Research article

Adrenalectomy stimulates hypothalamic proopiomelanocortin expression but does not correct diet-induced obesity

Hideo Makimura[1,2], Tooru M Mizuno[1,2], Joe Beasley[2,3], Jeffrey H Silverstein[2,3] and Charles V Mobbs*[1,2]

Address: [1]Fishberg Center for Neurobiology, Neurobiology of Aging Laboratories, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York, USA 10029, USA, [2]Department of Geriatrics and Adult Development, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York, 10029, USA and [3]Department of Anesthesiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, New York, 10029, USA

Email: Hideo Makimura - Hideo.Makimura@mssm.edu; Tooru M Mizuno - Tooru.Mizuno@mssm.edu; Joe Beasley - Joseph.Beasley@msnyuhealth.org; Jeffrey H Silverstein - Jeff.Silverstein@msnyuhealth.org; Charles V Mobbs* - Charles.Mobbs@mssm.edu

* Corresponding author

Abstract

Background: Elevated glucocorticoid production and reduced hypothalamic POMC mRNA can cause obese phenotypes. Conversely, adrenalectomy can reverse obese phenotypes caused by the absence of leptin, a model in which glucocorticoid production is elevated. Adrenalectomy also increases hypothalamic POMC mRNA in leptin-deficient mice. However most forms of human obesity do not appear to entail elevated plasma glucocorticoids. It is therefore not clear if reducing glucocorticoid production would be useful to treat these forms of obesity. We hypothesized that adrenalectomy would increase hypothalamic POMC mRNA and reverse obese phenotypes in obesity due to a high-fat diet as it does in obesity due to leptin deficiency.

Results: Retired breeder male mice were placed on a high-fat diet or a low-fat diet for two weeks, then adrenalectomized or sham-adrenalectomized. The high-fat diet increased body weight, adiposity, and plasma leptin, led to impaired glucose tolerance, and slightly stimulated hypothalamic proopiomelanocortin (POMC) expression. Adrenalectomy of mice on the high-fat diet significantly reduced plasma corticosterone and strikingly increased both pituitary and hypothalamic POMC mRNA, but failed to reduce body weight, adiposity or leptin, although slight improvements in glucose tolerance and metabolic rate were observed.

Conclusion: These data suggest that neither reduction of plasma glucocorticoid levels nor elevation of hypothalamic POMC expression is effective to significantly reverse diet-induced obesity.

Background

Chronically elevated cortisol levels caused by Cushing's syndrome leads to central obesity [1] while the reduction of cortisol caused by Addison's disease leads to decreased body weight [2]. Obesity in several rodent models is also associated with elevated corticosterone levels, and adrenalectomy reverses some of the phenotypes associated with these forms of obesity [3–7]. Furthermore, elevated corticosterone production can cause a metabolic syndrome [8]. These data suggest that reduction of glucocorticoid

production might therefore constitute a plausible clinical strategy to treat obesity. On the other hand, obesity in humans does not generally entail elevated plasma cortisol [9–13] and it is not clear how effective reduction of glucocorticoid production would be to reverse obesity if glucocorticoid production were otherwise normal. Nevertheless, pharmacological antagonism of glucocorticoid (type II) receptor by mifepristone (RU486) was reported to reverse high fat diet-induced obesity [14]. Since diet-induced obesity does not entail elevated glucocorticoid levels [15–17], this result suggests that reduction of glucocorticoid levels would reverse obesity even when glucocorticoid levels are not elevated.

Although the mechanisms through which adrenalectomy acts to reverse obesity is not entirely clear, it is plausible that the mechanisms entail enhanced hypothalamic POMC production [6]. Hypothalamic POMC mRNA is reduced in several forms of obesity [18–20]. Conversely, reversal of obese phenotypes by adrenalectomy is associated with a restoration of hypothalamic POMC mRNA tone in leptin-deficient *ob/ob* mice [6]. Furthermore, the melanocortin agonist MTII (which mimics the key anti-obesity product of POMC, alpha-MSH) reduces body weight in diet-induced obesity [21]. On the other hand, the effect of diet-induced obesity on hypothalamic POMC mRNA is somewhat complex and may depend on the genetic background [22,23], and duration of the diet [24,25] or composition [23] of the diet. Furthermore, the effect of adrenalectomy on hypothalamic POMC mRNA may also depend genetic background [6,26,27]. Nevertheless, we hypothesized that if adrenalectomy enhanced hypothalamic POMC mRNA in diet-induced obesity in mice, as in leptin-deficient mice, adrenalectomy would also reverse obese phenotypes in mice that became obese due to a high-fat diet. In the present study, we observed that adrenalectomy did indeed lead to a remarkable enhancement of hypothalamic POMC mRNA tone in mice made obese on a high-fat diet; surprisingly, however, adrenalectomy did not reverse diet-induced obesity.

Results
Effects diet and adrenalectomy on body weight, adipose weight and caloric intake
Prior to adrenalectomy, mice on the high-fat diet gained body weight about five-fold more rapidly than chow-fed mice in the first seven days on the high-fat diet (chow fed mice gained 0.68 ± 0.13 g compared to high-fat diet mice which gained 5.1 ± 0.2 g over the first seven days on the diet; $p < 0.05$). At the end of the five week period, mice on the high-fat diet, both sham-operated and adrenalectomized, exhibited a significant elevation of both body weight (Fig. 1A) and adipose weight (Fig. 1B) compared to the chow fed mice, both sham-operated and adrenalectomized. Although adrenalectomy initially caused a

greater loss of body weight in the diet-induced obese mice (high fat-diet sham-operated mice lost 1.2 ± 0.5 g seven days after surgery compared to diet adrenalectomized mice who lost 2.9 ± 0.3 g; $p < 0.05$), by two weeks after the surgery body weight in both groups had fully recovered body weight to pre-surgery levels. In the sham-operated mice, mass of food intake was significantly decreased in mice on the high fat diet compared to the chow-fed mice (Fig. 1C). Although the diet-induced obese mice consumed more (calculated) calories than chow-fed mice during the first week on the diet (during which the most rapid weight gain was observed), by the fifth week they were hypophagic both in terms of mass of food consumed (g/day) and in calculated calories consumed (calories/day) (the chow sham-operated mice consumed 16.1 ± 0.8 [calculated] cal/day compared to 12.9 ± 1.2 cal/day for the diet sham-operated mice; $p < 0.05$).

Surprisingly, adrenalectomy had no effect on body weight (Fig. 1A), adipose weight (Fig. 1B) or food intake (Fig. 1C) for mice on either the chow diet or the high fat diet, five weeks after initiation of the diet and three weeks after adrenalectomy.

Effects of diet and adrenalectomy on serum hormones, glucose tolerance and metabolic rate
Serum leptin was elevated in mice on the high-fat diet, concordant with the development of obesity, but adrenalectomy had no effect on leptin levels (Table 1). Neither insulin nor glucose was significantly influenced by either diet or adrenalectomy (Table 1). The high fat diet had no effect on serum corticosterone, but as expected adrenalectomy reduced serum corticosterone by over 70% in mice on both diets (Table 1). While this effect was significant, consistent with previous studies [6], adrenalectomy did not eliminate detectable corticosterone completely.

Glucose tolerance was strikingly impaired after only one week on the high fat diet (Fig. 2; $p < 0.0001$). Adrenalectomy slightly but significantly improved glucose tolerance on the high fat diet (Fig. 2; $p < 0.05$). Total heat production (not normalized to body weight) was significantly elevated by the high fat diet (Fig. 3A; $p < 0.0001$) and adrenalectomy led to a slight but statistical further increase in heat production in the diet-induced obese mice (Fib. 3B; $p < 0.002$). VO_2 (oxygen consumption normalized to body weight) was reduced in mice on the high fat diet (Fig. 4A; $p < 0.0001$) and as with total heat production adrenalectomy slightly but statistically increased VO_2 in the diet-induced obese mice group (Fig. 4B; $p < 0.05$). Therefore adrenalectomy led to a slight but statistical increase in metabolic rate as well as glucose tolerance in the diet-induced mice.

A.

B.

C.
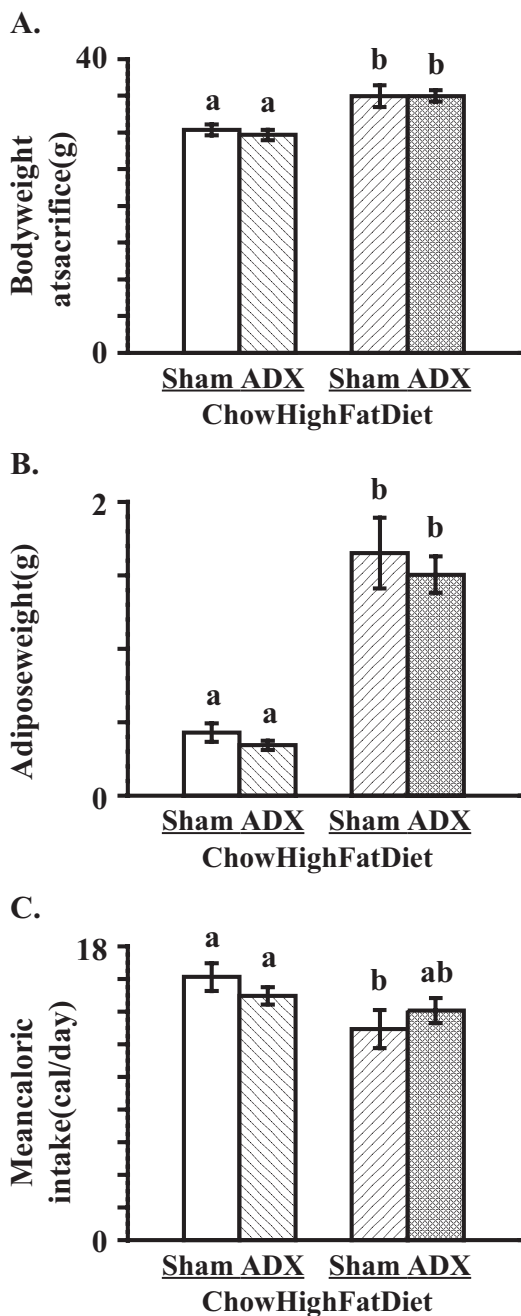
Figure 1
Effect of high fat diet and adrenalectomy on body weight, adipose weight and food intake. High fat diet increased both (A) body weight (g) and (B) epididymal white adipose weight (g); adrenalectomy did not significantly affect either parameter. High fat diet also decreased calculated daily caloric intake (calories/day) (C), and this was also not significantly influenced by adrenalectomy. Data are expressed as mean ± SEM. Groups with different letters are statistically different ($p < 0.05$) by ANOVA followed by Tukey-Kramer post hoc tests.

Effects of diet and adrenalectomy on pituitary POMC mRNA

Although diet did not influence pituitary POMC mRNA, adrenalectomy increased pituitary POMC expression in mice on both diets (Table 1). Together with the reduction in serum corticosterone, this induction of pituitary POMC indicates that the adrenalectomy produced a physiologically significant reduction in corticosterone tone.

Effects of diet and adrenalectomy on hypothalamic POMC mRNA

The high-fat diet led to a slight but significant increase in hypothalamic POMC mRNA in sham-operated mice (Figure 5A). Adrenalectomy produced a non-significant trend toward an increase in hypothalamic POMC mRNA in sham-operated mice, but a 5-fold induction of POMC mRNA in mice on the high-fat diet (Fig. 5A). The high-fat diet did not significantly influence hypothalamic AGRP in this study, but surprisingly adrenalectomy slightly stimulated hypothalamic AGPR mRNA levels (Fig. 5B).

Discussion

The high fat diet used in this study led to the development of obesity over the course of five weeks as expected (Fig. 1A). As observed in leptin-deficient *ob/ob* mice, adrenalectomy in diet-induced obese mice led to a striking increase in hypothalamic POMC mRNA (Fig. 5A). This elevation in hypothalamic POMC with only a very modest increase in the antagonist AGRP mRNA (Fig. 5B) would be expected to lead to significant amelioration of obese phenotypes, consistent with effects of adrenalectomy on other forms of obesity [3–7]. Indeed adrenalectomy initially led to a greater loss of body weight after surgery in the diet-induced obese mice (results in text above). Surprisingly, however, adrenalectomized mice regained all the weight lost after surgery (Fig. 1A), and adrenalectomy also failed to reduce food intake, adipose weight, or plasma leptin in the obese mice.

As has been observed in other models of obesity due to a high-fat diet, the diet-induced obese mice also became hypophagic compared to the chow fed mice and compared to the hyperphagia exhibited by mice in the first two weeks on a high fat diet [28] (Fig. 1C). The hypophagia of diet-induced obesity may be related to the modestly elevated hypothalamic POMC, whose product alpha-MSH is a known anorexic agent [20,29]. In turn, the induction of hypothalamic POMC by the high-fat diet might have been due in part to the elevation of leptin in diet-induced obese mice (Table 1). The further elevation in POMC expression observed after adrenalectomy of the obese mice could plausibly be due to elevated leptin combined with decreased glucocorticoids, since glucocorticoids may antagonize the effects leptin [30].

Table 1: Effect of high fat diet and adrenalectomy on serum hormones and pituitary POMC mRNA.

| Group	n	Glucose (mg/dl)	Insulin (ng/ml)	Corticosterone (ng/ml)	Leptin (ng/ml)	Pituitary POMC (%)
Chow/Sham	8	135 ± 7	1.7 ± 0.3	86 ± 14	1.8 ± 0.5	100 ± 38.7
Chow/ADX	10	148 ± 5	1.4 ± 0.3	11 ± 5.3*	1.6 ± 0.2	228 ± 36*
Diet/Sham	8	127 ± 13	1.9 ± 0.5	117 ± 23	11 ± 2.5*	154 ± 40
Diet/ADX	11	144 ± 6	1.6 ± 0.2	31 ± 7.0†	8.0 ± 1.5*	329 ± 50†

Effect of high fat diet and adrenalectomy on blood glucose (mg/dl), serum insulin (ng/ml), corticosterone (ng/ml), leptin peptide (ng/ml), and pituitary POMC mRNA (% chow sham-operated). Data are expressed as mean ± SEM. Groups with different symbols are statistically different (p < 0.05) by ANOVA followed by Tukey-Kramer post hoc test.

Tolerance to a glucose load was also impaired in mice after one week on the high-fat diet, suggesting the development of insulin resistance (Fig. 2). However, neither basal blood glucose nor serum insulin levels were elevated by the high fat diet at this time point (Table 1). We have observed in other studies that basal hyperinsulinemia is not detected until mice have been on the high-fat diet for at least two months (unpublished observations). Adrenalectomy led to only minor improvement in impaired glucose tolerance (Fig. 2) and it did not affect blood glucose or serum insulin levels (Table 1).

The key observation concerning food intake was that adrenalectomy did not significantly influence food intake on either diet. Mice on the high-fat diet ate less than the mice on the chow diet, both in terms of mass consumed and, surprisingly, in terms of total calculated caloric intake (Fig. 1C). Since caloric intake was calculated without normalization to body weight, for purposes of analyzing metabolic economy the most valid comparison of total caloric intake is to total caloric expenditure (total heat production) without normalization to body weight, as shown in Figure 3. Although the effect was small, the increase on the high-fat diet in total heat production without normalization to body weight was significant (statistical reliability derived in part from the many repeated measures, once every five minutes for 18 hours, that measuring metabolic rate entails). Similarly, the very small further increase in metabolic rate after adrenalectomy was significant. When metabolic rate was normalized to body weight, expressed as VO2 (Figure 4A), the effect of diet was to reduce this parameter, and adrenalectomy increased very slightly but significantly caused this measure of metabolic rate, normalized for body mass (Figure 4B). The effect of diet on normalized metabolic rate may be construed as suggesting that the failure of metabolic rate to scale proportionally to the increase in body weight on the high fat diet may have contributed to the maintenance of the obese state, especially since the calculated caloric intake on the high fat diet was less than on the chow fat diet.

The key conclusion to be drawn from the metabolic rate and food intake data is that effect of adrenalectomy on metabolic rate was marginal, and there was no effect of adrenalectomy on food intake, consistent with the lack of effect of adrenalectomy on other obese phenotypes. Nevertheless, with respect to the mechanism of diet-induced obesity, the present data seem to indicate that the diet-induced obese mice maintained their obesity despite apparently decreased caloric intake and increased caloric expenditure. Several explanations might account for this anomalous result. First, caloric intake is calculated from the caloric density as empirically measured and reported by the manufacturers of the diets; it remains possible that due to differences in these measurements, the true metabolically available calories may differ from these empirically measured calories. If so, it remains possible that the high-fat diet may actually include relatively more metabolically available calories than the chow diet, and thus that the mice on the high-fat diet in reality consumed more metabolically available calories than reflected by simple calculation of empirically determined caloric density. It is also possible that calories were absorbed more efficiently on the high-fat diet. In any case, none of these considerations compromise the basic observation that adrenalectomy failed to reverse diet-induced obesity.

Since adrenalectomy only reduced plasma corticosterone by about 70% in the obese mice, rather than completely eliminating the steroid from circulation, it remains possible that even further reduction of corticosterone would produce an effect on diet-induced obese phenotypes. On the other hand, using the same adrenalectomy protocol that produced a similar reduction, but not elimination, of plasma corticosterone levels, we observed a remarkable reversal of obese phenotypes in leptin-deficient *ob/ob* mice [6]. Furthermore, the reduction of corticosterone by adrenalectomy in the present study was sufficient to produce elevation of pituitary POMC mRNA (as well as hypothalamic POMC mRNA), demonstrating that the reduced levels were clearly producing physiological effects. Consistent with these results, high fat diet attenu-
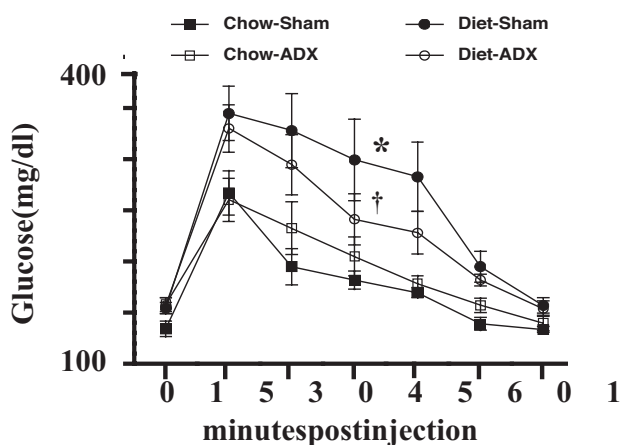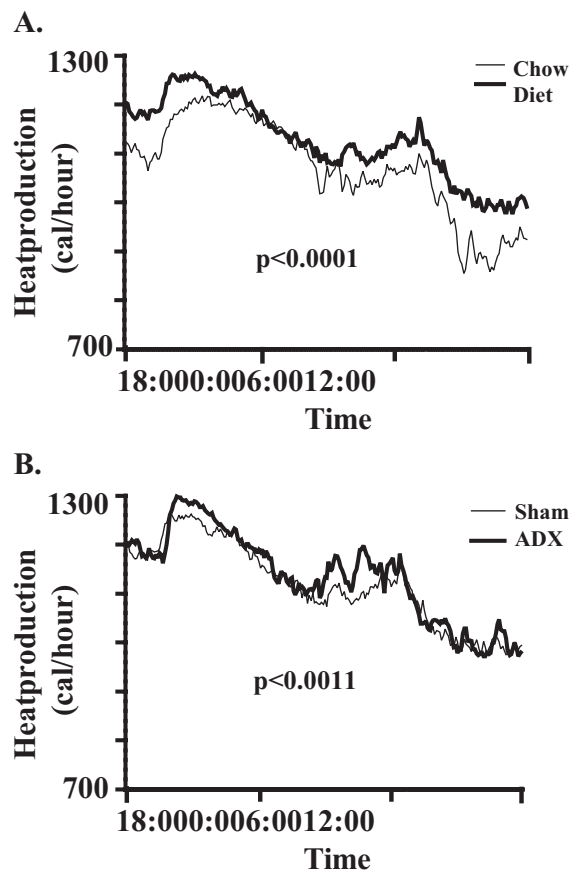
Figure 2
Effect of high fat diet and adrenalectomy on glucose tolerance. High fat diet led to impairments in glucose tolerance, which was slightly improved by adrenalectomy. *Sham-operated mice on a high fat diet exhibited significant impairments in glucose tolerance compared to chow sham-operated mice by ANOVA ($p < 0.0001$). †Adrenalectomized mice on a high-fat diet showed statistical improvements in glucose tolerance compared to diet sham-operated mice by ANOVA ($p < 0.05$). Data are expressed as mean ± SEM. Black square=chow sham-operated mice; white square=chow adrenalectomized mice; black circle=diet sham-operated mice; white circle=diet adrenalectomized mice.

Figure 3
Effect of high fat diet and adrenalectomy on heat production. Total heat production reflects metabolic rate without normalization to body weight. (A) High-fat diet increased heat production in the sham-operated mice (chow sham-operated vs. diet sham-operated) statistically by ANOVA ($p < 0.0001$). (B) Adrenalectomy increased heat production further in diet-induced obese mice (diet sham-operated vs. diet adrenalectomized) by ANOVA ($p < 0.002$). Data are plotted as an average, within each group, of every five-minute interval between 18:00 hours and 12:00 hours.

ates the effect of adrenalectomy to reverse obese phenotypes in *ob/ob* mice [31].

Conclusions

Diet-induced obesity led to significant increases in body weight and adiposity and to the development of glucose intolerance. Adrenalectomy reverses obese phenotypes including melanocortin tone in mice models of obesity in which plasma glucocorticoid levels are elevated. Consistent with results in ob/ob mice, adrenalectomy did stimulate hypothalamic POMC mRNA associated with marginal improvements in glucose tolerance and metabolic rate in mice made obese by the high-fat diet. However, these responses to adrenalectomy failed to reduce food intake, body weight, adiposity, or leptin in mice made obese by the high-fat diet, in which the obsese state was not associated with elevated glucocorticoid levels. Thus neither adrenalectomy nor remarkable enhance-

ment of hypothalamic POMC mRNA produced significant attenuation of the diet-induced obese phenotype. In contrast to other models of obesity, however, diet-induced obesity was not associated with either elevated glucocorticoid levels or reduced hypothalamic POMC mRNA. These data raise the possibility that potential anti-obesity drugs based on reducing glucocorticoid tone or elevating melanocortin tone may have limited efficacy in treating forms of obesity in which those systems are not perturbed.
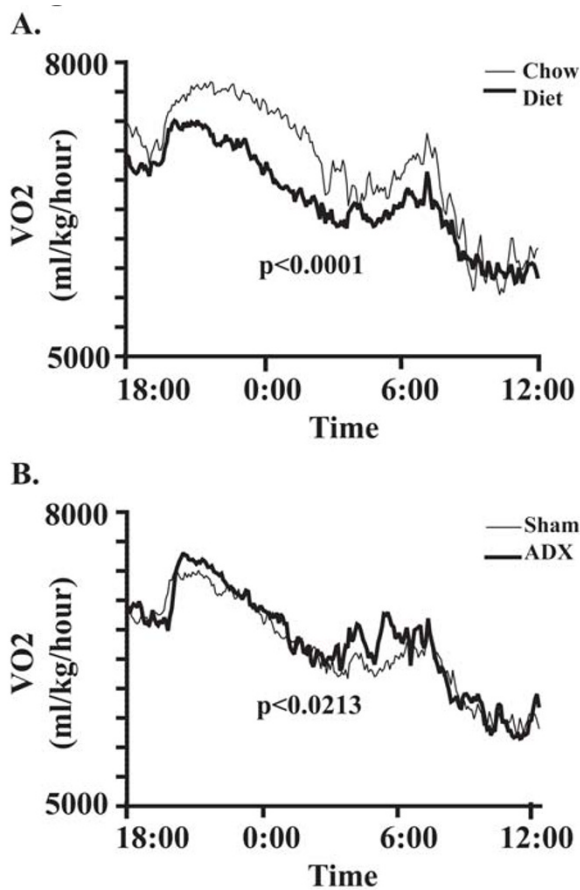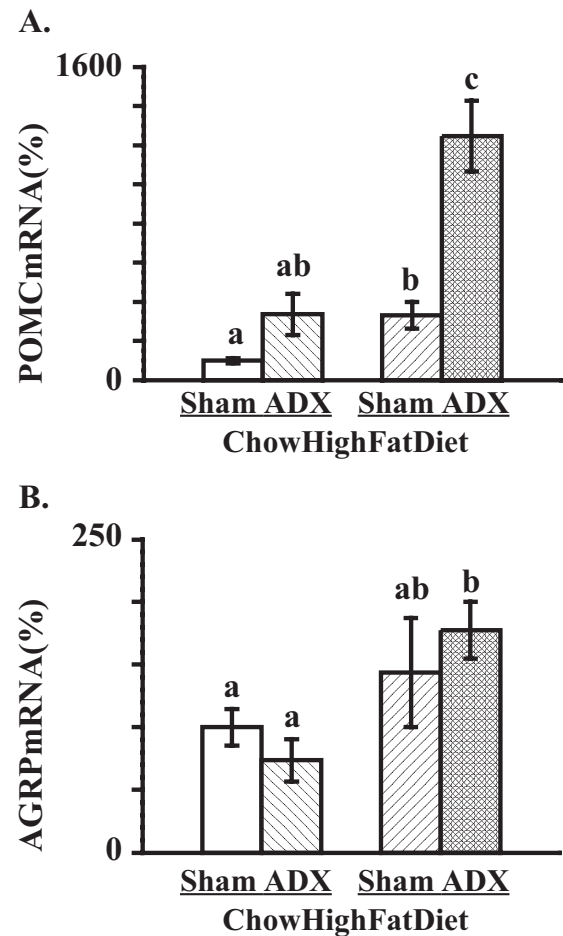
Figure 4
Effect of high fat diet and adrenalectomy on oxygen consumption. Oxygen consumption (VO_2) reflects metabolic rate normalized to body weight. (A) High-fat diet decreased VO_2 in the sham-operated mice (chow sham-operated vs. diet sham-operated) statistically by ANOVA ($p < 0.0001$). (B) Adrenalectomy increased VO_2 in diet-induced obese mice (diet sham-operated vs. diet adrenalectomized) by ANOVA ($p < 0.05$). Data are plotted as an average, within each group, of every five-minute interval between 18:00 hours and 12:00 hours.

Figure 5
Effect of high fat diet and adrenalectomy on hypothalamic POMC and AGRP mRNA. Hypothalamic (A) POMC and (B) AGRP mRNA as measured by Northern blot analysis. Data are expressed as mean percentage of chow sham-operated mice ± SEM. Groups with different letters are statistically different ($p < 0.05$) by ANOVA followed by Tukey-Kramer post hoc tests.

Methods
Animals
The appropriate Institutional Animal Review Board had approved all studies.

Male C57Bl/6J mice were obtained at 8 months of age from The Jackson Laboratory (Bar Harbor, ME) and were individually housed with free access to feed and water under 12:12 h light-dark cycle (lights on at 07:00 h). This age was chosen because body weight is stable at this age, facilitating interpretation of the expected opposing effects

of diet and adrenalectomy. The C57Bl/6J strain was chosen for susceptibility to obesity on a high fat diet [22,32–34]. Mice were placed on either a high fat diet (diet #D12492; Research Diets, New Brunswick, NJ) or regular rodent chow diet (diet #5053; Purina Mills, Richmond, IN) for a total of five weeks during which time both body weight and food intake was monitored daily. Cages were changed daily and a sieve was used to measure spilt food. The high fat diet (5.2 kcal/g) consisted of 20 kcal% protein, 20 kcal% carbohydrate and 60 kcal% fat, while the standard rodent chow diet (3.08 kcal/g) consisted of 23

kcal% protein, 64 kcal% carbohydrate and 11 kcal% fat. More specifically, the composition of the high fat diet was (per kg): casein, 80 mesh (200 g), L-cystine (3 g), maltodextrin 10 (125 g), sucrose (68.8 g), cellulose, BW200 (50 g), soybean oil (25 g), lard (245 g), mineral mix (10 g), dicalcium phosphate (13 g), calcium carbonate (5.5 g), potassium citrate (16.5 g), vitamin mix (10 g), choline bitartrate (2 g) and FD&C Blue Dye #1 (0.05 g).

After one week on their respective diets, tolerance to a glucose load was tested by injection of a glucose solution (2 mg/g body weight, i.p.) monitored for three hours after injection. Previous to the glucose tolerance test, food was removed from the cages for five hours from 09:00 to 14:00 hours. After two weeks on the high-fat diet, mice from both chow and high-fat diets were either bilaterally adrenalectomized or sham-operated as previously described [6]. All mice were then given one injection of dexamethasone (45 mg/kg body weight i.p.) to facilitate recovery from the surgery. The drinking water was replaced with normal saline (0.9% NaCl) to compensate for the loss of mineralocorticoids. Two weeks after the surgery when all mice had recovered body weight lost due to the stress from the surgery, another glucose tolerance test was performed, and after another week, all the mice were placed into metabolic cages in groups of eight in a balanced design. Metabolic rate was monitored continuously by indirect calorimetry for 18 hours as previously described [35,36]. Food and water were freely available in the metabolic cages. Briefly, air from each cage was sampled every five minutes and oxygen and carbon dioxide concentrations from each sample were measured independently. From the change in oxygen, VO_2 was calculated as oxygen consumption per hour normalized to body weight (ml/kg/hour). Heat production was then calculated using the following formula: Heat (cal/hour) = $((4.33+0.67 \times (VCO_2 /VO_2)) \times VO_2 \times (Body weight) \times 60)$. Therefore, the parameter VO_2 is normalized to body weight, whereas heat production is not normalized to body weight and so reflects total energy expenditure.

The mice were sacrificed toward the end of the light period at the end of the day in the metabolic cages (between 17:00 and 19:00 h) by decapitation after a brief exposure to carbon dioxide and trunk blood was collected; we have verified in pilot studies that this method of sacrifice does not increase plasma corticosterone above levels observed without carbon dioxide exposure. Brains for Northern blot analysis were quickly removed and the hypothalamus was dissected out, frozen on dry ice and stored at -70°C until use as described previously [Makimura, 2000 #5346; [Mizuno, 1999 #5302]. In addition, the anterior lobe of the pituitary was also removed and frozen on dry ice and stored at -70°C until use. Epididymal white adipose tissue was also removed, weighed

and frozen on dry ice and stored at -70°C until use. The four groups that resulted from this experimental design was as follows: chow sham-operated (n=8), chow adrenalectomized (n=10), diet sham-operated (n=8), and diet adrenalectomized (n=11). Successful adrenalectomy was verified by visual inspection at the time of sacrifice, as well as by the induction of pituitary proopiomelanocortin (POMC) mRNA levels and the reduction in serum corticosterone levels.

Blood chemistry
Blood glucose levels were measured by a Lifescan One-Touch II glucose meter (Johnson & Johnson, Mountain View, CA). Serum insulin and leptin peptide levels were assayed by ELISA with commercial kits (CRYSTAL CHEM INC., Chicago, IL) and serum corticosterone was assayed by RIA with a commercial kit (ICN Pharmaceuticals Inc., Costa Mesa Ca).

Northern blot analysis
Hypothalamic proopiomelanocortin (POMC) and agouti-related peptide (AGRP) mRNA and pituitary POMC mRNA were measured by Northern blot analysis as previously described [6]. Briefly, total RNA was extracted in TRIzol (GIBCO BRL, Gaithersburg, MD) and three micrograms of total RNA from hypothalamus, estimated by spectrophotometer and verified by gel electrophoresis, was subjected to Northern blot analysis to measure POMC and AGRP mRNA. Three micrograms of total RNA from anterior pituitary was used to measure pituitary POMC mRNA. Total RNA was denatured in a mixture of glyoxal and dimethyl sulfoxide for 1 hr at 50°C and electrophoresed for 60 minutes on a 10 mM sodium phosphate agarose gel. The RNA was transferred to an Immobilon membrane over night in 20 × standard saline citrate (SSC) by capillary action. The membrane was subsequently baked for several hours at 80°C and crosslinked with UV light at 33000 uJ/cm² energy. The membranes were prehybridized in a commercial Ultrahyb buffer (Ambion, Inc., Austin, TX) for 1 to 2 hours at 65°C. Hybridization was carried out in the same buffer with a single stranded internally labeled ³²P probe over night at 42°C. The membranes were washed in 1 × SSC/0.1% SDS for 15 minutes at room temperature twice followed by two more washes with 0.1 × SSC/0.1%SDS for 15 minutes at room temperature. Finally, the membrane was washed in 0.1 × SSC/0.1%SDS for 3 hours at 42°C, and exposed to a phosphoimager screen over night. The Northern blots were probed using single-stranded internally labeled DNA probes as described previously [6]. Membranes were reprobed and hybridized with ³²P-labeled probe encoding 18S ribosomal RNA and all signals were normalized to this 18S signal upon quantification. The total integrated densities of hybridization signals were determined by

phosphoimager (STORM 860, Molecular Dynamics, Sunnyvale, CA).

Statistical analysis

Statistical analysis was performed by a two-way analysis of variance (ANOVA) followed, when indicated by appropriate p-values (p < 0.05), by Tukey-Kramer post-hoc test, using the JMP statistical package implemented on the Macintosh operating system. For the glucose tolerance tests and metabolic rate measurements, a two-way ANOVA (treatment × time) was performed using the JMP statistical package.

Authors' contributions

Author 1 (HM) carried out the animal experiments, molecular assays, and statistical analysis and drafted the manuscript. Author 2 (TMM) participated in the animal surgeries. Author 3 (JB) carried out all the hormone assays. Author 4 (JHS) contributed to the hormone assays. Author 5 (CVM) conceived of the study, participated in the design and coordination of the studies, and helped draft the manuscript.

Acknowledgments

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