



The neutral cannabinoid CB₁ receptor antagonist AM4113 regulates body weight through changes in energy intake in the rat

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ABSTRACT

The aim of this study was to determine if the neutral cannabinoid CB₁ receptor antagonist, AM4113, regulates body weight in the rat via changes in food intake. We confirmed that the AM4113-induced reduction in food intake is mediated by CB₁ receptors using CB₁ receptor knockout mice. In rats, intraperitoneally administered AM4113 (2, 10 mg kg⁻¹) had a transient inhibitory effect on food intake, while body weight gain was suppressed for the duration of the study. AM4113-induced hypophagia was no longer observed once the inhibitory effect of AM4113 on body weight stabilized, at which time rats gained weight at a similar rate to vehicle-treated animals, yet at a lower magnitude. Pair-feeding produced similar effects to treatment with AM4113. Food intake and body weight gain were also inhibited in rats by oral administration of AM4113 (50 mg kg⁻¹). Dual energy x-ray absorptiometry (DEXA) was used to measure lean and fat mass. The AM4113 treated group had 29.3 ± 11.4% lower fat mass than vehicle-treated rats; this trend did not reach statistical significance. There were no differences in circulating levels of the endogenous cannabinoid 2-arachidonoyl glycerol (2-AG), glucose, triglycerides, or cholesterol observed between treatment groups. Similarly, 2-AG hypothalamic levels were not modified by AM4113 treatment. These data suggest that blockade of an endocannabinoid tone acting at CB₁ receptors induces an initial, transient reduction in food intake which results in long-term reduction of body weight gain.

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1. Introduction

The endogenous cannabinoid (CB) system consists of CB₁ and CB₂ receptors, endogenous ligands for these receptors, and the enzymes responsible for the synthesis and degradation of these ligands (Sugiura et al., 2002; Matias and Di Marzo, 2006). Anandamide and 2-arachidonoyl glycerol (2-AG) were the first endogenous cannabinoid ligands to be discovered, and, like the exogenous ligands for these receptors, they display a range of actions including those on food intake and appetite (Kirkham and Williams, 2001). Cannabinoids affect appetite and body weight by acting at CB₁ receptors, where agonist stimulation increases food intake (Cota, 2007). Treatment of rodents with a CB₁

antagonist/inverse agonist, such as rimonabant, causes reductions in daily food intake, but this effect is transient, and food intake soon returns to the level of vehicle-treated controls (Colombo et al., 1998; Hildebrandt et al., 2003; Vickers et al., 2003; Ravinet Trillou et al., 2003; Bensaïd et al., 2003; Liu et al., 2005). This observation led to the hypothesis that the effect of CB₁ receptor antagonist/inverse agonists on body weight is maintained through actions on peripheral metabolic pathways (Ravinet Trillou et al., 2003; Cota et al., 2003; Jbilo et al., 2005; Horvath, 2006; Nogueiras et al., 2008; Cota et al., 2009; Koolman et al., 2010).

Endogenous cannabinoid signaling could affect metabolism by acting on lipogenic pathways, or on pathways that affect lipolysis and energy expenditure. CB₁ receptors are expressed in liver (Osei-Hyiaman et al., 2005), adipose tissue (Bensaïd et al., 2003; Cota et al., 2003; Jbilo et al., 2005; Starowicz et al., 2008), gastrointestinal tract (Gómez et al., 2002; Duncan et al., 2005), pancreas (Starowicz et al., 2008) and skeletal muscle (Liu et al., 2005). In rodents, CB₁ receptor antagonist/inverse agonists increase *in vivo* resting energy expenditure (Liu et al., 2005; Herling et al., 2008; Kunz et al., 2008) and glucose uptake in isolated soleus muscle (Liu et al., 2005). CB₁ receptor agonists increase the expression of lipogenic transcription factors and *de novo* lipogenesis in liver (Osei-Hyiaman et al., 2005) and cultured adipocytes (Cota et al., 2003), effects that are blocked by a CB₁ receptor antagonist/inverse agonist.

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Pair-feeding studies, specifically designed to test the hypothesis that changes in metabolism maintain weight loss induced by a CB₁ receptor antagonist/inverse agonist, have produced conflicting results (Vickers et al., 2003; Ravinet Trillou et al., 2003; Thornton-Jones et al., 2006; Janiak et al., 2007; Irwin et al., 2008; Herling et al., 2008; Cota et al., 2009). In some studies, differences in body weight between pair-fed rodents, and rodents treated with a CB₁ receptor antagonist/inverse agonist implied the presence of an effect on metabolism (Ravinet Trillou et al., 2003; Herling et al., 2008; Cota et al., 2009). In others, pair-fed rodents weighed the same as treated animals, indicating that changes in body weight induced by a CB₁ receptor antagonist/inverse agonist result solely from the inhibition of food intake (Vickers et al., 2003; Thornton-Jones et al., 2006; Janiak et al., 2007; Irwin et al., 2008). The effects of a CB₁ receptor antagonist/inverse agonist on energy expenditure have also produced inconsistent findings. In one study, mice treated with SR141716 had higher basal oxygen consumption rates than vehicle-treated mice (Liu et al., 2005), but the body weight in these animals was the same as pair-fed mice. In another study, SR141716A significantly increased oxygen consumption in rat, but only for a brief time and only after the first treatment (Kunz et al., 2008).

We investigated whether the effect of a neutral CB₁ receptor antagonist, AM4113, on body weight in rat was due solely to effects on food intake or whether effects on metabolism may contribute to the effect. The potential advantage of a neutral CB₁ receptor antagonist is that the effects are specific to the pharmacological blockade of endogenous cannabinoid signaling (Chambers et al., 2007; Sink et al., 2008), without effects on constitutive receptor activity. Recently, it was shown that AM4113 inhibited food intake to a similar degree as the CB₁ receptor antagonist/inverse agonist AM251 in rat (Chambers et al., 2007; Sink et al., 2008), but unlike AM251, AM4113 did not potentiate vomiting in the ferret (Chambers et al., 2007) or promote nausea in rat (Sink et al., 2008). We examined the role that food intake plays in the actions of AM4113 on body weight by measuring food intake and body weight in rats that were pair-fed to an AM4113 treatment group. The effects of AM4113 on body composition and on fasting glucose and lipid levels were assessed using dual energy x-ray absorptiometry (DEXA) and blood analysis, respectively, to investigate which tissues and metabolic pathways were potentially altered by AM4113 administration. We also examined hypothalamic and plasma levels of the endogenous cannabinoid 2-AG following each treatment to determine whether AM4113 was modifying endocannabinoid levels as well as antagonizing CB₁ receptors to exert its effects. Furthermore, we investigated the effects of orally administered AM4113 to gain an insight into the therapeutic potential of a neutral cannabinoid antagonist.

2. Materials and methods

2.1. Compounds

The pyrazole-based neutral antagonist AM4113 (Chambers et al., 2007; Sink et al., 2008) was synthesized at Northeastern University. For intraperitoneal (i.p.) administration AM4113 was dissolved in DMSO using gentle heating and sonication before being diluted with Tween 80 and saline (4% DMSO; 1% Tween 80; 95% saline) to a final concentration of 2 and 10 mg ml⁻¹ (Chambers et al., 2007). Injections were administered to mice at 100 µl 10 g⁻¹ body weight and to rats at 100 µl 100 g⁻¹ body weight. For oral (p.o.) administration AM4113 was dissolved in 4% DMSO before being diluted with extra light olive oil (Safeway, Calgary, Canada) to a final concentration of 25 mg ml⁻¹ and was delivered in a volume of 200 µl 100 g⁻¹ body weight.

2.2. Animal experiments

Animals were individually housed in transparent plastic cages, with sawdust bedding, in a temperature-controlled room maintained on a 12-h:12-h light/dark cycle and were allowed access to water ad

libitum. Animal use for these studies was approved by the University of Calgary Animal Care Committee and all protocols were carried out in accordance with the guidelines of the Canadian Council on Animal Care.

2.3. Acute feeding experiments in CB₁ receptor knockout (–/–) and wild-type mice

Two breeding pairs of heterozygous CB₁^{+/-}C57BL/6N mice were obtained from B. Lutz (University Medical Center, Mainz, Germany) and bred at the University of Calgary to obtain CB₁^{-/-}C57BL/6N mice (Marsicano et al., 2002). Animals used in these studies were backcrossed to C57BL/6N for 6 generations and were used at the same age (10–16 weeks) and maintained under the same conditions as the wild-type mice. All CB₁^{-/-} mice were genotyped using established protocols and were confirmed as homozygous gene-deficient animals (CB₁^{-/-}C57BL/6N) prior to inclusion in the study.

Female CB₁ receptor knockout mice weighing between 20 and 30 g at the start of the study were placed on a medium fat, palatable diet (51.4% carbohydrate, 31.8% fat, 16.8% protein; 4.41 kcal/g; Diet # D12266B, Research Diets, New Brunswick, NJ, USA) at least 4 days prior to the experiment. Food was available for 18 h each day starting at 16:30 h (lights off 18:00 h). One day prior to the experiment mice were assigned to either vehicle (wild-type mice, mean body weight ± S.E.M.; 26.3 ± 1.2 g, n = 5; CB₁^{-/-} mice: 22.1 ± 0.4 g, n = 6) or 10 mg kg⁻¹ AM4113 (wild-type mice: 26.5 ± 1.1 g, n = 5; CB₁^{-/-} mice: 22.3 ± 0.5 g, n = 6) treatment groups. Mice were injected i.p. with 10 mg kg⁻¹ AM4113 or vehicle immediately prior to the addition of food and their food intake was measured 1, 2, 3 and 18 h after injection.

2.4. Fourteen-day chronic feeding study in rats

Male Sprague–Dawley rats (Charles River, Montreal, Quebec; 180–280 g at the start of the study) were used to examine the effect of i.p. administered AM4113 on food intake and body weight. Animals were fed chocolate flavored Ensure plus liquid diet (53.3% carbohydrate, 29% fat, 16.7% protein; 1.41 kcal/g) (Abbot Laboratories, Abbott Park, IL, USA) to promote food intake and control for spillage. Rats were habituated to testing and handling procedures for 7 days prior to the start of the study. Food and water were presented in drip free inverted glass bottles that attached to the outside of the cage. Food was available for 18 h each day starting at 16:00 h (lights off 16:00 h). Prior to the first day of treatment, rats were assigned to either vehicle (1 ml kg⁻¹, mean body weight ± SEM; 229 ± 14 g, n = 6), 2 mg kg⁻¹ (231 ± 12 g; n = 6), or 10 mg kg⁻¹ AM4113 (231 ± 11 g, n = 5) treatment groups. Treatments were given i.p. once each day, for 14 days, immediately prior to the addition of food. An additional group of rats was treated with vehicle and pair-fed (232 ± 15 g, n = 5) to rats in the 10 mg kg⁻¹ treatment group. Food intake was time delayed in these rats by 1 day. Food intake and body weight were measured daily.

On day 15, after the 6 h period of food deprivation, under light anesthesia with isoflurane, body composition of each animal was analyzed using dual energy x-ray absorptiometry (DEXA) in conjunction with Hologic QDR software for small animals (Hologic QDR 4500, Hologic, Inc., Bedford, MA, U.S.A.). After DEXA analysis was complete rats were decapitated and trunk blood was collected in heparinized and non-heparinized tubes for the collection of plasma and serum, respectively. Heparinized tubes were immediately centrifuged for 10 min at 13,000 RCF after which 500 µl of plasma was aliquoted and flash-frozen in liquid nitrogen. At the same time the hypothalamus from each animal was isolated and flash-frozen. The level of 2-AG in plasma and hypothalamic samples was determined using the liquid chromatography-mass spectrometry (triple quadrupole) method described below. Non-heparinized tubes were placed on ice for at least 30 min before being centrifuged and aliquoted into two 300 µl

serum samples. These samples were analyzed by Calgary Laboratory Services (Calgary, Alberta) for glucose, triglycerides, total cholesterol, and HDL cholesterol levels. Serum was analyzed for glucose (Glucoquant/HK reagent, Roche Diagnostics) using an enzymatic UV assay. Circulating triglycerides (Triglycerides GPO-PAP reagent, Roche Diagnostics) and cholesterol (Cholesterol CHOD-PAP Reagent and HDL-C Plus 3rd generation Reagent, Roche Diagnostics) were analyzed by enzymatic colorimetric assays using a Roche/Hitachi Modular P800. All samples were collected using coded tubes and were analyzed blindly.

2.5. 2-AG levels

Mixtures of the analytes and the deuterated analogs that had been stored at -80°C were further diluted in a 20 mg ml^{-1} solution of fatty acid free bovine serum albumin to simulate analyte-free plasma and in ethanol to make the calibration standards, quality control samples and reference samples, as previously described (Williams et al., 2007). Calibration standards, reference extraction and tissue samples were extracted using a modified version of the Folch extraction, as previously described (Folch et al., 1957; Williams et al., 2007; Wood et al., 2010). Frozen tissue samples were weighed prior to extraction. Calibration curves were constructed from the ratios of the peak areas of the analytes versus the internal standard. Chromatographic separation was achieved using an Agilent Zorbax SB-CN column ($2.1 \times 50\text{ mm}$, 5 mm) on a Finnigan TSQ Quantum Ultra triple quad mass spectrometer (Thermo Electron, San Jose, CA) with an Agilent 1100 HPLC on the front end (Agilent Technologies, Wilmington, DE) (Williams et al., 2007; Wood et al., 2010). The mobile phase consisted of 10 mM ammonium acetate, $\text{pH } 7.3$ (A) and methanol (B) in the following gradient: initial conditions are held at 10% B for 1 min , increased to 60% B in 0.5 min , then from 60% to 75% from 1.5 to 10 min , finally to 95% in 0.5 min (flow rate = 0.5 ml min^{-1}); the autosampler was kept at 4°C to prevent analyte degradation. Eluted peaks were ionized via atmospheric pressure chemical ionization in multiple reaction monitoring mode.

2.6. Seven-day orally administered AM4113 feeding study

Rats, weighing $400\text{--}530\text{ g}$ at the start of the study, were habituated to testing and handling procedures as described above. The day before the experiment rats were assigned to either vehicle (2 ml kg^{-1} , $459 \pm 23\text{ g}$, $n = 5$), 50 mg kg^{-1} ($457 \pm 24\text{ g}$, $n = 4$), or pair-fed ($459 \pm 21\text{ g}$, $n = 4$) treatment groups. Treatments were given orally once each day, for 7 days , immediately prior to the addition of food. Pair-fed rats were treated with vehicle. Food intake and body weight were measured daily during the treatment period and for a further 7 days following the cessation of treatment.

2.7. Statistics

Food intake and body weight data were analyzed using a 2-way mixed design ANOVA with time as the repeated measure. Significant differences were analyzed using Bonferroni's post hoc test at each time point. Food intake data are expressed as mean food intake ($\text{g} \pm \text{S.E.M.}$). Body weight data are expressed as mean weight change ($\text{g} \pm \text{S.E.M.}$) or as a mean percentage body weight of vehicle-treated rats. DEXA data was analyzed using a 1-way independent-measures ANOVA. Data are expressed as the mean fat (g) or lean (g) body mass $\pm \text{S.E.M.}$ 2-AG levels were analyzed in triplicate using a 1-way independent measures ANOVA and are presented as mean $\mu\text{g ml}^{-1}$ and mean $\text{ng g}^{-1} \pm \text{S.E.M.}$ in plasma and hypothalamus, respectively. Glucose and lipid profiles were analyzed using a 1-way ANOVA and are expressed as mean $\text{mmol l}^{-1} \pm \text{S.E.M.}$

3. Results

3.1. Feeding experiments in CB_1 receptor knockout and wild-type mice

AM4113 (10 mg kg^{-1}) significantly reduced food intake in wild-type mice, $F = 13.6$, $p = 0.006$ (Fig. 1A), at $2, 3$ and 18 h , $p < 0.05$. In comparison, there were no significant differences in food intake between vehicle and AM4113 treated CB_1 receptor knockout mice, $F = 0.6$, $p > 0.05$ (Fig. 1B).

3.2. Fourteen-day chronic feeding study

AM4113 produced a transient reduction in food intake (Fig. 2A) seen as an effect of treatment $F = 3.93$, $p = 0.026$, accompanied by a sustained reduction in weight gain (Fig. 2C), $F = 5.74$, $p = 0.006$. Post-hoc analysis revealed that food intake was significantly reduced on days 1 and 2 in 2 mg kg^{-1} treated rats ($p < 0.05$), and on days 2–4 in 10 mg kg^{-1} treated rats ($p < 0.01$), compared to vehicle controls (Fig. 2A). AM4113 (10 mg kg^{-1}) significantly ($p < 0.05$) reduced body weight gain on treatment days 5–14 (Fig. 2C). Body weight of rats treated with 2 and 10 mg kg^{-1} AM4113 stabilized at 5% and 10% less than vehicle-treated rats, respectively (Fig. 2B). The anorectic effect of AM4113 on food intake was lost at the same time as the effect of AM4113 on body weight stabilized (Fig. 2B; 2 mg kg^{-1} , day 3 and 10 mg kg^{-1} , day 5). Changes in body weight were similar between the pair-fed rats and the treated rats demonstrating that the effect of AM4113 on body weight was caused by reductions in food intake. When the anorectic effect of AM4113 was no longer observed (Fig. 2A), rats gained weight at a similar rate to vehicle-treated rats (Fig. 2C). DEXA analysis showed that lean body mass was comparable between treatment groups (Fig. 2D). There was a strong trend for fat mass (Fig. 2E) to be reduced in AM4113 treated rats and their pair-fed controls, although these differences were not statistically significant

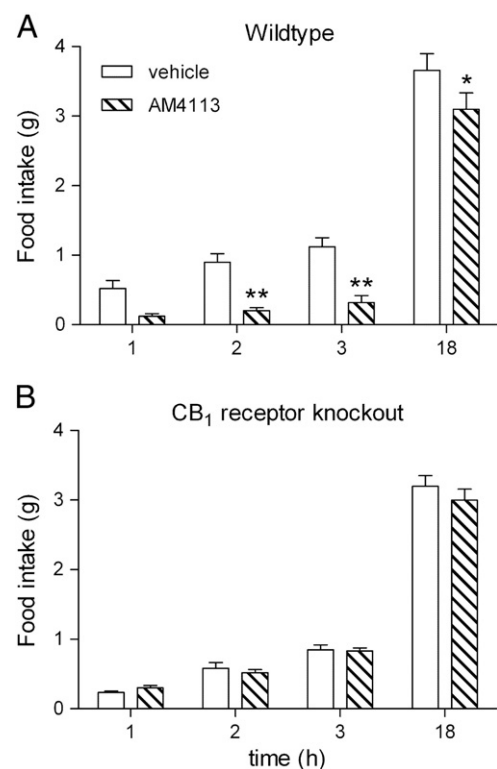


Fig. 1. The effect of AM4113 (10 mg kg^{-1} ; i.p.) on food intake compared to vehicle (4% DMSO; 1% Tween 80; 95% saline; i.p.) treated animals in wild-type (A) and CB_1 receptor knockout mice (B). Bars represent the mean \pm S.E.M., $n = 5\text{--}6$ per group. * $p < 0.05$ and ** $p < 0.01$ represent a significant difference to vehicle treatment analyzed by 2-way ANOVA followed by Bonferroni's post hoc test.

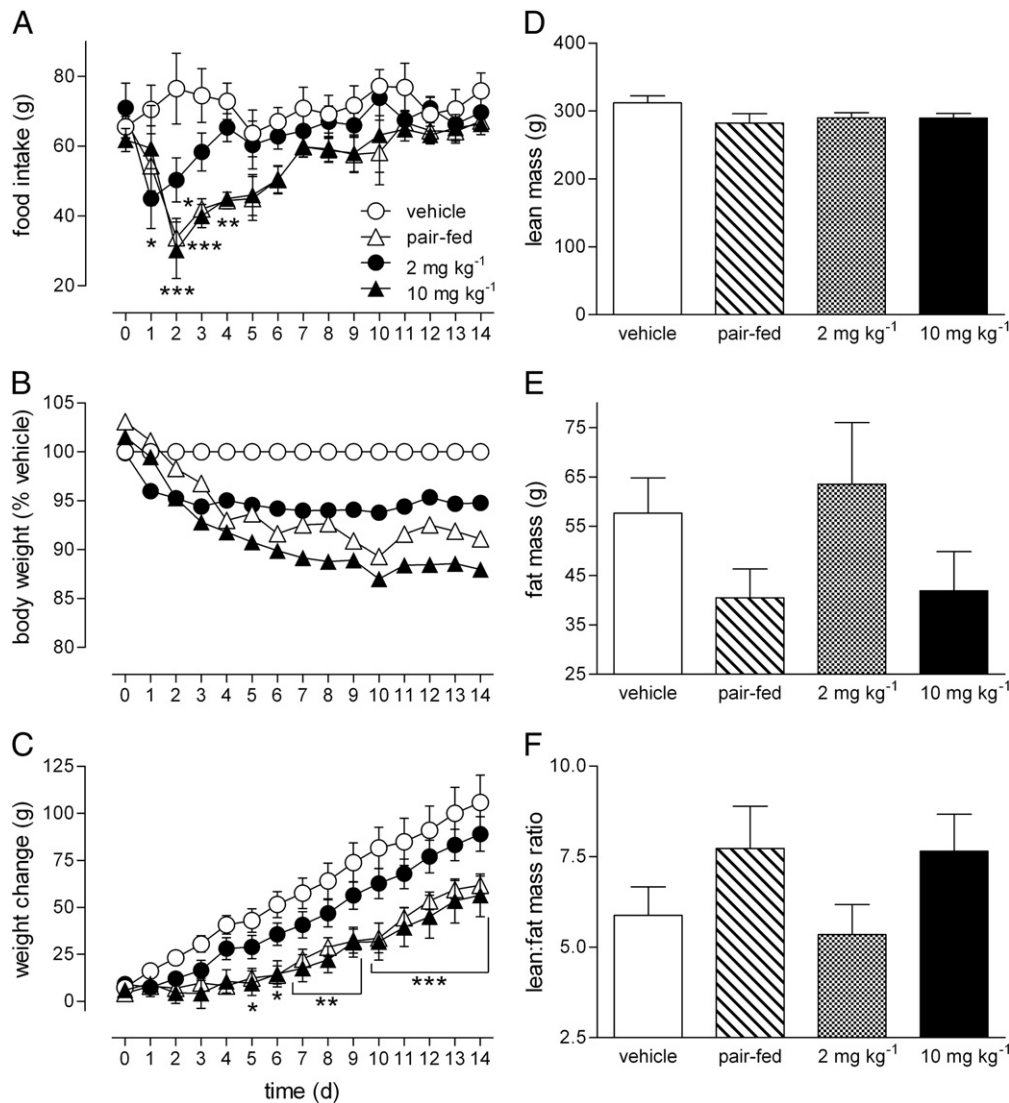


Fig. 2. The effect of daily AM4113 (2 or 10 mg kg⁻¹; i.p.) administration, over 14 days, on food intake (A), body weight (B) and weight change (C) in rats compared to vehicle (4% DMSO; 1% Tween 80; 95% saline; i.p.) and rats pair-fed to the 10 mg kg⁻¹ treated group. The effect of AM4113 (2 or 10 mg kg⁻¹), vehicle or being pair-fed on lean mass (D), fat mass (E) and the lean:fat mass ratio (F). Data points or bars represent the mean \pm S.E.M, $n = 5-6$ per group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ represent a significant difference to vehicle treatment analyzed by 2-way ANOVA (A–C) or 1-way ANOVA (D–F) followed by Bonferroni's post hoc test.

alone or when data were expressed as lean:fat mass ratio (Fig. 2F). Rats in the 10 mg kg⁻¹ treatment group and pair-fed rats had $29.3 \pm 11.4\%$ less fat mass, and $9.0 \pm 2.2\%$ less lean body mass, than vehicle-treated rats.

3.3. 2-AG levels and lipid profiles

Endogenous 2-AG and lipid profiles were examined in each group following the 14-day chronic feeding study. Following AM4113 (2 or 10 mg kg⁻¹) treatment, plasma and hypothalamic levels of 2-AG were comparable to those measured in vehicle-treated rats. Serum levels of glucose, triglycerides, total cholesterol, and HDL cholesterol in treated rats were also comparable to those in vehicle-treated rats. The results are summarized in Table 1.

3.4. The effect of orally administered AM4113 on food intake

Preliminary studies showed that 2 and 10 mg kg⁻¹ AM4113 administered orally produced transient reductions in food intake, that were not significant 24 h after treatment, compared with vehicle treatment (data not shown). Seven-day treatment with 50 mg kg⁻¹

AM4113 significantly reduced food intake and body weight change compared with vehicle-treated rats, with 2-way ANOVA revealing a treatment by time interaction (food intake, $F = 9.0$, $p < .0001$; body weight, $F = 2.5$, $p = .0003$) (Fig. 3A and C). Rats were orally treated with AM4113 on days 1–7 and post hoc analysis revealed that food intake was significantly reduced ($p < 0.05$) on days 1–5 and day 7. On days 8–14, following cessation of treatment, food intake increased to become comparable to that recorded in vehicle-treated control rats

Table 1

Plasma and brain 2-AG levels and serum lipid profiles following the 14 days i.p. chronic feeding study. 2-AG levels are presented as mean $\mu\text{g ml}^{-1}$ and mean $\text{ng g}^{-1} \pm \text{S.E.M.}$ in plasma and brain, respectively. Serum glucose and lipid profiles are expressed as mean $\text{mmol l}^{-1} \pm \text{S.E.M.}$

	Vehicle	2 mg kg ⁻¹	10 mg kg ⁻¹	Pair-fed
Plasma 2-AG ($\mu\text{g ml}^{-1}$)	18.1 ± 3.5	28.4 ± 7.9	12.7 ± 1.0	22.3 ± 4.6
Hypothalamic 2-AG (ng g^{-1})	5.1 ± 0.4	7.1 ± 0.7	6.4 ± 0.4	6.0 ± 0.4
Glucose (mmol l^{-1})	12.0 ± 1.2	12.0 ± 0.2	13.4 ± 0.8	11.5 ± 0.5
Triglycerides (mmol l^{-1})	1.4 ± 0.4	1.2 ± 0.3	1.4 ± 0.3	1.1 ± 0.3
Total cholesterol (mmol l^{-1})	1.7 ± 0.09	1.65 ± 0.1	1.65 ± 0.15	1.75 ± 0.12
HDL cholesterol (mmol l^{-1})	1.24 ± 0.07	1.19 ± 0.01	1.13 ± 0.11	1.33 ± 0.13

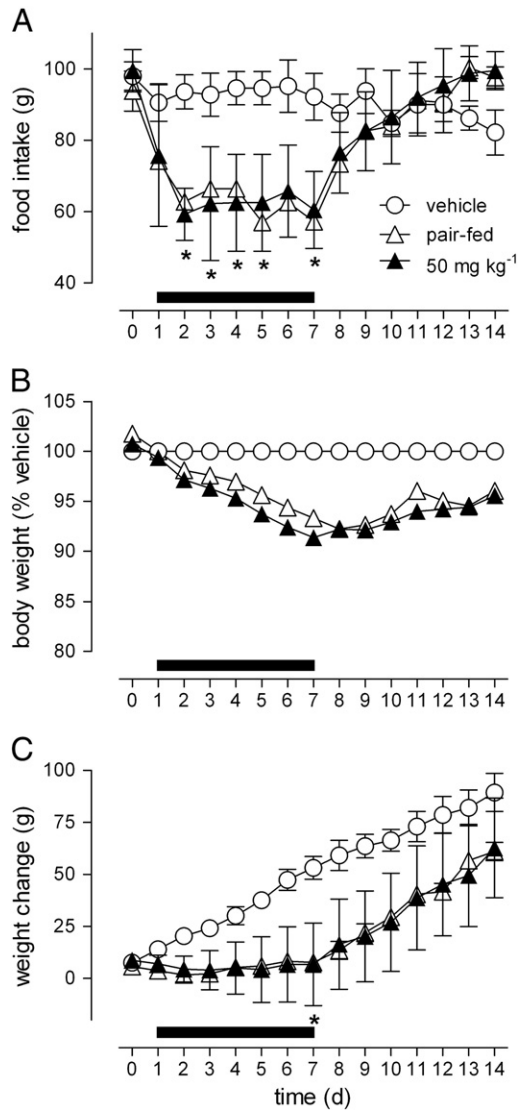


Fig. 3. The effect of daily AM4113 (50 mg kg⁻¹; p.o.) administration, over 7 days (treatments days denoted by the black horizontal bar) on food intake (A), body weight (B) and weight change (C) in rats compared to vehicle (4% DMSO; 96% olive oil; p.o.) and rats pair-fed to the 50 mg kg⁻¹ treated group. Data points represent the mean \pm S.E.M, $n = 4-5$ per group. * $p < 0.05$ represents a significant difference to vehicle treatment analyzed by 2-way ANOVA followed by Bonferroni's post hoc test.

(Fig. 3A). Body weight change was significantly ($p < 0.05$) reduced in AM4113 treated rats on day 7 (Fig. 3C). Comparison to the pair-fed rats showed that the effect of AM4113 on body weight (Fig. 3B and C) was associated with a reduction in food intake. The anorectic effect of AM4113 given orally was sustained throughout the 7-day treatment period, while body weight continued to drop, and did not stabilize, in AM4113 treated rats relative to vehicle-treated controls (Fig. 3B).

4. Discussion

We tested the hypothesis that endogenous cannabinoid signaling at CB₁ receptors regulates body weight by affecting food intake. Using CB₁ receptor knockout mice we confirmed that the neutral CB₁ receptor antagonist AM4113 reduced food intake through an action at CB₁ receptors. In rats, when administered intraperitoneally, AM4113 transiently reduced food intake and induced a sustained reduction in body weight gain in a dose-dependent manner. Oral administration of this compound also reduced food intake and body weight in rat, and a higher dose was required than with the i.p. route of administration.

Contrary to some of the literature, changes in metabolism did not appear to contribute to the actions of the CB₁ receptor antagonist on body weight, since pair-feeding produced almost identical effects to rats treated with AM4113. Chronic treatment with AM4113 had no effect on hypothalamic or circulating levels of the endogenous cannabinoid 2-AG in fasted rats, and there were also no significant differences in circulating levels of glucose, triglycerides, or cholesterol.

Following 14 days of daily, i.p. administration of AM4113, body weight was approximately 5% (2 mg kg⁻¹) and 10% (10 mg kg⁻¹) lower relative to vehicle-treated rats, which is comparable to the effect of CB₁ receptor antagonist/inverse agonists in animals (Colombo et al., 1998; Vickers et al., 2003; Chambers et al., 2006; Herling et al., 2007; Janiak et al., 2007) and humans (Despres et al., 2005; Van Gaal et al., 2005; Pi-Sunyer et al., 2006). Moreover, data from pair-fed rats showed that the effect of AM4113 on body weight had been, and continued to be, maintained by changes in food intake rather than through a metabolic action of the endocannabinoid system. Our interpretation of these data differs from the idea that differences in body weight are maintained by an effect on metabolism (Ravinet Trillou et al., 2003; Jbilo et al., 2005; Liu et al., 2005; Horvath, 2006; Osei-Hyiaman et al., 2006; Cota, 2007; Nogueiras et al., 2008; Herling et al., 2008; Cota et al., 2009).

Studies have shown significant differences in body weight between rodents treated with a CB₁ receptor antagonist/inverse agonist and their pair-fed controls (Ravinet Trillou et al., 2003; Herling et al., 2008) suggesting that the reduction in body weight is due to factors other than a reduction in food intake. In one of those studies the effect of rimonabant (SR14176A) was examined in diet-induced obese mice that were treated over 3 days (Ravinet Trillou et al., 2003). In another study, rats treated with rimonabant for 5 weeks demonstrated a lowered body weight and it was concluded that this was due to an increase in energy expenditure (Herling et al., 2008). However, it was also postulated that differences in body weight between pair-fed rats and rats treated with rimonabant could result from a reduced metabolic rate in pair-fed rats (Herling et al., 2008). Differences in energy expenditure between vehicle-treated rats and rats treated with rimonabant were significant on the first day of the study, but not at later times (Herling et al., 2008). A similar effect of rimonabant on energy expenditure had been reported previously in rats (Kunz et al., 2008) yet in another study this CB₁ receptor antagonist/inverse agonist had no effect on energy expenditure in mice (Koolman et al., 2010). These studies highlight the controversy (Vickers et al., 2003; Thornton-Jones et al., 2006; Janiak et al., 2007; Kunz et al., 2008) surrounding the proposed role of energy expenditure in weight change induced by changes in CB₁ receptor activity (Ravinet Trillou et al., 2003; Bensaid et al., 2003; Jbilo et al., 2005; Liu et al., 2005; Osei-Hyiaman et al., 2005; Horvath, 2006; Cota, 2007; Herling et al., 2008; Nogueiras et al., 2009; Cota et al., 2009).

In the present study, the inhibitory effect of AM4113 on body weight was initiated by a dramatic reduction in food intake. When the hypophagic effect of AM4113 was no longer observed, and food intake returned to control levels, body weight no longer continued to decrease. This resulted in a rate of growth in rats treated with AM4113 that paralleled that of vehicle-treated animals, although at a lower level (see Fig. 2B). These data support the notion that AM4113 had lowered defended body weight.

DEXA analysis showed that the effect of AM4113 on body weight was likely due to reduced fat mass as treated rats had a lower fat mass than vehicle-treated rats. Rimonabant (Ravinet Trillou et al., 2003; Doyon et al., 2006; Herling et al., 2008; Cota et al., 2009) and AM251 (Hildebrandt et al., 2003) also preferentially reduce fat mass compared with lean body mass. This suggests that the actions on fat mass may be due to blockade of endocannabinoid tone on CB₁ receptors as AM4113 is a neutral antagonist and, unlike rimonabant and AM251, has no effect on constitutive activity of the receptors. The

fact that weight loss induced by cannabinoid antagonists is fairly specific to reduced fat stores seems significant given the expression of CB₁ receptors on adipocytes (Bensaid et al., 2003), increased fat oxidation by rimonabant (Liu et al., 2005; Herling et al., 2008; Kunz et al., 2008), the proposed role of CB₁ receptors in lipogenesis (Cota et al., 2003; Osei-Hyiaman et al., 2005), and lipolysis (Jbilo et al., 2005). The observation that tolerance to the anorectic effect of CB₁ receptor antagonist or antagonist/inverse agonist is linked to existing energy stores, i.e., is delayed in heavier animals (Vickers et al., 2003; Doyon et al., 2006), seems to suggest that endogenous cannabinoid signaling plays a specific role in the maintenance and regulation of adipose tissue mass.

In humans, circulating levels of the endogenous cannabinoid 2-AG are positively correlated with intra-abdominal obesity (Bluher et al., 2006; Cote et al., 2007). In rat, 2-AG levels are increased in the hypothalamus of fasted animals and normalized by feeding (Kirkham et al., 2002). We examined circulating and hypothalamic levels of 2-AG to see if they were affected by AM4113 or weight loss in pair-fed rats. No differences were seen between treatments. More recently Martin-Garcia et al. (2010) showed that CB₁ receptor density and activity were decreased by chronic treatment with a CB₁ receptor antagonist in rats. Thus, it may be that CB₁ receptor down-regulation mediates tolerance to the anorectic effect of these compounds. CB₁ receptor antagonist/inverse agonist treatment has been reported to improve circulating glucose and cholesterol levels in humans (Despres et al., 2005; Van Gaal et al., 2005; Pi-Sunyer et al., 2006) and rodents (Doyon et al., 2006; Herling et al., 2007; Janiak et al., 2007; Irwin et al., 2008). These effects may be induced by the inverse agonist properties of these agents because in the current study these levels were not altered by neutral antagonism of CB₁ receptors by AM4113. CB₁ receptor expression in peripheral tissues (Cota, 2007), on vagal afferent fibers (Burdyga et al., 2004, 2006), and in areas of the brain that regulate food intake (Cota, 2007) may explain how endogenous cannabinoid signaling is able to regulate short- and long-term caloric intake. Furthermore, recent studies have reported that neutral antagonism of peripheral CB₁ receptors inhibits food reinforced behaviour (Randall et al., 2010) and food intake, and that receptors located on vagal afferents may not be involved in the mediation of these effects (Cluny et al., 2010).

In summary, we show that pharmacological blockade of endogenous cannabinoid signaling resulted in a new lower body weight that was defended by changes in food intake. Induced by a transient reduction in food intake, body weight was reduced and resumed a revised rate of growth. DEXA analysis showed that lower body weights in AM4113 treated rats resulted primarily from reductions in fat mass. In our study, there were no differences in circulating glucose and lipid levels, or levels of the endogenous cannabinoid 2-AG, between treatments. Future studies will be needed that examine if the effect of a CB₁ receptor antagonist at later time points to determine if circulating endogenous cannabinoid levels are related to fat mass in rodents, and if so, how this is affected by treatment with pharmacological tools which act at cannabinoid CB₁ receptors.

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References

- Bensaid M, Gary-Bobo M, Esclangon A, Maffrand JP, Le Fur G, Oury-Donat F, et al. The cannabinoid CB₁ receptor antagonist SR141716 increases Acip30 mRNA expression in adipose tissue of obese fa/fa rats and in cultured adipocyte cells. *Mol Pharmacol* 2003;63:908–14.
- Bluher M, Engeli S, Kloting N, Berndt J, Fasshauer M, Batkai S, et al. Dysregulation of the peripheral and adipose tissue endocannabinoid system in human abdominal obesity. *Diabetes* 2006;55:3053–60.
- Burdyga G, Lal S, Varro A, Dimaline R, Thompson DG, Dockray GJ. Expression of cannabinoid CB₁ receptors by vagal afferent neurons is inhibited by cholecystokinin. *J Neurosci* 2004;24:2708–15.
- Burdyga G, Varro A, Dimaline R, Thompson DG, Dockray GJ. Ghrelin receptors in rat and human nodose ganglia: putative role in regulating CB-1 and MCH receptor abundance. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G1289–97.
- Chambers AP, Koopmans HS, Pittman QJ, Sharkey KA. AM 251 produces sustained reductions in food intake and body weight that are resistant to tolerance and conditioned taste aversion. *Br J Pharmacol* 2006;147:109–16.
- Chambers AP, Vemuri VK, Peng Y, Wood JT, Olszewska T, Pittman QJ, et al. A neutral CB₁ receptor antagonist reduces weight gain in rat. *Am J Physiol Regul Integr Comp Physiol* 2007;293:R2185–93.
- Cluny NL, Vemuri VK, Chambers AP, Limebeer CL, Bedard H, Wood JT, Lutz B, Zimmer A, Parker LA, Makriyannis A, Sharkey KA, et al. A novel peripherally restricted cannabinoid receptor antagonist, AM6545, reduces food intake and body weight, but does not cause malaise, in rodents. *Br J Pharmacol* 2010;161:629–42.
- Colombo G, Agabio R, Diaz G, Lobina C, Reali R, Gessa GL. Appetite suppression and weight loss after the cannabinoid antagonist SR 141716. *Life Sci* 1998;63:L113–7.
- Cota D. CB₁ receptors: emerging evidence for central and peripheral mechanisms that regulate energy balance, metabolism, and cardiovascular health. *Diab Metab Res Rev* 2007;23:507–17.
- Cota D, Marsicano G, Tschöp M, Grubler Y, Flachskamm C, Schubert M, et al. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* 2003;112:423–31.
- Cota D, Sandoval DA, Olivieri M, Prodi E, D'Alessio DA, Woods SC, et al. Food intake-independent effects of CB₁ antagonism on glucose and lipid metabolism. *Obesity (Silver Spring)* 2009;17:1641–5.
- Cote M, Matias I, Lemieux I, Petrosino S, Almeras N, Despres JP, et al. Circulating endocannabinoid levels, abdominal adiposity and related cardiometabolic risk factors in obese men. *Int J Obes (Lond)* 2007;31:692–9.
- Despres JP, Golay A, Sjostrom L. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* 2005;353:2121–34.
- Doyon C, Denis RG, Baraboi ED, Samson P, Lalonde J, Deshaies Y, et al. Effects of rimonabant (SR141716) on fasting-induced hypothalamic-pituitary-adrenal axis and neuronal activation in lean and obese Zucker rats. *Diabetes* 2006;55:3403–10.
- Duncan M, Davison JS, Sharkey KA. Review article: endocannabinoids and their receptors in the enteric nervous system. *Aliment Pharmacol Ther* 2005;22:667–83.
- Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* 1957;226:497–509.
- Gómez R, Navarro M, Ferrer B, Trigo JM, Bilbao A, Del Arco I, et al. A peripheral mechanism for CB₁ cannabinoid receptor-dependent modulation of feeding. *J Neurosci* 2002;22:9612–7.
- Herling AW, Gossel M, Haschke G, Stengelin S, Kuhlmann J, Muller G, et al. CB₁ receptor antagonist AVE1625 affects primarily metabolic parameters independently of reduced food intake in Wistar rats. *Am J Physiol Endocrinol Metab* 2007;293: E826–32.
- Herling AW, Kilp S, Elvert R, Haschke G, Kramer W. Increased energy expenditure contributes more to the body weight-reducing effect of rimonabant than reduced food intake in candy-fed wistar rats. *Endocrinology* 2008;149:2557–66.
- Hildebrandt AL, Kelly-Sullivan DM, Black SC. Antiobesity effects of chronic cannabinoid CB₁ receptor antagonist treatment in diet-induced obese mice. *Eur J Pharmacol* 2003;462:125–32.
- Horvath TL. The unfolding cannabinoid story on energy homeostasis: central or peripheral site of action? *Int J Obes (Lond)* 2006;30(Suppl 1):S30–2.
- Irwin N, Hunter K, Frizzell N, Flatt PR. Antidiabetic effects of sub-chronic administration of the cannabinoid receptor (CB₁) antagonist, AM251, in obese diabetic (ob/ob) mice. *Eur J Pharmacol* 2008;581:226–33.
- Janiak P, Poirier B, Bidouard JP, Cadrouvele C, Pierre F, Gouraud L, et al. Blockade of cannabinoid CB₁ receptors improves renal function, metabolic profile, and increased survival of obese Zucker rats. *Kidney Int* 2007;72:1345–57.
- Jbilo O, Ravinet-Trillou C, Arnone M, Buisson I, Ribes E, Peleraux A, et al. The CB₁ receptor antagonist rimonabant reverses the diet-induced obesity phenotype through the regulation of lipolysis and energy balance. *FASEB J* 2005;19:1567–9.
- Kirkham TC, Williams CM. Endogenous cannabinoids and appetite. *Nutr Res Rev* 2001;14:65–86.
- Kirkham TC, Williams CM, Fezza F, Di Marzo V. Endocannabinoid levels in rat limbic forebrain and hypothalamus in relation to fasting, feeding and satiation: stimulation of eating by 2-arachidonoyl glycerol. *Br J Pharmacol* 2002;136:550–7.
- Koolman AH, Boks VW, Oosterveer MH, Jonas I, Kuipers F, Sauer PJ, et al. Metabolic responses to long-term pharmacological inhibition of CB₁-receptor activity in mice in relation to dietary fat composition. *Int J Obes (Lond)* 2010;34:374–84.
- Kunz I, Meier MK, Bourson A, Fisseha M, Schilling W. Effects of rimonabant, a cannabinoid CB₁ receptor ligand, on energy expenditure in lean rats. *Int J Obes (Lond)* 2008;32:863–70.
- Liu YL, Connoley JP, Wilson CA, Stock MJ. Effects of the cannabinoid CB₁ receptor antagonist SR141716 on oxygen consumption and soleus muscle glucose uptake in Lep(ob)/Lep(ob) mice. *Int J Obes (Lond)* 2005;29:183–7.

- Marsicano G, Wotjak CT, Azad SC, Bisogno T, Rammes G, Cascio MG, et al. The endogenous cannabinoid system controls extinction of aversive memories. *Nature* 2002;418:530–4.
- Martin-Garcia E, Burokas A, Martin M, Berrendero F, Rubi B, Kiesselbach C, et al. Central and peripheral consequences of the chronic blockade of CB1 cannabinoid receptor with rimonabant or taranabant. *J Neurochem* 2010;112:1338–13351.
- Matias I, Di Marzo V. Endocannabinoid synthesis and degradation, and their regulation in the framework of energy balance. *J Endocrinol Investig* 2006;29:15–26.
- Nogueiras R, Veyrat-Durebex C, Suchanek PM, Klein M, Tschop J, Caldwell C, et al. Peripheral, but not central, CB1 antagonism provides food intake-independent metabolic benefits in diet-induced obese rats. *Diabetes* 2008;57:2977–91.
- Nogueiras R, Diaz-Arteaga A, Lockie SH, Velasquez DA, Tschop J, Lopez M, et al. The endocannabinoid system: role in glucose and energy metabolism. *Pharmacol Res* 2009;60:93–8.
- Osei-Hyiaman D, Depetrillo M, Pacher P, Liu J, Radaeva S, Batkai S, et al. Endocannabinoid activation at hepatic CB1 receptors stimulates fatty acid synthesis and contributes to diet-induced obesity. *J Clin Invest* 2005;115:1298–305.
- Osei-Hyiaman D, Harvey-White J, Batkai S, Kunos G. The role of the endocannabinoid system in the control of energy homeostasis. *Int J Obes (Lond)* 2006;30(Suppl 1): S33–8.
- Pi-Sunyer FX, Aronne LJ, Heshmati HM, Devin J, Rosenstock J. Effect of rimonabant, a cannabinoid-1 receptor blocker, on weight and cardiometabolic risk factors in overweight or obese patients: RIO-North America: a randomized controlled trial. *JAMA* 2006;295:761–75.
- Randall PA, Vemuri VK, Segovia KN, Torres EF, Hosmer S, Nunes EJ, et al. The novel cannabinoid CB1 antagonist AM6545 suppresses food intake and food-reinforced behavior. *Pharmacol Biochem Behav* 2010.
- Ravinet Trillou C, Arnone M, Delgorge C, Gonalons N, Keane P, Maffrand JP, et al. Anti-obesity effect of SR141716, a CB1 receptor antagonist, in diet-induced obese mice. *Am J Physiol Regul Integr Comp Physiol* 2003;284:R345–53.
- Sink KS, McLaughlin PJ, Wood JA, Brown C, Fan P, Vemuri VK, et al. The novel cannabinoid CB1 receptor neutral antagonist AM4113 suppresses food intake and food-reinforced behavior but does not induce signs of nausea in rats. *Neuropsychopharmacology* 2008;33:946–55.
- Starowicz KM, Cristino L, Matias I, Capasso R, Racioppi A, Izzo AA, et al. Endocannabinoid dysregulation in the pancreas and adipose tissue of mice fed with a high-fat diet. *Obesity (Silver Spring)* 2008;16:553–65.
- Sugiura T, Kobayashi Y, Oka S, Waku K. Biosynthesis and degradation of anandamide and 2-arachidonoylglycerol and their possible physiological significance. *Prostaglandins Leukot Essent Fatty Acids* 2002;66:173–92.
- Thornton-Jones ZD, Kennett GA, Benwell KR, Revell DF, Misra A, Sellwood DM, et al. The cannabinoid CB1 receptor inverse agonist, rimonabant, modifies body weight and adiponectin function in diet-induced obese rats as a consequence of reduced food intake. *Pharmacol Biochem Behav* 2006;84:353–9.
- Van Gaal LF, Rissanen AM, Scheen AJ, Ziegler O, Rossner S. Effects of the cannabinoid-1 receptor blocker rimonabant on weight reduction and cardiovascular risk factors in overweight patients: 1-year experience from the RIO-Europe study. *Lancet* 2005;365:1389–97.
- Vickers SP, Webster LJ, Wyatt A, Dourish CT, Kennett GA. Preferential effects of the cannabinoid CB1 receptor antagonist, SR 141716, on food intake and body weight gain of obese (fa/fa) compared to lean Zucker rats. *Psychopharmacology (Berl)* 2003;167:103–11.
- Williams J, Wood J, Pandarinathan L, Karanian DA, Bahr BA, Vouros P, et al. Quantitative method for the profiling of the endocannabinoid metabolome by LC-atmospheric pressure chemical ionization-MS. *Anal Chem* 2007;79:5582–93.
- Wood JT, Williams JS, Pandarinathan L, Janero DR, Lammi-Keefe CJ, Makriyannis A. Dietary docosahexaenoic acid supplementation alters select physiological endocannabinoid-system metabolites in brain and plasma. *J Lipid Res* 2010;51:1416–23.