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Review

Increased adiposity on normal diet, but decreased susceptibility to diet-induced obesity in μ-opioid receptor-deficient mice

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Abstract

The mu-opioid receptor encoded by the *Oprm1* gene plays a crucial role in the mediation of food reward and drug-induced positive reinforcement, but its genetic deletion has been shown to provide food intake-independent, partial protection from diet-induced obesity. We hypothesized that mu-opioid receptor-deficient mice would show an even greater, intake-dependent, resistance to high-fat diet-induced obesity if the diet comprises a sweet component. We generated an F2 population by crossing the heterozygous offspring of homozygous female *Oprm1*$^{-/-}$ mice (on a mixed C57BL/6 and BALB/c genetic background) with male inbred C57BL/6 mice. Groups of genotyped wild-type (WT) and homozygous mutant (KO) males and females were fed either control chow or a high caloric palatable diet consisting of sweet, liquid chocolate-flavored Ensure together with a solid high-fat diet. Food intake, body weight, and body composition was measured over a period of 16 weeks. Unexpectedly, male, and to a lesser extent female, KO mice fed chow for the entire period showed progressively increased body weight and adiposity while eating significantly more chow. In contrast, when exposed to the sweet plus high-fat diet, male, and to a lesser extent female, KO mice gained significantly less body weight and fat mass compared to WT mice when using chow fed counterparts for reference values. Male KO mice consumed 33% less of the sweet liquid diet but increased intake of high-fat pellets, so that total calorie intake was not different from WT animals. These results demonstrate a dissociation of the role of μ-opioid receptors in the control of adiposity for different diets and sex. On a bland diet, normal receptor function appears to confer a slightly catabolic predisposition, but on a highly palatable diet, it confers an anabolic metabolic profile, favoring fat accretion. Because of the complexity of μ-opioid gene regulation and tissue distribution, more selective and targeted approaches will be necessary to fully understand the underlying mechanisms.

Keywords: Palatable diet; High-fat diet; *Oprm1*; Food intake; Body weight; Glucose clearance

Contents

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1. Introduction

Obesity is now recognized as a major health problem in most industrialized countries, with more than 20% of the adult population classified as clinically obese and about half the population as overweight in the USA (Mokdad et al., 2003). Childhood obesity continues to rapidly increase, and because of the strong correlation between body mass index and development of type 2 diabetes, every third child born in the USA is expected to develop type 2 diabetes later in life (Lee et al., 2006). In addition, obesity is a significant risk factor for cardiovascular disease, gall bladder disease, osteoarthritis, sleep and mental disorders and the general metabolic syndrome.

Although important genes responsible for monogenic childhood obesities have been discovered recently (Farooqi et al., 2003), the rapidly changing environment and lifestyle on the background of evolutionary engraved and perinatally imprinted physiological response patterns seems to be primarily responsible for the current obesity epidemic (Gluckman et al., 2007; Prentice et al., 2005). Food intake and energy expenditure are controlled by complex, redundant, and distributed neural and peripheral systems involving thousands of genes and reflecting the fundamental biological importance of adequate nutrient supply and energy balance, but it is not clear what the mechanisms are by which the modern environment overrides the physiological controls of appetite and homeostatic body weight regulation (Berthoud, 2007).

One of the environmental factors identified as a potential contributor to the development of obesity is the increased availability of palatable food and exposure to conditioned cues of such foods in the modern world (Young and Nestle, 2002). Food reward and sensory-specific satiety are thought to be important mechanism that guaranteed sufficient intake of energy and micronutrients from a variety of sources in times of restricted availability (Kelley, 2004). In modern times with plenty of palatable and energy dense foods, these evolutionary conserved mechanisms are maladaptive, leading to exaggerated energy intake (Rolls et al., 2002; Tordoff, 2002).

The opioid system has been recognized to play an important role in neural reward processes leading to addictive behavior such as self-administration of opioid agonists directly and other drugs of abuse such as nicotine and alcohol. Many of the neural structures involved in addictive behavior are also involved in food reward and opioid receptor antagonists attenuate both addictive drug taking and appetite for palatable food (Kelley, 2004; Levine and Billington, 2004). Thus, craving for palatable food could be considered as a form of addiction and the opioid system seems to play a crucial role (Colantuoni et al., 2002; Spangler et al., 2004).

Among the opioid receptors, the mu-opioid system has been most strongly implicated in reward processes. Administration of the selective mu-opioid receptor agonist [D-Ala^2, *N*-MePhe^4, Gly-ol^5]-enkephalin (DAMGO) into the nucleus accumbens of rats and mice results in a voracious feeding response in completely satiated animals (Kelley et al., 2002; Will et al., 2003), and administration of the irreversible mu-opioid receptor antagonist β-funaltrexamine into the nucleus accumbens reduces sucrose intake for several days in rabbits (Ward et al., 2006). These effects could be due to mu-opioid receptor stimulation-induced amplification of hedonic impact (liking) of a particular food in a subset of nucleus accumbens neurons (Pecina and Berridge, 2005), the production of a selective increase in preference for recently sampled flavors (Woolley et al., 2007), and increased motivation to obtain highly palatable foods (wanting) (Mathon et al., 2006; Papaleo et al., 2007). Consistent with these observations, both mu-opioid receptor and enkephalin-deficient mice show decreased motivation to eat when the animals are required to press a bar to obtain food (Hayward and Low, 2007; Papaleo et al., 2007). Furthermore, mu-opioid receptor-deficient mice exhibit diminished food-anticipatory running wheel activity (Kas et al., 2004), and reduced drug reward as indicated by decreased cocaine conditioned place preference (Hall et al., 2004) and decreased alcohol consumption (Koob et al., 2003).

Given these observations, antagonism to mu-opioid receptor function is thus a plausible strategy to prevent obesity induced by easy availability of highly palatable and energy dense foods in the modern environment. Few studies have focused on this aspect and there are considerable differences in outcome. In one study, mu-opioid receptor-deficient mice had increased body weight in adulthood without a detectable change in regular chow intake, indicating reduced energy expenditure or partitioning of food into fat storage (Han et al., 2006). In contrast, male mu-opioid receptor-deficient mice were more resistant to high-fat diet-induced obesity than wild-type mice, an effect that was mainly due to increased fat oxidation but not hyperphagia (Tabarin et al., 2005). In a pharmacological study, high-fat diet-induced increases in body weight and adiposity seen in control

animals were almost abolished by chronic oral treatment with a non-morphinian general antagonist for mu, kappa, and delta opioid receptors, and the effect was due to both a reduction in food intake and stimulation of fat oxidation (Statnick et al., 2003). Finally, the strong, dose-dependent anorectic response produced by another non-selective opioid receptor antagonist was diminished in mu-opioid receptor-deficient mice, supporting an important role for the mu-opioid receptor in the control of food intake (Zhang et al., 2006).

Given these variable outcomes and implied mechanisms of action, the aim of the present study was to further characterize the response of mu-opioid receptor-deficient mice to dietary manipulations. Specifically, we wanted to test the response to a diet consisting of a choice of solid high-fat chow and sweet liquid diet (Ensure), as sweet taste perception is one of the most salient activators of reward circuits. Furthermore, we wanted to compare male and female knockout mice as gender differences have been reported in the motivation to bar press for food in endorphin-deficient mice (Hayward and Low, 2007).

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Pennington Biomedical Research Center Institutional Animal Care and Use Committee. Female μ-opioid receptor-deficient knockout (KO) mice, homozygous for the *Oprm1*^{tm1Loh} mutation on a (BALB/cJ × C57BL/6J)F1 genetic background (Loh et al., 1998) were obtained from Dr. H. Loh at the University of Minnesota, and mated to C57BL/6J male mice, purchased from the Jackson Laboratory (Bar Harbor, ME) to generate *Oprm1*$^{+/-}$ heterozygous male and female mice. These F1 mice were intercrossed to generate an F2 population. After genotyping, representative wild-type (WT, *Oprm1*$^{+/+}$) or mutant (KO, *Oprm1*$^{-/-}$) male and female mice were selected for the dietary obesity studies.

Sex and genotype matched KO and WT mice were housed in groups of 2 to 3 in shoebox cages in the Pennington Biomedical Research Center vivarium at a temperature of 21–23 °C and a 12 h light–dark cycle (lights on 07:00, off at 19:00). Food and water were provided ad libitum.

2.2. Diets

Mice used for breeding and weaned progeny were fed standard pelleted mouse chow (# 5015, LabDiet, Richmond, IN) containing 19.8 kcal% protein, 25.3 kcal% fat and 54.9 kcal% carbohydrate. The F2 mice selected for the dietary obesity experimental and control groups were switched at 8 weeks of age to a powdered version of the same chow diet provided in 2 oz. glass jars (Unifab, Kalamazoo, MI). These jars are covered with a stainless steel disk with 6 circular holes and a stainless steel lid securing the disc to facilitate the accurate measurement of food intake. At 12 weeks of age, mice were randomly assigned to one of two feeding paradigms. The control groups consisted of male and female mice continuing to be fed the same powdered 5015 chow diet. Male and female mice in the experimental group were switched to a powdered high-fat (HF) diet (D12492, Research Diets, NJ) containing 20 kcal% protein, 60 kcal% fat and 20 kcal% carbohydrate provided in a jar and were also given access to liquid sweet chocolate-flavored Ensure (Ross Products Div., Abbott Laboratories, Columbus, OH) containing 14 kcal% protein, 22 kcal% fat and 64 kcal% carbohydrate in sipper tubes (Sarstedt Inc., Newton, CT). Tap water was freely available to all mice.

2.3. Dietary obesity phenotyping paradigm

Male and female KO and WT mice were adapted to feeding powdered mouse 5015 chow in glass jars for a 4-week baseline period from 8 to 12 weeks of age. Food intake of chow diet was measured every two to three days. At 12 weeks of age, the mice were stratified for by gender into 4 weight-matched groups (males and females, high-fat diet + Ensure and chow).

HF diet consumption was measured periodically for 2–3 days over an 8-week period. Body weights were recorded on the same days as food intake was measured and continued to be measured twice weekly until the end of the 16-week experiment. Body composition was first determined in all mice at 10 weeks of age using nuclear magnetic resonance (NMR) spectroscopy (Minispec, Brucker Optics, TX) and again at 16, 18, 20, 24 and 28 weeks of age.

During the last week of palatable diet or chow exposure, the serological response of mice to a glucose challenge was determined in 16 h food- (and Ensure — as appropriate) deprived animals.

2.4. Measurement of food intake

Powdered chow or HF diet was presented in glass jars with spill-proof lids. On given days, food intake was measured by weighing the jars at the beginning and end of a 24 h period, around 09:00, to the nearest 0.05 g. Spillage was low and if it did occur was subtracted from the intake. The HF diet was changed every other day for freshness.

Chocolate-flavored Ensure was presented in 50 ml glass cylinders with a rubber stopper at one end and a sipper stainless steel sipper tube at the other end. To measure intake, drinking tubes were weighed at the beginning and end of a 24 h period to the nearest 0.05 g. The initial dripping from the spout when the tubes were inserted into the cages was corrected for. Intakes of both diets were expressed in kcal using the energy density provided by the supplier and estimated to a per mouse basis by dividing the group intake by the number of mice in each cage.

2.5. Measurement of body composition

Fat mass and fat free mass was measured with whole body NMR spectroscopy (Minispec, Brucker Optics, The Woodlands, TX). The average of three successive fat mass measurements was taken. Body weight was measured independently and adiposity determined (mean fat mass divided by body weight expressed as a percentage). Fat free mass was calculated

as the body weight minus fat mass. All biometric parameters in the text are presented as mean ± S.E.M.

2.6. Glucose tolerance test

Blood glucose was measured from a baseline (0 min) blood sample (~1 μl) was taken from overnight (16–17 h) food- and Ensure-deprived mice from a nicked tail using a Glucometer (Lifescan One Touch Ultra, Lifescan, Milipitas, CA). Immediately thereafter, glucose (1.5 mg/gm body weight of α-D glucose from a 150 mg/ml stock solution) was injected intra-peritoneally. Blood glucose was measured from the tail at 10, 30, 60, and 120 min after glucose injection.

2.7. Genotyping

Genomic DNA from all F2 mice was prepared from tail snips after Proteinase K digestion and phenol–chloroform extraction. Two independent PCR assays were used to genotype at the *Oprm1* locus to distinguish between wild-type (WT, $Oprm1^{+/+}$), mutant (KO, $Oprm1^{-/-}$) and heterozygous ($Oprm1^{+/-}$) mice. One PCR assay amplifies a 492 bp amplicon from Neomycin (Neo) gene associated with the targeted $Oprm1^{tm1Loh}$ allele. The second PCR assay amplifies a 550 bp amplicon derived from the wild-type, but not the targeted *Oprm1* gene. KO mutant mice, therefore, were identified after detection of the Neo gene and no amplification of the *Oprm1* gene fragment. WT mice were scored on the basis of amplification of the *Oprm1* gene fragment and no amplification of the Neo gene fragment. Heterozygous mice could generate both neo and *Oprm1* amplicons after PCR. Primer oligonucleotide sequences used for Neo amplification were 5′-AGGATCTCCTGTCAT CTCACCTTGCTCCTG-3′ and 5′-AAGAACTCGTCAAGAAGGGCGATAGAAGGCG-3′. Primer oligonucleotide sequences used to amplify the *Oprm1* gene fragment were 5′-AGGAGCTTGCTATC TTCT-GTA-3′ and 5′-CTATAAAAGGGCAAGCCTGAC-3′. PCR amplicons were visualized by gel electrophoresis on 4% NuSieve agarose gels and ethidium bromide staining.

3. Results

3.1. Food intake

There was a small but significant difference in chow intake during the 4-week long baseline period, with the KO mice eating less than WT mice (15.06 ± 0.22 vs. 17.17 ± 0.33 kcal/day; $n=9$ for each group, $P<0.05$). Although a trend for reduced food intake was also observed in KO females relative to WT females (16.13 ± 1.48 vs. 16.65 ± 0.52 kcal/day, respectively; $n=6$ for each group), this difference was not statistically significant.

The mean energy intake during HF + Ensure feeding measured over the first 8 weeks of the dietary obesity experiment revealed significant differences in dietary preferences and continued sexual dimorphism dependent on the *Oprm1* genotype. Although the overall total kcal/day energy intake of male KO and WT mice was not significantly different (14.28 ± 0.7 vs. 15.49 ± 0.74 kcal/day; Fig. 1); a significant difference was observed between these mice with respect to the relative amounts of liquid Ensure and solid HFD consumed. Male KO mice derived 5.65 ± 0.25 kcal/day of energy from Ensure consumption, significantly less than the 8.48 ± 0.48 kcal/day of energy consumed by WT males ($P<0.001$). The energy intake derived from the HF diet was also significantly different but in the opposite direction. Male KO mice derived significantly greater energy from the solid diet than did WT males (8.64 ± 0.53 vs. 7.06 ± 0.50; $P<0.05$). No significant differences were observed in either the total kcal or the individual consumption of Ensure or HF diet between WT and KO females (Fig. 1).

3.2. Body weight and composition

The body weight of male and female KO and WT mice did not significantly differ prior to exposure to the palatable HF + Ensure diet. However, at 10 weeks of age there was a significant increase in fat mass and adiposity observed in KO males relative to WT males (Table 1). Continued feeding of chow diet to a

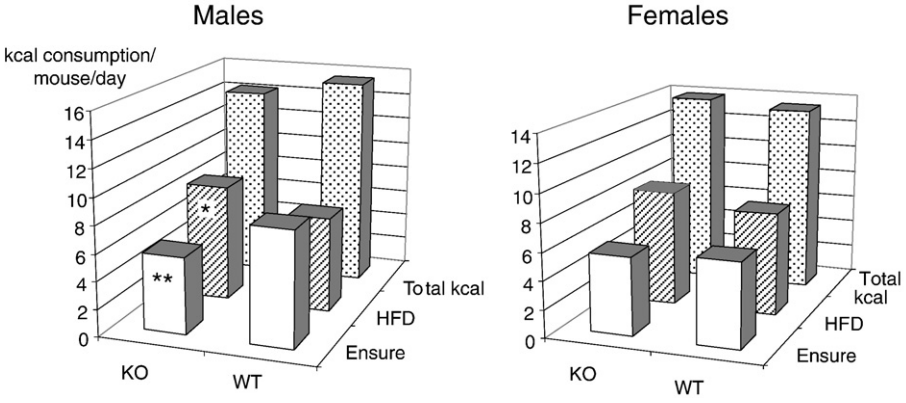

Fig. 1. Mean daily kcal intake for KO and WT males and females. Data are presented as mean intake measured over an eight week period from the start of high-fat (HF) + Ensure feeding when the mice were 12 weeks of age. Ensure data (kcal consumption) are shown as the white bars, HF diet intake (kcal consumption) are shown as diagonal stripped bars and the total kcal consumption (HF diet kcal + Ensure kcal) are shown as the stippled bars. Symbols; asterisk '*' and '**' indicate $P<0.05$ and $P<0.01$, respectively, between the two genotypic groups.

Table 1
Body weight and composition of male and female KO and WT mice at 10 weeks of age

	Sex	KO	WT	P
		N=12	N=9	
Body weight (g)	Male	29.62±0.80	28.38±0.49	ns
Fat mass (g)	Male	2.86±0.33	1.53±0.27	0.006
Fat free mass (g)	Male	26.75±0.60	26.84±0.42	ns
Adiposity (%)	Male	9.50±0.89	5.35±0.86	0.003
		N=12	N=7	
Body weight (g)	Female	23.51±0.39	22.80±0.7	ns
Fat mass (g)	Female	1.97±0.16	1.95±0.12	ns
Fat free mass (g)	Female	21.54±0.39	20.85±0.66	ns
Adiposity (%)	Female	8.37±0.65	8.58±0.50	ns

Mice were weaned at 4 weeks, group housed and maintained on 5015 chow diet. Data are presented as mean±S.E.M. Symbols; *N*, identifies the number of animals in each group; *P*, probability, identifies the significance associated between the two genotypic groups; ns, not significant, indicates a *P* value>0.05.

subset of these mice to 28 weeks of age revealed that the difference in adiposity (attributable to differences in fat mass) persisted but was only statistically significant at 10 and 16 weeks of age (Fig. 2). The basis for the lack of significance at later time points is due to the increased variance of fat mass (and adiposity) from the three WT males associated with this group (*N*=6 for the KO chow fed males). Despite these differences in body composition, no significant differences in body weight were observed between chow fed KO and WT male mice.

In contrast to the observed differences in fat mass and adiposity in 10-week old KO and WT males, there were no differences in body composition seen in mutant and wild-type females. Continued chow feeding of these mice (*N*=5 for KO and *N*=3 for WT) revealed no significant differences in body weight or fat free mass but significant differences in fat mass and adiposity were seen between the two genotypic groups at 16, 18, 20, 24 and 28 weeks of age (Fig. 2). The fat mass of KO females is 1.5–2.2 fold greater than in WT females.

During 16 weeks of exposure to the HF+Ensure diets, KO and WT mice of both genders gained a significantly greater amount of weight and fat mass as compared to chow fed mice (Fig. 2). Despite differences in fat mass and adiposity observed between KO and WT chow fed mice, no significant differences in body weight or adiposity are seen between the two genotypic groups upon feeding with the palatable diet. After 16 weeks of HF+Ensure feeding, the body weights of KO and WT males reached 47.7±1.8 g and 51±1.8 g, respectively (*N*=6 for both groups). The fat mass was determined to be 15.1±1.2 g and 16.2±0.8, respectively in the same mice.

In mutant and wild-type females, a similar observation was seen. After 16 weeks of HF+Ensure feeding, the body weights of KO and WT females were 37.3±3.5 (*N*=7) and 39.6±4.0 (*N*=4), respectively. The fat mass in the same mice was 12.0±2.8 and 14.6±2.8 g, respectively.

Given the observed differences in body composition apparent in chow fed mice and to better determine the selective effects of palatable diets on the *Oprm1*$^{-/-}$ KO genotype, we expressed the changes in body weight and body composition of

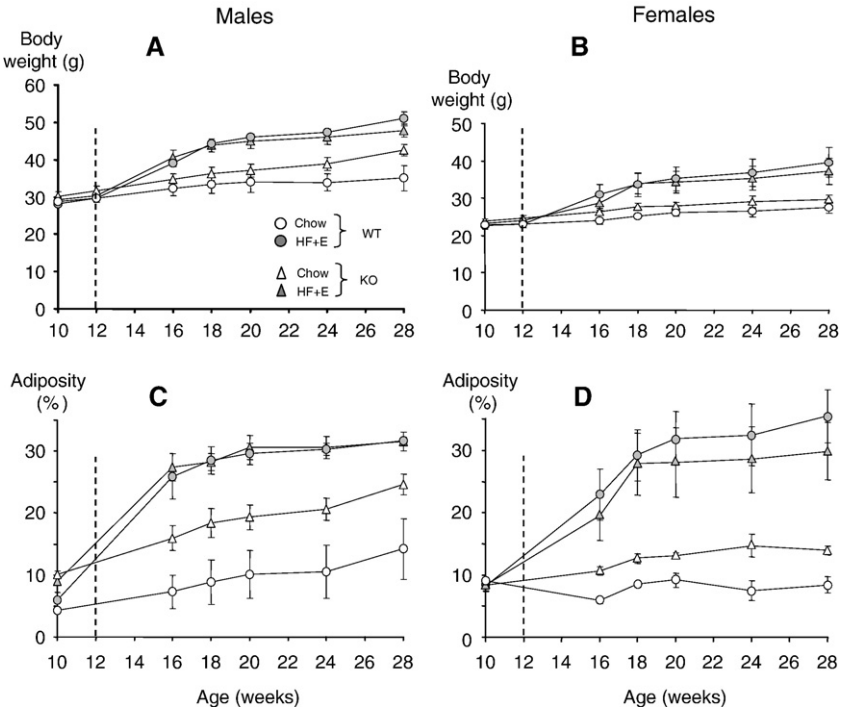

Fig. 2. Body weight and adiposity of male (A, C) and female (B, D), KO mutant (triangles) and WT (circles) mice fed chow diet throughout the 28-week period (open symbols) or exposed to a palatable diet consisting of high-fat pellets and liquid chocolate Ensure for 16 weeks from week 12 (dashed line) onwards (filled symbols). Data are presented as mean±S.E.M. Chow groups: male KO (*n*=6), male WT (*n*=3), female KO (*n*=5), female WT (*n*=3); Palatable (HF+Ensure) diet groups: male KO (*n*=6), male WT (*n*=6), female KO (*n*=7), female WT (*n*=4).

the HF+Ensure mouse genotypic groups as a percentage of the age-matched baseline (chow) values (Fig. 3).

This analysis revealed that male KO mice gained significantly less body weight, fat mass and adiposity than WT males on the palatable diet. The significant difference in fat mass- and adiposity-gain first seen after 4 weeks of HF+Ensure feeding precedes the difference in body weight gain seen after a further 4 weeks of exposure to the palatable diet. There is no significant difference in fat free mass throughout this feeding study except that after 16 weeks of HF+Ensure feeding, the KO males showed significantly less relative fat free mass gain than wild-type males.

In female mice, we observed a greater degree of variance possibly attributable to the smaller group size ($N=4$) of the WT female HF+Ensure group and the inherent epigenetic variability associated with high-fat feeding of mice (Koza et al., 2006). Thus, no significant difference in HF+Ensure-specific relative gain was seen for body weight, fat mass or fat free mass. Upon calculation of relative adiposity gains, a significant difference was observed in female mice that were exposed to the palatable diet for 12 and 16 weeks. The data suggest that HF+Ensure induced adiposity gain in female KO mice is significantly reduced relative to WT females and that this difference is delayed relative to the difference seen in male KO and WT mice (Fig. 3).

3.3. Glucose tolerance

There is no significant difference in circulating glucose levels associated with the *Oprm1* genotype in overnight fasted male or female mice on either HFD+Ensure or chow diets after 15 weeks of dietary intervention (130±12.5 and 119.3±8.3 mg/dl, respectively in KO and WT HF+Ensure fed males and 134.7±18.6 and 110±37.3 mg/dl, respectively in KO and WT chow fed males; 103.9±8.8 and 123.5±3.1 mg/dl in KO and WT HF+Ensure fed females and 91.4±14.5 and 91.3±7.2 mg/dl, respectively in KO and WT females). However, upon a glucose challenge, significant differences in glucose clearance

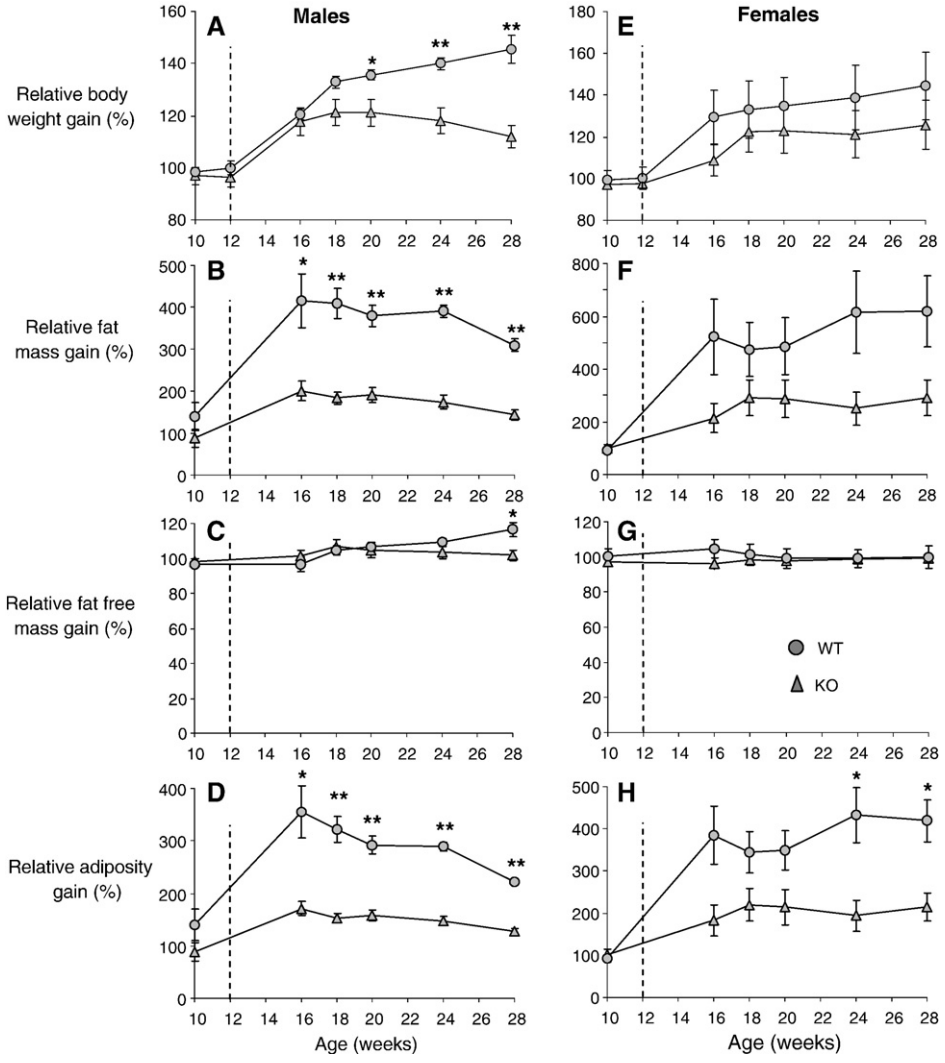

Fig. 3. Body weight and body composition responses to HF+Ensure diet exposure expressed as percent of continued chow feeding. Data are presented as mean±S.E.M. The relative effect of HF+Ensure feeding on body weight gain, fat mass, fat free mass and adiposity of male (panels A–D) and females (panels E–H) KO (triangles) and WT (circles) mice are shown. Male KO ($n=6$), male WT ($n=6$), female KO ($n=7$), female WT ($n=4$). Symbols; asterisk '*' and '**' indicate $P<0.05$ and $P<0.01$, respectively, based on ANOVA and adjusting for repeated measures.

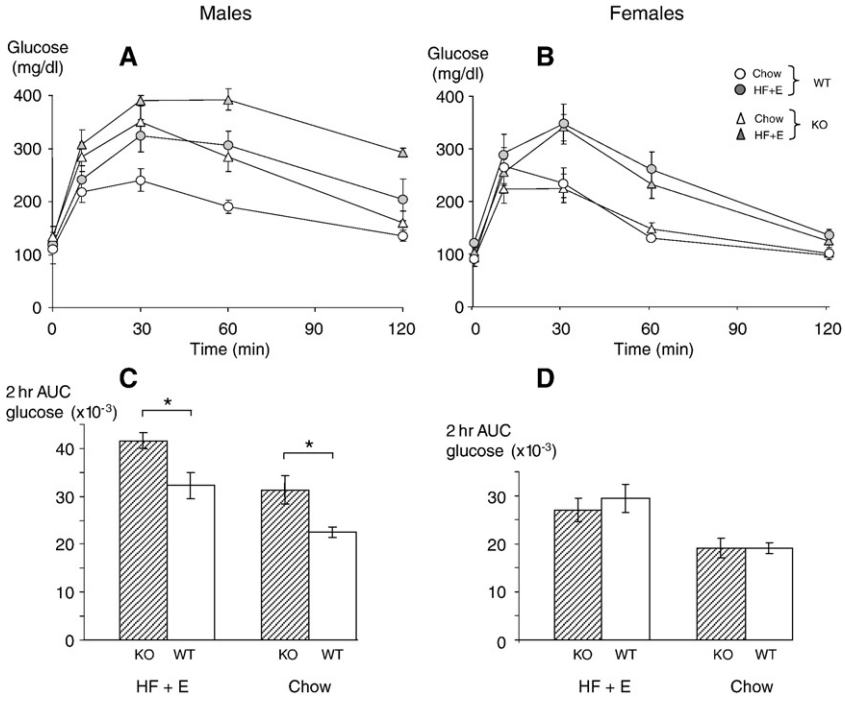

Fig. 4. Glucose clearance rates in male (panels A and C) and female (panels B and D) KO (triangles) and WT (circles) mice fed a chow (open symbols) or HF + Ensure (E) (filled symbols) measured after a glucose challenge. Data are presented as mean ± S.E.M. Glucose excursion is shown in panels A and B, and the 2 h area under the curve (AUC) is shown in panels C and D. Diagonal stripped bars identify KO mice and white bars identify WT mice. Asterisk '*' signifies $P<0.05$, between the indicated groups.

were observed between KO and WT male mice on both diets (Fig. 4). Whereas both male and female mice demonstrate the expected significant increases in glucose clearance associated with HF + Ensure feeding, the effect of the *Oprm1*$^{-/-}$ mutation is to significantly increase the time associated with glucose clearance in males on both chow and HF + Ensure diets. The glucose clearance rate of chow fed KO males is statistically equivalent to the glucose clearance rate of HF + Ensure fed WT mice (Fig. 4C). Although dietary effects on glucose clearance are also seen in female mice, there is no significant effect associated with the *Oprm1* genotype on either dietary feeding paradigm.

4. Discussion

4.1. Normal chow diet

Our finding that *Oprm1*-deficient KO mice, particularly males, on a regular chow diet show increased body weight in adulthood confirms an earlier observation by Han et al. (2006). In addition we show here that adiposity is almost 2-fold higher in chow fed KO as compared to WT male mice. This is surprising given the opposite observation by Tabarin et al. (2005) and the large body of literature supporting an appetite stimulating activity of mu-opioid receptor signaling. Using an independent mu-opioid receptor knockout model, these investigators reported no differences in body weight, adiposity, chow intake, and locomotor activity, but a slight difference in respiratory quotient during the light phase, indicating a shift to preferential fat oxidation in the fed state. Han et al. (2006) speculated that disinhibition of NPY mRNA in the arcuate nucleus might be responsible, but this hypothesis was not directly tested and if true, one would expect at least some increase in food intake. In the absence of differences in food intake in two of the studies (Han et al., 2006; Tabarin et al., 2005) and a slightly lower chow intake in males in our study, this seems an unlikely explanation. Increased adiposity in our study may result from changes in energy partitioning away from lipid oxidation and/or decreased energy expenditure through thermogenesis and/or physical activity. Such effects could result from absent mu-opioid signaling in autonomic output pathways (Nason and Mason, 2006; Tsuchida et al., 2004), primary afferents from the gut (Grundy et al., 2004), and peripheral tissues such as the gastrointestinal tract, adipose tissue, liver (Chakass et al., 2007), and muscle, which all express the mu-opioid receptor (Wittert et al., 1996). The discrepancy in this phenotype between the Tabarin et al. study on one hand, and Han et al. and our study on the other hand might be explained by differences in the genetic background, differences in the knockout strategy (see below), subtle differences in the experimental protocols, such as individual vs. group housing, or combinations of these differences. Tissue-specific knockouts will be necessary to dissect this paradoxical and disparate phenotype displayed on chow diet.

We have also looked at female mice and found that the same trend towards higher body weight and adiposity was present in female knockout mice, although less pronounced than in males, suggesting modulation of the underlying mechanism by steroid hormones.

4.2. Response to highly palatable diet

Because the majority of studies suggested that the opioid system is specifically involved in the control of palatable food intake (Bodnar, 2004; Levine and Billington, 2004; Yeomans and Gray, 2002), we hypothesized that mu-opioid receptor-deficiency would attenuate or even prevent the development of obesity induced by the highly palatable chocolate Ensure–high-fat diet. Our results demonstrate that it did not completely prevent, but significantly reduce diet-induced body weight and fat mass gain. It is clear that the body weight and adiposity responses to the palatable diet are confounded by the higher baseline levels in knockout compared to wild-type animals. However, the much smaller response to the diet is unlikely due to a simple ceiling effect, as obesity attributable to homozygosity for leptin deficiency (*Lep*^{ob} mutants) or heterozygosity at the A^{y} mutant allele in B6.Cg-*A*^{y}/+ mice can result in considerably higher body weight and adiposity than seen in high-fat diet+Ensure fed *Oprm1*-deficient mice, using identical methods to measure body composition in mice housed in the same vivarium (Zuberi, data not shown).

In contrast to the phenotype displayed on chow diet, these findings confirm earlier observations by Tabarin et al. in male mice (Tabarin et al., 2005) using a simple high-fat diet. During 10 weeks of high-fat feeding, their male WT mice increased body weight by about 7 g and adiposity by about 19%, while our male WT mice on the high-fat+Ensure diet increased body weight by about 16 g and adiposity by about 24%, demonstrating once again the obesigenic power of palatable diets. These dramatic increases were significantly and similarly blunted in male KO mice in both studies. The much greater increase in body weight of WT mice in our study suggests that the addition of chocolate-flavored Ensure further enhanced the obesigenic capacity of this high-fat diet.

Perhaps most surprisingly, both the present and the earlier study by Tabarin et al. did not find significant decreases in high-fat diet+Ensure food intake in mu-opioid receptor-deficient mice. Interestingly, we did observe a significant reduced food in KO males when feeding on chow diet that was not observed in KO females. Given the large body of literature implicating this receptor in appetitive, hedonic, and motivational aspects of food intake(Bodnar, 2004; Mathon et al., 2006; Papaleo et al., 2007), we expected a clear decrease in palatable food intake in KO animals. Male mutants did decrease intake of Ensure by 33%, but because they compensated by eating more high-fat diet, the total energy intake was almost the same in KO and WT animals. Tabarin et al. (2005) reported that high-fat diet intake in individually housed mice did not differ for mutant and wild-type mice (Tabarin et al., 2005). Thus, when considering the effects of total caloric intake of palatable diets on the weight gain and adiposity of mutant and wild-type mice, the feed efficiency (amount of calories consumed to gain a given amount of body weight) of mutant mice was reduced in both studies by about 50%. Our studies further support a role for the mu-opioid receptor in selecting between liquid sweet diets and solid high-fat pellets in male but not female mice.

Because gene expression of mitochondrial enzymes involved in fatty acid oxidation was increased in muscle of mutant male mice, at least some of the reduced food efficiency is likely caused by increased muscle fat oxidation and reduced deposition of fat into adipose tissue (Tabarin et al., 2005). However, since no changes in locomotor activity and daytime rectal body temperature were detected, it is not clear how the extra energy derived from this increased fat oxidation in muscle dissipated (Tabarin et al., 2005). It should be noted that no fat leakage in the stool was detected in either genotype, making it unlikely that the mu-opioid receptor regulates dietary fat absorption, thus altering the energy bioavailability of consumed foods.

The absence of effects on total palatable diet intake in mu-opioid receptor-deficient mice is in stark contrast to the outcome of chronic pharmacological studies in rats. In one study, energy intake was reduced throughout the 16 weeks of daily oral treatment with a non-selective opioid receptor antagonist (LY255582) in Long Evans rats, and pair-feeding recapitulated much of the decreased body weight and adiposity seen in drug-treated rats (Statnick et al., 2003). Other chronic pharmacological studies also point to decreased fat intake as the major primary effect (Cole et al., 1995; Mandenoff et al., 1982), and the anorectic response produced by another non-selective opioid receptor antagonist was diminished in mu-opioid receptor-deficient mice (Zhang et al., 2006).

The reasons for the discrepant outcomes of genetic vs. pharmacological manipulation are not known, but several explanations are possible. The pharmacological attempts to block mu-opioid receptor signaling are not very selective and even when only targeting the mu receptor may be unable to block the different receptor gene splice variants (Kvam et al., 2004; see discussion below). Blockade may also not be sustained at a sufficient level throughout the treatment period. On the other hand, genetic gene ablation may cause compensatory mechanisms to alter the basic properties of energy balance regulation. Eventually, only more selectively targeted and inducible knockout strategies will be able to reveal the complex actions of this receptor.

4.3. Effects on glucose clearance

Our data suggest that *Oprm1*-deficient males, but not females, are less able to respond to a glucose challenge than WT mice. As well as being sex-specific, the phenotype also appears to be independent of adiposity in that it is found in both chow fed and high-fat+Ensure fed animals and KO and WT males on the high palatability diet have similar fat mass or body weight. The mu-opioid receptor plays an important role in the regulation of glucose and insulin homeostasis. Activation of mu-opioid receptors improves insulin sensitivity in obese rats (Tzeng et al., 2007) and genetic loss of the receptors promotes insulin resistance in mice (Cheng et al., 2003). Polymorphisms in the *Oprm1* gene are associated with susceptibility to type 2 diabetes in one African–American population (Gallagher et al., 2006). Oral administration of metformin does not affect plasma glucose in *Oprm1*-deficient diabetic mice at 8–10 weeks of

age but does lower glucose levels by ~21% in wild-type diabetic mice. Additional experiments are necessary to determine if there is an *Oprm1* genotype effect on the circulating levels of insulin in chow and HF + Ensure fed mice to determine if the differences in glucose clearance are associated with altered insulin sensitivity or due to differences in glucose utilization. Interestingly, we find a sex-specific effect of the *Oprm1* genotype on glucose clearance that has not been reported before. Sex-specific effects of high-fat diet-induced obesity and diabetes are well-known. These data suggest an interaction between *Oprm1* and endocrine/hormonal differences between male and female mice.

4.4. Regulation of the μ-opioid receptor gene

The genetic regulation of the murine mu-opioid receptor gene, *Oprm1* is complex. To date, it contains 19 known alternatively spliced exons (Doyle et al., 2007a,b; Kvam et al., 2004) capable of expressing greater than 27 splice variants encoding at least 11 morphine-binding isoforms of the receptor. Differences are also apparent between the rodent and human *Oprm1* genes. One alternatively spliced exon (exon 19) contains a putative nuclear localization domain that is conserved between mouse and rat but is not conserved in humans suggesting altered activities associated with the protein between species. *Oprm1* is expressed centrally throughout the brain and ubiquitously in peripheral tissues, including the gut, pancreas, adipose tissue, liver and muscle (Su et al., 2002). What remains to be determined is whether the expression profile of the gene and its splice variants in different tissues is altered in a tissue-specific and dietary-dependent manner. Thus, the gene demonstrates considerable complexity in transcript regulation and tissue-specific function. The *Oprm1*^{tm1Loh} mutant allele is one of nine targeted alleles in the *Oprm1* gene (www.informatics.jax.org). This specific mutation deletes six of the seven transmembrane spanning regions of the protein, ablating protein expression and function in all tissues of the mouse. Thus, interpretation of the resulting phenotypes that arise in these mutant mice are confounded by the possible phenotypic contributions of *Oprm1*-deficiency in different tissues and on the tissue-specific expression of adaptive mechanisms that are induced as a consequence of specific mutant alleles.

In summary, our study in mu-opioid receptor-deficient mice did not find the expected effect on intake of palatable food, but a sizable attenuation of dietary-induced obesity. Because this receptor is so widely distributed, information of how it affects food reward and intake may be better obtained from the phenotyping of mutant mice derived from more selective tissue-specific loss of function mutagenic strategies.

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