO mice. Notably, we identified down-regulation of the miR-466 family in ATMs from obese mice following SR141716A treatment. The main target genes of the miR-466 family are Klf4 and Stat6. Previous studies revealed a key role for KLF4 in regulating M2-macrophage polarization (41, 42). Furthermore, a loss of function study of KLF4 in mice bearing myeloid-specific deletion of KLF4 exacerbated DIO severity and metabolic dysfunction (43). Current literature remains limited on the role of the miR-466 family in inflammatory responses. Indeed a recent report from our laboratory defined a miR-466--TGF-β2 axis in which downregulation of miR-466 led to induction of the cytokine TGF-β2 and subsequent induction of immunosuppressive Foxp3+ regulatory T cells (44). In addition, miR-466l has been shown to be induced upon acute inflammation and play a role in resolution, suggesting the miR-466 family plays various roles in inflammatory processes (45, 46). Thus, our studies identified for the first time in ATMs that miR-466 downregulation may play a critical role in the induction of KLF4 and STAT6, and subsequently M2 polarization following CB1 blockade.

Furthermore, using pathway analysis, we identified the unique interaction between altered miR in the HFD+SR group and
molecules involved in the neuroimmune guidance cue pathway. Our data uncovered that therapeutic properties of SR141716A, in attenuation of adipose tissue inflammation, may involve targeting Netrin-1 and its receptor Unc5b in ATMs through miR-762-mediated regulation of AGAP2. Recent studies demonstrated the novel role of the neuroimmune guidance cue, Netrin-1 and its receptor Unc5b in regulating the chemotactic behavior of ATMs (17). Indeed, less accumulation of ATMs in adipose tissue of HFD-fed Netrin-1 knockout mice was identified when compared to HFD-fed wild type mice (17). The key regulatory role of Netrin-1 in leukocyte transmigration into tissues (intestine, kidney, and atherosclerosis plaques) has also been identified (47-49). Conversely, abundant expression of Netrin-1 in residual macrophages of atherosclerotic plaques, exacerbate the disease state by attracting more macrophages to the site of inflammation (50). Additionally, higher expression of Netrin-1 in endothelial cells of Apolipoprotein E knockout, and low-density lipoprotein receptor knockout mice ameliorates atherosclerosis by inhibiting leukocyte recruitment (51). Thus, conditional deletion of Netrin-1 and Unc5b in macrophages is required to eliminate the off-target effect of Netrin-1 deletion in other cells such as endothelial and epithelial. Our studies suggest that SR141716A may attenuate chronic low-grade inflammation in adipose tissue, by targeting Netrin-1 pathways exclusively in macrophages.

Recently, the key functional role of Netrin-1 was established as a monocyte chemoattractant signal, identified by monitoring the migration pattern of macrophages into and out of atherosclerotic plaques as well as adipose tissue of DIO phenotype (52, 53). Utilizing the fluorescent microsphere monocyte labeling technique in HFD-fed Netrin-1 knockout mice, these mice demonstrated less retention of macrophages in adipose tissue and more emigration into draining mesenteric lymph nodes when compared to wild-type mice (17). We demonstrated herein that cultured peritoneal macrophages in harvested medium from 3T3-L1 adipocytes have reduced expression of miR-762, increased expression of Agap2, decreased secretion of Netrin-1, and show higher migration to CCL19 in presence of SR141716A when compared to DMSO, despite equivalent expression of CCR7. Together, our studies suggested that SR141716A treatment alters miRs that target Netrin-1 signaling in ATMs leading to lesser retention of macrophages in adipose tissue.

Although the role of Netrin-1 in ATM retention is well documented, the underlying mechanisms remain poorly defined. Herein, for the first time we identified the key role of miR-762 and AGAP-2 in regulation of Unc5b in ATMs. In an earlier study, the association of AGAP-2 and Unc5b in a P53-dependent manner was identified in a cancer cell line (54). In the current study, we also identified that SR141716A treatment leads to miR-762 downregulation in vivo in ATMs and in vitro in cultured peritoneal macrophages leading to induction of AGAP-2, which downregulates Unc5b expression and Netrin-1 activation. We also demonstrated direct binding of miR-762 to the 3’UTR of the Agap2 gene. Overexpression of miR-762 in SR-treated macrophages reversed SR-mediated migration towards CCL19. Thus, SR141716A treatment targets both Netrin-1 and Unc5b in ATMs through alterations in miR expression.

This study provides new insights into the role of SR141716A in the induction of activity for Netrin-1 and Netrin-1 related receptors. However, further investigation is required for uncovering the potential effect of SR141716A on other neuronal immune guidance cue families such as Slit, Ephrin, Semaphorin and their related receptors in ATMs.

Experimental Procedures

Animals, diet and treatment
C57BL/6J male mice (Jackson Laboratory, Bar Harbor, ME), were fed HFD consisting of 60% Kcal from fat (D12492, Research Diets Inc, New Brunswick, NJ) for 12 weeks before starting the intervention treatment with SR141716A. Additionally, age-matched C57BL/6J male mice were fed a low fat diet (LFD) consisting of 10% Kcal from fat (D12450J, Research Diets Inc., New Brunswick, NJ) to develop lean controls. Only male mice were used due to their increased susceptibility to
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DIO development (55, 56). SR141716A was obtained from NIDA and administered at a dose of 10mg/kg daily by oral gavage for 4 weeks, beginning after 12 weeks of diet and ending after 16 weeks. A pair-fed control group was used (HFD-PFSR) in which food intake was restricted to the amount of daily food intake of the HFD+SR group. All mice were maintained in an AAALAC-accredited animal facility at the University of South Carolina, School of Medicine (Columbia, SC). All procedures were performed according to NIH guidelines under protocols approved by the Institutional Animal Care and Use Committee.

Analytical procedures

Body composition in mice was analyzed by dual-energy X-ray absorptiometry (DEXA, LUNAR, Madison, WI). The mice were anesthetized and placed in the prone position on the specimen tray to allow scanning of the entire body. For food intake measurements, mice were given a defined amount of large intact food pellet weekly, or daily for the HFD-PFSR group. Food weight was measured using a balance with a precision of 0.01 g. Solid food intake was corrected for any visible spillage.

Assessment of metabolic parameters

Measurements of blood glucose, insulin, total cholesterol, and triglycerides were performed in animals fasted for 5 hours. Blood was collected from the tip of the tail. The glucose level in whole blood was measured with a glucometer (Bayer, Leverkusen, Germany). Insulin concentration was determined in isolated plasma using an ELISA kit (Abcam, Cambridge, MA). Total cholesterol (Genzyme, Kent, United Kingdom), and triglycerides (Pointe Scientific, Canton, Michigan) were determined by colorimetric enzymatic reaction according to the manufacturer’s instructions (57). To quantify insulin resistance, the homeostatic model assessment of insulin resistance index (HOMA-IR) was calculated as previously described (58).

Tissue collection

After 4 weeks of treatment, mice were euthanized for tissue collection. Tissues were removed, weighed, and immediately processed to single cell suspension, snap-frozen in liquid nitrogen and stored at −80°C, or fixed in 10% formalin.

Adipocyte and adipose tissue macrophage purification

Epididymal fat pads of mice were excised and placed in gentleMACS C Tubes (Miltenyi Biotec, San Diego, CA) containing digestion medium (HBSS, 2 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO), and 2% BSA), followed by homogenization utilizing a gentleMACS Dissociator (Miltenyi Biotec). After incubation at 37°C for 30 min with gentle shaking, the cell suspension was filtered through a 100-µm filter and then spun at 300g for 5 min to separate floating adipocytes from the stromal vascular fraction (SVF) pellet. Isolation of F4/80+ ATMs from the SVF was performed using EasySep™ FITC Positive Selection Kit following the manufacturer’s protocol (STEMCELL Technologies, Vancouver, BC). Single-cell suspensions were first incubated with Fc receptor blocker consisting of rat IgG anti-mouse CD16/32 antibody to prevent non-specific binding of FITC-conjugated antibodies to Fc receptors (Biolegend, Cat#101301). Next, FITC-anti-F4/80 antibodies (Clone: BM8, BioLegend, San Diego, CA) were used to label the cells for positive selection. Isolated cells were lysed for RNA analysis in Qiazol (Qiagen, Valencia, CA) and stored at -80°C until RNA purification. Flow cytometry of the collected flow-through was used to evaluate selection efficiency, which was routinely >90% (Supplementary Figure 3).

RNA Purification, cDNA Synthesis, and quantitative RT-PCR

ATM RNA was isolated by miRNeasy mini kit (Qiagen). For RNA extraction from whole adipose tissue, 1 mg of adipose tissue was homogenized in RNA-Solv Reagent with OBI’s HiBind® technology reagent (Omega Bio-tek, Norcross, GA) and total RNA was isolated as previously described (57). RNA quality was verified by NanoDrop 2000C (ThermoScientific, Waltham, MA). Purified RNA (0.5–1 µg) was reverse-transcribed using miScript cDNA Synthesis Kit (Qiagen), and qRT-PCR analysis was conducted using SsoAdvanced™ Universal SYBR® Green Supermix kit (Bio-Rad,
Hercules, CA). Fold change in mRNA expression was calculated using the comparative cycle method ($2^{-\Delta\Delta Ct}$).

**miR expression profiling and analysis**

The unique expression profile of miRs was assessed in F4/80+ cells isolated from adipose tissue by Affymetrix Gene Chip miRNA 4.0 array platform. The array contains 3100 murine-specific probes from Sanger miRBase. Total RNA were 3′-end labeled with Flash Tag biotin HSR hybridization technique (Genisphere, Hatfield, PA) and was carried out according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Microarray CEL files were assessed for quality, normalized, and converted to CHP files by using the algorithm RMA-DABG in the software Affymetrix Expression Console Version 1.4.1.46. CHP files were further analyzed using Affymetrix Transcriptome Analysis Console Version 3.1.0.5 for annotation and differential expression. Expression of Log2 fold change (Log2 FC) was calculated and mean normalized expression was visualized in the form of a heat-map. miRs were considered differentially expressed if the Log2 FC was greater than +/- 1.5.

**Bioinformatics analysis**

The differentially expressed miR target genes were assessed by miR target prediction algorithms miRwalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) and miRmap (http://mirmap.ezlab.org). To carry out an enrichment analysis of predicted target genes of miRs in biological pathways, the commercially available analysis tool, Ingenuity Pathway Analysis (IPA, Qiagen) was used. IPA predicts the top affected canonical pathways, causal connections between differentially altered miRs and their target genes, downstream effectors, and upstream regulators. The Molecular Activity Predictor (MAP) feature of IPA was performed to predict the downstream effect of the differentially expressed miRs which were overlaid to the dataset including miR probes, fold change and p value. Gene ontology was assessed in Cytoscape platform using the CluGo app (59).

**ELISA assays**

TNFα, IL-6, and IFNγ ELISA kits were purchased from BioLegend. Netrin-1 ELISA kits were purchased from USCN Life Science (Houston, TX). Concentrations were measured in cell supernatants using mouse standards according to the manufacturer's guidelines.

**Migration**

The migration of macrophages to CCL19 (500 ng/ml, R&D Systems) was assessed by FluoroBlock permeable inserts (Corning, Tewksbury, MA) and Cytation5 imaging (BioTek, Winooski, VT). Peritoneal macrophages were harvested from mice primed with 1 ml of 3% (wt/vol) thioglycolate (i.p.) to elicit peritoneal exudates with macrophage number peaking on day 4. Macrophages were collected in euthanized mice by intraperitoneal wash. For tracking migration towards chemoattractants, macrophages were labeled with DilC12(3) fluorescent dye (Corning, Tewksbury, MA). Macrophages were treated with conditioned media from 3T3-L1 adipocytes as described and treated either with SR141716A (10^{-6} M) or DMSO as the vehicle control (60, 61).

**Western blot analysis**

Western blot analyses were carried out according to standard protocols with antibodies raised against Netrin-1 (1:1000, MAB1109, R&D Systems, Minneapolis, MN) or γ-tubulin (1:10000, T5326, Sigma-Aldrich, St. Louis, MO), which was used as a loading control.

**Transfection and reporter gene assay**

For miR-466 targeting of Klf4, RAW264.7 macrophages were transfected with 5–10 µg of pmirGLO or pmirGLO-KLF4-UTR or pmirGLO-KLF4-ScrUTR plasmids using Effectene Transfection reagent from Qiagen and following the protocol of the company (Qiagen). Reporter construct was generated containing mouse KLF4 UTR DNA sequences. To this end, we used pmirGLO reporter vector from Promega (Promega Corporation, Madison, WI). pmirGLO reporter vector contains two luciferase genes, 1) firefly luciferase reporter gene (luc2) that generates luminescence in the absence of microRNA and 2) Renilla luciferase reporter gene (hRluc-neo fusion protein coding region).
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that generates luminescence in presence of microRNA. Mouse KLF4-specific UTR region complementary to mouse miR-466F was cloned into pmiRGO vector and these were designated as pmiRGO- KLF4-UTR or pmiRGO-KLF4-Scramble UTR scramble (pmiRGO- KLF4-ScrUTR). The details of the KLF4 sequences cloned into pmiRGO are as described as follows. Both nucleotides of normal and scramble miR-466F-specific KLF4 UTR regions contain Pmel and XbaI restriction sites.

miR-466 sense target sequence: 5′-AAACTAGCGGCCGCTAGTCACAACACACGCACACT-3′, miR-466 antisense target sequence: 5′-CTAGAGTGTCGTTGTTGTTG TGACTAGCGGCCGCTAGTTT-3′, miR-466 scramble sense target sequence: 5′-AAACAAGCGGCCGCTAGTCACAACTTCTGTGCACT-3′, miR-466 scramble antisense target sequence: 5′-CTAGAGTGTGCTGTCTTGTTG TGACTAGCGGCCGCTAGTTT-3′. Two days post transfection with plasmids, RAW264.7 cells were replated in triplicate in 96-well plate (75 µl/well) and the cells were treated with vehicle (DMSO) or SR 141716A (10 µM) and incubated for 24 h at 37°C, 5% CO₂. Following treatments with vehicle or SR, luciferase assays were performed using Dual-Glo Luciferase Assay system from Promega and following the protocol of the company (Promega Corporation, Madison, WI). In brief, equal volume (75 µl/well) of Dual-Glo reagent was added to each well and thoroughly mixed. The cells were incubated for 10–15 minutes at room temperature to allow cells to lyse. Firefly luciferase activity was measured by reading the sample luminescence using Victor² (Perkin Elmer). After first reading of the samples, Dual-Glo Stop & Glo reagent (75 µl/well) was added to each well, mixed thoroughly, and incubated for 10–15 minutes. Renilla luminescence was measured by reading the sample luminescence using Victor² (Perkin Elmer). Ratio of luminescence from experimental samples to luminescence from the control reporter was calculated. Luminescence ratio was then normalized to the ratio of control wells. Relative luminescence ratio was calculated from the normalized ratios and values were expressed as “normalized-fold induction.”

For miR-762 targeting of Agap2, bone marrow derived macrophages (BMDM) were transfected with a plasmid containing a Renilla luciferase (transfection efficiency control) and 3′UTR AGAP-2 (Gene Accession: NM_001301014.1 UTR Length: 808 bp (-383 - 787 bp) in a Firefly luciferase reporter gene. Cloning details was as follows: the complete plasmid is 7097 bp, Vector: Pezx-MT06, Promoter: SV40, Antibiotic Ampicillin, 5′ Cutting Site: AsiSI, EcoRI, BsiWI3′, Cutting Site: XhoI,SpeI with sequencing Primers (Forward: 5′-GATCCGCGAGATCCTGAT-3′, and Reverse: 5′-CCTATTGGCGTTACTATG-3′ (GeneCopeia™, Rockville, MD). Cell cultures were transfected with miR-762 miRCURY LNA™ microRNA Inhibitor (5nM) (Exiqon, Woburn, MA). Lipofectamine® RNAiMAX Transfection Reagent (ThermoFisher Scientific, Waltham, MA) was used for delivery of oligos into the cell.

Rescue experiments

Rescue experiments were performed by overexpressing miRs then treating with SR141716A. Thioglycollate-elicited peritoneal macrophages were isolated from naïve mice then transfected with miR-466f, miR-466j, miR-762, or AllStars negative control (“Ctrl miR”) miScript mimics (Qiagen). Lipofectamine 3000 was used as the transfection reagent (ThermoFisher Scientific). The macrophages were then cultured for 24h in conditioned medium from 3T3-L1 adipocytes + 10uM SR141716A or DMSO vehicle. For the cells transfected with miR-762, cells were plated in FluoroBlock inserts (Corning 351152). Complete DMEM medium containing 500ng/mL CCL19 (BioLegend, 587802) was added to the bottom well 12h prior to harvest to induce migration.

Statistical Analysis

For the in vivo mouse experiments, 10 mice were used per experimental group, unless otherwise specified. For in vitro assays, all experiments were triplicated. Most experiments were repeated at least twice to see consistency. Statistical analyses were performed using GraphPad Prism Version 7.000 (GraphPad Software, La Jolla, CA). Body weight, body
composition outcomes, and metabolic outcomes were analyzed using a repeated measures two-way ANOVA. For statistical differences, one-way ANOVA was calculated for each experiment. Tukey’s post-hoc test was performed to analyze differences between groups. A p-value of ≤ 0.05 was considered statistically significant.

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Conflicts of interest
The authors declare no conflicts of interest.

Data materials availability
Microarray data will be deposited to NCBI’s GEO database upon acceptance for publication.
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FOOTNOTES

Abbreviations: Cannabinoid receptor 1, (CB1), Diet-Induced Obesity, (DIO), high-fat diet (HFD), low-fat diet (LFD), SR141716A-treated DIO group (HFD+SR), Pair-fed to SR141716A group (PFSR), Adipose Tissue Macrophages (ATMs), Visceral Adipose Tissue (VAT), Stromal Vascular Fraction (SVF), microRNA (miR)
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Figure 1: SR141716A ameliorates DIO phenotype by inducing weight loss. DIO was induced in mice by 12 weeks of purified diet feeding. After week 12, mice were treated orally with either SR141716A (SR) (10mg/kg/day) or Vehicle (Veh) (0.1% Tween 80) for 4 weeks. (a) Experimental timeline. (b) Experimental groups and description of purified diet. (c) Daily body weight of each group of mice is shown for the duration of treatment. (d) Weekly energy intake during the 4-week intervention. For (c-d) n=9-10. Data are shown as mean ± SD. Data were analyzed using two-way ANOVA with a Tukey post hoc test. p<0.05 if alphabetical characters differ between the groups being compared.
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Figure 2: SR141716A intervention treatment ameliorates metabolic dysfunction in DIO phenotype.

Figure 2. SR141716A intervention treatment ameliorates metabolic dysfunction in DIO phenotype. Fasted metabolic parameters assessed before intervention (“pre”) and on the day before euthanasia (Day 27, “post”). (a) Fasting blood glucose (b) Serum insulin concentration (c) HOMA insulin resistance index (HOMA-IR) = fasting insulin (µU/ml) x fasting glucose (mmol/l) /22.5. (d) Total cholesterol concentration. (e) Triglyceride concentration. Data are shown as mean ± SD. Data were analyzed using two-way ANOVA with a Tukey post hoc test. p<0.05 at the “Post” timepoint if alphabetical characters differ between the groups being compared., n=10, except n=9 for HFD+SR.
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Figure 3. SR141716A treatment of DIO mice reduces adipose inflammation and alters miR expression in ATMs.

Figure 3. SR141716A treatment of DIO mice reduces adipose inflammation and alters miR expression in ATMs. Adipose inflammatory markers and changes in miR expression in ATMs were assessed following SR141716A treatment in DIO mice. (a-b) Adipose tissue expression of genes for polarization markers iNOS (M1, Nos2) and Arginase-1 (M2, Arg1) (c) Cluster analysis of up- and down-regulated miRs (Log2 fold change of at least ±1.5) in F4/80+ ATMs represented in venn diagrams. (d) Venn diagrams of dysregulated miRs that are independent of appetite. (e) Heatmap of modified miRs due to the effect of SR141716A treatment independent of its effect on calorie restriction. Data are shown as
mean ± SD. For (a) data were analyzed using one-way ANOVA. Groups with different alphabetical characters are significantly different from each other (p < 0.05).
Figure 4. Downregulation of the miR-466 family following SR141716A treatment promotes M2 polarization in ATMs. Differentially expressed miRs in ATMs due to SR treatment were analyzed for overlap with macrophage polarization genes. (a) IPA analysis from filtered miRs revealed the miR-466 family targets M2 transcription factors. (b) qRT-PCR for the miR-466 family in ATMs. (c) qRT-PCR for Klf4 in ATMs. (d) qRT-PCR for Stat6 in ATMs. For (e-i) peritoneal macrophages from naïve mice were transfected with a negative control LNA (Ctrl LNA) or miR-466f inhibitor LNA (Anti-miR-466f) or miR-466j* inhibitor LNA (Anti-miR-466j*), then cultured in 3T3-L1 adipocyte conditioned medium. (e) qRT-PCR for Klf4. (f) qRT-PCR for Stat6. For (g-i) cytokines were measured in cell supernatant by ELISAs. (g) TNF-α ELISA. (h) IL-6 ELISA. (i) INF-γ ELISA. Data are shown as mean ± SD. For (b-d) data with different alphabetical characters are significantly different (p<0.05) by post-hoc one-way ANOVA. For (e-i) *p<0.05, **p<0.01, ****p<0.0001 vs. Ctrl LNA by post-hoc one-way ANOVA.
Figure 5. SR141716A treatment reduces miR-762 in ATMs to promote negative regulation of Netrin-1-mediated retention via induction of AGAP-2. ATMs from different treated groups were examined for changes in the expression of miRs that target neuroimmune guidance cues. (a) IPA analysis of differentially expressed miRs following SR treatment overlayed with the neuroimmune guidance cue canonical pathway. The MAP module was applied for down-stream effect prediction. IPA analysis revealed AGAP-2 is targeted by downregulated miRs. (b) qRT-PCR expression validation of miR-762 in ATMs. (c) qRT-PCR of the miR-762 target Agap2 in ATMs. (d) Western blot of Netrin-1 (Ntn-1) in ATMs. (e) qRT-PCR of Ntn1 in ATMs. (f) qRT-PCR of Unc5b in ATMs. (g) Luciferase reporter assay of BMDM transfected with the 3’UTR of Agap2 followed by either Ctrl-LNA or Anti-miR-762 LNA. Luminescence was measured 24hr after transfection with LNA. Data are shown as mean ± SD. Data with different alphabetical characters are significantly different (p<0.05) by post-hoc one-way ANOVA. ****p<0.0001, *p< 0.05 by two-tailed Student’s t-test.
CB1 blockade reduces obesity-induced inflammation by miRNA

Figure 6. Treatment of macrophages with SR141716A in vitro promotes M2 polarization and inhibits macrophage retention.
Figure 6. Treatment of macrophages with SR141716A in vitro reduces the miR-466 family and miR-762 expression, promotes M2 polarization and inhibits macrophage retention. Thioglycollate-elicited peritoneal macrophages were isolated from naïve mice then cultured in 3T3-L1 adipocyte conditioned medium containing SR141716A (10μM) or DMSO for 24 hours. (a) qRT-PCR miRs. (b-d) qRT-PCR of Klf4, Stat6, and Agap2. (e) Netrin-1 ELISA from cell supernatants. For (f-g) macrophages were stained with DilC12(3) and treated with SR141716A or DMSO. Migration rate towards CCL19 (500 ng/ml) was assessed by seeding 2.5x10^5 macrophages in serum reduced conditioned medium from differentiated 3T3L-1 adipocytes in fluoroblock plates. 12 hours later, cell migration was quantified using Cytation5 imaging and gene expression analysis was conducted for CCR7. (f) Number of cells migrated towards CCL19. (g) qRT-PCR expression of Ccr7. Data are shown as mean ± SD. *p<0.05, **p<0.01, ****p<0.0001 vs. DMSO vehicle control by two-tailed Student’s t-test.
CB1 blockade reduces obesity-induced inflammation by miRNA

Figure 7. Overexpression of miR-466f, miR-466j, and miR-762 reverses effects of SR141716A treatment in macrophages.

Thioglycollate-elicited peritoneal macrophages were harvested from naïve mice then transfected with a scrambled negative control miR oligonucleotide (Ctrl miR), miR-466f, miR-466j or miR-762 mimics, then cultured for 24h in conditioned medium from 3T3-L1 adipocytes + 10uM SR141716A (SR) or DMSO vehicle. (a) qRT-PCR of miR-466f, miR-466j, Klf4, and Stat6 in macrophages transfected with miR-466f or miR-466j +/- SR. For (b), luciferase reporter assay was performed in RAW264.7 macrophages transfected with pmirGLO, pmirGLO-KLF4-UTR, or pmirGLO-KLF4-ScrUTR then incubated 24h with SR141716A (10uM) or DMSO vehicle. (b) Firefly luciferase activity was measured by luminescence and normalized to pmiR-Glo + DMSO negative control. For (c-d) 5 x 10^5 peritoneal macrophages were plated in fluoroblock inserts then transfected with miR oligonucleotides and cultured 24h in conditioned medium from 3T3-L1 adipocytes + 10uM SR141716A (SR) or DMSO vehicle. At 12h of culture, medium containing 500ng/mL CCL19 was added to the bottom well and migrated cells were harvested at 24h for migration analysis and qRT-PCR. (c) qRT-PCR of miR-762, Agap2, Nn1, and Ccr7 in macrophages transfected with miR-762 +/- SR. (d) Number of cells migrated towards CCL19. Data are mean +/- SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 by one-way ANOVA.
Role of microRNA in CB1 antagonist-mediated regulation of adipose tissue macrophage polarization and chemotaxis during diet-induced obesity
Pegah Mehrpouya-Bahrami, Kathryn Miranda, Narendra P. Singh, Elizabeth E. Zumbrun, Mitzi Nagarkatti and Prakash S. Nagarkatti

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