Original Article

MOLECULAR METABOLISM

Peripheral cannabinoid-1 receptor blockade restores hypothalamic leptin signaling

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ABSTRACT

Objective: In visceral obesity, an overactive endocannabinoid/CB_1 receptor (CB_1R) system promotes increased caloric intake and decreases energy expenditure, which are mitigated by global or peripheral CB_1R blockade. In mice with diet-induced obesity (DIO), inhibition of food intake by the peripherally restricted CB_1R antagonist JD5037 could be attributed to endogenous leptin due to the rapid reversal of hyperleptinemia that maintains leptin resistance, but the signaling pathway engaged by leptin has remained to be determined.
Methods: We analyzed the hypothalamic circuitry targeted by leptin following chronic treatment of DIO mice with JD5037.
Results: Leptin treatment or an increase in endogenous leptin following fasting/refeeding induced STAT3 phosphorylation in neurons in the arcuate nucleus (ARC) in lean and JD5037-treated DIO mice, but not in vehicle-treated DIO animals. Co-localization of pSTAT3 in leptin-treated mice was significantly less common with NPY^+ than with $POMC^+$ ARC neurons. The hypophagic effect of JD5037 was absent in melanocortin-4 receptor (MC4R) deficient obese mice or DIO mice treated with a MC4R antagonist, but was maintained in $NPY^{-/-}$ mice kept on a high-fat diet.
Conclusions: Peripheral CB_1R blockade in DIO restores sensitivity to endogenous leptin, which elicits hypophagia via the re-activation of melanocortin signaling in the ARC.

Keywords Peripheral CB1 blockade; Leptin resistance; POMC; NPY; Diet-induced obesity

1. INTRODUCTION

Visceral obesity and its metabolic complications, commonly called the metabolic syndrome, represent a growing public health concern worldwide [1]. Accumulating evidence supports the pathogenic role of an overactive endocannabinoid/CB_1 receptor (CB_1R) system in obesity/metabolic syndrome [2–4]. Indeed, the CB_1R antagonist/inverse agonist rimonabant was effective in reducing body weight in obese/overweight people and also improved the associated insulin resistance, fatty liver, and dyslipidemia [5], but the therapeutic development of this class of compounds was halted due to neuropsychiatric side effects mediated by blockade of CB_1R in the central nervous system [6]. More recent evidence indicates that activation of CB_1R in peripheral tissues, including adipose tissue [7], liver [8], skeletal muscle [9], the endocrine pancreas [10], and proinflammatory macrophages [11], contributes to visceral adiposity and its metabolic complications, and its selective blockade by CB_1R antagonists/inverse agonists with limited brain penetrance can improve the obese phenotype in animal models of diet-induced metabolic syndrome without eliciting behaviors attributable to blockade of CB_1R in the CNS [11–13].

In a previous study, we reported that the peripherally restricted CB_1R inverse agonist JD5037 was as effective as its brain penetrant parent compound SLV319 (ibipinabant) in normalizing all of the metabolic consequences of a high-fat diet (HFD), including the normalization of body weight as well as causing transient but pronounced hypophagia [13]. The latter effect was surprising in view of the dominant role of central neural circuits in the control of food intake. To resolve this paradox, we posited that the hypophagic effect of chronic blockade of peripheral CB_1R is mediated by endogenous leptin, as a result of the rapid reversal of the hyperleptinemia and associated leptin resistance of diet-induced obese (DIO) mice [13]. Reversal of the hyperleptinemia, in turn, could be attributed to reduced leptin production due to direct inhibition of CB_1R in adipocytes and sympathetic nerve endings in adipose tissue, as well as increased leptin clearance in the kidney, due to inhibition of CB_1R in renal proximal tubular cells [13].

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The ability of peripherally generated leptin to reach its hypothalamic receptors reconciles the paradox of modulating a centrally regulated function using a drug restricted to the periphery. The question remains: which neural circuits are activated by endogenous leptin, once the leptin resistance in DIO mice is reversed by treatment with a peripherally restricted CB_1R inverse agonist. Generally, leptin coordinates the activity of the appetitive neural circuitry primarily via promoting the activity of pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons [14–16] and subsequent anorexigenic signaling by α-melanocyte-stimulating hormone (α-MSH) via the melanocortin 4 receptor (MC4R) [17] and simultaneously inhibiting the activity of orexigenic neuropeptide Y (NPY)/agouti-related peptide (AgRP) neurons in the arcuate nucleus (ARC) [18,19]. Therefore, we examined the relative role of these two pathways in the anorexigenic effect of endogenous leptin in DIO mice chronically treated with the peripheral CB_1R antagonist JD5037. The results indicate that resensitization of DIO mice to leptin is reflected in increased leptin-induce phosphorylation of STAT3 in both POMC and NPY neurons, with the former playing a key role in the anorexigenic and weight reducing actions of endogenous leptin, as reflected in the absence of these effects in MC4R knockout mice or DIO mice treated with an MC4R antagonist.

2. MATERIALS AND METHODS

2.1. Animals and experimental protocol

The experimental protocol was approved by the Institutional Animal Care and Use Committees of the NIAAA and Hebrew University of Jerusalem. Male 6-week old $NPY^{-/-}$ mice (*129S-Npy^{tm1Rpa}/J*) and their littermate controls were obtained from the Jackson Laboratory. Adult, male, genetically obese $MC4R^{-/-}$ mice (*MC4R^{tm1Lowl}/J*) were generated by heterozygote mating using wild-type littermates as controls. Mice were maintained under a 12 h light/dark cycle and fed *ad libitum*. To generate DIO, C57Bl6/J and $NPY^{-/-}$ mice were fed a HFD (Research Diet, D12492; 60% calories from fat, 20% from protein, and 20% from carbohydrates), with age-matched lean controls receiving a standard laboratory diet (STD, NIH-31 rodent diet) for 12–14 weeks. To achieve normoleptinemia in DIO mice, we adapted a protocol described by Knight et al. [20]. Briefly, leptin-deficient *ob*/*ob* mice were implanted with an osmotic minipump (model 2001D, Alzet Osmotic Pumps; Durect, Cupertino, CA) delivering leptin dissolved in phosphate-buffered saline (PBS) at a rate of 150 ng/h for 12 weeks, during which time they were fed a HFD to induce DIO. Control groups of wild-type mice on HFD and *ob*/*ob* mice on STD were also implanted with minipumps delivering PBS at the same rate. Pumps were replaced every 28 days. HFD-induced obese C57Bl/6J, $NPY^{-/-}$ and normoleptinemic *ob*/*ob* mice, and genetically obese $MC4R^{-/-}$ mice on STD were treated daily with JD5037 (3 mg/kg/day, po) or vehicle (Veh; 4% DMSO + 1% Tween80 in normal saline) for 7 days. Body weight and food intake were monitored daily. Mice were euthanized by cervical dislocation under anesthesia, and their brains and trunk blood were collected for further analyses.

2.2. Leptin sensitivity

Leptin sensitivity was assessed in lean and DIO mice and DIO mice treated daily with JD5037 (3 mg/kg po.) for 7 days followed by twice daily treatment with leptin (3 mg/kg, ip) or vehicle for an additional 4 days. One hour after the last dose of leptin or vehicle, mice were anesthetized, perfused via the left ventricle with 5 mL of 0.9% saline for 1 min followed by 60–80 mL of cold 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 15 min at room temperature. Then, the perfused brains were removed, post-fixed in the same fixative for 6 h at 4 °C and further processed for immunohistochemical analyses.

2.3. Immunohistochemistry

After fixation, the brains were cryoprotected with 0.1 M phosphate buffer (pH 7.4) containing 20% sucrose for 72 h and then rapidly frozen in isopentane pre-cooled to −70 °C with dry ice. Serial coronal sections (30 μm) were cut using a cryostat through the brain region containing the ARC. After inactivating the endogenous peroxidase activity with 0.6% hydrogen peroxidase (Sigma–Aldrich, St. Louis, MO), sections were incubated separately with avidin and biotin solutions (Vector Lab, Burlingame, CA) for blocking nonspecific binding of endogenous biotin, biotin-binding protein, and lectins. Then, the sections were incubated free-floating in 0.01 M PBS (pH 7.4) containing 2% normal donkey serum (Jackson ImmunoResearch Labs, West Grove, PA), 0.3% Triton X-100 (Sigma–Aldrich, St. Louis, MO) and rabbit anti-pSTAT3 antibody (1:1,500; Cell Signaling, Beverly, MA) or rabbit anti-c-Fos antibody (1:10,000; Santa Cruz Biotechnology, CA) for 43 h at 4 °C. The immunoreaction product was visualized using the Vectastain elite ABC kit (Vector Lab., Burlingame, CA) and 3′,3′-diaminobenzidine (Sigma–Aldrich, St. Louis, MO) as a chromogen. After thorough washes, sections were mounted on gelatin-coated slides. Following dehydration in ethanol, sections were cleared in xylene and coverslipped in Permount® (Fisher Scientific, Fair Lawn, NJ). Staining for pSTAT3 and c-Fos were visualized using a bright field light source and captured with a digital camera mounted on an Olympus BX41 microscope.

Sections earmarked for double labeling with pSTAT3+POMC or pSTAT3+NPY were processed according to the indirect immunofluorescence method of Coons [21]. Briefly, following washes in PBS, the sections were incubated free-floating in PBS containing Triton X-100, blocking serums and two primary antibodies: rabbit anti-pSTAT3 (1:500; Cell Signaling, Beverly, CA) and chicken anti-POMC (1:1,000; Abcam, Cambridge, MA) or rabbit anti-pSTAT3 (1:500; Cell Signaling) and chicken anti-NPY (1:2,000; Novus Biologicals) for 43 h at 4 °C. Then, the sections were incubated at room temperature in PBS containing Triton X-100, blocking serum, and donkey anti-Rabbit Alexa Fluor 594 (1:250; Invitrogen) for 1 h, and then Triton X-100, blocking serum, and goat anti-chicken Alexa Fluor 488 (1:250; Invitrogen) for another hour. After thorough washes in PBS, all sections were mounted on gelatin-coated microscope slides, coverslipped with Vectashield (Vector Lab.), and analyzed using a Zeiss LSM700 confocal microscope. The localization of the immunoreactive signals was identified using Hof's mouse brain atlas. Six corresponding sections in the ARC, organized in a consecutive rostral to caudal sequence from −0.94 mm to −2.92 mm relative to the bregma, were counted for pSTAT3 and c-Fos positive cells and analyzed for co-localization of pSTAST3 with POMC or NPY positive cells. Cell counts from 4 mice per group were obtained from both sides of the brain in each section. pSTAT3-, c-Fos-, POMC-, and NPY-positive cells as well as double labeled cells were scored in each ARC section from vehicle and leptin-treated animals. The percentage of double-positive $pSTAT3^+$ POMC or $pSTAT3^+$ NPY cells was also determined by cell counting.

2.4. Blood biochemistry

Serum leptin levels were measured by an ELISA kit (Millipore, Billerica, MA, and R&D Systems, Minneapolis, MN).

2.5. Chronic infusion of SHU-9119

To assess whether hypothalamic MC4R mediates the response to peripheral CB_1R antagonism in DIO mice, vehicle (saline) or the MC4R

antagonist SHU-9119 (Tocris; 24 nmol/day) was delivered over 7 days by intracerebroventricular (icv) infusion using osmotic minipumps. The effects of simultaneous 7-day treatment with daily oral doses of vehicle or JD5037 (3 mg/kg, po.) on body weight, food intake, and serum leptin levels were determined as described above.

2.6. Fasting/refeeding paradigm

To determine whether peripheral CB_1R blockade resensitizes endogenous leptin action, we used a fasting/refeeding paradigm. Briefly, male, 6–7 week old C57Bl/6J mice were kept on STD or HFD for 5 weeks. During the last week of feeding, the mice were treated with either vehicle or JD5037 (3 mg/kg, po.) for 1 week. Following the last dose, the mice were fasted for 16 h and then food was introduced for an additional 4 h before sacrifice. The hypothalamus and trunk blood were collected from each mouse, and the phosphorylation of STAT3 was determined by western blotting, while serum leptin levels were measured by an ELISA kit (Millipore, Billerica, MA).

2.7. Endocannabinoid measurements

Mice were euthanized and the brain and hypothalamus were rapidly dissected and kept at −80 °C until processed. Arachidonoyl ethanolamide (AEA) and 2-arachidonoylglycerol (2-AG) in brain cortex and hypothalamus were extracted, purified, and quantified by the stable isotope dilution LC-MS/MS method described previously [13].

2.8. Materials

Rimonabant was obtained from the National Institute of Drug Abuse Drug Supply Program. JD5037, kindly provided by Jenrin Discovery, Inc. [22]. For oral administration by gavage, rimonabant and JD5037 were dissolved in 4% DMSO + 1% Tween-80 in saline. SHU-9119 was purchased from Tocris and was dissolved in saline to be administered by an Alzet osmotic minipump (Durect, Cupertino, CA) at the rate of 24 nmol/day as described previously [23,24].

2.9. Statistics

Data are expressed as mean ± SEM. Unpaired two-tailed Student's t-test was used to determine differences between vehicle- and drug-treated groups. Results in multiple groups were compared by one- or two-way ANOVA followed by a Bonferroni or Sidak's multiple comparisons test, as appropriate (GraphPad Prism v6 for Windows). Significance was set at $P < 0.05$.

3. RESULTS

3.1. Hyperleptinemia is required for the hypophagic and weight reducing effects of peripheral CB_1R blockade

We have previously demonstrated that the weight reducing and hypophagic effects of JD5037 in DIO mice develop rapidly within 7 days of treatment and are associated with a similar rapid reversal of hyperleptinemia and leptin resistance [13]. Here we sought to further test whether hyperleptinemia is, in fact, a prerequisite of the efficacy of peripheral CB_1R blockade. To this end, we tested the effects of JD5037 in obese mice with substantially different leptin status using an approach based on that developed by Knight et al. [20], as detailed in the Methods section and illustrated in Figure 1A and B.

Treatment with a daily oral dose of JD5037 (3 mg/kg) for 14 days failed to significantly affect body weight, food intake, and plasma leptin levels in obese but normoleptinemic animals (*ob/ob* mice on HFD chronically infused with leptin) and was similarly ineffective in aleptinemic *ob/ob* mice. On the other hand, and in accordance with our previous findings [13], JD5037 treatment alleviated hyperleptinemia, caused transient

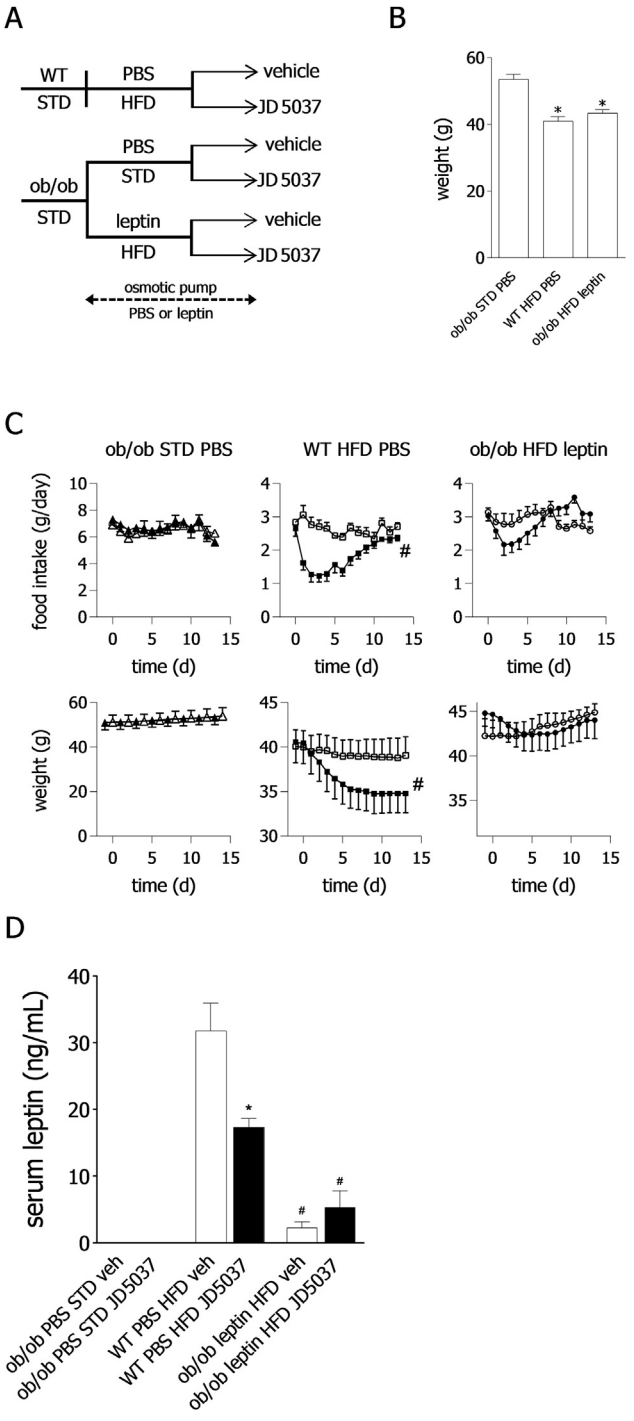

Figure 1: Effects of peripheral CB_1R blockade on food intake and body weight in obese mice with different leptin status. (A) Experimental protocol yielding obese mice with aleptinemia (*ob/ob* mice on standard diet [STD] and chronically infused with PBS), normoleptinemia (*ob/ob* mice on high-fat diet [HFD] infused with leptin), or hyperleptinemia (wild-type mice on HFD, infused with PBS). Horizontal line indicates the period of leptin or PBS infusion. **(B)** Mean body weights at the end of the pretreatment period. n = 10, 13, and 11 for *ob/ob* STD, *ob/ob* HFD + leptin and WT HFD + PBS groups, respectively. $^{*}P < 0.0001$ as compared to *ob/ob* STD + PBS group. **(C)** Daily food intake and body weight of vehicle-treated (open symbols) and JD5037-treated animals (filled symbols), n = 6 in all 6 groups. $^{\#}P < 0.001$ for the effect of JD5037 vs vehicle treatment. **(D)** Serum leptin concentrations on day 14 of JD5037 or vehicle treatment, n = 4–6 mice per group. Leptin was undetectable in sera of *ob/ob* mice infused with PBS. $^{\#}P < 0.0001$ relative to vehicle-treated WT mice.

hypophagia, and significantly reduced body weight in wild-type DIO mice (Figure 1C and D). Furthermore, the weight-reducing effect of JD5037 in the last group could be counteracted by daily treatment with a pharmacologic dose of leptin which also reversed the JD5037-induced decrease in plasma leptin (Supplementary Fig. 1). Together, these findings support the notion that the anorectic and weight reducing effects of peripheral CB_1R blockade in DIO are contingent on hyperleptinemia and its drug-induced reversal.

3.2. Peripheral CB_1R blockade restores hypothalamic leptin sensitivity

Next, we analyzed leptin-induced STAT3 phosphorylation in the diencephalon, a marker of leptin signaling, to test whether peripheral CB_1R blockade restores leptin sensitivity in the mediobasal hypothalamus. As illustrated in Figure 2A, B, leptin treatment robustly increased STAT3 phosphorylation in the ARC of lean control mice with a few responsive cells also present in the adjacent ventromedial hypothalamus (VMH). Although the number of $pSTAT3^+$ cells in the VMH was increased in vehicle-treated DIO mice compared to lean control mice, leptin treatment was without any effect on hypothalamic pSTAT3 in DIO mice, similar to findings by others [20,25]. In contrast, in DIO mice treated with JD5037 for 7 days, leptin treatment robustly increased STAT3 phosphorylation, similarly to its effect in lean controls. To determine the effects of leptin treatment, HFD status and peripheral CB_1R blockade on neuronal activity in the ARC, we examined the expression of c-Fos protein in consecutive brain sections used to evaluate pSTAT3 expression. We found that the leptin-induced increase in c-Fos expression in the ARC of lean mice was completely absent in vehicle-treated DIO mice but was partially restored in JD5037-treated DIO mice (Figure 2C, D). Thus, peripheral CB_1R blockade restores hypothalamic leptin-induced STAT3 phosphorylation and neural activation in DIO mice.

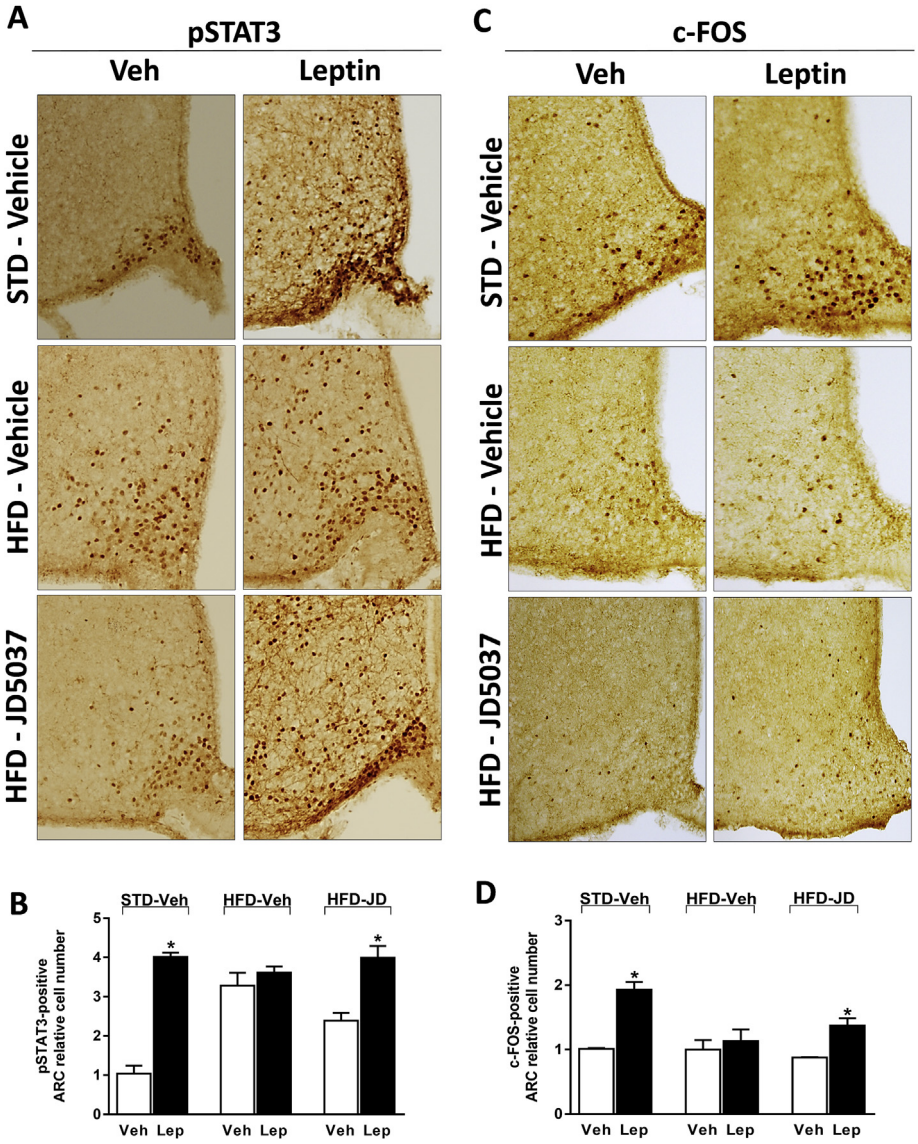

Figure 2: Peripheral CB_1R blockade restores hypothalamic leptin-induced STAT3 phosphorylation in DIO mice. Effect of leptin (3 mg/kg, ip twice daily for 4 days) on tyrosine^{705}-phosphorylated STAT3-positive ($pSTAT3^+$) neurons **(A)** or c-Fos expression **(C)** in hypothalamic coronal sections obtained from lean and DIO C57Bl/6J mice treated for 7 days with either vehicle or JD5037 (3 mg/kg, po). Quantification of the number of $pSTAT3^+$ cells **(B)** or c-Fos^+ cells **(D)** in the ARC. Data are mean ± SEM from 4 animals in each group. $^*P < 0.05$ relative to the corresponding control group treated with vehicle (Veh).

Analyses of leptin sensitivity typically involve the use of pharmacological doses of leptin even in lean mice. We therefore tested whether changes in the phosphorylation status of STAT3 are also detectable in response to changes in the plasma levels of endogenous leptin within its physiological range. Leptin plasma levels drop during fasting and increase upon refeeding [26]. We therefore tested the effects of overnight fasting followed by brief refeeding on plasma leptin levels and pSTAT3/STAT3 status in the mediobasal hypothalamus. As shown in Figure 3, plasma leptin level at the end of the fasting period was low in lean mice, was about ten times higher in vehicle-treated DIO mice,

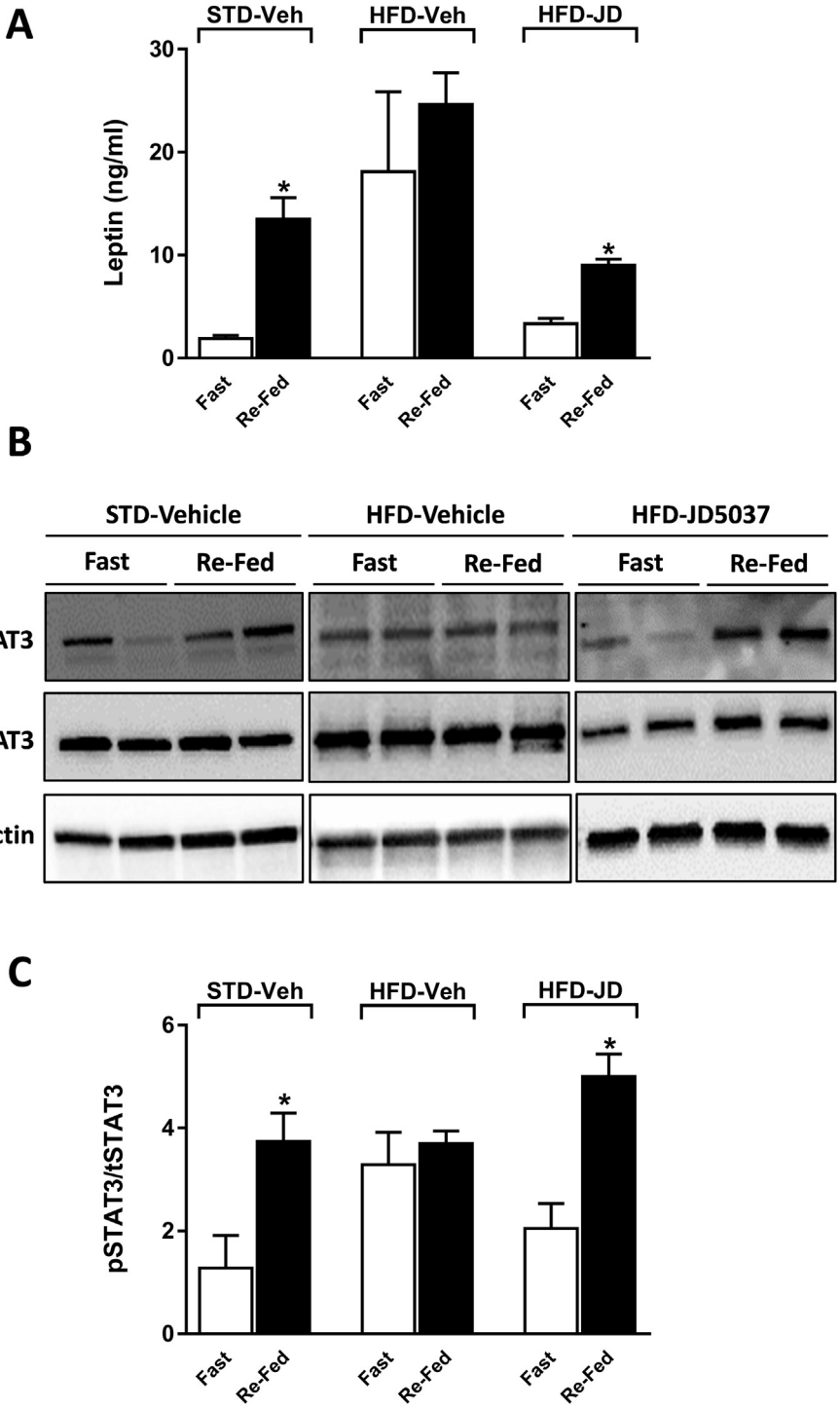

Figure 3: Peripheral CB_1R blockade restores sensitivity to endogenous leptin. Wild-type C57Bl/6J mice on STD or HFD were treated orally for one week with 3 mg/kg/day JD5037 or vehicle. After the last dose the mice were fasted for 16 h, then provided access to food for 4 h, after which they were sacrificed and brain tissue and plasma were collected for quantifying plasma leptin **(A)**, and hypothalamic pSTAT3/STAT3 **(B** and **C)**, as detailed in Methods. Quantitation of pSTAT3 in panel B was done by densitometry, and values are expressed as the ratio of pSTAT3/STAT3. Data are mean ± SEM from 5 animals in each group. $^*P < 0.05$ relative to the corresponding value in the fasted group.

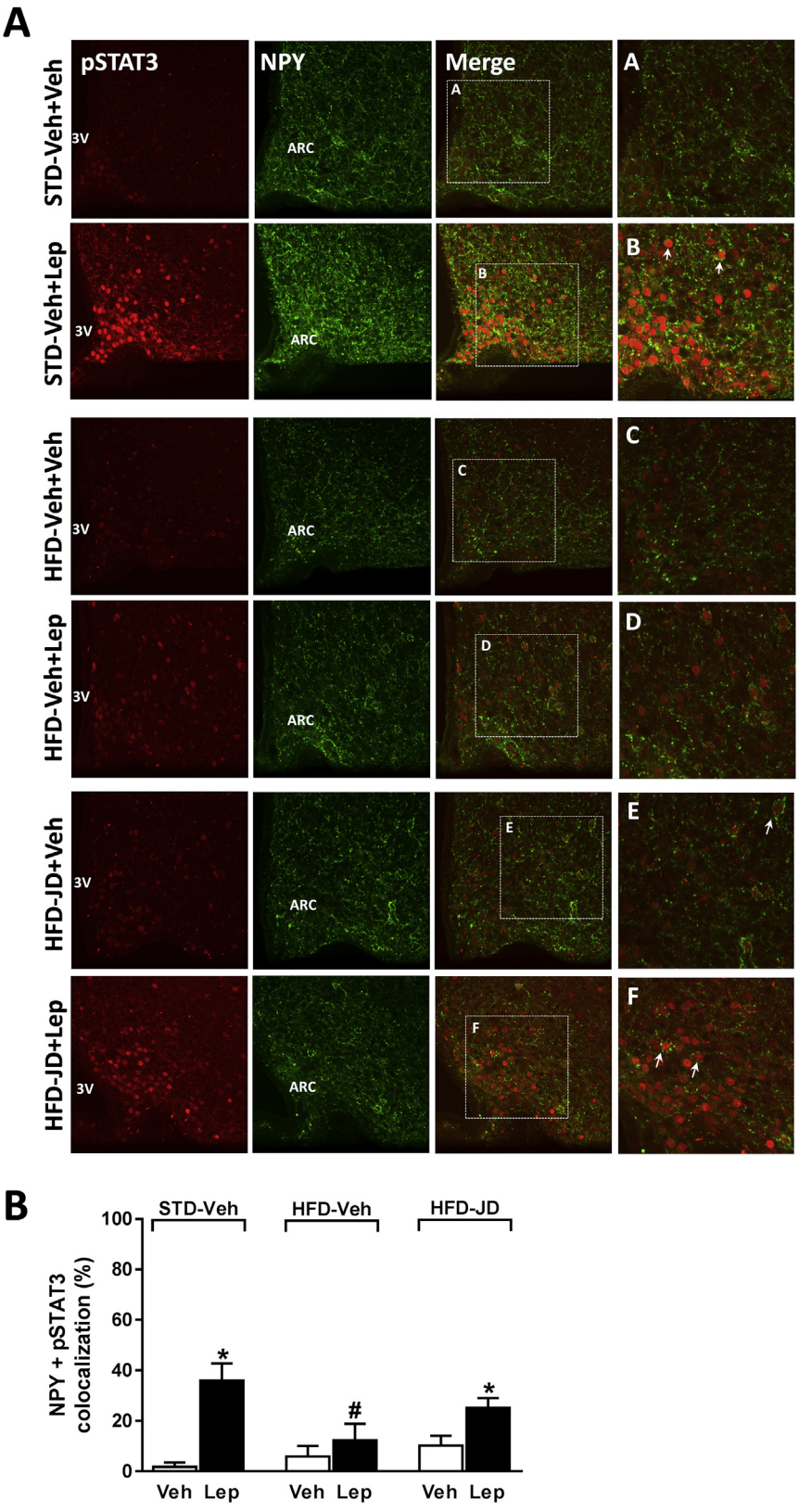

Figure 4: Peripheral CB_1R blockade increases leptin-induced tyrosine^{705}-STAT3 phosphorylation in NPY-positive neurons. Representative photomicrographs showing tyrosine^{705}-pSTAT3^{+} neurons (red) and NPY^{+} neurons (green) in the hypothalamic ARC 45 min after the last injection of leptin (3 mg/kg, ip. twice daily for 4 days) or vehicle **(A)**. Insets (A–F): Examples of NPY^{+} neurons coexpressing pSTAT3. Quantitation of the percentage of pSTAT3^{+} NPY neurons in coronal brain sections **(B)**. pSTAT3^{+} NPY neurons were counted from four brain sections from each group. $^{*}P < 0.05$ relative to the corresponding control group treated with Veh. $^{\#}P < 0.05$ relative to the leptin-treated mice in the STD-Veh group.

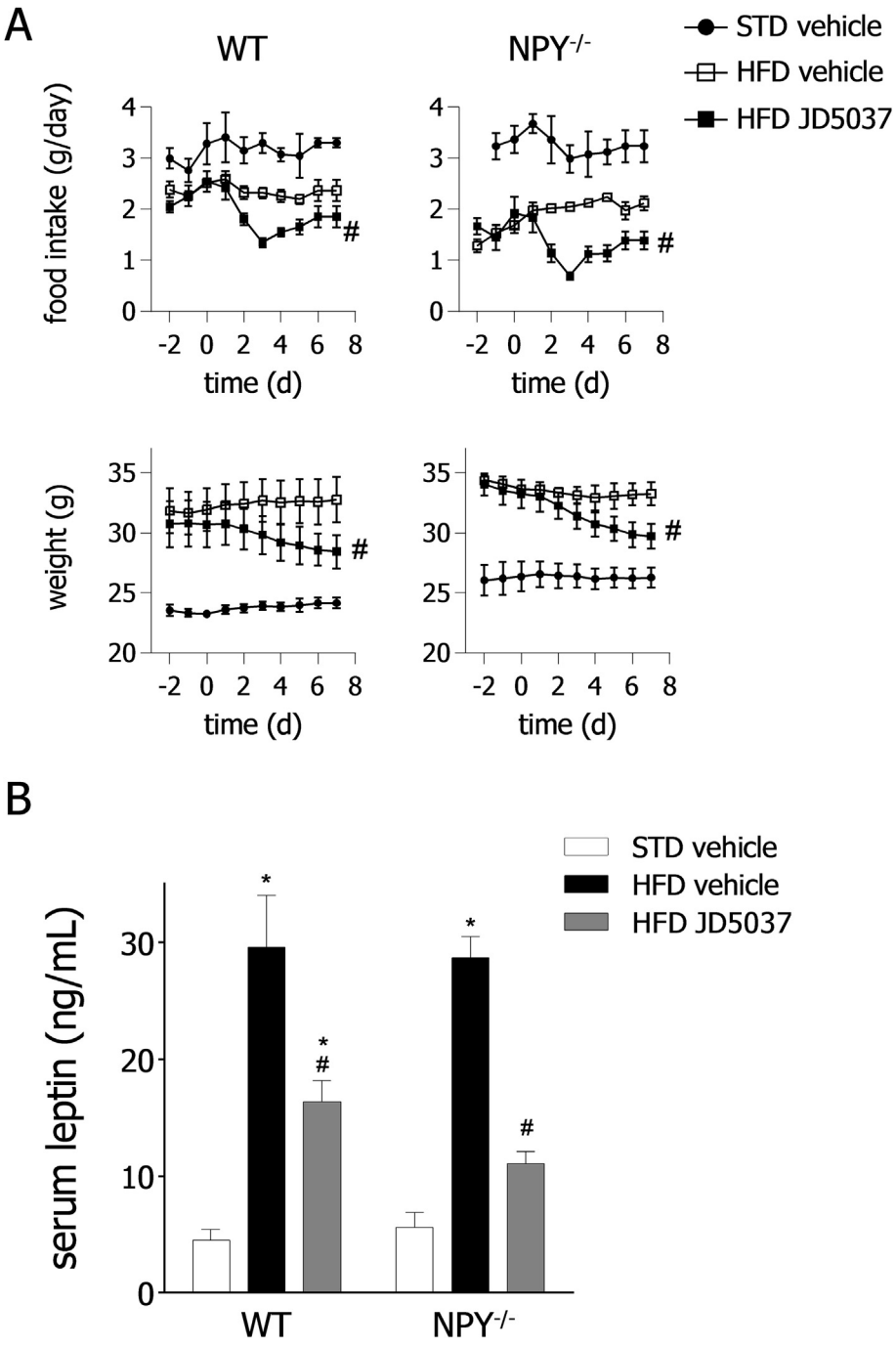

Figure 5: Peripheral CB_1R blockade is equieffective in wild-type and $NPY^{-/-}$ mice on HFD. (A) Body weight and food-intake of WT and $NPY^{-/-}$ mice kept on STD or HFD and treated with JD-5037 or vehicle. $^{*}P < 0.01$, JD5037 vs vehicle, n = 5 in all groups except for STD groups (n = 4) and $NPY^{-/-}$ HFD JD-5037 treated (n = 4). **(B)** Serum leptin concentrations on day 7 of JD5037 treatment. $^{*}P < 0.01$, vs STD; $^{\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs HFD vehicle group. Numbers of observations were as in **A**.

and returned to near control levels in DIO mice treated for one week with daily doses of 3 mg/kg JD5037. Refeeding elicited significant, several-fold increases in plasma leptin in both lean mice and DIO mice treated with JD5037, but no significant further increase was evident in vehicle-treated DIO mice. STAT3 phosphorylation quantified using Western blots showed parallel changes, being significantly increased upon refeeding in lean and in DIO mice treated with JD5037 but not in vehicle-treated DIO animals. This indicates that JD5037 treatment of obese mice reverses their resistance to endogenous leptin.

3.3. Inhibition of NPY signaling by leptin is not involved in the weight reducing and hypophagic effects of peripheral CB_1R blockade in DIO mice

In the above experiments, JD5037 treatment restored leptin responsiveness in the ARC of DIO mice and normalized their body weight. These effects of leptin can be mediated either by inhibition of orexigenic NPY/AgRP neurons or activation of anorexigenic POMC/CART neurons in the ARC [27,28]. We first explored the possibility that the restored effects of leptin that result from peripheral CB_1R

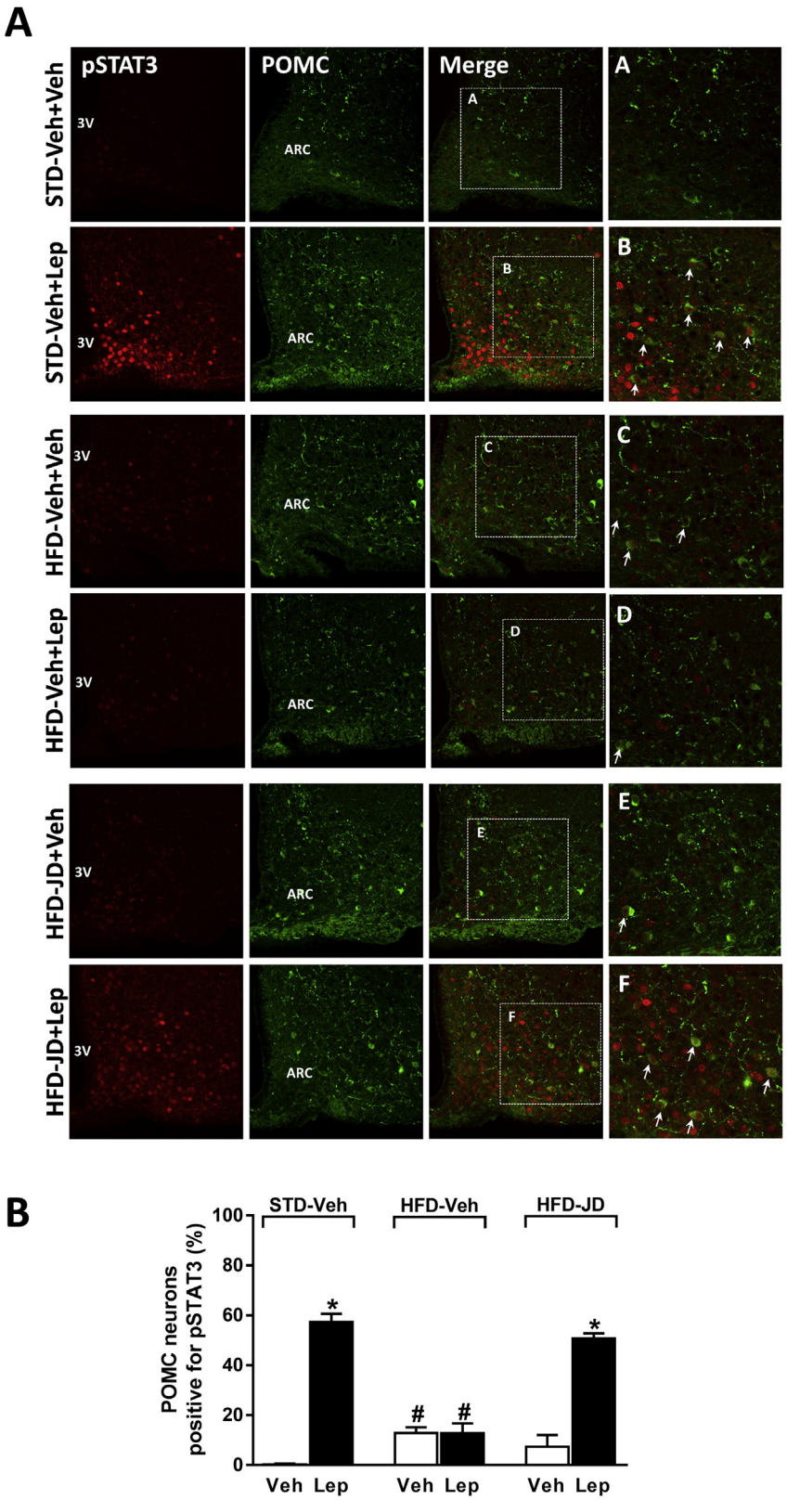

Figure 6: Peripheral CB_1R blockade increases leptin-induced tyrosine^{705}-STAT3 phosphorylation in POMC-positive neurons. Representative photomicrographs showing tyrosine^{705}pSTAT3^{+} neurons (red) and POMC-positive neurons (green) in the hypothalamic ARC 45 min after the last injection of leptin (3 mg/kg, ip twice daily for 4 days) or vehicle **(A)**. Insets (A—F): examples of POMC^{+} neurons co-expressing pSTAT3. Quantitation of the percentage of pSTAT3^{+} POMC neurons in coronal brain sections **(B)**. pSTAT3^{+} POMC neurons were counted from four brain sections from each group. $^{*}P < 0.05$ relative to the corresponding control group treated with Veh. $^{\#}P < 0.05$ relative to the vehicle + leptin-treated mice in the STD-Veh group.

blockade involve the NPY/AgRP pathway. As shown in Figure 4, leptin induced detectable STAT3 phosphorylation in about one third of NPY-positive neurons in lean mice, as assessed by double immunohistochemistry and confocal microscopy. HFD suppressed leptin-activated STAT3 phosphorylation in NPY-positive neurons, and this suppression was only partially reversed by chronic treatment with JD5037 (3 mg/kg, po) for 7 days (Figure 4A, B).

To further test the functional role of altered NPY signaling in mediating the weight reducing and hypophagic effects of JD5037, we treated HFD-induced obese NPY$^{-/-}$ mice and their HFD-fed wild-type littermates with JD5037 (3 mg/kg, po) for 7 days and monitored their body weight, food intake, and serum leptin levels. Compared to wild-type mice on STD, both wild-type and NPY$^{-/-}$ mice on HFD displayed similar significant increases in body weight (Figure 5A), visceral adiposity (Supplementary Fig. 2B), and serum leptin levels (Figure 5B). JD5037 caused robust decreases in body weight, food intake, and serum leptin levels in NPY$^{-/-}$ animals, which were similar or, in the case of serum leptin, even slightly greater than its parallel effects in wild-type DIO littermates (Figure 5A,B, and Supplementary Fig. 2). These findings argue against the involvement of NPY in the weight reducing effect of JD5037, although they do not rule out the possible involvement of AgRP.

3.4. Peripheral CB_1R blockade restores hypothalamic leptin sensitivity via POMC signaling

Since leptin activates the anorexigenic POMC/CART neurons in the ARC [14,16], we next explored whether peripheral CB_1R blockade affects this neuronal pathway. Consistent with previous reports [28], leptin administration stimulated STAT3 phosphorylation in POMC-positive neurons of lean mice, as assessed by double immunohistochemistry and confocal microscopy (Figure 6). The percentage of POMC^{+}/pSTAT3^{+} neurons in the ARC (57.4 $\pm$ 3.3%) was significantly higher than the percentage of NPY^{+}/pSTAT3^{+} neurons (see Figure 4, 35.9 $\pm$ 6.8%, $P < 0.01$). Leptin-stimulated STAT3 phosphorylation in these neurons was completely abrogated in DIO mice, although basal pSTAT3 levels were significantly increased. Leptin-induced STAT3 phosphorylation was fully restored in DIO mice treated with JD5037 (3 mg/kg, po.) for 7 days (Figure 6) prior to leptin treatment, suggesting that peripheral CB_1R blockade enhances hypothalamic leptin signaling via the activation of anorexigenic POMC neurons.

The anorexigenic effect of leptin is believed to be mediated by α-MSH acting on MC4R [29]. To explore the functional role of POMC-positive neurons in mediating the hypophagic effect of JD5037, we treated STD-fed obese MC4R$^{-/-}$ mice and their HFD-fed littermate controls with JD5037 (3 mg/kg, po.) for 7 days and monitored their body weight, food intake, and serum leptin levels. As shown in Figure 7, the

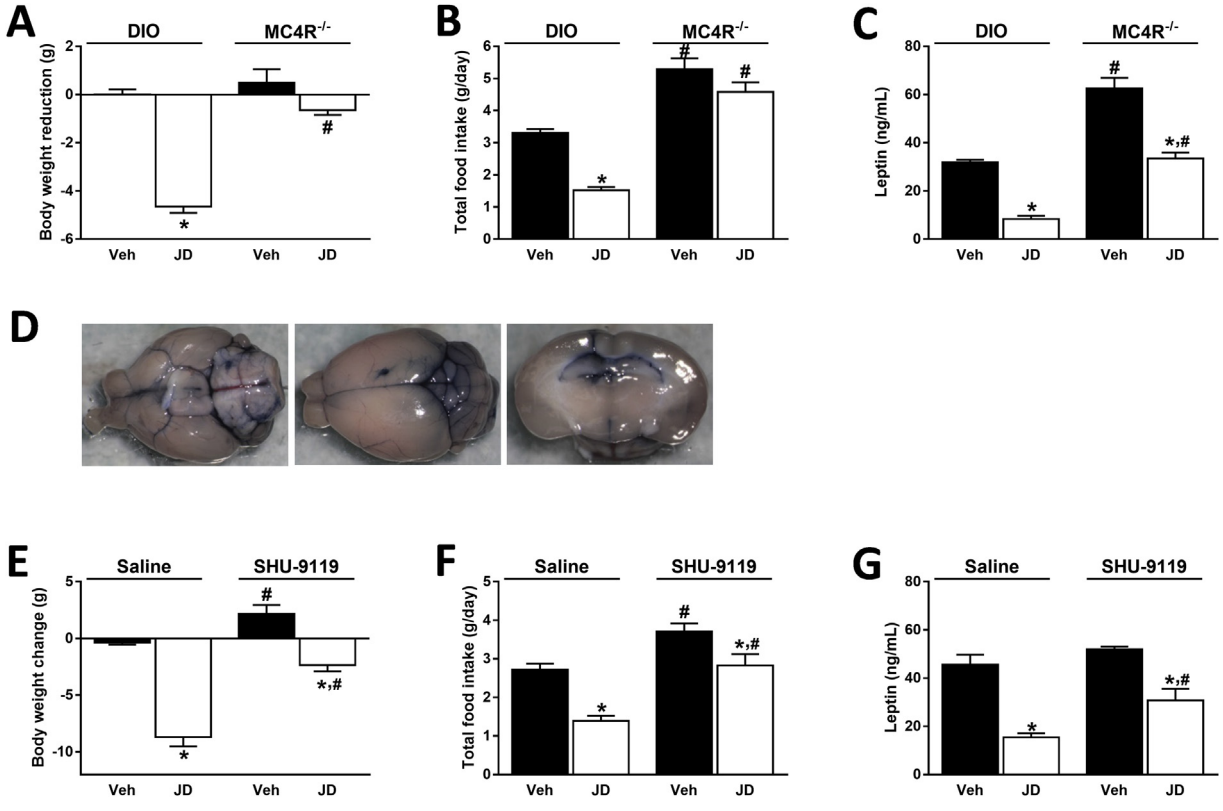

Figure 7: MC4R deactivation abolishes the hypophagic and weight reducing effects of peripheral CB_1R blockade. JD5037 (3 mg/kg, po, for 7 days) reduced body weight **(A)** and food intake **(B)** in DIO mice but not in MC4R$^{-/-}$ animals. Absolute body weights of DIO mice are: 42.3 $\pm$ 0.7 g (Veh) vs 36.4 $\pm$ 0.9 g (JD5037), and of MC4R$^{-/-}$ mice: 43.2 $\pm$ 1.5 (Veh) vs 42.3 $\pm$ 1.8 g (JD5037). Serum leptin levels were similarly reduced by JD5037 in both mouse strains, although the net values were much greater in MC4R$^{-/-}$ mice **(C)**. Treatment of DIO mice with SHU-9119 (24 nmol/day, icv via osmotic minipump, for 7 days) attenuated the reductions in body weight, food intake and serum leptin levels induced by JD5037 (3 mg/kg/day, po, for 7 days) (**E**–**G**). Absolute body weights in saline-infused DIO mice are 50.0 $\pm$ 1.6 g (Veh) vs 41.9 $\pm$ 0.8 g (JD5037), and in SHU-9119-infuced DIO mice are 53.8 $\pm$ 0.9 g (Veh) vs 49.5 $\pm$ 1.2 g (JD5037). **D**, representative pictures of a cannulated brain demonstrating the location of the cannula and the injection site of SHU-9119 into the 3rd ventricle (blue staining). Data are obtained from 4 to 5 animals in each group. $^{*}P < 0.05$ relative to the corresponding obese group treated with Veh; $^{\#}P < 0.05$ relative to the corresponding DIO group treated with either Veh or JD5037.

weight reducing and hypophagic effects of JD5037 observed in DIO mice were absent in MC4R$^{-/-}$ mice (Figure 7A, B). Although JD5037 treatment reduced serum leptin levels in both mouse strains, the absolute levels remained significantly higher in MC4R$^{-/-}$ compared to DIO mice (Figure 7C). To further test the role of MC4R in mediating the increased hypothalamic signaling of leptin in DIO mice treated with JD5037, we continuously infused via a minipump the potent MC4R antagonist, SHU-9119 (24 nmol/day, icv) to HFD-fed animals that were simultaneously treated with daily oral doses of JD5037 (3 mg/kg) or vehicle for 7 days. The correct location of the icv infusion of SHU-9119 into the 3rd ventricle was verified postmortem by the infusion of toluidine blue (Figure 7D). In the presence of SHU-9119, the effects of JD5037 on body weight, food intake, and serum leptin levels were significantly attenuated (Figure 7E–G). Unlike these results, treatment of MC4R$^{-/-}$ mice with the globally acting CB$_1$R inverse agonist rimonabant resulted in reductions in body weight, food intake and serum leptin levels (Figure 8A–C).

3.5. Effects of high-fat diet and peripheral CB$_1$R blockade on hypothalamic endocannabinoids

Our laboratory has earlier documented an inverse relationship between leptin and hypothalamic endocannabinoids levels and a role in appetite regulation [30]. We therefore measured the tissue levels of AEA and 2-AG in the cortex and hypothalami of lean and DIO mice as well as DIO mice chronically treated with JD5037 (3 mg/kg, po.). As seen in Supplementary Figure 3, keeping mice on HFD resulted in a significant increase in hypothalamic 2-AG and a smaller, not statistically significant increase in AEA levels. In contrast, treatment of DIO mice with JD5037 significantly reduced AEA, but not 2-AG levels. These changes were specific for the hypothalamus, as brain endocannabinoid levels remained unchanged by either diet or drug treatment.

4. DISCUSSION

Rimonabant, the first-in-class, globally acting CB$_1$R antagonist/inverse agonist is an anti-obesity agent that not only reduced body weight but also improved all the associated metabolic abnormalities [5]. Although neuropsychiatric side-effects led to its withdrawal from the market, this was not unexpected in view of the known obligatory role of endocannabinoids and CB$_1$R in the mesolimbic dopaminergic reward pathway in mediating both natural and drug reward [31]. Subsequent evidence that non-brain-penetrant CB$_1$R antagonists are, as or nearly as, effective as rimonabant in improving metabolic end points could be attributed to the presence of CB$_1$R at low yet functionally relevant levels in tissues involved in metabolic control and their upregulation in obesity. On the other hand, the efficacy of peripheral CB$_1$R blockade in reducing food intake in DIO mice was surprising in view of the dominant role of a central neural circuitry in appetite control. The absence of a similar effect in mice with defective leptin signaling or in wild-type DIO mice treated with a leptin antagonist supported the hypothesis that the hypophagic effect of peripheral CB$_1$R blockade is mediated by endogenous leptin, due to the rapid reversal of hyperleptinemia and the associated leptin-resistant state [13]. This notion is further supported by the present findings that the ability of peripheral CB$_1$R blockade to reduce food intake and body weight is abrogated by clamping serum leptin levels of DIO mice at the normal physiologic range (Figure 1), or by the infusion of exogenous leptin to counter the leptin lowering effect of JD5037 in hyperleptinemic DIO mice (Supplementary Figure 1). These observations are also in agreement with the concept of Knight et al. [20] that hyperleptinemia is both necessary and sufficient for maintaining leptin resistance in DIO.

The present findings further indicate that once the leptin resistance of DIO mice is reversed by peripheral CB$_1$R blockade, endogenous leptin acts in the hypothalamus primarily via activating POMC neurons resulting in MC4R activation, as indicated both by increased STAT3 phosphorylation in POMC neurons and a parallel increase in c-Fos expression in the ARC. The dominant role of melanocortin signaling is further supported by the failure of peripheral CB$_1$R blockade to reduce food intake and body weight in obese MC4R$^{-/-}$ mice and its reduced efficacy in DIO mice simultaneously exposed to a MC4R antagonist. In contrast, the hypophagic effect of JD5037 is fully maintained, if not increased, in HFD-fed NPY$^{-/-}$ mice compared to their HFD-fed wild-type littermates, which is similar to the maintained hypophagic effect of rimonabant in overnight fasted NPY$^{-/-}$ mice [30]. The preferential activation of POMC neurons is also indicated by the significantly greater co-localization of pSTAT3 with POMC than with NPY expressing arcuate neurons following leptin challenge of JD5037-treated DIO mice. Nevertheless, the co-expression of pSTAT3 with some NPY neurons could reflect their inhibition by leptin that would result in reduced AgRP expression and, as a consequence, disinhibition of melanocortin signaling. This mechanism would be retained in NPY$^{-/-}$ mice as gene deletion of NPY does not affect the AgRP content of these neurons [32].

Refeeding after fasting was associated with a significant increase in serum leptin and a parallel increase in hypothalamic pSTAT3 in lean and JD5037-treated DIO mice, but not in vehicle-treated DIO mice. This is compatible with the role of pSTAT3 signaling in the metabolic effects of endogenous leptin, as proposed previously [33,34]. The HFD-

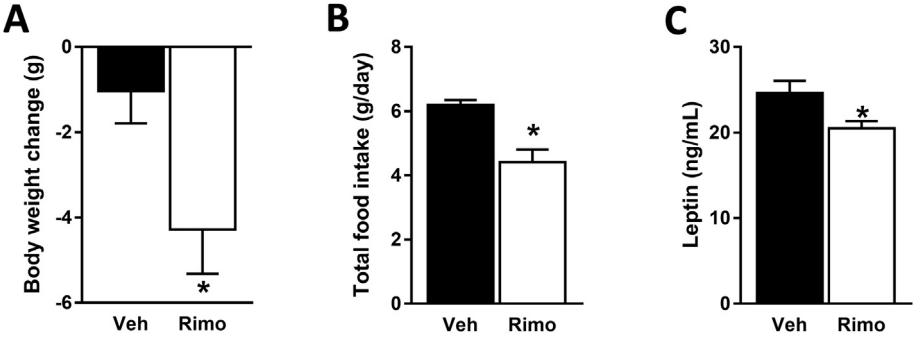

Figure 8: MC4R deactivation does not abolish the hypophagic effect of global CB$_1$R blockade. Rimonabant (3 mg/kg, po., for 7 days) reduced body weight **(A)**, food intake **(B)** and serum leptin levels **(C)** in MC4R$^{-/-}$ mice. Absolute body weights are 52.5 ± 2.9 g (Veh) vs 49.6 ± 2.3 g (rimonabant). Data are mean ± SEM from 4 to 5 animals in each group. *$P < 0.05$ relative to the corresponding control group treated with Veh.

induced increase in the baseline level of hypothalamic pSTAT3 (Figure 2) is also compatible with recent evidence that ARC neurons in DIO mice remain sensitive to endogenous leptin in leptin-resistant states, elicited by high-fat diets [35,36], or chronic central infusion of leptin [37]. However, our findings also indicate that at the high circulating levels of leptin associated with DIO, leptin receptors are maximally activated, so that any further increase in leptin either from endogenous or exogenous sources results in no change in either pSTAT3 or food intake. The apparent dissociation between functional leptin resistance and continued leptin signaling in ARC neurons in DIO may be related to the control of endogenous reactive oxygen species in the hypothalamus [38].

Although the role of the LepR/pSTAT3/αMSH/MC4R pathway in the acute metabolic effects of leptin is well established [17,33,39], genetic inactivation of STAT3 in POMC neurons reduced, but did not abolish, the hypophagic effect of leptin [40], whereas STAT3 inactivation in AgRP/NPY neurons also attenuated leptin-induced hypophagia [41]. This suggests that both POMC and AgRP/NPY neurons could be targets of the acute metabolic effects of leptin. Although STAT3 phosphorylation following chronic peripheral CB_1R blockade could be detected in a small proportion NPY^+ neurons (Figure 4), genetic deletion of NPY did not affect the acute metabolic response of DIO mice to leptin (Figure 5). Nevertheless, these findings do not rule out the role of the AgRP/NPY pathway in the hypophagic effects of leptin under different conditions. Indeed, recent findings indicate that AgRP neurons drive consummatory feeding but are dispensable for reward-driven feeding of palatable foods, such as a HFD used in the present study [42].

It was reported that young pre-obese $MC4R^{-/-}$ mice retain sensitivity to exogenous leptin [29] and to leptin receptor antagonist [35], whereas older animals with manifest obesity do not respond to such treatments. This implies that there is an age and/or obesity-dependent switch towards the POMC pathway to convey the central effects of leptin on energy metabolism [35]. Whether such a shift is predominantly age or obesity related remains to be determined, but it is noteworthy that the age-related decrease in central leptin sensitivity was found to be promoted by concurrent obesity [43]. Nevertheless, the above findings are in good agreement with the present observations that once peripheral CB_1R blockade resensitizes the mediobasal hypothalamus to leptin, the hormone signals primarily via the melanocortin system to reduce food intake and body weight in obese animals.

Earlier findings indicate that both peripheral and central CB_1R are involved in the orexigenic effects of endocannabinoids. Peripherally restricted CB_1R antagonists inhibit food intake in HFD-fed mice [12,13,44] in a leptin-dependent manner [13], but failed to inhibit the hunger-induced increase in food intake in STD-fed lean mice, which could still be inhibited by rimonabant in a leptin-independent manner [13]. Similarly, JD5037 treatment failed to reduce food intake and body weight in $MC4R^{-/-}$ mice, whereas rimonabant retained its efficacy. It could be argued that intact leptin signaling via MC4R mediates the hypophagic effect of peripheral CB_1R blockade, but not the effect of rimonabant, which, in these animals, is likely due to blockade of CB_1R in the CNS. Indeed, pharmacological blockade of MC4R was reported to cause a delayed increase in hypothalamic endocannabinoid levels [45], and a possible similar increase in the hypothalami of $MC4R^{-/-}$ mice may contribute to their hyperphagia via increased CB_1R activation, which would be antagonized by rimonabant.

Paradoxically, altered central CB_1R activity may also contribute to the hypophagic effect of peripheral CB_1R blockade. Our laboratory has earlier reported that leptin-deficient *ob/ob* mice have elevated 2-AG levels in the hypothalamus, whereas leptin treatment of these animals preferentially reduces hypothalamic AEA [30]. Interestingly, the same pattern was evident in the present study, in which HFD feeding increased hypothalamic 2-AG (in agreement with the findings of others [46,47]), and JD5037 treatment decreased hypothalamic AEA levels. This is compatible with the hypothesis that the hypophagic effect of JD5037 is mediated by endogenous leptin [13], in part through reducing central AEA/CB_1R signaling.

Hyperleptinemia is thought to be responsible for maintaining a leptin resistant state in obesity [20], and we have earlier demonstrated that peripheral CB_1R blockade reverses the hyperleptinemia of DIO mice by antagonizing leptin production in adipose tissue and promoting leptin clearance by the kidney [13]. Because circulating leptin can reach the mediobasal hypothalamus [48], normalization of plasma leptin may directly lead to resensitization of hypothalamic leptin receptors, resulting in increased signaling by endogenous leptin. Alternatively, circulating leptin may regulate the sensitivity of hypothalamic leptin receptors indirectly, through vagal afferent neurons. Leptin receptors are expressed in vagal afferents innervating the stomach and duodenum, and their activation was reported to promote satiety [49,50], whereas their genetic deletion led to hyperphagia and obesity [51]. Others found that catabolic changes in visceral fat depots due to overexpression of uncoupling protein-1 (UCP1) lead to leptin-dependent hypophagia, which can be abolished by vagal deafferentation [52]. Increased energy expenditure by CB_1R blockade also involves increased UCP1 expression in white adipocytes [53], and vagal deafferentation was found to abolish the hypophagic effect of rimonabant [54]. Taken together, these findings could suggest that an interaction between endocannabinoids and leptin at the level of vagal sensory afferents plays a role in the resensitization of hypothalamic leptin signaling by peripheral CB_1R blockade. Studies are in progress to explore this intriguing possibility.

AUTHOR CONTRIBUTIONS

JT, AD, GS, ZL, RC, and YK conducted the experiments and analyzed the data. MLR provided material and analyzed the data. JT and GK designed and supervised the experiments and wrote the manuscript.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.06.010.

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