Responses to the cannabinoid receptor-1 antagonist, AM251, are more robust with age and with high-fat feeding

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Abstract

Endocannabinoids (EC) are involved in regulating energy homeostasis, particularly in promoting hyperphagia and the consumption of a palatable diet. We have previously shown that rats given a high-fat (HF) diet display a transient hyperphagia that is normalized by a process partially dependent on leptin. We now propose that the induction of this hyperphagia is mediated, at least partially, by the EC signaling system. Obesity, including diet-induced and age-related, is associated with dysregulation of the EC system, and obese rodent models are hypersensitive to a cannabinoid-1 (CB1) receptor antagonist. This suggests that aged rats will be more responsive to the anorectic effects of a CB1 receptor antagonist. To test this, we examined the responsiveness to CB1 receptor antagonist, AM251, in young and aged rats during two experimental paradigms. First, we administered

AM251 simultaneously with the introduction of an HF diet. Second, AM251 treatment began after the establishment of diet-induced obesity. Responses were measured by changes in body weight and composition, calorie intake, serum leptin, and biochemical indicators. The results demonstrated three key findings. 1) CB1 receptor activity contributes to the hyperphagia seen with the introduction of an HF diet. 2) Increased AM251 sensitivity and efficacy is increased with age and HF feeding, with the greatest responsiveness observed in HF-fed, aged rats. 3) AM251 sensitivity is elevated to a greater extent with HF diet than with established obesity. Thus, both age and an HF diet are associated with enhanced anorectic responses to AM251, but the underlying mechanism of these responses remains speculative.

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Introduction

Endocannabinoids (ECs), such as anandamide and 2-arachidonoylglycerol, act through G-protein-coupled cannabinoid-1 (CB1) receptors in the brain and in various peripheral organs to increase both energy intake and adipogenesis (Pagotto *et al.* 2005, Bellocchio *et al.* 2008, Valassi *et al.* 2008). CB1 receptor knockout mice, compared to wild-type littermates, consume less food after being fasted (Vickers *et al.* 2003). These knockout mice also display reduced adiposity levels and resistance to high-fat (HF) diet-induced obesity, even when they consume the same amount of food as controls (Matias *et al.* 2006).

Recent evidence indicates that the EC system may function to promote hyperphagia and the consumption of highly palatable food. Administration of exogenous CBs in rats increases food intake, and this effect can be blocked by pre-treatment with a CB1 receptor antagonist (Vickers *et al.* 2003). In addition, studies have shown that administration of a CB1 receptor antagonist preferentially reduces calorie intake on a highly palatable diet compared to a standard chow diet

(Arnone *et al.* 1997, Simiand *et al.* 1998, Hildebrandt *et al.* 2003). We have previously shown that when rats are provided an HF diet *ad libitum*, they immediately and spontaneously experience an increase in calorie intake (Judge *et al.* 2008). This hyperphagia normalizes to pre-HF diet levels usually within a week, depending on the diet and rat strain. We previously demonstrated that this normalization, at least partially, is leptin dependent, and thus, prolonged in leptin-resistant rats (Zhang *et al.* 2007). Moreover, this hyperphagia is heightened and prolonged in aged rats who also display leptin resistance (Judge *et al.* 2008). In addition to leptin resistance, we propose that this hyperphagia is, at least partially, mediated by overactive CB1 receptors in aged rats. To test this hypothesis, we examined the efficacy of a CB1 receptor antagonist, AM251, in young and aged rats with the introduction of HF feeding.

In addition, obese animals with established obesity demonstrate enhanced responsiveness to CB1 receptor antagonists compared with lean animals. Other evidence suggests that both a highly palatable diet and obesity raise central and peripheral EC levels (Harrold *et al.* 2002, Higgs

et al. 2002, Matias *et al.* 2006, Bellocchio *et al.* 2008). Conversely, CB1 antagonist anorectic responses can be enhanced in lean animals after EC tone is increased, for example, when animals are exposed to a highly palatable diet or acutely fasted (Matias & Di Marzo 2006). Collectively, this suggests that there is higher EC tone in the obese state. If so, then CB1 receptor blockade will result in greater physiological responses. To this end, we tested the responsiveness to AM251 in young and aged rats with established HF diet-induced obesity.

Materials and Methods

Animals

Male Fisher 344×Brown Norway rats of 4 and 29 months of age were obtained from the National Institute for Aging. Rats were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals. Rats were housed individually on a 12 h light:12 h darkness cycle (lights on from 0700 to 1900). Protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Experimental design

Experiment 1: AM251 treatment simultaneous with introduction of HF diet Young ($n=24$, 4 months of age) and aged ($n=30$, 29 months of age) rats initially weighing 305.85 ± 6.1 and 551.70 ± 7.3 g respectively, were provided a chow (15% fat; 3.3 kcal/g diet 2018; Harlan Teklad, Madison, WI, USA) diet *ad libitum* until the experiment began. Approximately 1 week prior to the initiation of the experiment, the rats were all given mock injections over a 2-day protocol during which they were introduced to both the i.p. injection procedures and HF diet (60% fat; 5.2 kcal/g D12492; Research Diets, New Brunswick, NJ, USA). After the mock injections, rats were fed chow diet *ad libitum* and allowed to rest 1 week before experimentation began.

On the first day of the experiment, all rats were acutely fasted for 2 h prior to the onset of the darkness cycle. Just before the darkness cycle, the rats received an i.p. injection of either vehicle (7.7% DMSO, 4.6% Tween 80, 87.7% saline) or 0.83 mg/kg AM251 (dissolved in 7.7% DMSO, 4.6% Tween 80, 87.7% saline; Cayman Chemical, Ann Arbor, MI, USA). Injection solutions were prepared fresh each day. At the time of the injection, either chow or HF diet was given to the rats and they were allowed *ad libitum* access to the food for the remainder of the week-long experiment. The rats were given the i.p. injection of vehicle or AM251 for 6 consecutive days an hour before the onset of the darkness cycle. Body weight and food intake were recorded daily. At the conclusion of the injection period, serum was drawn by tail nicking, and the rats were allowed to rest for ~1 week. For the next phase of the experiment, a crossover design was employed such that the

rats previously receiving vehicle now received 2.78 mg/kg AM251 and the rats previously receiving AM251 now received vehicle using the same experimental design (Fig. 1A). Again, serum was collected by tail nicking at the conclusion of this portion of the experiment.

Experiment 2: leptin or AM251 treatment in rats with established dietary obesity Young ($n=29$, 4 months of age) and aged ($n=32$, 29 months of age) rats initially weighing 311.5 ± 5.4 and 559.5 ± 6.3 g respectively, were provided a chow or HF diet *ad libitum* for 60 days. Body weight and food intake were recorded daily. On day 60, a 14-day osmotic minipump (model 2002, Durect, Cupertino, CA, USA), administering either saline or 0.1 mg/day leptin, i.p. was implanted in a subcutaneous pocket on the dorsal surface of the rat. After the infusion period, the rats were allowed to recover for ~2 weeks before AM251 injections began. A crossover design was employed, in that rats previously receiving saline now received 0.45 mg/day AM251, and those rats previously receiving leptin now received vehicle with all groups maintained on their respective diets, *ad libitum* (Fig. 1B). Rats were administered either vehicle or AM251, daily, by i.p. injection for 6 consecutive days, and afterwards, the rats were killed for tissue analysis. Blood was collected by cardiac puncture.

Body composition

Body composition was determined by time domain-nuclear magnetic resonance measurements on restrained but awake and alert animals (TD-NMR Minispec, Bruker Optics, The Woodlands, TX, USA). The MiniSpec provides three components of body composition (fat, free body fluid, and lean tissue) by acquiring and analyzing TD-NMR signals from all protons in the sample area. Validation of TDNMR methodology has been provided (Tinsley *et al.* 2004).

Figure 1 Schematic representation of experimental groups for both young and aged rats in experiment 1 (A) and experiment 2 (B).

Serum leptin

Serum was collected by tail nicking or cardiac puncture after termination of AM251 treatment. Serum leptin was measured using rat RIA kits (Linco Research, Inc., St Charles, MO, USA).

Tissue harvesting and preparation

Rats were killed by thorocotomy under a ketamine (75 mg/kg), xylazine (7 mg/kg) cocktail anesthesia, and the circulatory system was perfused with 30 ml cold saline. The epididimal, perirenal, and retroperitoneal white adipose tissues (EWAT, PWAT, and RTWAT respectively), hypothalamus, and brown adipose tissue (BAT) were excised. Protein concentrations were determined using the DC protein assay kit (Bio-Rad).

Western analysis

Protein homogenate was separated on an SDS-PAGE gel and electrotransferred to PVDF membranes, as previously described (Zhang *et al.* 2007). Immunoreactivity was assessed with antibodies specific to phospho-signal transducer and activator of transcription-3 (pSTAT3 Tyr705, Cell Signaling, Danvers, MA, USA), phospho-acetyl Co-A carboxylase (P-ACC, Upstate, Billerica, MA, USA), or uncoupling protein-1 (UCP-1, Linco Research, Inc.).

Statistical analysis

Data were analyzed by *t*-test (paired and unpaired) one-way ANOVA, and two-way ANOVA. When the mean was significant, a *post-hoc* test (Bonferroni) was applied to determine the individual differences between the means. A value of $P < 0.05$ was considered significant.

Figure 2 Change in calorie intake (A and C) and body weight (B and D) in young rats during daily i.p. 0.83 or 2.78 mg/kg AM251 administration. Data represents mean $\pm$ s.e.m. of chow-fed with vehicle treatment ($n=6$, open triangles), chow-fed with AM251 treatment ($n=6$, closed triangles), HF-fed with vehicle treatment ($n=6$, open circles), and HF-fed with AM251-treatment ($n=6$, closed circles). (A) AM251 treatment did not reduce calorie intake in young rats, regardless of dietary treatment. Inset: HF diet stimulated an increase in cumulative calorie intake ($^{\dagger}P$ value < 0.001) that was unchanged by AM251. (B) Only the HF-fed vehicle-treated young rats gained weight during the experimental period. *P value < 0.01 for the gain in body weight in HF-fed vehicle-treated compared to all other groups starting at day 2 by *t*-test. (C) AM251 treatment inhibited the HF diet-induced hyperphagia. Inset: 5-day cumulative calorie intake was significantly reduced by AM251 treatment in chow-fed ($^*P < 0.05$) and HF-fed ($^{**}P < 0.01$) rats. (D) AM251 treatment caused body weight loss in young rats on both diets ($P < 0.05$ for the difference between AM251-treated rats and their diet-matched controls, starting on day 1). $^{**}P < 0.05$ for the difference between HF-fed AM251-treated and chow-fed AM251-treated rats on days 1–3.

Results

Experiment 1: AM251 treatment simultaneous with introduction of HF diet

When rats are first introduced to an HF diet, they display an immediate hyperphagia accompanied by an increase in body weight (Judge *et al.* 2008). With either age or leptin resistance, this HF-induced hyperphagia is further exacerbated (Judge *et al.* 2008, Scarpace & Zhang 2009). In this experiment, we tested responsiveness to two doses, 0.83 and 2.78 mg/kg per day, of AM251 in young and aged rats with and without simultaneous introduction of an HF diet.

As expected, chow-fed, vehicle-treated young rats maintained a steady calorie intake and body weight throughout the study, whereas HF-fed, vehicle-treated rats demonstrated a transient hyperphagia with a peak calorie intake of ~100 kcal, a 38% increase in cumulative food consumption, and a steady gain in body weight (Fig. 2, open symbols, open bars). While the low dose (0.83 mg/kg per day) of AM251 in young rats did not significantly reduce either calorie intake (Fig. 2A) or body weight (Fig. 2B) in chow-fed rats, this dose combined with HF feeding resulted in a 22% reduction in peak calorie intake (Fig. 2A) and a prevention of HF diet-induced weight gain (Fig. 2B). Similarly, cumulative food consumption was unchanged in the AM251 chow-fed but diminished with AM251 in the HF-fed animals (Fig. 2A, inset). Adiposity levels, lean mass, and the fat-to-lean mass ratio were not affected by diet or this lower dose of drug treatment in these young rats (Table 1). However, despite no overall increase in adiposity with this short-term HF feeding, there was a nearly 50% increase in serum leptin levels in the young rats, which was unchanged by AM251 treatment (Table 1).

The higher dose (2.78 mg/kg per day) of AM251 caused a complete inhibition of the HF diet-induced hyperphagia in young rats (Fig. 2C). Calorie intake was reduced on both diets, with the reduction in cumulative calorie intake during the 5-day treatment period being 21% in chow-fed and 27% in HF-fed young rats with AM251 treatment (Fig. 2C inset).

None of the vehicle-treated, but all of the rats treated with the high dose of AM251 immediately lost body weight compared to diet-matched controls by day 1, regardless of dietary treatment (Fig. 2D). Moreover, the AM251-mediated body weight loss was greater with HF feeding beginning a day 1 with an overall reduction in body weight of ~10 g in chow- and 16 g in HF-fed young rats by day 5 compared with diet-matched controls (Fig. 2D). Body composition was unchanged in the young rats with this dose of AM251 (Table 1).

In the aged rats, the vehicle-treated, chow-fed animals maintained a stable body weight and calorie intake while the HF-fed, vehicle-treated rats demonstrated a hyperphagia of ~150 kcal, nearly 50% more than that observed in the young rats (Fig. 3, open symbols). Unlike treatment in the young rats, the low dose (0.83 mg/kg) of AM251 caused a 16% reduction in the HF-induced peak in calorie intake, and reduced the normal period of hyperphagia from 30 days (Judge *et al.* 2008) to 6 days (Fig. 3A). Similarly, the 5-day cumulative calorie intake was reduced by 24% in chow-fed and 20% in HF-fed aged rats with AM251 treatment (Fig. 3A, inset). HF feeding resulted in a considerable gain in body weight in the vehicle-treated rats (Fig. 3B). Peripheral AM251 treatment reduced body weight beginning at day 1 in chow-fed and partially prevented body weight gain by day 1 in the HF-fed rats. By the end of the 5-day period, body weight differed by 17 g in chow-fed and 22 g in HF-fed aged rats compared with diet-matched controls (Fig. 2B). In addition, AM251 treatment with HF feeding improved the fat-to-lean mass ratio by 14% compared with diet-matched controls, while whole body adiposity and lean mass were unchanged (Table 2). Interestingly, this low dose of AM251 significantly blunted the HF-diet-induced increase in serum leptin levels in the aged rats (Table 2).

The high dose of AM251 in aged rats completely inhibited HF-induced hyperphagia. Interestingly, the absolute level of calorie intake in both chow- and HF-fed aged rats was nearly equivalent by day 5 (Fig. 3C). However, when compared to their diet-matched controls, the response to AM251-treatment in the HF-fed aged rats was considerably greater

Table 1 Adiposity, lean mass, and serum leptin levels after respective AM251 doses in young rats during hyperphagia. Data represents mean ± S.E.M. of 6–8 rats per group analyzed by two-way ANOVA

	Adiposity (g)	Lean mass (g)	Fat/lean mass ratio	Serum leptin (ng/ml)
Experimental group				
Young rats, 0.83 mg/kg AM251				
Chow-vehicle	70.0 ± 3.5	191.6 ± 9.3	0.37 ± 0.01	6.0 ± 1.1
Chow-AM251	71.7 ± 2.7	190.6 ± 5.1	0.38 ± 0.01	6.1 ± 1.2
HF-vehicle	73.2 ± 4.5	200.3 ± 10.0	0.36 ± 0.01	12.5 ± 1.6 ^a
HF-AM251	70.7 ± 1.7	196.2 ± 6.5	0.36 ± 0.00 ^b	11.5 ± 1.5 ^a
Young rats, 2.78 mg/kg AM251				
Chow-vehicle	93.2 ± 5.6	230.0 ± 10.4	0.40 ± 0.01	No data available
Chow-AM251	89.7 ± 2.9	222.6 ± 5.3	0.40 ± 0.01	No data available
HF-vehicle	79.4 ± 3.9	211.9 ± 7.2	0.37 ± 0.01 ^a	13.1 ± 3.1
HF-AM251	73.7 ± 4.2 ^a	200.2 ± 11.9	0.37 ± 0.01 ^a	8.4 ± 1.5

^aIndicates the difference with HF feeding from corresponding chow-fed rats.
^bIndicates the difference with AM251 treatment from corresponding diet-matched rats.

than that in the young rats with a reduced cumulative calorie intake of 76% compared with 29% respectively (Fig. 3C, inset). This higher dose of AM251 caused a parallel reduction in body weight in chow- and HF-fed aged rats. However,

if these rats are compared with their diet-matched controls, the AM251 responsiveness is dramatically augmented in the HF-fed aged rats, with body weight differing by ~ 27 and 58 g in chow-fed and HF-fed aged rats respectively (Fig. 3D). Body composition analysis revealed that adiposity, lean mass, and serum leptin were not significantly different with this high-dose AM251 treatment in chow-fed, whereas the fat-to-lean mass ratio was improved by 9% (Table 2). In contrast, drug treatment coupled with HF feeding reduced adiposity by 23%, the fat-to-lean mass ratio by 19%, and serum leptin levels by 73%, whereas lean mass was unchanged (Table 2). In essence, the HF diet-induced elevation in adiposity and serum leptin levels and the decrease in fat-to-lean mass ratio were reversed with high-dose AM251 treatment in aged rats (Table 2).

Experiment 2: AM251 treatment after prolonged chow or HF feeding

In this second experiment, the responsiveness to AM251 was examined in young and aged rats with established obesity as a result of 60 days of HF feeding as compared with chow-fed animals. Prior to examining AM251 responsiveness, but after 60 days of chow or HF feeding, leptin responsiveness was examined in both age groups by peripheral leptin infusion. The HF-fed young rats demonstrated a partial leptin resistance and all the aged rats displayed a complete leptin resistance (Fig. 4). The absolute reduction in body weight on day 10, on which the rats displayed the greatest response to the leptin treatment, was reduced by both HF feeding and age (young chow-fed: 15.5 ± 3.1 g, young HF-fed: 11.8 ± 1.2 g, aged chow-fed: 9.7 ± 2.3 g, and aged HF-fed: 4.3 ± 1.5 g).

After a 2-week recovery period, responsiveness to AMP251 was examined. The young chow-fed rats did not respond to the 7-day AM251 treatment (0.45 mg/day) with a

Figure 3 Change in calorie intake (A and C) and body weight (B and D) in aged rats during daily i.p. 0.83 or 2.78 mg/kg AM251 administration. Data represents mean $\pm$ S.E.M. of chow-fed and vehicle-treated ($n=6$, open triangles), chow-fed and AM251-treated ($n=7$, closed triangles), high-fat fed and vehicle-treated ($n=7$, open squares), and high-fat fed and AM251-treated ($n=8$, closed squares). (A) AM251 treatment significantly reduced calorie intake in HF-fed rats beginning on day 2. * P value <0.01 for the difference in calorie intake between HF-fed vehicle-treated and AM251-treated aged rats. Inset: 5-day cumulative calorie intake is reduced in all AM251-treated aged rats (* $P<0.05$, ** $P<0.001$) $^{\dagger}P$ value <0.001 for the increase in cumulative calorie intake with HF-feeding compared to treatment-matched chow-fed rats. (B) AM251 treatment caused a reduction in body weight compared to diet-matched, vehicle-treated aged rats (* P value <0.01). (C) AM251 completely inhibited HF diet-induced hyperphagia and inhibited chow intake in aged rats. Inset: 5-day cumulative calorie intake is reduced with AM251 treatment on both diets (* $P<0.001$, $^{\dagger}P$ value <0.001 for the increase in cumulative calorie intake with HF-feeding compared to treatment-matched chow-fed rats). (D) AM251 reduced body weight of both chow- and HF-fed aged rats beginning at day 1 ($P<0.001$ for difference between AM251-treated and diet-matched vehicle-treated rats at all days during experimental period, by t -test).

Table 2 Adiposity, lean mass, and serum leptin levels after respective AM251 doses in aged rats during hyperphagia. Data represents mean ± S.E.M. of 6–8 rats per group analyzed by two-way ANOVA

	Adiposity (g)	Lean mass (g)	Fat/lean mass ratio	Serum leptin (ng/ml)
Experimental group				
Aged rats, 0.83 mg/kg AM251				
Chow-vehicle	154.7 ± 5.5	305.7 ± 5.3	0.51 ± 0.01	24.2 ± 1.9
Chow-AM251	139.1 ± 7.4	304.0 ± 7.7	0.46 ± 0.02	20.7 ± 2.2
HF-vehicle	184.4 ± 6.3	318.8 ± 4.3	0.55 ± 0.02	70.0 ± 9.8 ^a
HF-AM251	149.8 ± 9.8	321.6 ± 11.6	0.47 ± 0.03 ^b	40.8 ± 5.9 ^b
Aged rats, 2.78 mg/kg AM251				
Chow-vehicle	148.8 ± 5.6	295.7 ± 5.3	0.50 ± 0.01	18.0 ± 2.4
Chow-AM251	136.1 ± 4.8	296.9 ± 5.8	0.46 ± 0.01 ^b	14.6 ± 1.8
HF-vehicle	163.9 ± 10.4	307.8 ± 9.0	0.54 ± 0.02	50.8 ± 10.5 ^a
HF-AM251	126.3 ± 8.5 ^b	292.5 ± 10.0	0.43 ± 0.02 ^{a,b}	13.9 ± 3.9 ^b

^aIndicates the difference with HF feeding from corresponding chow-fed rats.
^bIndicates the difference with AM251 treatment from corresponding diet-matched rats.

significant decrease in cumulative calorie intake, although they displayed a decrease in body weight (Fig. 5A and B), suggesting an increase in energy expenditure accounts for the decrease in body weight. Similar to the responses with short-term HF feeding, the anorectic responses to AM251 were more efficacious with HF feeding compared to chow feeding. Cumulative calorie intake was reduced by 23% in HF-fed AM251-treated rats, but there was no effect on cumulative calorie intake in chow-fed rats (Fig. 5A). Initially, the young rats on both the chow and HF diets responded to the 7-day AM251 treatment (1.2 mg/kg per day or 0.45 mg/rat per day) with similar decreases in body weight, but beginning at day 5, the chow-fed animals started to regain the lost weight, such that by the end of the treatment period, the decrease in body weight was nearly double in the HF-fed compared with the chow-fed rats. This is indicated by the positive slope of the body weight change in chow-fed compared with the negative slope in the HF-fed rats (Fig. 5B, dotted boxes). The reduction in body weight in AM251-treated versus vehicle-treated on day 7 was 60% greater in HF-fed compared with

chow-fed young rats (Fig. 5B). In addition, there was a decrease in adiposity level in AM251-treated young rats on both chow- and HF-diet compared with an increase in adiposity in the vehicle-treated young rats (Table 3). Similar to the young rats, the responses of AM251 were more pronounced in aged rats with HF feeding compared with chow feeding. In contrast to the young, the aged rats also displayed robust responses to daily AM251 (0.8 mg/kg per day or 0.45 mg/rat per day) treatment in the chow-fed rats. The cumulative calorie intake was significantly reduced by AM251 treatment in by 30% in chow-fed young rats, with an even greater effect in HF-fed aged rats of 45% (Fig. 6A). Similarly, AM251 treatment reduced body weight by day 7 by 21 and 34 g in chow- and HF-fed aged rats respectively, when compared to vehicle-treated, diet-matched controls (Fig. 6B). Both adiposity and lean body mass levels were also reduced in aged rats treated with AM251 compared with their diet-matched, vehicle-treated controls (Table 4).

Biochemical indicators

In this second experiment in rats with established obesity, several indicator of energy homeostasis were assessed. Several neuropeptides, including leptin signals through phosphorylation of STAT3 (Levin *et al.* 2004). At death, hypothalamic pSTAT3 levels were elevated with age and with HF feeding in both young and aged rats (Tables 3 and 4). This is consistent with previous reports where HF feeding increased hypothalamic pSTAT3 levels compared to chow-fed controls (Wilsey *et al.* 2003, Scarpace & Zhang 2007). More interestingly, whereas AM251 treatment raised pSTAT3 levels in both young and aged chow-fed rats, it only reached significance in aged rats (Tables 3 and 4). Phosphorylation of ACC leads to fatty acid oxidation and the breakdown of adipose tissue to increase available substrate (Abu-Elheiga *et al.* 2000). Both HF feeding and age decreased pACC levels in PWAT (Tables 3 and 4), consistent with the

Figure 4 Decrease in body weight at day 10 following peripheral vehicle or leptin infusion in chow or HF fed rats. Data represent the difference in body weight from pretreatment values and is the mean ± S.E.M. of chow-fed young (n=8), HF-fed young (n=8), chow-fed aged (n=6), and HF-fed aged (n=8). High-fat feeding and to a greater extent, age caused a reduced responsiveness to peripheral leptin infusion (**P*<0.05 for the difference with HF feeding in young rats; ***P*<0.01 for the difference with age, regardless of dietary treatment. Delta body weight in all groups was significantly different from pretreatment (day 0) by paired *t*-test).

Figure 5 Change in cumulative calorie intake and body weight and in young rats during 7-day daily i.p. administration of AM251 (0.45 mg/rat per day; 1.2 mg/kg per day). Data represents mean $\pm$ S.E.M. of chow- or HF-fed and vehicle-treated ($n=8$, closed symbols), chow- or HF-fed and AM251-treated ($n=7$, open symbols). (A) The combination of HF feeding and AM251 treatment caused a reduction in cumulative calorie intake. ** P value <0.01 for the difference with AM251 treatment in HF-fed rats. (B) AM251-induced body weight reduction was similar in chow- and HF-fed young rats.

positive energy balance under both of these conditions. Surprisingly, AM251 treatment also caused a significant reduction in pACC levels in aged rats, despite the state of negative energy balance (Table 4).

Table 3 Change in body composition and biochemical markers of young rats during daily i.p. vehicle or AM251 injections. Data represents mean $\pm$ S.E.M. of 4–8 rats per group analyzed by two-way ANOVA. Adiposity and lean mass were determined prior to and after 7-day vehicle or AM251 daily injections; biomolecular marker levels of all young, chow-fed and vehicle-treated groups are set to 1.00 and S.E.M. adjusted accordingly

	Chow		HF	
	Vehicle	AM251	Vehicle	AM251
Change in adiposity (g)	3.8 $\pm$ 1.2	–2.1 $\pm$ 1.8 [†]	3.5 $\pm$ 0.9	–3.6 $\pm$ 1.6 [†]
Change in lean mass (g)	–0.4 $\pm$ 1.6	–4.6 $\pm$ 2.2	0.7 $\pm$ 1.3	–2.2 $\pm$ 3.4
Hypo STAT3	1.00 $\pm$ 0.08	1.32 $\pm$ 0.07	1.79 $\pm$ 0.11*	1.72 $\pm$ 0.09*
PWAT pACC	1.00 $\pm$ 0.19	1.31 $\pm$ 0.29	0.47 $\pm$ 0.16	0.29 $\pm$ 0.13*
BAT UCP-1	1.00 $\pm$ 0.08	1.19 $\pm$ 0.07	1.42 $\pm$ 0.05*	1.53 $\pm$ 0.10*

* $P<0.05$, indicates the difference with HF feeding from corresponding chow-fed rats. [†] $P<0.05$, indicates the difference with AM251 treatment from corresponding vehicle-treated and diet-matched rats.

The elevation of UCP1 protein levels in BAT is often used as a marker of energy expenditure (Judge *et al.* 2008). In accordance with previous reports, in the present study HF feeding increases UCP1 levels in both young and aged rats (Wilsey *et al.* 2003, Zhang *et al.* 2007, Judge *et al.* 2008). In addition, the current study found that AM251 tended to increase UCP1 levels in BAT in chow-fed young and aged rats, although these increases did not reach significance (Tables 3 and 4).

Comparisons of young and aged rats with chow and HF feeding

In addition to the comparisons with diet, the responsiveness of AM251 with age was compared. Figure 7 summarizes the loss in body weight in AM251-treated compared to vehicle-treated rats for each diet and dose of AM251 across ages. Based on the data presented in Figs 2 and 5, it appears that the 0.83 mg/kg per day dose is submaximal, whereas the 2.78 mg/kg per day of AM251 is maximal. With chow feeding, only the aged rats responded to the low dose of AM251 suggesting increased sensitivity to AM251 with age (Fig. 7A). Second, the responsiveness to this dose with in HF-fed rats of both ages is increased indicating increased sensitivity with HF feeding (Fig. 7A). Comparison of the maximal dose (Fig. 7B), indicates that maximum efficacy is increased with HF feeding and further augmented with age.

Comparisons with age were made in rats with established obesity as described in experiment 2. In this case, comparisons across age were complicated by the dosing regime. In this second experiment, the same dose of AM251 per animal (0.45 mg/rat per day) was administered to rats of both ages. Because of the considerable differences in body weights between young and aged rats, the dose if recalculated based on rodent body weight yields 1.20 and 0.80 mg/kg per day respectively, for young and aged rats. Thus, direct comparisons cannot be made between the young and old rats. However, the more robust body weight reduction was observed in the aged rats, which received the smaller dose per kilogram of body weight. With AM251 treatment, the HF-fed aged rats with established obesity lost 65% greater body weight compared with the corresponding chow-fed rats (Fig. 7C). Finally, comparison among aged

Figure 6 Change in cumulative calorie intake and body weight in aged rats during 7-day daily i.p. administration of AM251 (0.45 mg/rat per day; 0.8 mg/kg per day). Data represents mean $\pm$ S.E.M. of chow- or HF-fed and vehicle-treated ($n=8$, closed symbols), chow- or HF-fed and AM251-treated ($n=7$, open symbols). (A) Cumulative calorie intake was reduced by AM251 administration, regardless of dietary treatment (** P value <0.001). (B) AM251 treatment induced a loss of body weight in aged rats that was enhanced with prolonged HF feeding. * P value <0.001 for the difference with diet and AM251 treatment in aged rats. ** P value <0.001 for the difference in change in body weight in both dietary groups.

rats between those first introduced to the HF diet and those HF fed rats with established obesity indicates that low-dose AM251 treatment evoked more robust body weight reduction in the latter (Fig. 7A and C, right pairs of bars).

Table 4 Change in body composition and biochemical markers of aged rats during daily i.p. vehicle or AM251 injections. Data represents mean $\pm$ S.E.M. of 4–8 rats per group analyzed by two-way ANOVA. Adiposity and lean mass were determined prior to and after 7-day vehicle or AM251 daily injections; biomolecular marker levels of all young, chow-fed and vehicle-treated groups are set to 1.00 and S.E.M. adjusted accordingly

	Chow		HF	
	Vehicle	AM251	Vehicle	AM251
Change in adiposity (g)	-4.9 ± 1.6	$-18.1 \pm 2.2^{\dagger}$	-7.2 ± 1.7	$-25.0 \pm 1.9^{\dagger}$
Change in lean mass (g)	-0.7 ± 1.3	$-7.6 \pm 1.3^{\dagger}$	-0.4 ± 1.8	$-10.3 \pm 1.8^{\dagger}$
Hypo STAT3	1.26 ± 0.10	$1.87 \pm 0.25^{\dagger}$	$2.35 \pm 0.24^*$	2.08 ± 0.10
PWAT pACC	0.42 ± 0.06	$0.20 \pm 0.03^{\dagger}$	$0.22 \pm 0.04^*$	0.11 ± 0.02
BAT UCP-1	0.91 ± 0.04	1.29 ± 0.12	$1.79 \pm 0.15^*$	$1.78 \pm 0.09^*$

* $P<0.05$, indicates the difference with HF feeding from corresponding chow-fed rats. $^{\dagger}P<0.05$, indicates the difference with AM251 treatment from corresponding vehicle-treated and diet-matched rats.

Discussion

The present study demonstrates that the responsiveness of CB1 receptor antagonist was enhanced with both HF-feeding and age, with both increased sensitivity and maximum efficacy. These enhanced responses were observed with respect to both body weight reduction or prevention and the anorectic response. The latter includes the time course of food consumption as well as the overall reduction in cumulative calorie intake during HF feeding compared to chow feeding. Moreover, this same pattern was observed in both rats first introduced to HF feeding and in HF-fed rats with established obesity. In the former, the impact of AM251 on the normal hyperphagic response to HF feeding was evaluated. With age, this hyperphagia is elevated and prolonged and provides a sensitive model to evaluate AM251 responsiveness. Only the high dose was able to diminish this hyperphagia in young rats, whereas in the aged, the lower dose decreased the degree of hyperphagia and substantially shortened the hyperphagia period, clearly demonstrating increased sensitivity with age. On the other hand, the high dose of AM251 eliminated the hyperphagic period in all cases consistent with the idea that this dose maximally inhibited the CB1 receptor. Collectively, these data suggest that CB1 receptor activity contributes to the hyperphagia observed with the introduction of an HF diet.

In addition, the HF-feeding induced increase in AM251 sensitivity appears to be additive with age, with the greatest degree of responsiveness observed in aged-HF fed rats. In HF-fed young rats, enhancement in AM251 antagonism is modest. These data are consistent with previous reports that CB1 receptor antagonists preferentially block consumption of a more palatable diet as compared with standard chow diet (Arnone *et al.* 1997, Simiand *et al.* 1998, Hildebrandt *et al.* 2003). With age, the AM251 antagonist response is greatly magnified, with this antagonistic response is further exacerbated in HF-fed aged rats. Because both age and HF diet-induced obesity are associated with leptin resistance, these data are consistent with a positive relationship between CB1 receptor antagonist efficacy and leptin resistance. Leptin is believed to downregulate EC levels in the brain. Thus, in

Figure 7 Change in body weight in AM251-treated rats compared to vehicle-treated rats on day 5 of the respective experiments. (A) 0.83 mg/kg AM251 treatment during hyperphagia reduced body weight in young HF-fed and aged chow- and HF-fed rats. **P* value <0.05 for the difference in HF-fed compared to chow-fed young rats. ***P* value <0.001 for the difference with age during HF-feeding. (B) 2.78 mg/kg AM251 treatment during hyperphagia caused an increase in sensitivity with HF feeding in aged rats. **P* value <0.001 for the difference with HF feeding in aged rats. ***P* value <0.001 for the difference with age during HF-feeding. (C) 0.45 mg/kg per day during prolonged chow or HF feeding caused a significant reduction in body weight in aged rats, which was enhanced with HF feeding. **P* value <0.001 for the difference with HF feeding in aged rats. ***P* value <0.001 for the difference in age on an HF diet.

leptin resistant animals, the EC system may be free from leptin downregulation, leading to hyperactive CB1 receptors (Di Marzo *et al.* 2001). Under these circumstances, leptin resistance would expect to be associated with enhanced AM251 responsiveness. However, the present study provides no direct relationship between leptin resistance and CB1 receptor activity.

Moreover, the increase in AM251 efficacy with HF feeding appears to be influenced by diet and either the duration of that diet or established obesity more so than related to the presence of leptin resistance. Young rats with AM251 treatment displayed similar responsiveness regardless of the duration of HF feeding. However, in aged rats the efficacy of AM251 was enhanced to a greater degree with long-term HF feeding and established obesity compared with the introduction of the HF diet, suggesting both HF diet and established obesity may have separate roles. Because HF feeding in the aged rats does not appear to further increase resistance to peripherally infused leptin, both the long-term chow-fed and HF-fed rats were completely resistant to peripherally infused leptin (data not shown, (Judge *et al.* 2008)), but does increase the degree of obesity, these findings indicate the HF-diet enhanced AM251 efficacy, at least in aged rats, is independent of leptin resistance.

The present data cannot rule out the role of the presence of leptin resistance as a contributing factor in the young HF-fed rats. Recent data indicate that some obese states lacking normal leptin function are associated with elevated EC levels (Di Marzo *et al.* 2001, Maccarrone *et al.* 2005, Engeli 2008). In fact, hypothalamic EC levels are significantly elevated in Zucker rats, which have defective leptin receptors; db/db mice, which lack leptin receptors; and ob/ob mice, which lack leptin (Di Marzo *et al.* 2001). In each of these conditions, exogenous administration of leptin is effective in reducing EC levels back to those matching lean littermates (Di Marzo *et al.* 2001). Other evidence also suggests that EC levels are elevated in animals with both diet-induced and genetic obesity, but status of the CB1 receptor activity with obesity is unclear (Di Marzo *et al.* 2001, Osei-Hyiaman *et al.* 2005). In some cases, receptor levels are reciprocally related to EC levels, thus diminished with HF feeding. These later data do not support our findings of enhanced AM251 efficacy in the presence of an HF diet, and the mechanism underlying the increased efficacy with an HF diet or with age remains speculative.

One interesting observation is that the HF-fed rats displayed elevated UCP1 protein levels in BAT even in the aged-HF fed rats. Perhaps, there is a component of BAT thermogenesis that is contributing to energy homeostasis in the aged HF-fed rats that is absent in aged chow-fed rats, and blockade of this component by AM251 contributes to the increased efficacy with age, though additional experiments are necessary to solidify these speculations.

In summary, both age and an HF diet are associated with enhanced CB1 receptor antagonist efficacy, with the greater response in the aged rats, and the greatest enhancement in HF

fed aged rats. The enhanced efficacy with age and an HF diet appear to be more related to the diet, than the presence of leptin resistance, and the degree of obesity may play a role. In addition, CB1 receptor activity appears to contribute to the hyperphagia observed with the introduction of an HF diet.

However, the underlying mechanism of the enhanced CB1 receptor antagonist responsiveness remains speculative.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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