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Dynorphin Knockout Reduces Fat Mass and Increases Weight Loss during Fasting in Mice

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Endogenous opioids, particularly dynorphins, have been implicated in regulation of energy balance, but it is not known how they mediate this *in vivo*. We investigated energy homeostasis in dynorphin knockout mice (*Dyn*$^{-/-}$ mice) and probed the interactions between dynorphins and the neuropeptide Y (NPY) system. *Dyn*$^{-/-}$ mice were no different from wild types with regards to body weight and basal and fasting-induced food intake, but fecal output was increased, suggesting decreased nutrient absorption, and they had significantly less white fat and lost more weight during a 24-h fast. The neuroendocrine and thermal responses to fasting were at least as pronounced in *Dyn*$^{-/-}$ as in wild types, and there was no stimulatory effect of dynorphin knockout on 24-h energy expenditure (kilocalories of heat produced) or physical activity. However, *Dyn*$^{-/-}$ mice showed increased circulating concentrations of 3,4-dihydroxyphenlacetic acid and 3,4-dihydroxyphenylglycol, suggesting increased activity of the sympathetic nervous system. The respiratory exchange ratio of male but not female *Dyn*$^{-/-}$ mice was reduced, demonstrating increased fat oxidation. Interestingly, expression of the orexigenic acting NPY in the hypothalamic arcuate nucleus was reduced in *Dyn*$^{-/-}$ mice. However, fasting-induced increases in pre-prodynorphin expression in the arcuate nucleus, the paraventricular nucleus, and the ventromedial hypothalamus but not the lateral hypothalamus were abolished by deletion of Y_1 but not Y_2 receptors. Therefore, ablation of dynorphins results in increases in fatty acid oxidation in male mice, reductions in adiposity, and increased weight loss during fasting, possibly via increases in sympathetic activity, decreases in intestinal nutrient absorption, and interactions with the NPYergic system. (*Molecular Endocrinology* 21: 1722–1735, 2007)

THE WORLDWIDE PREVALENCE of obesity and type 2 diabetes are increasing at an alarming rate, necessitating urgent understanding of the mechanisms that regulate energy balance and glucose homeostasis in health and disease. A major site of regulation of these processes is the hypothalamus, mediated by numerous interacting orexigenic and anorexigenic molecules. The challenge is to know not only the identity and functions of each of the individual molecules involved but also to understand how these molecules interact.

Pharmacological and brain mapping studies suggest that the endogenous opioid system may be involved in the regulation of energy balance and glucose homeostasis. This system is composed of a family of three peptides: endorphins, enkephalins, and dynorphins, which act predominantly on μ, δ, and κ opioid receptors, respectively (1). Administration of opioid receptor agonists to humans and animals results in robust and sustained increases in food intake and body weight, whereas opioid receptor antagonists have the opposite effect (1). Opioids are thought to regulate glucose homeostasis via actions within the central nervous system (2, 3), albeit peripheral actions cannot be excluded because opioids have also been detected in the endocrine pancreas (4).

The dynorphins, a family of five peptides (dynorphin A, dynorphin B, dynorphin 32, α-neo-endorphin, and β-neo-endorphin) and their κ opioid receptors are strongly implicated in the effects of opioids on feeding

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Abbreviations: BAT, Brown adipose tissue; DHPG, 3,4-dihydroxyphenylglycol; DOPAC, 3,4-dihydroxyphenlacetic acid; DXA, dual-energy x-ray absorptiometry; *Dyn*$^{-/-}$, dynorphin knockout mice; NPY, neuropeptide Y; RER, respiratory exchange ratio; UCP-1, uncoupling protein 1; VO_2, oxygen consumption; WAT, white adipose tissue; WATi, inguinal white adipose tissue; WATm, mesenteric white adipose tissue.

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and body weight. Dynorphins are predominantly expressed within the central nervous system, and peripheral expression is limited to a few tissues such as the adrenal gland and testis (5), and dynorphin immunoreactivity has been demonstrated in rat pancreatic islets (4). Dynorphin expression has been detected within leptin-responsive neurons in the dorsomedial nucleus and the arcuate nucleus of the hypothalamus (6). Twenty-four-hour fasting is associated with a significant increase in expression of pre-prodynorphin mRNA in areas of the hypothalamus involved in energy homeostasis (7, 8). Moreover, the obesity syndrome of leptin-deficient *ob/ob* mice is associated with a 5-fold increase in dynorphin peptide level in the dorsomedial hypothalamus (9) and a significant increase in sensitivity to κ opioid receptor-preferring ligands with respect to glucoregulatory functions (10). Selective blockade of κ opioid receptors significantly reduces fasting-induced hyperphagia in rats (11) and reduces food intake and body weight in obese rodent models (12, 13). The specific and long-lasting κ opioid antagonist nor-binaltorphamine reduces food intake and body weight in *fa/fa* rats (13) and in rats with diet-induced obesity (12).

Although pharmacological studies and analysis of hypothalamic expression of dynorphins provide clues as to the role of these peptides, they lack the specificity and long-term effectiveness to definitively demonstrate the role of endogenous dynorphins in energy balance and glucose homeostasis *in vivo*. Moreover, most studies into the possible role of dynorphins in energy homeostasis have focused on food intake, which is only one of multiple determinants of energy balance. To address these questions, we generated pre-prodynorphin knockout mice that are deficient in all endogenous dynorphin opioids ("dynorphin knockouts") and then investigated feeding as well as hormonal and metabolic determinants of energy balance and glucose homeostasis under different conditions (chow diet, fasting, glucose or insulin injection, and high-fat feeding).

A question of major relevance to the possible role of dynorphins in long-term regulation of energy balance and glucose homeostasis *in vivo* is their interaction with other regulators of these processes, especially neuropeptide Y (NPY) and its five known receptors Y_1, Y_2, Y_4, Y_5, and Y_6 (14). Many factors with significant effects on energy balance act at least partially via interaction with the NPY system, notably leptin (15), ghrelin (16), glucocorticoids (17), and melanocortins (18). Some evidence suggests that the NPY system induces effects on energy balance in part through the κ opioid system. When NPY is injected into the paraventricular nucleus of rats, it induces hyperphagia and decreases uncoupling protein 1 (UCP-1) mRNA expression in brown adipose tissue (BAT), and these effects are partially or completely abolished by peripheral or central injection of specific κ opioid antagonists (19–21). Other evidence suggests the κ opioid and NPYergic systems induce effects on energy balance independently of each other, because intracerebroventricular administration of nor-binaltorphimine or a monoclonal antibody to NPY both inhibit fasting-induced hyperphagia, whereas coadministration of these substances results in additive inhibition of hyperphagia (11). To probe for functional interactions between dynorphins and the NPY system in the long-term regulation of energy homeostasis *in vivo*, we investigated hypothalamic expression of NPY in our dynorphin knockout mice ($Dyn^{-/-}$), as well as the impact of Y receptor knockout on the fasting-induced increase in pre-prodynorphin expression.

RESULTS

No Effect of Dynorphin Knockout on Breeding

The proportion of breeding pairs that produced live offspring was similar between genotypes (9 of 14 or 64.3% of $Dyn^{-/-}$ breeding pairs *vs.* 14 of 22 or 63.6% of wild-type breeding pairs), both genotypes produced litters at the same rate with similar numbers of pups, and there was no effect of genotype on the viability of pups born or the proportion of live male *vs.* female pups (data not shown).

Effect of Dynorphin Knockout on Body Weight and Food Intake

The body weight of male and female $Dyn^{-/-}$ mice from weaning to 14 wk of age was indistinguishable from that of wild-type mice (Fig. 1, A and B). However, when fasted for 24 h, male and female $Dyn^{-/-}$ mice lost significantly more weight than wild-type animals (Fig. 1, C and D). Actual weight loss was 4.11 $\pm$ 0.15 g in male knockouts *vs.* 3.42 $\pm$ 0.16 g in wild types (means $\pm$ SEM of at least 14 male mice per group; $P < 0.01$), and, in female knockout mice, it was 3.04 $\pm$ 0.15 *vs.* 2.65 $\pm$ 0.11 g in wild types (means $\pm$ SEM of at least 27 female mice per group; $P < 0.05$). Whereas the amount of food eaten (Fig. 1, E and F, *black columns*) and daily water intake (data not shown) by $Dyn^{-/-}$ mice was no different from that of wild-type mice, $Dyn^{-/-}$ mice of both genders had a significantly greater amount of ground, uneaten food spilled on the cage floor (Fig. 1, E and F, *white columns*). This shows that, although dynorphin knockout does not affect actual food intake, it significantly affects eating behavior. Whereas there was no effect of dynorphin knockout on daily fecal output in the 48 h of refeeding (data not shown), this parameter was significantly increased in both male and female $Dyn^{-/-}$ mice under nonfasted conditions (male knockouts, 1.67 $\pm$ 0.10 *vs.* 1.18 $\pm$ 0.09 g/d in wild types, means $\pm$ SEM of 7–13 mice per group, $P < 0.01$; female knockouts, 1.30 $\pm$ 0.09 *vs.* 0.98 $\pm$ 0.06 g/d in wild types, means $\pm$ SEM of six mice per group, $P < 0.05$). There was no evidence of loose feces or diarrhea in the knockouts. Together, these findings suggest that nutrient absorption was reduced by dynorphin knockout.

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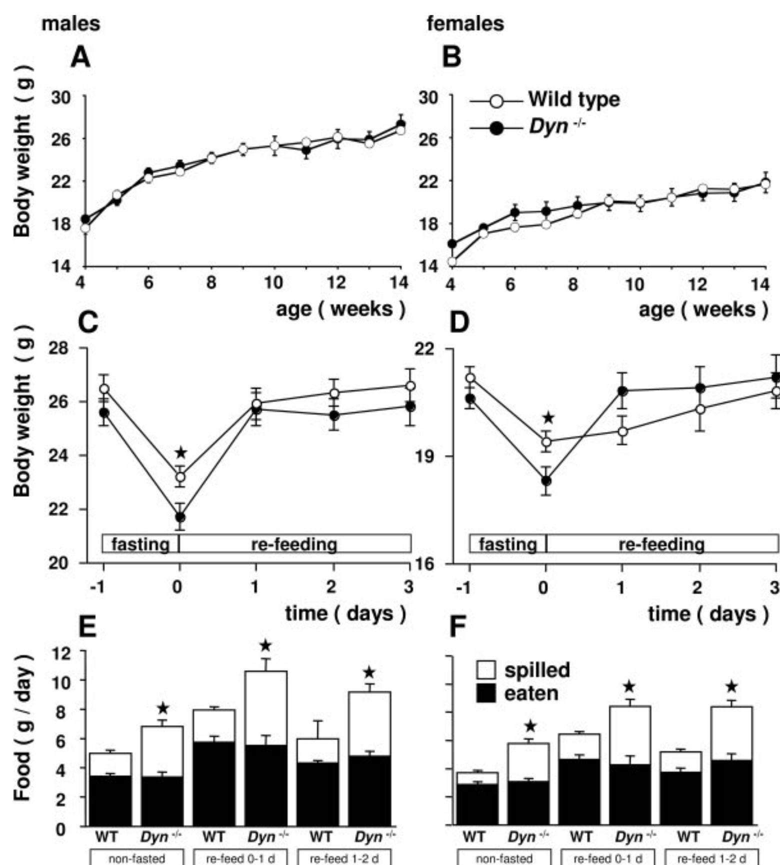

Fig. 1. Effect of Dynorphin Knockout on Basal and Fasting-Induced Changes in Body Weight and Food Intake

Body weight from weaning to 14 wk of age in male (A) and female (B) wild-type (WT) and dynorphin knockout mice (*Dyn*$^{-/-}$). Body weight and eating behavior in male (C and E) and female (D and F) wild-type and *Dyn*$^{-/-}$ mice in response to 24-h fasting and up to 72-h refeeding. Data are means ± SEM of at least 14 male and at least 27 female mice per group. *, $P < 0.05$ *vs.* same-sex wild-type control mice for body weight or food spillage at the corresponding time point.

Effect of Dynorphin Knockout on Body Composition

Under nonfasted conditions, the white adipose tissue (WAT) deposits of male and female *Dyn*$^{-/-}$ mice were significantly lighter than those of wild-type mice. This was true of visceral fat [mesenteric WAT (WATm)], as well as sc fat [inguinal WAT (WATi)], as seen from comparison of the *white columns* in Fig. 2A–D. In male but not female *Dyn*$^{-/-}$ mice, fasting reduced the weight of WATm and WATi depots to values significantly less than those of fasted wild-type animals (compare Fig. 2A–D, *black columns*). We chose to show data for WATm and WATi as examples of visceral and sc fat depots, but the same effect of dynorphin knockout was also observed for the epididymal or periovarian and the retroperitoneal WAT depots (data not shown). Figure 2E shows that female *Dyn*$^{-/-}$ mice have significantly less total body fat [as determined by dual x-ray absorptiometry (DXA)] than wild types, consistent with results obtained by weighing individual fat depots (Fig. 2, B and D). When fat masses (determined by dissection or DXA) from fasting mice were normalized to values in nonfasted mice of the same genotype and gender, there was no significant difference between genotypes (data not shown). This means that differences in fat mass between fasted dynorphin knockout and wild-type mice are attributable to dynorphin knockout mice having less fat overall, even in the nonfasted state. Under nonfasting conditions, there is no significant difference between female mice of either genotype with respect to total body lean mass (compare Fig. 2F, *white columns*). However, after 24-h fasting, lean mass was significantly reduced in female dynorphin knockout but not in female wild-type-mice (compare Fig. 2F, *black columns*), consistent with the greater fasting-induced decrease in body weight observed in the knockouts (Fig. 1D). When lean mass was normalized to body weight, there was no statistically significant effect of fasting or genotype (wild-type fed, 72.9 ± 1.1; wild-type fasted, 76.2 ± 1.4; *Dyn*$^{-/-}$ fed, 76.2 ± 1.8; *Dyn*$^{-/-}$ fasted, 75.0 ± 0.6% of body weight, means ± SEM of at least nine female mice per group), demonstrating that the reduced lean mass of fasted *Dyn*$^{-/-}$ mice (Fig. 2F) was proportional to the greater decrease in body weight in those animals (Fig. 1D). Our observation of reduced fat mass in nonfasted dynorphin knockout mice relative to wild-type litter-

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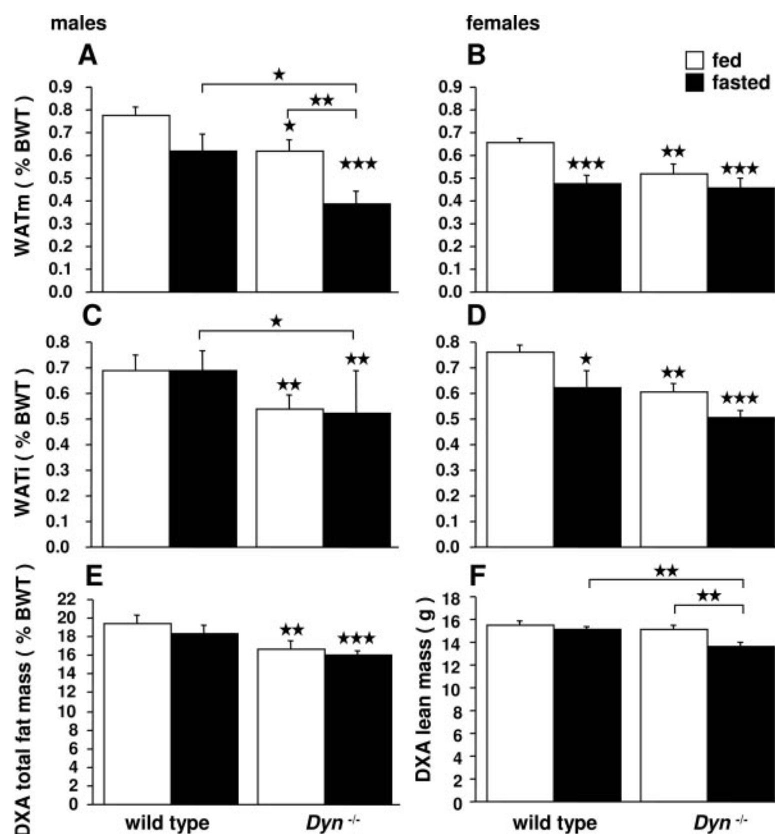

Fig. 2. Effect of Dynorphin Knockout on Basal and Fasting-Induced Changes in Body Composition

Weight [as a percentage of body weight (BWT)] of WATm and WATi deposits in male (A and C) and female (B and D) wild-type and dynorphin knockout mice ($Dyn^{-/-}$), either in the fed state or after 24-h fasting. Total body fat mass as a percentage of body weight (E) and total body lean mass (F) as determined by DXA in female wild-type and $Dyn^{-/-}$ mice either in the fed state or after 24-h fasting. Data are means ± SEM of at least six male mice and at least nine female mice per group. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs.* fed wild-type control mice of the same gender or the comparison shown by *horizontal bars*.

mates might appear at odds with the fact that body weight was not reduced in these mice. However, this apparent discrepancy may be reconciled by the marked and significant increase in total bone mineral content observed in $Dyn^{-/-}$ mice compared with wild-type littermates (our unpublished observations). These findings highlight the importance of measuring individual body compartments as we have done when assessing energy balance in mice, because differences in the mass of individual body compartments do not always translate to differences in gross body weight.

Effect of Dynorphin Knockout on the Neuroendocrine Response to Fasting

Fasting in normal animals is associated with neuroendocrine adaptations, notably activation of the hypothalamo-pituitary-adrenal axis, and inhibition of the hypothalamo-pituitary-thyroid, -somatotropic, and -gonadotropic axes (22–30). These hormonal changes contribute to energy economy in times of insufficient food intake. Under fasted conditions, male and female wild-type mice exhibited the expected increase in serum concentrations of corticosterone, and the expected decreases in circulating concentrations of free T_4 and IGF-I, as shown in Fig. 3A–F. In addition, fasting in male wild-type mice significantly reduced circulating concentrations of testosterone (data not shown). In male and female $Dyn^{-/-}$ mice, these hormonal responses to fasting were at least as pronounced as those observed in wild-type animals (Fig. 3A–F). These data suggest that the increased fasting-induced loss of body weight observed in dynorphin-deficient mice is not attributable to defects in fasting-induced activation or inhibition of these hypothalamo-pituitary axes. Interestingly, nonfasting serum corticosterone levels were significantly increased in male but not female $Dyn^{-/-}$ mice (Fig. 3, A and B).

Effect of Dynorphin Knockout on Physical Activity and Energy Metabolism

We investigated whether changes in physical activity or energy expenditure and thermogenesis could account for the decreased fat mass or body weight of dynorphin knockout mice under nonfasted or fasted conditions. There was no significant difference between genotypes with respect to oxygen consumption

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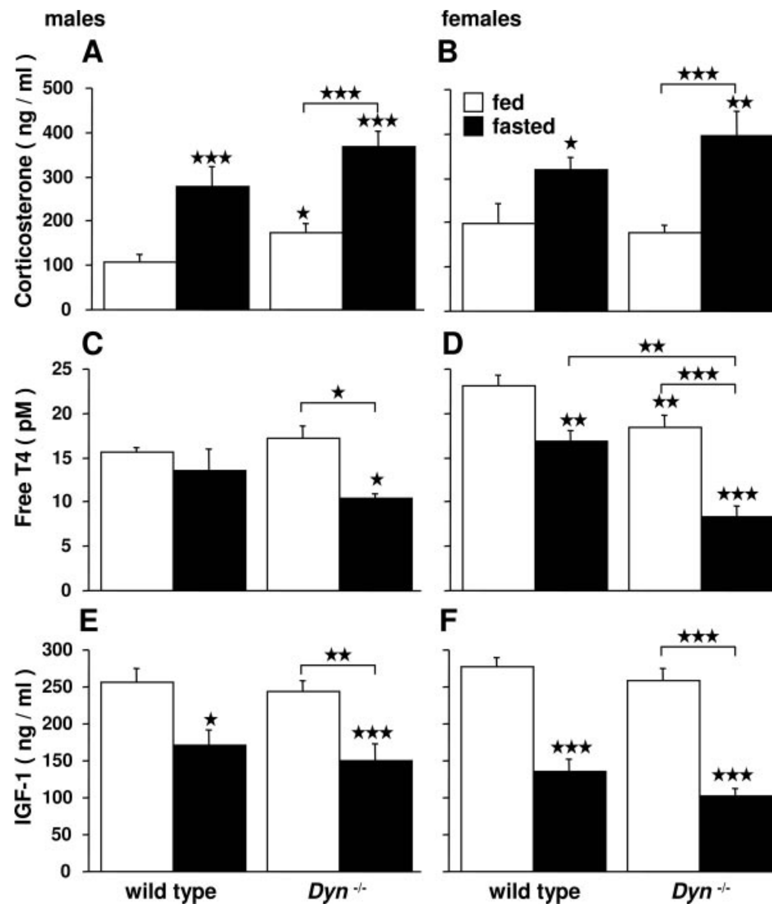

Fig. 3. Effect of Dynorphin Knockout on the Neuroendocrine Response to Fasting

Effect of 24-h fasting on serum concentrations of corticosterone (A and B), free T_4 (C and D), and IGF-I (E and F) in male (A, C, and E) and female (B, D, and F) wild-type and dynorphin knockout mice ($Dyn^{-/-}$). Data are means ± SEM of at least six male mice and at least 11 female mice per group. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ *vs.* fed wild-type control mice of the same gender or the comparison shown by *horizontal bars*.

(VO_2) as determined by indirect calorimetry (males, 3250 ± 50 ml O_2/24 h in $Dyn^{-/-}$ mice *vs.* 3120 ± 50 ml O_2/24 h in wild types; females, 3580 ± 120 ml O_2/24 h in $Dyn^{-/-}$ mice *vs.* 3600 ± 60 ml O_2/24 h in wild types; data are means ± SEM of at least eight male and at least seven female mice). There was no significant stimulatory effect of dynorphin knockout on energy expenditure (kilocalories of heat produced) (Fig. 4, A and B), with female $Dyn^{-/-}$ mice actually showing a significant decrease in heat production during the dark phase (Fig. 4B) ($P < 0.05$). Fasting led to a significant reduction in rectal temperature (by ~1.5 C) in male and female wild-type and dynorphin mice, and temperature was not significantly different between $Dyn^{-/-}$ and wild-type mice (data not shown), arguing against increased thermogenesis as a mechanism for the increased fat or weight loss in $Dyn^{-/-}$ mice. In keeping with this, there was no significant effect of dynorphin deficiency on the weight of interscapular BAT determined as an absolute weight or percentage of body weight in male or female mice (data not shown), and mRNA levels of UCP-1, expressed as a ratio of glyceraldehyde 3-phosphate dehydrogenase mRNA, in BAT were unaffected by dynorphin knockout (male wild type, 1.3 ± 0.1; male $Dyn^{-/-}$, 1.4 ± 0.1; female wild type, 1.9 ± 0.1; female $Dyn^{-/-}$, 1.8 ± 0.2; means ± SEM of four to five mice per group). Dynorphin knockout significantly reduced physical activity in female but not male mice, particularly during the dark phase (Fig. 4, C and D). Notably, the respiratory exchange ratio (RER) of male but not female $Dyn^{-/-}$ mice was significantly reduced compared with that measured in wild types, indicative of increased lipid as opposed to carbohydrate oxidation in the knockouts (31) (Fig. 4, E and F). Together, these data suggest that the decreased adiposity observed in male $Dyn^{-/-}$ mice may be attributable to increased fat oxidation.

Effect of Dynorphin Knockout on Glucose Homeostasis

Nonfasted and fasting-induced serum glucose concentrations in $Dyn^{-/-}$ mice were not significantly different from corresponding values in wild-type controls

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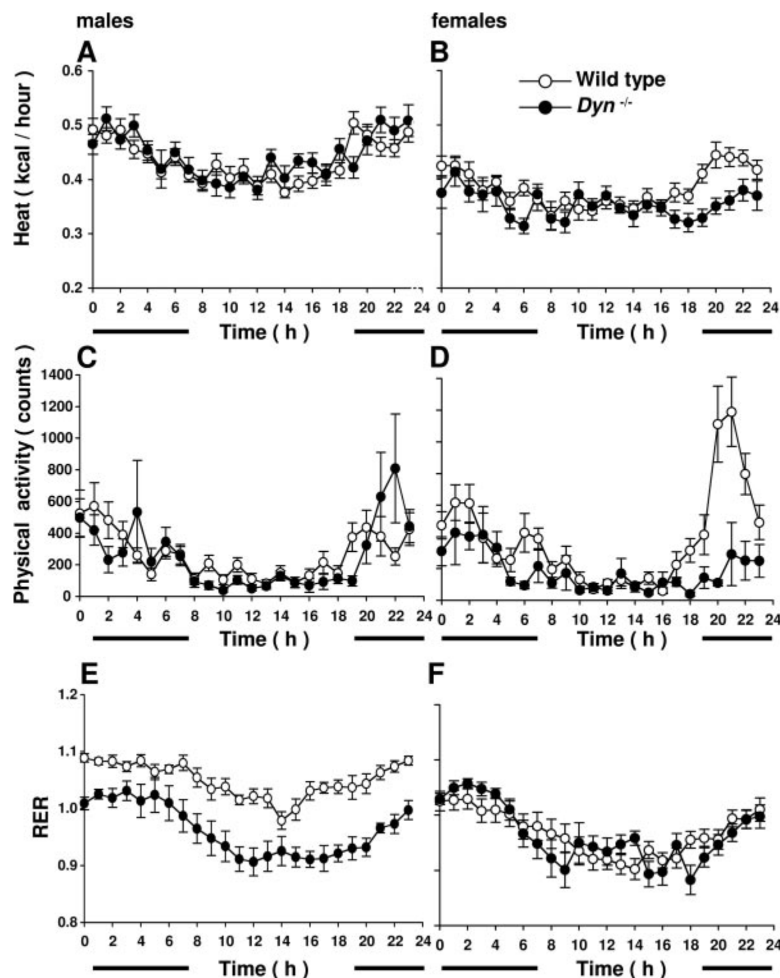

Fig. 4. Effect of Dynorphin Knockout on Energy Expenditure, Physical Activity, and RER

Energy expenditure (A and B), physical activity (C and D), and RER (E and F) over 24 h in male (A, C, and E) and female (B, D, and F) wild-type and dynorphin knockout mice ($Dyn^{-/-}$). Data are means ± SEM of at least eight male and at least seven female mice per group. *Horizontal bars* under x-axis represent dark phase.

(data not shown). After ip glucose injection, male and female dynorphin knockout mice were indistinguishable from wild types regarding dynamic changes in serum glucose (data not shown). There was no significant difference between genotypes with respect to area under the glucose response curves, either expressed as absolute values (males, 1260 ± 100 in knockouts *vs.* 1380 ± 50 mM/120 min in wild types; females, 1160 ± 60 in knockouts *vs.* 1120 ± 30 mM/120 min in wild types; means ± SEM of 8–12 male and 17–20 female mice per group) or normalized with respect to basal fasting serum glucose (males, 330 ± 40 in knockouts *vs.* 410 ± 50 mM/120 min in wild types; females, 200 ± 50 in knockouts *vs.* 310 ± 30 mM/120 min in wild types).

We also performed insulin tolerance tests because evidence suggests a role of opioids in the counterregulatory response to hypoglycemia (2, 3). There was no significant effect of genotype on basal (*i.e.* 5–6 h fasted) or ip insulin-induced glucose levels (Table 1). In wild-type mice, serum glucagon concentrations increased 2- to 4-fold by insulin injection (Table 1). Dynorphin knockout mice had fasting serum glucagon levels 3- to 4-fold lower than wild types, and insulin injection induced markedly significant increases in glucagon to values not significantly different from insulin-injected wild types (Table 1). There was no significant effect of insulin injection or genotype on circulating concentrations of corticosterone (Table 1) nor on the circulating concentrations of norepinephrine, epinephrine, or dopamine (data not shown). However, insulin injection led to significant increases in serum concentrations of 3,4-dihydroxyphenlacetic acid (DOPAC) (Table 1), the main product produced by oxidative deamination of dopamine, the precursor to norepinephrine and epinephrine (32). This finding is consistent with an insulin-induced increase in dopamine turnover. The stimulation of serum DOPAC levels by insulin was significantly more pronounced in $Dyn^{-/-}$ than in wild-type mice (Table 1). Serum concentrations of 3,4-dihydroxyphenylglycol (DHPG), the main product produced by oxidative deamination of noropineph-

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Table 1. Effect of Dynorphin Knockout on the Response to ip Insulin Injection

Parameter	Males				Females			
	Wild type		$Dyn^{-/-}$		Wild type		$Dyn^{-/-}$	
	Saline	Insulin	Saline	Insulin	Saline	Insulin	Saline	Insulin
Serum glucose (mM)	11.7 ± 1.1	4.7 ± 0.5[aaa]	10.2 ± 0.6	4.2 ± 0.6[aaa,bbb]	9.4 ± 0.6	3.1 ± 0.8[aaa]	9.2 ± 0.2	3.4 ± 0.6[aaa,bbb]
Serum glucagons (pM)	27.1 ± 6.2	54.0 ± 7.7	8.2 ± 3.7[a]	88.6 ± 25.0[a,bbb]	33.1 ± 21.4	136.3 ± 25.9[a]	7.0 ± 2.2	167.2 ± 55.8[a,bbb]
Serum cortico (ng/ml)	239 ± 28	358 ± 77	335 ± 41	432 ± 103	424 ± 28	379 ± 18	394 ± 9	581 ± 110
Serum DOPAC (nM)	19.4 ± 1.1	22.6 ± 0.7	20.6 ± 2.9	27.6 ± 3.0[b]	20.7 ± 0.4	32.9 ± 1.7[a]	28.7 ± 1.6	43.9 ± 7.2[aaa,bb,c]
Serum DHPG (nM)	1.85 ± 0.06	1.50 ± 0.14	2.43 ± 0.26	2.27 ± 0.43[c]	1.78 ± 0.18	1.48 ± 0.14	2.30 ± 0.11[a]	2.60 ± 0.21[aa,ccc]

Data are means ± SEM of three to five mice per group. cortico, Corticosterone. Mice were fasted 5–6 h before injection, and blood samples were taken 45 min later.
[a] $P < 0.05$, [aa] $P < 0.01$, [aaa] $P < 0.001$ *vs.* saline-injected wild-type mice of the same gender.
[b] $P < 0.05$, [bb] $P < 0.01$, [bbb] $P < 0.001$ *vs.* saline-injected $Dyn^{-/-}$ mice of the same gender.
[c] $P < 0.05$, [cc] $P < 0.05$, [ccc] $P < 0.001$ *vs.* insulin-injected wild-type mice of the same gender.

rine and epinephrine (32), were not significantly affected by insulin injection, but levels of this metabolite were significantly higher in knockout compared with wild-type mice (Table 1). Almost all DHPG in the circulation is produced by deamination of norepinephrine leaking from vesicles in sympathetic nerves (32), so increased serum levels of DHPG suggest increased turnover of norepinephrine in the sympathetic nervous system. Together, these data suggest activation of the sympathetic nervous system in $Dyn^{-/-}$ mice.

Effect of Dynorphin Knockout on Diet-Induced Obesity

We hypothesized that $Dyn^{-/-}$ mice might be less prone to diet-induced obesity than wild types. In wild-type and dynorphin knockout mice alike, consumption of the high-fat diet from weaning onward led to marked increases in daily caloric consumption, did not have any significant effect on body weight, but resulted in marked and significant increases in weight of all WAT deposits studied (illustrated by the increase in weight of the sum of WAT deposits shown in Table 2), as well as significant increases in BAT weight, serum leptin concentrations, and hepatic triglyceride concentration and content, with a tendency to reduced serum testosterone levels (Table 2). Interestingly, whereas dynorphin knockout mice on the chow diet spilled significantly more food on the cage floor than wild types, this spillage phenotype was not seen when animals were fed the high-fat diet (Table 2). In male but not female wild-type mice, the high-fat diet significantly increased nonfasting serum insulin levels, and this effect of dietary fat was not observed in $Dyn^{-/-}$ mice (Table 2). During glucose tolerance tests in high-fat-fed animals, dynamic changes in serum glucose levels were not significantly different between knockout and wild-type mice (data not shown), and there was no significant effect of genotype on area under the glucose response curves (males, 1290 ± 180 in knockouts *vs.* 1320 ± 40 mM/120 min in wild types; females, 1770 ± 150 in knockouts *vs.* 1450 ± 140 mM/120 min in wild types; means ± SEM of four to seven mice per group). Whereas fat feeding significantly increased serum free fatty acid concentrations in wild-type animals, no such increase was observed in fat-fed $Dyn^{-/-}$ mice (Table 2), suggesting decreased fatty acid output into the circulation or increased fatty acid oxidation, consistent with the reduced RER observed in male $Dyn^{-/-}$ mice (31) (Fig. 4E). Male but not female $Dyn^{-/-}$ mice showed significant increases relative to wild types in circulating concentrations of corticosterone under conditions of both chow and high-fat feeding (Table 2), consistent with data shown in Fig. 3, A and B, suggesting activation of the hypothalamo-pituitary-adrenal axis specifically in male knockouts. Fat feeding in $Dyn^{-/-}$ but not wild-type mice resulted in marked decreases in circulating concentrations of free T_4 and IGF-I, suggesting greater suppression of the hypothalamo-pitu-

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Table 2. Effect of a High-Fat Diet from Weaning to 14 wk of Age on Wild-Type and $Dyn^{-/-}$ Mice

Parameter	Males				Females			
	Wild type		$Dyn^{-/-}$		Wild type		$Dyn^{-/-}$	
	Chow-fed	Fat-fed	Chow-fed	Fat-fed	Chow-fed	Fat-fed	Chow-fed	Fat-fed
Food eaten (kcal/d)	7.4 ± 0.7	12.1 ± 0.8[aaa]	6.7 ± 0.7	9.9 ± 0.9[b]	6.3 ± 0.7	8.0 ± 0.7	6.6 ± 0.5	9.7 ± 0.6[a,b]
Food spilled (kcal/d)	5.0 ± 0.8	2.5 ± 0.4	10.6 ± 0.8[aaa]	2.9 ± 1.3[bbb]	3.4 ± 0.8	2.2 ± 0.2	8.6 ± 0.7[aaa]	1.2 ± 0.4[bbb]
Body weight (g)	26.4 ± 0.4	27.0 ± 0.6	25.6 ± 0.5	27.3 ± 1.0	21.0 ± 0.3	20.4 ± 0.5	20.7 ± 0.3	22.3 ± 1.0
Total WAT weight (% BWT)	2.38 ± 0.11	4.10 ± 0.22[aaa]	1.89 ± 0.11[a]	4.18 ± 0.34[aaa,bbb]	2.16 ± 0.07	3.18 ± 0.24[aaa]	1.80 ± 0.12[a]	4.39 ± 0.84[aaa,bbb,ccc]
BAT weight (% BWT)	0.30 ± 0.02	0.40 ± 0.03[aaa]	0.26 ± 0.01	0.42 ± 0.05[a,b]	0.29 ± 0.01	0.33 ± 0.02	0.28 ± 0.01	0.36 ± 0.02[bbb]
Serum leptin (ng/ml)	3.4 ± 0.3	8.6 ± 1.0[aaa]	3.0 ± 0.4	12.4 ± 2.6[aaa,bbb,c]	4.4 ± 0.2	8.3 ± 0.9[aaa]	5.6 ± 0.8	9.5 ± 0.8[aaa,bb]
Serum insulin (pM)	154 ± 19	225 ± 19[a]	154 ± 12	158 ± 46	177 ± 28	170 ± 37	174 ± 28	136 ± 34
Hepatic triglyceride (mmol/g)	6.4 ± 0.6	22.6 ± 4.1[aaa]	8.8 ± 1.0	18.9 ± 2.3[aaa,bb]	14.7 ± 2.0	36.3 ± 6.4[aa]	20.1 ± 2.9	51.9 ± 12.1[aaa,bb]
Hepatic triglyceride (mmol/liver)	6.6 ± 0.8	25.2 ± 4.8[aaa]	8.8 ± 1.1	17.8 ± 2.3[aa,b]	12.9 ± 1.8	28.7 ± 5.5[a]	17.8 ± 2.6	38.2 ± 9.5[aa,b]
Serum free fatty acid (mM)	0.70 ± 0.09	1.36 ± 0.13[aaa]	0.66 ± 0.07	0.88 ± 0.06[c]	1.07 ± 0.06	1.69 ± 0.18[aaa]	1.04 ± 0.11	1.16 ± 0.10[c]
Serum cortico (ng/ml)	109 ± 16	113 ± 20	178 ± 25[a]	252 ± 72[aa,cc]	198 ± 45	224 ± 61	176 ± 17	217 ± 32
Serum-free T_4 (pM)	15.7 ± 0.5	16.2 ± 1.4	17.2 ± 1.3	11.4 ± 1.5	23.0 ± 1.3	22.0 ± 3.8	18.5 ± 1.2[a]	10.0 ± 2.0[aa,b,cc]
Serum IGF-I (ng/ml)	248 ± 20	249 ± 16	230 ± 20	122 ± 37[aaa,bb,ccc]	277 ± 12	237 ± 20	258 ± 16	179 ± 33[aa,b]
Serum testosterone (nM)	8.4 ± 4.8	2.7 ± 0.8	7.2 ± 4.2	3.3 ± 2.3	ND	ND	ND	ND

Data are means ± SEM of 6–14 mice per group. BWT, Body weight; cortico, corticosterone; ND, Not determined.

[a] $P < 0.05$, [aa] $P < 0.01$, [aaa] $P < 0.001$ *vs.* chow-fed wild-type mice of the same gender.

[b] $P < 0.05$, [bb] $P < 0.01$, [bbb] $P < 0.001$ *vs.* chow-fed $Dyn^{-/-}$ mice of the same gender.

[c] $P < 0.05$, [cc] $P < 0.01$, [ccc] $P < 0.001$ *vs.* fat-fed wild-type mice of the same gender.

itary-thyroid and -somatotropic axes by the high-fat diet, in keeping with data shown in Fig. 3, C and D, for free T_4 in response to fasting. Together, these findings show that dynorphin deficiency does not protect against diet-induced increases in caloric intake and fat deposition, although it does alter some of the effects of fat feeding on circulating free fatty acid concentrations and neuroendocrine status.

Crosstalk between the Dynorphin and the NPYergic System

We showed previously that pre-prodynorphin mRNA is coexpressed with NPY mRNA in the hypothalamic arcuate nucleus (33). To investigate functional relationships between dynorphin and the NPYergic system, we measured NPY mRNA expression in the hypothalamus of dynorphin knockout mice. As shown in Fig. 5, dynorphin knockout significantly reduced expression of NPY mRNA in the hypothalamic arcuate nucleus (77.6 ± 3.7% of wild-type values in dynorphin knockout mice *vs.* 100 ± 3.4% in wild types; means ± SEM of five to six mice per group; $P < 0.001$). There was no significant effect of dynorphin knockout on NPY mRNA expression in any other region of the hypothalamus (data not shown). Next we investigated whether the fasting-induced increase in hypothalamic pre-prodynorphin expression involves the action of receptors for NPY. Indeed, whereas fasting in wild-type mice led to an almost 2-fold increase in pre-prodynorphin mRNA levels in the arcuate nucleus, paraventricular nucleus, and ventromedial hypothalamus, this effect of fasting was completely abolished in Y_1 receptor knockout mice (Fig. 6, A–C). The involvement of Y receptors in fasting-induced increases in pre-prodynorphin mRNA expression is specific for Y_1 receptors, because Y_2 receptor knockout mice responded in a way indistinguishable from wild types (Fig. 6, A–C). In the lateral hypothalamus, the fasting-induced increase in prodynorphin mRNA expression was not affected by knockout of Y_1 or Y_2 receptors (Fig. 6D).

DISCUSSION

This work demonstrates that lack of dynorphin significantly reduces fat mass and leads to significantly greater weight loss during 24-h fasting. These effects may be attributable to an increased propensity to oxidize fat, because male dynorphin knockout mice exhibited significant decreases in RER relative to wild types. Additionally, high-fat feeding increased serum free fatty acid concentrations in wild-type but not in knockout mice, suggesting increased serum free fatty acid utilization. Dynorphin knockout may promote fat oxidation by stimulation of sympathetic activity, as indicated by increased serum concentrations of the catecholamine breakdown products DOPAC and DHPG. The lean phenotype of dynorphin knockout

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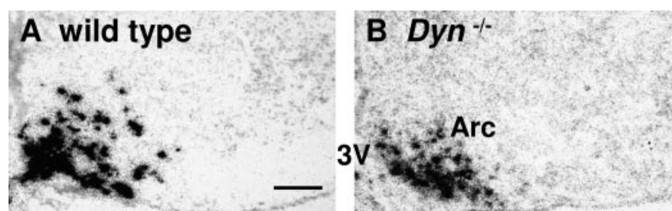

Fig. 5. Effect of Dynorphin Knockout on Hypothalamic NPY Expression

NPY mRNA levels in the hypothalamic arcuate nucleus of wild-type (A) *vs.* dynorphin knockout ($Dyn^{-/-}$; B) mice. *Scale bar*, 40 μm. 3V, Third cerebral ventricle; Arc, arcuate nucleus of the hypothalamus.

mice occurred in the absence of significant effects on basal- or fasting-induced feeding, without blunting of the neuroendocrine response to fasting, and in the absence of increases in physical activity, energy expenditure, or thermogenesis. However, it is possible that dynorphin knockout reduced the intestinal absorption of nutrients, because daily fecal output was significantly increased in dynorphin knockout mice, thereby contributing to negative energy balance and fat loss relative to wild-type littermates.

Our data support a long-term role of dynorphin in the regulation of the hypothalamo-pituitary-adrenal axis, because circulating corticosterone levels were significantly increased in male but not female $Dyn^{-/-}$ mice. Exogenous administration of dynorphin or a κ opioid receptor agonist to adult rats has been shown to increase plasma concentrations of ACTH and corticosterone, probably by an indirect mechanism such as stimulation of CRH release, as reviewed recently (34). In keeping with an inhibitory role of dynorphin on the hypothalamo-pituitary-adrenal axis, big dynorphin (a prodynorphin-derived peptide consisting of dynorphin A and dynorphin B) induced anxiolytic-like behavior (35) in male mice.

Besides increased serum concentrations of the catecholamine breakdown products DOPAC and DHPG, suggesting activation of the sympathetic nervous system, our finding of increased serum corticosterone levels suggests that $Dyn^{-/-}$ mice have generally increased stress responses. Dynorphin knockout could stimulate activity of the sympathetic nervous system via central and peripheral mechanisms. Intracerebroventricular injection of dynorphin decreases mean arterial pressure, and intracerebroventricular injection of the κ opioid receptor antagonist nor-binaltorphimine increases mean arterial pressure and renal sympathetic nerve activity, in keeping with central regulation of these sympathetically mediated processes (36). *In vitro*, dynorphin A blocks ischemia-induced release of noradrenaline from isolated cardiac mitochondrial/synaptosomal fractions via action at opioid receptors (37), suggesting peripheral mechanisms of action.

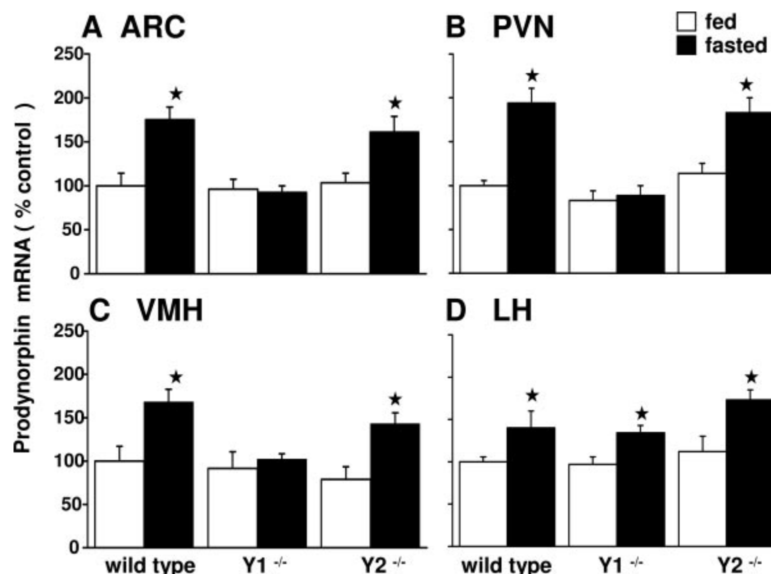

Fig. 6. Role of Y_1 and Y_2 Receptors in Fasting-Induced Increases in Hypothalamic Pre-Prodynorphin Expression

Effect of 24-h fasting in male wild-type, Y_1 receptor knockout ($Y1^{-/-}$), or Y_2 receptor knockout ($Y2^{-/-}$) mice on prodynorphin mRNA levels (expressed as a percentage of fed wild-type values) in the arcuate nucleus of the hypothalamus (ARC; A), the hypothalamic paraventricular nucleus (PVN; B), the ventromedial hypothalamus (VMH; C), and the lateral hypothalamus (LH; D). Data are means ± SEM of five to six mice per group. *, $P < 0.05$ *vs.* fed wild-type control mice.

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However, because dynorphins are predominantly expressed within the central nervous system and peripheral expression is limited to a few tissues such as the adrenal gland, testis (5), and pancreas (4), it is likely that dynorphin knockout affects sympathetic activity mainly via central mechanisms.

Opioid receptor agonism has been shown to stimulate the GH axis, whereas nonspecific opioid receptor blockade with naloxone or naltrexone inhibits it (38). Our findings show that effects of opioids on the GH axis may be mediated by the dynorphin system, because dynorphin knockout in male and female mice significantly reduced the serum concentrations of IGF-I (the main effector of the growth-promoting effects of GH) under high-fat-fed conditions. Despite this inhibition of serum IGF-I levels in fat-fed dynorphin knockout mice, there was no significant effect of knockout on serum IGF-I levels or lean body mass or body weight in nonfasted animals.

Although pharmacological studies have suggested a role of dynorphins or κ opioid receptors in the regulation of food intake (1, 11–13, 19–21), our data from dynorphin knockout mice argue against a long-term role of dynorphins in this process. Moreover, our demonstration of a lean phenotype in dynorphin knockout mice in the absence of decreased food intake illustrates an important concept: that food intake is not the only determinant of energy balance. Indeed, in diet- or stress-induced obesity in rats or mice, the decreased body weight gain observed after chronic opioid or κ opioid receptor blockade was disproportionately greater than the effects on food intake, which were modest (12) or nonsignificant (39). Collectively, these data show that long-term blockade of the dynorphin system can induce weight loss via effects independent of food intake.

Body weight or body fat cannot change unless there is an imbalance in energy dynamics (energy in *vs.* energy out), and this includes the amount of nutrients that are absorbed from food. The daily fecal output of male and female dynorphin knockout mice was greater than that of wild-type littermates, suggesting that nutrient absorption was reduced in these animals. Previous work has shown marked inhibition of intestinal transit (rate of transit) by opioids, an effect that appears to be mediated in the brain (40). Therefore, it is possible that dynorphin knockout decreased nutrient absorption by increasing intestinal transit and reducing the time that food spends in transit, thereby resulting in significantly increased fecal output and reduced body fat in the face of no effect on food intake and no stimulatory effects on energy expenditure. In keeping with this, it has been shown that interventions that increase intestinal transit (*i.e.* fiber feeding or treatment with methotrexate) lead to impaired nutrient absorption and a reduced ability to sustain body weight after a 24-h fast (41, 42).

Whereas dynorphin knockout enhanced weight loss and percentage body fat loss during fasting, it did not prevent the obesity syndrome (increases in caloric intake, percentage body fat, serum leptin concentration, and hepatic triglyceride concentration and content) that occurs in response to chronic consumption of a high-fat diet. This suggests that endogenous dynorphins are not involved in mediation of diet-induced obesity. Studies testing the role of different endogenous factors in energy homeostasis or screening possible weight loss pharmaceuticals frequently use diet-induced obesity as an experimental model. However, because weight loss drugs are designed to promote weight loss in combination with improved diet and regular exercise and are not designed to prevent weight gain during consumption of a rich diet, models of caloric restriction (such as short-term fasting or long-term caloric restriction in obese animals) provide greater insights into the possible benefits of different agents for weight loss.

The marked food-grinding phenotype of our dynorphin knockout mice on a chow diet, and the fact that it was not observed when animals were fed the obesogenic diet, is noteworthy. The exact reasons for food grinding in mice are not clear (43), but mice are known to dissect some foods, such as seeds, to avoid unpalatable or toxic components, which may result in discarding up to 95% of the mass of food processed (44). Thus, it would seem likely that, when presented with a palatable food, animals eat a greater proportion of the food and spill less of it than less palatable foods. Indeed, our wild-type animals on a chow diet spilled more than 50% of their food in the cage floor, whereas on a high-fat high-sucrose diet, spillage was reduced to around 25%. Our data suggest that dynorphin knockout may induce altered perceptions of palatability, with reduced perceived palatability of chow food but not of food that is high in fat and sucrose, in keeping with a proposed role of opioids, notably the κ opioid-dynorphin system, in ingestion of palatable foods (1).

Pharmacological studies have suggested a role of opioids in mediating the counter-regulatory response to hyperglycemia (2, 3). Systemic administration of the nonspecific opioid receptor antagonist naloxone increases serum glucose levels under basal conditions as well as after insulin-induced hypoglycemia, in association with increases in circulating concentrations of glucagon, cortisol, and epinephrine (2). Intracerebroventricular injections of micro doses of opioid receptor agonists and antagonists suggest that the effects of opioids on the glucose counter-regulatory response occur within the central nervous system (3). However, our detailed investigation revealed no significant effect of dynorphin knockout on glucose or insulin tolerance. Contrarily to the increases in basal- and insulin-induced serum glucagon levels seen in dogs acutely infused with naloxone (2), our dynorphin knockout mice showed a significant decrease in basal serum glucagon levels with no difference from wild types with respect to insulin-induced serum glucagon. The most prominent physiological role of glucagon is to stimulate hepatic glucose production to help main-

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tain euglycemia in states of rapid glucose utilization or fasting, but it is also known to inhibit feeding via effects on the liver and brain (45). Therefore, it is possible that the lower basal serum glucagon levels seen in our dynorphin knockout mice may have contributed to the enhanced food-grinding behavior of these animals. This work demonstrates that any involvement of opioids in glucose homeostasis is not mediated via the dynorphin system.

This study also reveals important interactions between the dynorphin and the NPYergic system because dynorphin knockout significantly reduced expression of NPY mRNA in the hypothalamic arcuate nucleus. Additionally, the fasting-induced increases in pre-prodynorphin mRNA levels in the hypothalamic arcuate nucleus, paraventricular nucleus, and ventromedial hypothalamus were abolished by deletion of Y_1 but not Y_2 receptors for NPY. Together, we propose that negative energy balance increases the expression of NPY in the hypothalamus, and this acts specifically via Y_1 receptors to stimulate hypothalamic expression of dynorphins. Dynorphins also stimulate hypothalamic NPY expression (in keeping with the reduced NPY mRNA levels in the arcuate nucleus of dynorphin knockout mice), so the augmented expression of dynorphins and NPY are reciprocally reinforced. Because we now know that lack of dynorphins or NPY both promote fat loss [dynorphins via stimulation of fat oxidation possibly through increased sympathetic activity and NPY via effects on appetite, neuroendocrine pathways, and metabolic rate (Ref. 15)], we conclude that dynorphins act in concert with NPY to oppose continued weight loss during negative energy balance.

Interestingly, the fasting-induced increase in preprodynorphin expression in the lateral hypothalamus, unlike the increases seen in the hypothalamic arcuate nucleus, paraventricular nucleus, and ventromedial hypothalamus, was not abolished by deletion of Y_1 receptors. This finding may be attributable to the fact that, compared with other areas of the hypothalamus, the lateral hypothalamus exhibits lower expression of Y_1 receptors (46) and prodynorphin (33). The precise role of the lateral hypothalamus in energy homeostasis is not clear, but the lateral hypothalamus was formerly known as the "feeding center" because its ablation led to anorexia and wasting and stimulation of this area led to hyperphagia and obesity, whereas the ventromedial hypothalamus was formerly known as the "satiety center" because ablation or stimulation led to the opposite set of responses (47). Our current data further suggest that dynorphin and Y_1 receptors in the lateral hypothalamus play a differential role in the response to negative energy balance compared with the ventromedial hypothalamus and other hypothalamic nuclei.

Our study revealed marked sexual dimorphisms in expression of dynorphin knockout, with some phenotypes (*e.g.* reduced fat mass and reduced RER) being more pronounced in male mice and other phenotypes (*e.g.* increases in serum DOPAC and DHPG levels and reduction in serum T_4 levels) being more pronounced in females. Estrogen is known to alter the endogenous opioid system in several hypothalamic regions rich in estrogen receptors (notably the ventromedial hypothalamus), as reviewed previously (48). Additionally, progesterone receptors are coexpressed with dynorphin in several regions of the hypothalamus, notably the arcuate nucleus (49). Moreover, blocking the opioid system with naloxone results in significant reductions in locomotor activity in male rats and ovariectomized female rats, and this effect is abolished by estrogen replacement in ovariectomized females (48). Therefore, it is likely that the effects of dynorphin deficiency are similarly influenced by differences in circulating estrogen levels between male and female mice.

In summary, this work has revealed a significant role of endogenous dynorphins in regulating body fat and in attenuating weight loss during negative energy balance, possibly via reduced intestinal nutrient absorption and sympathetically mediated increases in fat oxidation. Moreover, these effects of dynorphins occur at least partially via interactions with the NPYergic system. Because deficiency of dynorphins or NPY promote weight and fat loss via divergent mechanisms [dynorphins via stimulation of fat oxidation and NPY via effects on appetite, neuroendocrine pathways, and metabolic rate (15)], it will be of considerable interest to see whether dual deletion of dynorphins and NPY in double knockout mice is able to produce even greater enhancements in weight loss and fat loss than that seen in individual knockouts, paving the way for possible treatment of obesity via dual pharmacological manipulation of the dynorphin and NPYergic systems.

MATERIALS AND METHODS

Animals

Dynorphin knockout mice were generated as previously published (50). This knockout leads to deletion of the pre-prodynorphin gene, the common precursor of all five dynorphin peptides. Generation of the Y_1 and Y_2 receptor knockout mice were published previously (17, 51). All knockout mice and wild-type littermates were on a mixed C57BL/6–129/SvJ background. All research and animal care procedures were approved by the Garvan Institute/St. Vincent's Hospital Animal Experimentation Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific Purpose. Mice were housed under conditions of controlled temperature (22 C) and illumination (12-h light, 12-h dark cycle, lights on at 0700 h). Unless otherwise stated, mice were fed a normal chow diet *ad libitum* (6% calories from fat, 21% calories from protein, 71% calories from carbohydrate, 2.6 kcal/g; Gordon's Specialty Stock Feeds, Yanderra, New South Wales, Australia). Some mice were fed a high-fat diet supplemented with fat and sucrose (46% calories from fat, 21% calories from protein, 33% calories from carbohydrate, 4.72 kcal/g) from weaning onward. The diet was based on the composition of Rodent Diet catalog no. D12451 (Research Diets, New Brunswick, NJ), with the exception that safflower oil and copha were used in place of soybean oil and lard. The high-fat diet was

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available *ad libitum* unless otherwise stated. Body weight was determined once a week at the same time each week from weaning (4 wk) until 14 wk of age.

Feeding Studies

Mice were transferred from group housing on soft bedding to individual cages with paper towel bedding and allowed to acclimatize for two to four nights. At 11 wk of age, 24-h food and water intake were determined as the average of triplicate readings taken over 3 consecutive days. Actual food intake was calculated as the weight of pellets taken from the food hopper minus the weight of food spilled in the cage. Fecal weight was also determined in triplicate during these analyses. At 12 wk of age, the effect of 24-h fasting on body weight was determined. Food and water consumption and fecal output were determined as described above after 1 d and then again after 2 d of refeeding, and body weight was also tracked during the first 3 d of refeeding. Mice were put back onto soft bedding after completion of feeding studies.

Glucose Tolerance Tests

At 13 wk of age, mice were fasted for 24 h, and then glucose tolerance tests were performed by ip injection of a 10% D-glucose solution (1.0 g/kg) with tail blood sampling (~20–50 μL) at −10, 0, 15, 30, 60, 90, and 120 min after injection. Serum was stored at −20 C for subsequent analysis of glucose and insulin as described below.

Temperature Measurements

At 13–14 wk of age, body temperature was measured in a subset of mice at approximately 1400 h with a thermometer connected to a rectal probe (BAT-10 multipurpose thermometer; Physitemp Instruments, Clifton, NJ). Temperature readings were taken within 10 sec of removing the mouse from its cage. Half of the mice were fasted for 24 h before temperature measurements.

Tissue Collection and Quantification of UCP-1 Expression and Hepatic Triglycerides

At 14 wk of age, freely fed animals were culled between 1200 and 1500 h by cervical dislocation, followed by decapitation for collection of trunk blood. Some animals were completely fasted for 24 h before cull. Serum was stored at −20 C for subsequent analysis as described below. The interscapular BAT was removed, weighed, and frozen until analysis for UCP-1 mRNA levels, standardized with respect to glyceraldehyde 3-phosphate dehydrogenase mRNA, as described previously (52). WAT depots [right inguinal, right epididymal or periovarian (gonadal), right retroperitoneal, and mesenteric] were removed and weighed. The weight of these WAT depots were summed together and expressed as total WAT weight. Liver was removed, weighed, and frozen until extraction of total lipids as described previously (53) and subsequent analysis of hepatic triglyceride content using a commercial triglyceride assay from Roche Diagnostics (Mannheim, Germany).

Analysis of Body Composition

Separate mice were either allowed to feed *ad libitum* or were fasted for 24 h before anesthesia with 100 mg/kg ketamine and 20 mg/kg xylazine (Parke Davis-Pfizer, Sydney, New South Wales, Australia; and Bayer, Leverkusen, Germany) and then scanned for whole-body lean and fat mass using DXA (Lunar PIXImus2 mouse densitometer; GE Healthcare, Waukesha, WI).

Insulin Tolerance Tests

For a subset of 14-wk-old mice, food was removed from cage hoppers at 0830 h and, 5–6 h later, a dose of insulin (2 IU/kg, Actrapid; Novo Nordisk, Baulkham Hills, New South Wales, Australia) or isotonic saline vehicle was injected into the peritoneal cavity. Mice were culled by cervical dislocation, followed by decapitation 45 min later, trunk blood was collected into chilled tubes and allowed to clot on ice for 2 min, and then serum was immediately collected and frozen at −80 C for subsequent determination of glucose, glucagon, corticosterone, and catecholamines as described below.

Determination of Metabolic Rate, RER, and Physical Activity

Metabolic rate was measured by indirect calorimetry in 14- to 16-wk-old male and female $Dyn^{-/-}$ and wild-type mice using an eight-chamber open-circuit calorimeter (Oxymax Series; Columbus Instruments, Columbus, OH). Mice were housed individually in specially built Plexiglas cages (20.1 × 10.1 × 12.7 cm). Temperature was maintained at 22 C, with an air-flow of 0.6 l/min. Food and water were available *ad libitum*. Mice were acclimatized to the cages for 24 h before beginning recordings and then monitored for 24 h. VO_2 was measured every 27 min, and the RER was calculated as VCO_2/VO_2. Energy expenditure (kilocalories of heat produced) was calculated as calorific value (CV) × VO_2, where CV is 3.815 + 1.232 × RER. Ambulatory activity was measured using an OPTO-M3 sensor system (Columbus Instruments), whereby ambulatory counts were a record of consecutive adjacent photobeam breaks. Cumulative ambulatory counts in the x- and y-axes were recorded every minute. Data for the 24-h monitoring period was averaged for 1-h intervals for VO_2, RER, heat, and activity.

Expression Levels of Dynorphin and NPY mRNA

Brains were collected between 1000 and 1400 h from wild-type and dynorphin knockout mice with free access to chow food for subsequent determination of NPY mRNA expression as described previously (54). In addition, chow-fed wild-type, Y_1 and Y_2 receptor knockout mice were either allowed free access to food or fasted for 24 h and were then killed between 1000 and 1400 h by cervical dislocation and decapitation. Brains were immediately removed and frozen on dry ice and then stored at −80 C until analysis of pre-prodynorphin mRNA expression as described previously (33).

Serum Analyses

Some serum hormone levels were determined with commercial RIA kits from Linco Research (St. Louis, MO) (glucagon, leptin, insulin), ICN Biomedicals (Costa Mesa, CA) (corticosterone, free T_4, testosterone), and Bioclone Australia (Marrickville, Australia) (IGF-I). Serum glucose and free fatty acid levels were determined with a glucose oxidase kit (Trace Scientific, Melbourne, Victoria, Australia) and a kit from Wako (Osaka, Japan), respectively. Serum catecholamines (noradrenaline, adrenaline, and their precursor dopamine) and the main metabolites produced by degradation of noradrenaline (DHPG) and dopamine (DOPAC) were determined by gas chromatography-mass spectrometry as described previously (55, 56).

Statistical Analysis

All data are expressed as means ± SEM. Differences among groups of mice were assessed by ANOVA or repeated-measures ANOVA, followed by Fisher's *post hoc* comparisons if appropriate (StatView version 4.51; Abacus Concepts,

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Berkeley, CA). Data for serum glucagon levels were log10 transformed before statistical analysis. Data for heat production were analyzed by analysis of covariance (heat *vs.* body weight). Statistical significance was defined as $P < 0.05$.

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