Inhibition of the CD36 receptor reduces visceral fat accumulation and improves insulin resistance in obese mice carrying the BDNF-Val66Met variant

Obesity-induced metabolic dysfunctions increase the risk for vascular diseases, including type II diabetes and stroke. Managing obesity is of interest to address the worldwide health problem; however, the role of genetic variability in human obesity development and specific targets for obesity-related metabolic disease have not been thoroughly studied. A SNP in the brain-derived neurotrophic factor (BDNF) gene that results in the substitution of a valine with a methionine at codon 66 (Val66Met) occurs with a high frequency in humans. This study addressed the effect of genetic variability in developing obesity and the efficacy of the inhibition of cluster of differentiation 36 (CD36), a multifunctional receptor implicated in obesity and insulin resistance, in WT mice and mice with the BDNF-Val66Met variant. CD36 inhibition by salvonionic acid B (SAB) in diet-induced obese WT mice reduced visceral fat accumulation and improved insulin resistance. The benefit of SAB was abrogated in CD36 knockout mice, showing the specificity of SAB. In addition, mice with the Val66Met variant in both alleles (BDNF-M/M) fed a high-fat diet exhibited extreme obesity with increased CD36 gene and protein levels in macrophages. Chronic SAB treatment in BDNF-M/M mice significantly decreased visceral fat accumulation and improved insulin resistance. Notably, the effect of SAB was greater in the extremely obese BDNF-M/M mice compared with the WT mice. The study demonstrated a link between BDNF Val66Met and elevated CD36 expression and suggested that CD36 inhibition may be a potential strategy to improve metabolic dysfunctions and to normalize risk factors for vascular diseases in the obese population.

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This article was selected as one of our Editors’ Picks.

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Obesity is a precipitating cause for the development of a cluster of metabolic disorders, including insulin resistance (IR), dyslipidemia, and type II diabetes (1, 2). These conditions are well-known risk factors for vascular diseases, including stroke, and represent a serious global health concern (3, 4). Whereas tight glycemic control was suggested to slow down the progression of diabetes (5–7), this approach has not been fully translated into practice (8). It has been generally assumed that individual genetic makeup influences energy expenditure and metabolism. However, the impact of inter-individual genetic variability on the development of human obesity and IR is relatively unknown. Thus, validating molecular targets that underlie these metabolic disturbances, based on genetic makeup, would facilitate interventional strategies for individuals that are prone to develop obesity and obesity-associated metabolic disorders.

There is a strong link between high fat intake and obesity-associated metabolic disorders. CD36, a class B scavenger receptor, is a multifunctional receptor and is involved in innate immunity, inflammation, and lipid metabolism. CD36 has been reported as a key factor in innate immunity and phagocytosis in resolution phase after ischemic stroke (9). In addition, CD36 uptake long-chain fatty acids and oxidized lipids and has been identified as a fat-sensing gustatory receptor in the taste buds (10–12). CD36 is also highly expressed in the duodeno-jejunum of small intestine, the main site of fat absorption (13). Highly expressed in monocytes, macrophages, and adipocytes, CD36 has been implicated in the development of IR and diabetes. CD36 in plasma (soluble CD36), contained within microparticles, has been identified as a novel marker of IR in diabetes (14, 15), suggesting CD36 as a potential target for intervention in metabolic disorders associated with obesity.

Brain-derived neurotrophic factor (BDNF) is an anorexigenic neurotrophin that regulates appetite and body weight (16). Deficiencies of BDNF expression in heterozygous and conditional KO mice display an obese phenotype as well as...
aggressive and abnormal locomotor activity (17–19). Haploinsufficiency of BDNF was associated with hyperphagia and severe obesity in patients (20). In addition, a mutation in TrkB, the receptor for BDNF, causes severe obesity and developmental delays in humans (21) that closely link BDNF deficiency with the obese phenotype. The BDNF gene in humans is polymorphic. An extensively studied BDNF polymorphism is rs6265, in which a methionine is substituted for a valine at codon 66 (Val66Met). The BDNF Val66Met SNP occurs in a high frequency in humans, with 20–30% of the United States population and nearly 70% of Asians carrying the Met on either one or both alleles (22, 23). The rs6265 SNP is linked to eating disorders and obesity in children and adults and also deficits in memory function (24–27). Moreover, mice harboring the human Val66Met SNP in both alleles (BDNF<sup>M/M</sup>) are obese and anxious compared with WT littermates (BDNF<sup>V/V</sup>) (28, 29), suggesting that the obesity-prone BDNF<sup>M/M</sup> mice fed a high-fat diet provide an appropriate animal model for diet-induced obesity in humans carrying the BDNF Met allele.

Previously, we reported that salvianolic acid B (SAB), a polyphenol compound extracted from the roots of <i>Salvia miltiorrhiza</i> Bunge, is a CD36 antagonist that blocks the uptake of oxidized lipids in macrophages (30). With CD36 as a potential target for intervention in metabolic disorders, the purpose of the current study is to address the <i>in vivo</i> effect of CD36 inhibition on improving obesity-associated metabolic dysfunctions and the efficacy of SAB on genetically obesity-prone mice that carry the BDNF Met allele. Here we report that CD36 inhibition normalizes metabolic dysfunction in diet-induced obese mice with greater benefits in BDNF SNP carriers. The greater reduction of metabolic dysfunction in BDNF SNP mice suggests that individual genetic makeup should be considered for intervention strategies for obesity and its associated metabolic dysfunction.

**Results**

**SAB attenuates macrophage oxidized low-density lipoprotein (oxLDL) uptake in physiological conditions**

The uptake of oxLDL is a major function of macrophage CD36. Compared with vehicle (Veh) treatment, SAB treatment significantly reduced macrophage oxLDL uptake in a dose-dependent manner in macrophages (Fig. 1B). SAB solution is highly acidic (pH ~2.0). For chronic <i>in vivo</i> treatment using an Alzet pump, we tested the stability of neutralized SAB at pH 7.0 with different durations of incubation at 37 °C. Neutralized SAB incubated at 37 °C for up to 4 weeks did not diminish the inhibitory effect of oxLDL uptake in macrophages (Fig. 1C). The results demonstrated that SAB is a suitable CD36 inhibitor for chronic administration <i>in vivo</i>.

**SAB reduces insulin resistance and visceral fat accumulation in obese mice**

The effects of SAB on body weight gain and obesity-associated metabolic dysfunction were investigated in diet-induced obese C57BL/6 mice. There were no differences in starting body weight between groups that received an Alzet pump containing either Veh or SAB (Veh 23.9 ± 0.5 g versus SAB 23.3 ± 0.6 g). SAB did not affect the rate of body weight gain during an 8-week high-fat diet (HFD) intervention (Fig. 2A). SAB-treated mice accumulated less visceral fat after 8 weeks of HFD intervention (Fig. 2B). SAB mice showed a tendency of lower fasting blood glucose (Fig. 2C, p = 0.0675) and cleared blood glucose significantly faster (Fig. 2D). Peritoneal macrophages isolated from SAB-treated mice had significantly reduced oxLDL uptake (Fig. 2E), indicating that chronic SAB administration effectively inhibits CD36 function <i>in vivo</i>. The results demonstrate that SAB counteracts visceral obesity and insulin-associated metabolic dysfunction.

**The absence of CD36 abolishes SAB-induced benefits in obese mice**

To address whether the pharmacological inhibition of CD36 by SAB has off-target effects, we determined the effect in CD36 knockout (KO) mice (C57BL/6 background) that were chronically treated with SAB while being fed a HFD. Body weight gains were similar between Veh- and SAB-treated CD36 KO mice (Fig. 3A). SAB treatment did not improve visceral fat accumulation, fasting blood glucose level, and glucose clearance/IR and visceral fat accumulation (Fig. 3, B–D). There were no differences in oxLDL uptake in peritoneal macrophages isolated from Veh- or SAB-treated mice (Fig. 3E), suggesting that SAB counteracts IR and visceral obesity through CD36 function.

**BDNF<sup>M/M</sup> mice display extreme obesity and severe metabolic dysfunctions with elevated CD36 expression**

Body weight of BDNF<sup>V/V</sup> and BDNF<sup>M/M</sup> male mice at 6 weeks of age were similar (BDNF<sup>V/V</sup> 23.1 ± 1.4 g versus BDNF<sup>M/M</sup> 23.9 ± 2.6 g). BDNF<sup>M/M</sup> mice fed normal diet gained body weight significantly faster than BDNF<sup>V/V</sup> mice as they aged (Fig. 4A). HFD intervention accelerates the degree of weight gain in BDNF<sup>M/M</sup> mice. Both male and female BDNF<sup>M/M</sup> mice receiving a HFD gained body weight significantly faster than BDNF<sup>V/V</sup> mice (Fig. 4B). BDNF<sup>M/M</sup> mice fed HFD for 8 weeks had higher fasting blood glucose levels in males (Fig. 4C) and delayed blood glucose clearance in both sexes (Fig. 4D), indicating the development of IR. CD36 mRNA and protein expression levels were increased in macrophages isolated from male BDNF<sup>M/M</sup> mice, compared with male BDNF<sup>V/V</sup> mice (Fig. 4, E and F). Soluble CD36 (sCD36) levels in plasma were not significantly different between BDNF<sup>V/V</sup> and BDNF<sup>M/M</sup> mice, but the association between sCD36 and IR of BDNF<sup>M/M</sup> mice was significantly different from that of BDNF<sup>V/V</sup> mice (Fig. 4G). The results show that genetically obesity-prone BDNF<sup>M/M</sup> mice with increased CD36 expression can be used to model diet-induced human obesity.

**SAB reduces visceral fat accumulation and improves IR in obese BDNF<sup>M/M</sup> mice**

The effect of SAB on counteracting obesity-associated metabolic dysfunction was investigated in BDNF<sup>V/V</sup> and BDNF<sup>M/M</sup> male mice fed a HFD. BDNF<sup>M/M</sup> mice gained body weight much faster than BDNF<sup>V/V</sup> mice with HFD intervention (Fig. 5A). Compared with Veh-treated mice, SAB treatment slowed down body weight gains in BDNF<sup>M/M</sup> mice (Fig. 5A). SAB treatment reduced visceral fat accumulation with a greater reduction in BDNF<sup>M/M</sup> mice (Fig. 5B). The treatment did not affect
quadriceps weight (Fig. 5C), suggesting that the reduction of body weight was largely from visceral fat accumulation. Due to extreme obesity, glucose levels were measured up to 3 h after glucose challenge for glucose tolerance tests. SAB treatment cleared glucose significantly faster in both genotypes, but the improvement was greater in BDNFM/M mice (Fig. 5D). SAB-treated BDNFM/M mice showed a tendency of decreased fasting insulin level without statistical significance, and SAB treatment significantly decreased the homeostasis model assessment–estimated insulin resistance (HOMA-IR) level (Fig. 5E). Daily food consumption was not different between Veh- and SAB-treated BDNFM/M mice (Fig. 5F). The results suggest that individuals with the BDNF Met allele would have a greater benefit in normalizing metabolic dysfunction by the pharmacological inhibition of CD36.

To address effects of SAB on lipid uptake on adipose and hepatic tissue in HFD-fed BDNFM/M mice, we performed cellular lipid staining using Oil Red O. SAB-treated animals showed less lipid accumulation in both adipose (Fig. 6A) and hepatic tissue (Fig. 6B). Quantification of the extent of lipid accumulation showed that SAB treatment attenuated the intensity as well as the size of lipid droplets in adipose tissue and hepatic tissue of obese mice (Fig. 6C). We further determined the effect of SAB on tissue inflammation. The presence of F4/80-positive macrophage infiltration in adipose and hepatic tissue and crownlike structure in adipose tissue (Fig. 6D, inset) were decreased in animals treated with SAB (Fig. 6, D and E).
SAB treatment significantly decreased CD36, F4/80, and monocyte chemoattractant protein-1 (MCP-1) gene expressions in adipose tissue of HFD-fed BDNFM/M mice (Fig. 6F). Interleukin-1β and tumor necrosis factor α gene levels showed a tendency to decrease by SAB treatment, but it was not statistically significant (Fig. 6F). The results show that SAB treatment counteracts obesity-induced macrophage infiltration and inflammation in adipose and hepatic tissues.

**Discussion**

Obesity is a precipitating cause for the development of IR and type II diabetes. Despite increasing awareness that genetics influence developing human obesity and its associated metabolic disorders, underlying pinning events based on individual genetic variability are not well understood. With accumulating evidence of CD36 involvement in the development of obesity and IR (31, 32), the current study addressed the effect of CD36 inhibition on visceral obesity and IR in diet-induced obese mice and the efficacy of pharmacological intervention on genetically obesity-prone mice. Key findings from the study include that chronic inhibition of CD36 by SAB effectively attenuated visceral obesity and improved glucose clearance in obese C57BL/6 mice. Importantly, mice harboring the human BDNF Met allele (BDNF<sup>M/M</sup>) that were fed a HFD developed extreme obesity and IR and had elevated expression of CD36. Chronic SAB treatment effectively normalized metabolic dysfunction in BDNFM/M mice and decreased adipose tissue inflammation. This is the first study that links CD36 function to obesity/IR and investigates the effect of CD36 inhibition in genetically at-risk populations that carry the BDNF Val<sup>66Met</sup> allele.

SAB is a hydrophilic component derived from danshen, a dried root of *Salvia miltiorrhiza* Bunge (33). Previously, we identified SAB as an effective agent to reduce macrophage uptake of modified LDL in a CD36-dependent manner (30). Literature has shown a close link between CD36 and lipid metabolism, insulin signaling pathways, and inflammation (34,
By showing SAB’s ability to block CD36 lipid uptake for 4 weeks in physiological conditions (pH 7.0 at 37 °C), the current study optimized SAB’s stability in physiological conditions for chronic in vivo administration using the Alzet pump (Fig. 1). Moreover, the effects of SAB on reducing visceral obesity, improving IR, and reducing oxLDL uptake in macrophages of SAB-treated obese mice also support a link between macrophage CD36 and obesity-associated metabolic dysfunctions. The absence of SAB’s beneficial effects in CD36 KO mice (Fig. 3) confirms the specificity of SAB as a CD36 antagonist.

Because individual genetic makeup influences energy expenditure and metabolism, this study addressed the effect of the BDNF Val66Met genetic variant (rs6265) on developing obesity-associated metabolic disturbance. The variant commonly occurs with high frequency in humans, and association of the Met allele with a higher body mass index implied potential involvement of the variant in obesity (25, 36). Use of mice with the human BDNF Val66Met knock-in in both alleles (BDNF^{M/M}) showed that BDNF^{M/M} mice gain body weight faster even when they were fed a normal diet (18, 28, 29). We observed that BDNF^{M/M} mice become extremely obese when they are exposed to a HFD, modeling diet-induced obesity in humans. Intriguingly, whereas both male and female BDNF^{M/M} mice were extremely obese, female BDNF^{M/M} mice had lower fasting blood glucose levels and better glucose clearance compared with their male counterparts (Fig. 4, C and D). The observed sex difference is likely due to differential fat distribution and a protective estrogen effect associated with females (37, 38).

There are reciprocally implicated roles between CD36 and BDNF in obesity and metabolic disorders. BDNF is an anorexigenic neurotrophin that attenuates appetite and body weight (16). Neurons from BDNF^{M/M} mice exhibited reduced activity-dependent BDNF secretion (28). On the other hand, with high affinity toward oxidized lipids and advanced glycation end products, CD36 has been implicated in the development of IR and diabetes (39–41). We previously reported increased CD36...
mRNA levels in the brains of 9-month-old BDNFM/M mice (42). In this study, we observed that BDNFM/M mice fed a HFD develop extreme obesity even at younger ages (i.e., 14 weeks old), which is accompanied with elevated expression of CD36 in the brain. Studies reported that CD36 expression occurs in a PPARγ-dependent manner (43, 44). CD36 internalizes modified/oxidized LDLs, generates intracellular PPARγ ligands, and further activates PPARγ and CD36 transcription. The increased CD36 expression leads to more internalization of CD36 ligands, thereby escalating a feed-forward interaction. The excess circulating lipids in obese male BDNFM/M mice likely intensify the interaction of these ligands with CD36, suggesting the involvement of CD36 in the progression of obesity and metabolic disturbances. Although we found that sCD36 levels in BDNFM/M mice were similar to those in BDNFV/V mice, the slope of correlation analyses between sCD36 level and the degree of IR were significantly different between BDNFV/V and BDNFM/M mice (Fig. 4G). Both BDNFV/V and BDNFM/M did not show significant correlation, but BDNFM/M mice had lower p values and higher R² values compared with BDNFV/V mice (BDNFV/V versus BDNFM/M, p value 0.7082 versus 0.1206, R² 0.0163 versus 0.4133). Our findings support the view of CD36 involvement in obesity and obesity-associated metabolic disorders.

Based on the observation that BDNFM/M mice develop extreme obesity, IR, and up-regulated macrophage CD36 expression, the study postulated that the inhibition of CD36 would provide greater benefits counteracting obesity-related metabolic dysfunctions in BDNFM/M mice. As predicted, SAB benefits in BDNFV/V mice were less pronounced, including slower body weight gain, less visceral obesity, higher quadriceps ratio, and improved insulin sensitivity and HOMA-IR level compared with the obese BDNFM/M mice (Fig. 5). The greater benefit of SAB observed in BDNFM/M mice suggests that genetic variability in populations that are at risk for developing obesity and its associated metabolic dysfunctions is an important factor to consider for intervention strategies. The notion of genetic variability is particularly noteworthy in Asians, a population that exhibits a high prevalence of the BDNF SNP (around 70%) (22, 23). Despite Asians having a significantly lower mean body mass index than other ethnic groups, studies have shown that the exposure of this population to a Western-style HFD profoundly increases the risk of developing IR and type II diabetes by 1.5–2-fold, which is higher than other ethnic
groups (45–47). In addition, a significantly higher incidence of undiagnosed diabetes in Asians (48) suggests that a treatment strategy targeting populations genetically at risk for developing obesity-associated metabolic dysfunction may be beneficial.

In summary, the study demonstrates that CD36 underlies the progression of obesity-associated metabolic dysfunctions, and using an intervention strategy that inhibits CD36 may have therapeutic utility in preventing visceral obesity and metabolic dysfunctions in BDNF SNP carriers. Thus, the study suggests that intergenetic variability should be considered in developing strategies to counteract obesity and obesity-associated metabolic dysfunction.

**Experimental procedures**

**Preparation of salvianolic acid B**

SAB was purchased from Chengdu Biopurify Phytochemicals (Sicuhan, China) (tested >98% purity) or Alfa Chemistry (Stony Brook, NY) (tested >97% purity). The efficacy of each lot of SAB was routinely checked using an in vitro 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate–conjugated oxLDL (DiI-oxLDL) uptake test described below. SAB was dissolved in PBS to make a stock solution (5 mM), and the solution was left unmodified (pH 2.0) or adjusted to pH 7.0, depending on experimental conditions. The stock solutions were diluted further and passed through 0.22-μm syringe filters before applying to macrophages.

**Ex vivo oxLDL uptake assay in macrophages**

SAB solutions (pH 2.0 or 7.0) were prepared and preincubated at 37 °C to test stability before applying to the cells. Peripheral cells were collected by lavage from naive mice, Veh-treated HFD-fed mice, or SAB-treated HFD-fed mice. Cells were plated in a 24-well plate in macrophage-SFM medium (Thermo Fisher Scientific) and incubated overnight to allow cells to attach. The next day, cells were treated with prepared SAB solutions for 30 min and then treated with 2 μg/ml DiI-oxLDL (Alfa Aesar, Tewksbury, MA) for 24 h. Cells without DiI-oxLDL served as a negative control. After 24 h of incubation, cells were harvested and analyzed using a flow cytometer (Accuri C6, BD Biosciences, San Jose, CA).

**Study design for in vivo experiments**

The use of animals and procedures was approved by the institutional animal care and use committee (IACUC) of Weill Cornell Medicine and in accordance with the IACUC, National Institutes of Health, and ARRIVE (49) guidelines. A total of 17 C57BL/6, 8 CD36 KO, 10 male and 7 female BDNFV/V, and 27 male and 6 female BDNFM/M mice were used for this study. Experiments were conducted in a randomized manner. Animals were randomly assigned to either the vehicle or treatment group. The experiments were not completely blinded due to the visualization of different degrees of obesity that were dependent on the genotype.

**Diabetes-induced obesity model**

Four different mouse lines were used for this study: C57BL/6, CD36 KO, BDNFV/V, and BDNFM/M. CD36 KO, BDNFV/V, and BDNFM/M mice were backcrossed 11 times with C57BL/6 mice (99.99% C57BL/6). The mice were housed at the institute’s animal facility, which monitors and maintains temperature, humidity, and 12-h light/dark cycles. A maximum of five mice
were housed in a cage with an individual ventilating system and irradiated bedding (1/800 Bed o’Cobs, The Andersons, Maumee, OH). Sterilized regular food (PicoLab rodent diet 5053, LabDiet, St. Louis, MO) or a HFD (S3282, 35.7% carbohydrate, 36.0% fat, and 20.5% protein, 60% kcal from fat, Bio-Serv, Flemington, NJ) and water were freely accessible to mice in their cage. To induce obesity, animals were fed a HFD for 8 weeks, and body weight of the animals was measured weekly to confirm their body weight gain. Daily food consumption was measured during the last week of 8 weeks of HFD intervention. Animals were individually housed, and the food consumption of each animal was measured daily and averaged.

**Chronic administration of SAB**

Implantation of an Alzet pump (model 2004, Durect Corp., Cupertino, CA) was performed in 6-week-old mice that were then fed a HFD for 8 weeks. Pumps were filled with either Veh (PBS) or neutralized SAB (50 mg/kg/day). To implant the pumps, mice were anesthetized, and an incision of the appropriate length was made in the dorsal subcutaneous skin. Subcu-

![Figure 6. Effect of SAB treatment on adipose and hepatic tissue inflammation in HFD-fed BDNFM/M mice.](image-url)

A and B, representative image of foam cell staining in adipose (A) and hepatic (B) tissues. Lipids were stained with Oil Red O. C, SAB-treated obese animals showed less optical density (mean intensity) and smaller lipid droplet size. D and E, SAB intervention decreased macrophage accumulation in adipose (D) and hepatic (E) tissues. F, SAB-treated animals showed decreased CD36 and F4/80 level and lower inflammatory gene expressions in adipose tissue. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Error bars, S.E.
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taneous dead space was created, and the filled pump was inserted into the dead space and placed in a position such that the pump did not lie directly beneath the incision. The pumps were replaced with new ones after 4 weeks. Visceral fat and quadriceps from left hind leg were collected at the time of sacrifice and weighed.

Real-time RT-PCR analysis

Relative mRNA levels were quantified with real-time RT-PCR using fluorescent TaqMan technology as described previously (9). Peritoneal cells were collected by lavage, and cell pellets were stored at −80 °C until use. Total RNA from the cell pellets was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA), and total RNA from adipose tissues was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen). A total of 0.5 μg for peritoneal cells and 0.2 μg for adipose tissue were reverse-transcribed using QuantiTech reverse transcription kits (Qiagen). Gene-specific PCR primers and probes were purchased (Life Technologies, Inc.): CD36 (Mm00441242_m1), interleukin-1β (Mm00802529_m1), monocyte chemoattractant protein-1 (MCP-1; Mm00441242_m1), interleukin-1β (Mm00434228_m1), tumor necrosis factor α (Mm00443258_m1), and Rn18s (Mm03928990_g1). Rn18s was used as an internal control for sample normalization. The PCR was performed using FastStart Universal Probe Master Mix (Roche Applied Science, Penzberg, Germany) in an Applied Biosystems 7500 fast real-time PCR system (Life Technologies). Gene expression was presented as the Rn18s normalized value according to the expression, 2(Rn18s threshold cycle – CD36 threshold cycle).

Western blot analysis

Peritoneal cell pellets were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA), supplemented with 1 mM phenylmethylsulfonyl fluoride and complete Mini protease inhibitor and Phospho Stop (Roche Applied Science). Cell lysates were centrifuged, and supernatants were used for Western blot analyses. Protein concentration was determined using a Bio-Rad DC protein assay, and a total of 2 μg of protein from cells were separated on NuPAGE 4–12% BisTris gels (Thermo Fisher Scientific) and transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked in blocking buffer (LI-COR, Lincoln, NE) for 1 h and then incubated with anti-mouse goat α-actin (1:10,000, sc-8432, Santa Cruz Biotechnology, Inc., Dallas, TX) antibody in blocking buffer at 4 °C. Membranes were then washed with TBS containing 0.05% Tween 20, followed by incubation with appropriate secondary antibodies conjugated with Alexa Fluor 680 (1:5000, A21088, lot 1722382, Life Technologies) or IRDye® 680RD (1:5000, 926-68071, lot C50520-02, LI-COR) in blocking buffer for 1 h. Protein bands were visualized using the Odyssey imaging system (LI-COR). Western blots were performed in multiple gels. To normalize interblot variability, identical samples were loaded in each blot as an internal control, and the density of the internal standard sample was used to standardize samples in multiple blots.

Plasma sCD36 and insulin level measurement and HOMA-IR level calculation

Trunk bloods from the mice were collected at the time of sacrifice, and plasmas were obtained from the blood. Plasmas were stored at −80 °C until use. Soluble CD36 concentrations were measured by the ELISA method using a mouse soluble CD36 ELISA kit (Aviscera Bioscience, Inc., Santa Clara, CA), according to the manufacturer’s protocol. Plasma insulin levels were determined in overnight-fasted mice using a mouse insulin ELISA kit (ALPCO Diagnostics), according to the manufacturer’s protocol. All samples were duplicated, and average numbers were used. HOMA-IR level was calculated using the HOMA2 Calculator (OCDEM, Oxford, UK).

Glucose tolerance test

The glucose tolerance test was performed at 7 weeks of HFD intervention. Baseline blood glucose concentrations (fasting blood glucose) were measured in overnight-fasted animals using a glucometer (Ascensia Contour, Bayer, Whippany, NJ). d-Glucose (2 mg/g body weight) was injected intraperitoneally, and blood glucose levels were measured at multiple time points.

Adipose and hepatic tissue histology

Visceral fat and liver were collected at the time of sacrifice and stored at −80 °C until use. Tissues were sectioned at a 14-μm thickness using a cryostat (Leica Biosystems, Buffalo Grove, IL). For Oil Red O staining, sections were fixed in methanol for 5 min and stained with Oil Red O solution for 30 min at room temperature. Then sections were rinsed with methanol briefly, followed by PBS. Immunohistochemical staining for macrophages was performed with monoclonal rat anti-F4/80 antibody (1:100, MCA497GA, lot 1610, Bio-Rad). After methanol fixation, the sections were quenched with 0.3% H2O2 and blocked with 1% BSA and 10% normal goat serum for 1 h. The sections were labeled with rat anti-F4/80 -actin (1:10,000, sc-8432, Santa Cruz Biotechnology, Inc., Dallas, TX), anti-mouse CD36 (1:1000, AF2516, lot VYQ0108041, R&D Systems, Minneapolis, MN) or anti-broad spectrum of species, including anti-mouse goat β-actin (1:10,000, sc-8432, lot G2508, Santa Cruz Biotechnology, Inc., Dallas, TX) antibody in blocking buffer at 4 °C. Membranes were then washed with TBS containing 0.05% Tween 20, followed by incubation with appropriate secondary antibodies conjugated with Alexa Fluor 680 (1:5000, A21088, lot 1722382, Life Technologies) or IRDye® 680RD (1:5000, 926-68071, lot C50520-02, LI-COR) in blocking buffer for 1 h. Protein bands were visualized using the Odyssey imaging system (LI-COR). Western blots were performed in multiple gels. To normalize interblot variability, identical samples were loaded in each blot as an internal control, and the density of the internal standard sample was used to standardize samples in multiple blots.

Statistics

Statistical analyses between two groups were done by t tests. For multiple-group comparison, one-way ANOVA was performed. In analyses that involve more than one factor (i.e. effect of drug (Veh versus SAB) and effect of genotype (BDNF<sup>V/v</sup> versus BDNF<sup>Em/M</sup>)), two-way ANOVA was used to analyze the effect of each factor and interaction between them. Post hoc Bonferroni correction was used for multiple comparisons for significance at p < 0.05. Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA).
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Author contributions—J. Y. performed experiments, analyzed the data, and wrote the manuscript. K. W. P. performed CD36 expression experiments. S. C. designed the study and wrote the manuscript.

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