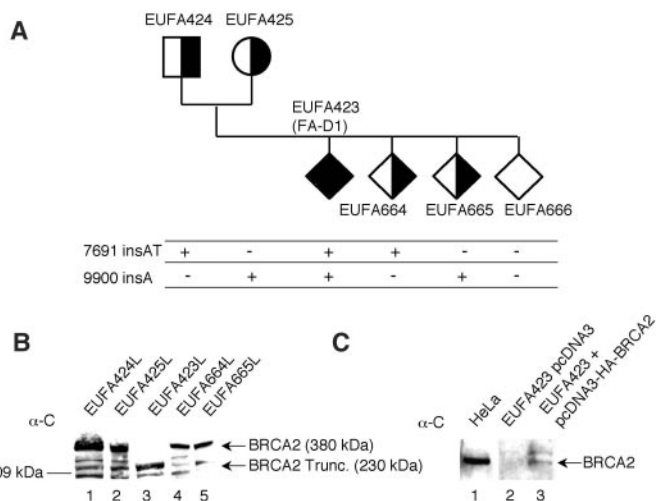


Fig. 3. Segregation of *BRCA2* mutant alleles in the EUFA423 pedigree. (A) The proband with FA subtype D1 is EUFA423. Genomic DNA was prepared from lymphoblasts from the indicated family members and was sequenced for *BRCA2* mutations. (B) Expression of mutant *BRCA2* polypeptides in lymphoblasts derived from EUFA423 kindred. Proteins were immunoblotted with Ab-2. (C) EUFA423F was transfected either with pcDNA3-empty vector or pcDNA3-HA-*BRCA2* (28, 29), and stable G418-resistant cells were isolated. Cell lines were analyzed by immunoblot with Ab-2 and by the MMC chromosome breakage assay (Table 2).



cancer risk as high as 70% by age 70. Other variant *BRCA2* alleles, such as the polymorphic stop codon ter3326, appear to cause no increased cancer risk (18) but may cause FA in the compound heterozygous state. The smallest known cancer-associated deletion removes only 224 amino acids from the COOH-terminus of *BRCA2* (24). Due to the unavailability of clinical records, we were unable to assess the cancer risk of the *BRCA2* mutant alleles in these FA families (Table 1).

FA patients with biallelic *BRCA2* mutations share clinical features with FA patients from other subtypes (i.e., congenital abnormalities, abnormal skin pigmentation, bone marrow failure, and cellular sensitivity to MMC) (25) (table S1). These similarities suggest that *BRCA2* and other FA proteins cooperate in a common DNA damage response pathway, the FA/*BRCA* pathway (Model, fig. S3A). According to this model, DNA damage activates the monoubiquitination of FANCD2, thus targeting FANCD2 to DNA repair foci containing *BRCA1* and *BRCA2* (26). Previous studies have indicated that FA-B cells lack FANCD2 monoubiquitination, whereas FA-D1 cells express monoubiquitinated FANCD2 (8) (fig. S3B). *BRCA2* may function upstream in the pathway, by promoting FA complex assembly and FANCD2 activation, and/or downstream in the pathway, by transducing signals from FA proteins to RAD51 and the homologous recombination machinery (27). The precise molecular function(s) of *BRCA1* and *BRCA2* in this pathway remain to be elucidated.

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Materials and Methods
Figs. S1 to S3
Table S1

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Activation of Central Melanocortin Pathways by Fenfluramine

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D-fenfluramine (d-FEN) was once widely prescribed and was among the most effective weight loss drugs, but was withdrawn from clinical use because of reports of cardiac complications in a subset of patients. Discerning the neurobiology underlying the anorexic action of d-FEN may facilitate the development of new drugs to prevent and treat obesity. Through a combination of functional neuroanatomy, feeding, and electrophysiology studies in rodents, we show that d-FEN-induced anorexia requires activation of central nervous system melanocortin pathways. These results provide a mechanistic explanation of d-FEN's anorexic actions and indicate that drugs targeting these downstream melanocortin pathways may prove to be effective and more selective anti-obesity treatments.

Drugs that increase the activity of central serotonin (5-hydroxytryptamine, 5-HT) have been widely used as appetite suppressants (1-3). However, these drugs often elicit unwanted side effects because they target multiple 5-HT pathways and receptors. A notable

example is d-FEN, a drug that blocks the reuptake of 5-HT and stimulates its release (3). In the mid-1990s, d-FEN was prescribed to millions of people in the United States for weight loss, frequently in combination with the sympathomimetic phentermine, but was

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withdrawn from clinical use in 1997 by the Food and Drug Administration after reports of adverse cardiopulmonary events (4).

Delineation of the central nervous system (CNS) pathways through which d-FEN mediates its effects on food intake may lead to the development of new appetite suppressant drugs that have fewer side effects. As a first step toward this goal, we performed a dose-response study to identify a threshold dose of d-FEN (Sigma) that reduced feeding behavior in rats without inducing other gross behavioral abnormalities (fig. S1) (5). We used Fos-like immunoreactivity (FOS-IR) to correlate anorexic efficacy with neuronal activation patterns in the brain (6). As previously reported, d-FEN induced FOS-IR in a dose-dependent manner in many brain regions associated with energy homeostasis (7), including the arcuate nucleus of the hypothalamus (ARC) and the paraventricular nucleus of the hypothalamus (PVN). ARC FOS-IR expression was most prominent in the lateral regions, where pro-opiomelanocortin (POMC) neurons reside. These neurons are a potential target for d-FEN action because they receive direct input from 5-HT dorsal raphe nucleus neurons (8) and project to regions associated with energy regulation (9–11).

To determine whether d-FEN activates POMC neurons, we performed dual-labeling immunohistochemical experiments using antisera to α -melanocyte stimulating hormone (α -MSH), a POMC-derived anorexic peptide. Threshold anorexic doses of d-FEN significantly and consistently induced FOS-IR in α -MSH-immunoreactive (α -MSH-IR) neurons throughout the ARC [average coexpression, d-FEN = 45%, saline = 14%; $t(6) = 14.9$, $P < 0.001$] (Fig. 1, A to C). A similar pattern of d-FEN-induced FOS-IR was observed in ARC cells expressing POMC mRNA (12). These data strongly suggest that threshold anorexic doses of d-FEN activate POMC neurons in the ARC.

We hypothesized that 5-HT receptors (5-HTRs) are expressed in POMC neurons and that action at these receptors mediates a component of the anorexic effect of d-FEN. 5-HT_{2C}Rs, formerly called 5-HT_{1C}Rs, are a strong candidate for this action because they are expressed in the ARC (13). Moreover, 5-HT_{2C}R-deficient mice are hyperphagic, obese, and refractory to threshold anorexic doses of d-FEN (14, 15). We found that up to 80% of α -MSH neurons express 5-HT_{2C}R mRNA (Fig. 1, D to F) and that the pattern of coexpression was greatest in the caudal ARC.

If d-FEN exerts its anorexic effect through action at 5-HT_{2C}Rs, then direct activation of 5-HT_{2C}Rs should mimic the effects of d-FEN. Indeed, rats treated with the 5-HT_{2C/1B}R agonist 1-(*m*-chlorophenyl)piperazine (mCPP, Sigma) decreased their acute food intake (fig. S1) and showed increased induction of FOS-IR in a pattern consistent with d-FEN-induced FOS-IR expression in the ARC and PVN (fig. S2). Moreover, threshold anorexic doses of mCPP [5.0 mg/kg intravenously (iv)] in-

duced similar significant FOS-IR in α -MSH neurons in the ARC [average coexpression, mCPP = 54%, saline = 14%; $t(5) = 15.8$, $P < 0.001$]. These data indicate that d-FEN and mCPP-induced FOS-IR in POMC neurons may involve activation of 5-HT_{2C}Rs.

To investigate whether d-FEN directly activates POMC neurons, we performed electrophysiology studies on hypothalamic slices from transgenic mice expressing green fluorescent protein (GFP) under control of the POMC promoter (5, 16). d-FEN induced a doubling of the spontaneous firing rate in POMC neurons, an effect reversed by drug washout (Fig. 2A). Moreover, POMC neurons depolarized in response to administration of d-FEN, 5-HT, or two 5-HT_{2C}R agonists, mCPP and MK 212 (Fig. 2B). These data support the notion that central 5-HT systems directly activate POMC neurons.

On the basis of these findings, we hypothesized that d-FEN stimulates the release of 5-HT in the ARC and that the neurotransmitter then binds to 5-HT_{2C}Rs

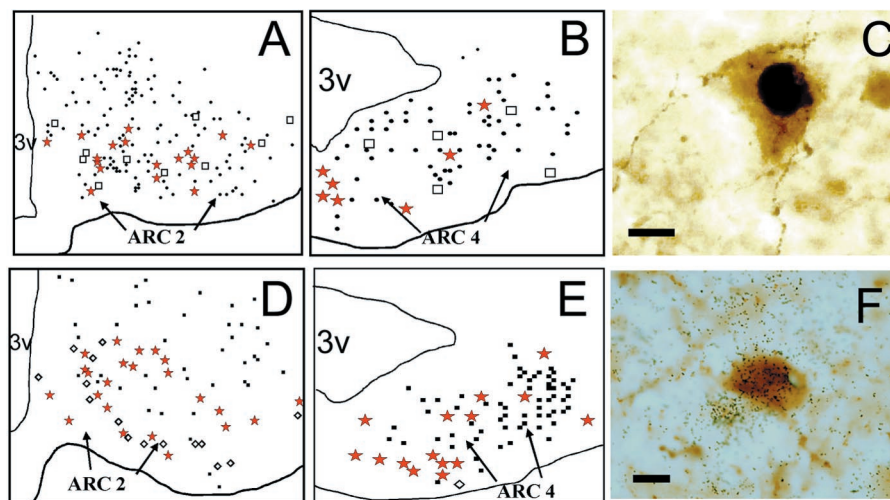


Fig. 1. d-FEN activates ARC α -MSH neurons that express 5-HT_{2C}Rs. Pathogen-free adult male Sprague-Dawley rats ($n = 3$ to 6 per group; 250 to 325 g) were individually housed with water and rat chow pellets available ad libitum in a light-controlled (12 hours on/12 hours off) and temperature-controlled environment [21.5° to 22.5°C (26)]. Rats were perfused and brains were processed for histochemistry (6). Multiple markers for α -MSH-expressing neurons were examined [α -MSH-IR, CART-IR, and ³⁵S-labeled POMC cRNA riboprobe (9)] for coexpression with FOS-IR or 5-HT_{2C}R mRNA. Each combination assessed yielded comparable results. Data were collected from four levels of the ARC, which were identified on the basis of expression patterns of cellular neurochemical markers detailed in a rat brain atlas [atlas fig. 27 = ARC 1; atlas fig. 30 = ARC 2; atlas fig. 33 = ARC 3; atlas fig. 37 = ARC 4; 3v, third ventricle (6, 27)]. Examples of α -MSH coexpression patterns with FOS-IR and 5-HT_{2C}R mRNA in ARC 2 and ARC 4 were transposed into drawings by concurrently visualizing brain sections with a 10 \times objective of a microscope and a computer screen. The architecture and marker expression of the cells was then transposed into a drawing. (A and B) Rats were injected with d-FEN (1.0 mg/kg, iv). Two hours later, rats were perfused and brains were removed. FOS-IR (•), α -MSH (□), and coexpression of FOS-IR and α -MSH (red stars) are shown at ARC 2 and ARC 4 levels. d-FEN significantly induced FOS-IR in α -MSH neurons at all four levels of the ARC compared to saline treatment ($P < 0.001$). (C) α -MSH-IR neurons (brown cytoplasm) contain FOS-IR (black nuclei). (D and E) α -MSH neurons express 5-HT_{2C}R mRNA [5-HT_{2C}R riboprobe was described in (28)]. α -MSH (◇), 5-HT_{2C}R (■), and coexpression of α -MSH and 5-HT_{2C}R (red stars) are shown at ARC 2 and ARC 4 levels. (F) α -MSH-IR neurons (brown cytoplasm) contain ³⁵S-labeled 5-HT_{2C}Rs (cluster of black grains). Scale bar, 10 μ m (C, F).

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expressed in POMC neurons. This in turn could stimulate the release of α -MSH, which acts on effector neurons expressing melanocortin 4 receptors (MC4-R) and melanocortin 3 receptors (MC3-R). MC4-Rs have been identified as critical regulators of food intake, energy expenditure, and neuroendocrine function [e.g., (17–20)]. MC3-Rs have also been implicated in energy homeostasis (21, 22). To test whether

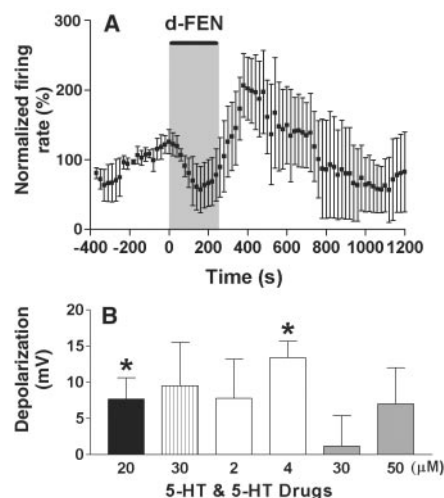
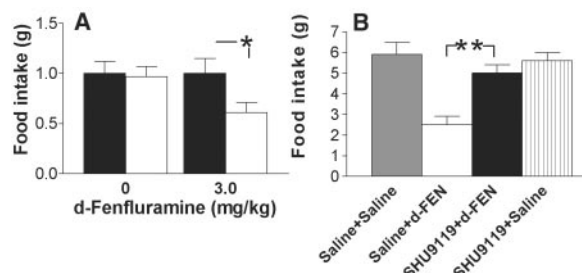


Fig. 2. 5-HT drugs increase the frequency of action potentials and depolarize POMC cells. Standard electrophysiological techniques were applied to coronal slices of hypothalamus from male POMC-EGFP transgenic mice (20 to 25 g) (5, 16, 26). (A) Using a loose-cell-attached mode, d-FEN (20 μ M) induced a doubling of the mean (\pm SEM) POMC neuron firing rate ($n = 3$). This effect was reversed with drug washout. (B) Mean (\pm SEM) peak depolarization of POMC-EGFP neurons ($n = 4$ to 8 per dose) bathed with 20 μ M d-FEN (black bar), 30 μ M 5-HT (hatched bar; Sigma), 2 or 4 μ M mCPP (white bars), or 30 or 50 μ M MK 212 (gray bars; Sigma), using a conventional whole-cell mode ($*P < 0.05$).

Fig. 3. Melanocortin receptor antagonists attenuate the anorectic effect of d-FEN. (A) Male A^y mice (black bars) and their wild-type littermates (white bars) (4 weeks old, Jackson Laboratories) were individually housed as described in Fig. 1 (26). Mice were treated with d-FEN [0 or 3.0 mg/kg intraperitoneally (ip)] at the onset of the dark cycle and wet chow mash intake was measured 50 min after treatment (15). d-FEN significantly reduced mash intake in wild-type but not A^y mice ($*P < 0.05$). No differences in mash intake were observed after saline treatment. (B) Rats, housed as described in Fig. 1, were stereotaxically implanted with a guide cannula aimed at the third cerebral ventricle [1.5 to 2 mm posterior to bregma in the midline and 8 to 8.2 mm below the skull surface (25, 26)]. Two weeks later, rats were fasted for 16 hours with water available ad libitum. Animals ($n = 5$ to 6 per group) were pretreated with saline or SHU9119 (0.3 nmol icv; Phoenix Pharmaceuticals) and treated with saline or d-FEN (1 mg/kg ip). Intake of rat chow pellets was measured for the next hour. Animals pretreated with vehicle and treated with d-FEN showed significant reductions in food intake ($P < 0.001$). However, pretreatment with SHU9119 significantly attenuated d-FEN-induced anorexia ($**P < 0.01$). SHU9119 did not alter food intake when given alone.



genetic or pharmacological blockade of MC4-Rs/MC3-Rs reduces the anorectic efficacy of d-FEN, we administered d-FEN to A^y mice, an obese strain resulting from ectopic overexpression of agouti, an endogenous melanocortin receptor antagonist (23–25). We observed that young A^y mice exhibited significantly attenuated responses to the anorectic actions of d-FEN compared to wild-type littermates (Fig. 3A). Similarly, we found that administration of the MC4-R/MC3-R antagonist SHU9119 to rats before d-FEN significantly decreased the anorectic response to d-FEN (Fig. 3B) (25). Thus, MC4-Rs/MC3-Rs are likely downstream mediators of d-FEN-activated neurons.

To confirm that 5-HT_{2C}R action on POMC neurons is upstream of MC-Rs, we administered the MC4-R/MC3-R agonist MT II [1 nmol intracerebroventricularly (icv)] to 5-HT_{2C}R-deficient mice and wild-type littermates before divergence of body weight (5). Relative to mice given vehicle alone, treatment with MT II caused a significant reduction in food intake up to 8 hours after injection. No differences in food intake at vehicle or drug treatment were observed between genotypes, nor were there genotype-specific differences in baseline POMC mRNA expression in the ARC (fig. S3).

Our data show that components of the anorectic effects of d-FEN are mediated through the central melanocortin system, which is now well established as a fundamental regulator of food intake and body weight in rodents and humans. d-FEN is an indirect 5-HT agonist, thereby increasing 5-HT activity throughout 5-HT pathways in the CNS. It is noteworthy that our findings indicate that genetic or pharmacological blockade of MC4-Rs/MC3-Rs is sufficient to attenuate the anorectic efficacy of

threshold doses of d-FEN. Our results suggest that drugs targeting these downstream melanocortin pathways may act in part in a similar manner to d-FEN to decrease food intake and body weight, and may yield fewer 5-HT-related side effects. Finally, these data indicate that 5-HT_{2C}Rs are expressed on many POMC neurons, and add the CNS 5-HT system to the growing list of metabolic signals that converge on melanocortin neurons in the ARC.

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