

# Dysregulation of Striatal Dopamine Signaling by Amphetamine Inhibits Feeding by Hungry Mice

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## Summary

Amphetamine (AMPH) releases monoamines, transiently stimulates locomotion, and inhibits feeding. Using a genetic approach, we show that mice lacking dopamine (DA-deficient, or DD, mice) are resistant to the hypophagic effects of a moderate dose of AMPH (2  $\mu$ g/g) but manifest normal AMPH-induced hypophagia after restoration of DA signaling in the caudate putamen by viral gene therapy. By contrast, AMPH-induced hypophagia in response to the same dose of AMPH is not blunted in mice lacking the ability to make norepinephrine and epinephrine (*Dbh*<sup>-/-</sup>), dopamine D<sub>2</sub> receptors (*D2r*<sup>-/-</sup>), dopamine D<sub>1</sub> receptors (*D1r*<sup>-/-</sup>), serotonin 2C receptors (*Htr2c*<sup>-/-</sup>), neuropeptide Y (*Npy*<sup>-/-</sup>), and in mice with compromised melanocortin signaling (*A<sup>y</sup>*). We suggest that, at this moderate dose of AMPH, dysregulation of striatal DA is the primary cause of AMPH-induced hypophagia and that regulated striatal dopaminergic signaling may be necessary for normal feeding behaviors.

## Introduction

Amphetamine (AMPH) was widely prescribed for the treatment of obesity between 1937 and 1970 and has served as a prototype for the development of subsequent anorexic drugs, such as phentermine. Acute, systemic AMPH releases the biogenic amines dopamine (DA), norepinephrine (NE), epinephrine (Epi), and serotonin (5-HT), flooding the synaptic cleft with excess ligand (Sulzer et al., 1995). In addition to reducing food intake, AMPH increases blood pressure, locomotor activity, oxygen consumption, and heart and respiratory rates and can result in dry mouth, sweating, enlargement of the pupils, and slowed gastrointestinal transit time. Humans treated with AMPH report euphoria, increased attention, feelings of confidence, restlessness, difficulty sleeping, and headaches, while some may become anxious, irritable, hostile, and aggressive. Because of the effects of

AMPH on human appetite and body weight, the mechanisms of AMPH-induced hypophagia and weight loss have been investigated extensively.

A substantial literature is consistent with the hypothesis that systemic AMPH reduces appetite by release of catecholamines within the perifornical lateral hypothalamus (pflH) (Baptista et al., 1993; Chen et al., 2001; Kuo, 2003; Leibowitz, 1975a, 1975b; Leibowitz and Shor-Posner, 1986; Wellman, 1990). In support of this hypothesis, AMPH, DA, NE, and Epi, as well as pharmacological agonists of dopamine D<sub>2</sub> and  $\beta$ -adrenergic receptors, reduce food intake when injected locally into the pflH (Booth, 1968; Leibowitz and Rossakis, 1979), and local D<sub>2</sub> and  $\beta$ -adrenergic antagonists can stimulate feeding in sated animals (Leibowitz, 1970b; Parada et al., 1988b). Finally, pflH D<sub>2</sub> and  $\beta$ -adrenergic antagonists attenuate the hypophagic effect of locally injected AMPH (Leibowitz, 1975a).

Here, we sought to investigate the mechanism of AMPH-induced hypophagia by using a genetic approach. We tested whether AMPH-induced hypophagia requires activation of DA and  $\beta$ -adrenergic receptors (Leibowitz, 1975a, 1975b) by using mice that lack the endogenous  $\beta$ -adrenergic ligands NE and Epi (*Dbh*<sup>-/-</sup>), dopamine D<sub>2</sub> receptors (*D2r*<sup>-/-</sup>), or dopamine D<sub>1</sub> receptors (*D1r*<sup>-/-</sup>). We also tested three mouse models with genetic modification of hypothalamic feeding effectors: neuropeptide Y (*Npy*<sup>-/-</sup>) and serotonin 2C receptor (*Htr2c*<sup>-/-</sup>) knockout and agouti (*A<sup>y</sup>*) mice. Finally, we tested mice that lack the ability to make DA (DA-deficient, or DD, mice), before and after restoration of DA signaling to the caudate-putamen (CPU) by virally mediated gene transfer. The results of these experiments, along with a review of the literature, lead us to a new hypothesis to account for the hypophagia induced by a moderate dose of AMPH and the role of striatal DA in feeding behavior.

## Results and Discussion

The general strategy used in most of the studies reported here to assess the effects of AMPH on food intake and locomotion is depicted in Figure 1A. For these studies, we chose to use a moderate dose of AMPH (2  $\mu$ g/g) that results in a substantial, though not absolute, reduction in food intake during the first hour after treatment (Figure 1B) as well as significant locomotor activation (Figure 1C).

### NE, Epi, D<sub>2</sub>, and D<sub>1</sub> Receptors Are Not Required for AMPH-Induced Hypophagia

Because both  $\beta$ -adrenergic and dopamine D<sub>2</sub> receptor antagonists infused directly into the pflH were able to attenuate the hypophagia caused by a low dose of AMPH, it has been suggested that the function of either of these receptors is required for the suppressive effects of AMPH on eating (Leibowitz, 1975a). To test the importance of  $\beta$ -adrenergic signaling, we examined mice lacking dopamine  $\beta$ -hydroxylase (*Dbh*<sup>-/-</sup>), which is essential

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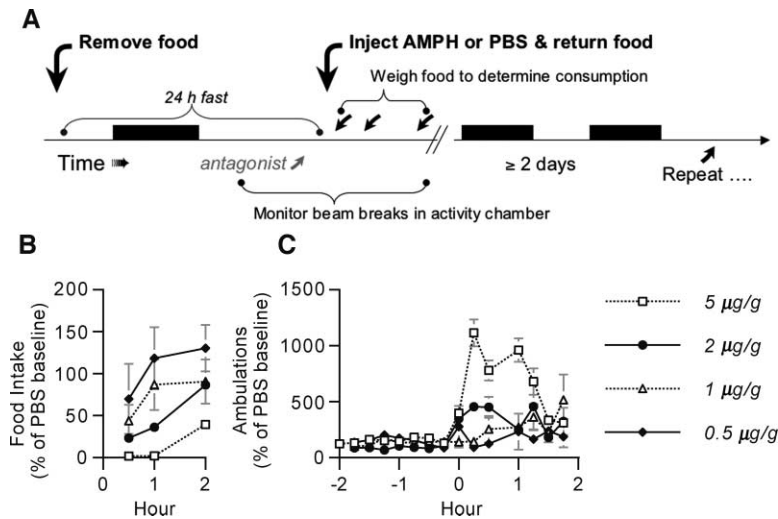


Figure 1. Procedure for Tests of AMPH-Induced Hypophagia

(A) Food was removed and mice were transferred to a new home cage 24 hr prior to the beginning of each test. Because of hyperphagia in response to fresh chow pellets, pellets from each cage were bagged separately and returned to the same mice for testing. After ~22 hr of fasting, mice were transferred to the activity chamber to acclimate. Activity data were collected as ambulations (consecutive breaks to two separate beams) per 15 min interval. Two hours later, mice were weighed, injected with PBS or AMPH and returned to the activity chamber. Mice were always observed during the first 30 min after AMPH administration and were usually observed at intervals throughout the test. Weighed food was returned immediately after injection, then removed, weighed, and returned at 0.5, 1, and 2 hr. The calculated difference in weight was assumed to represent food intake by the

mouse. Water was available at all times. After 2 hr, mice were returned to the home cage where food was available ad libitum. Tests were separated by a minimum of 2 days. On the first 2 testing days, mice were acclimated to the procedure and given only PBS. AMPH or PBS was given in counterbalanced order on the last 2 testing days.

(B) Because mice sensitize to repeated treatment with AMPH, separate groups of C57BL/6J mice were used for each dose of AMPH. Data for each mouse were converted to a percentage of the fasting-induced intake of the same mouse following PBS injection. Doses of 1 (n = 8), 2 (n = 12), or 5 (n = 16) µg/g AMPH reduced fasting-induced feeding in the first 30–60 min after treatment, with the effect of 5 µg/g persisting through the second hour. Lower doses of 0.8 (n = 8, data not shown), 0.5 (n = 7), and 0.2 (n = 12, data not shown) µg/g AMPH resulted in some stimulation of feeding behavior, particularly late in the testing interval. In a separate study (data not shown), we observed no difference between male and female mice in the hypophagic response to 2 or 5 µg/g AMPH.

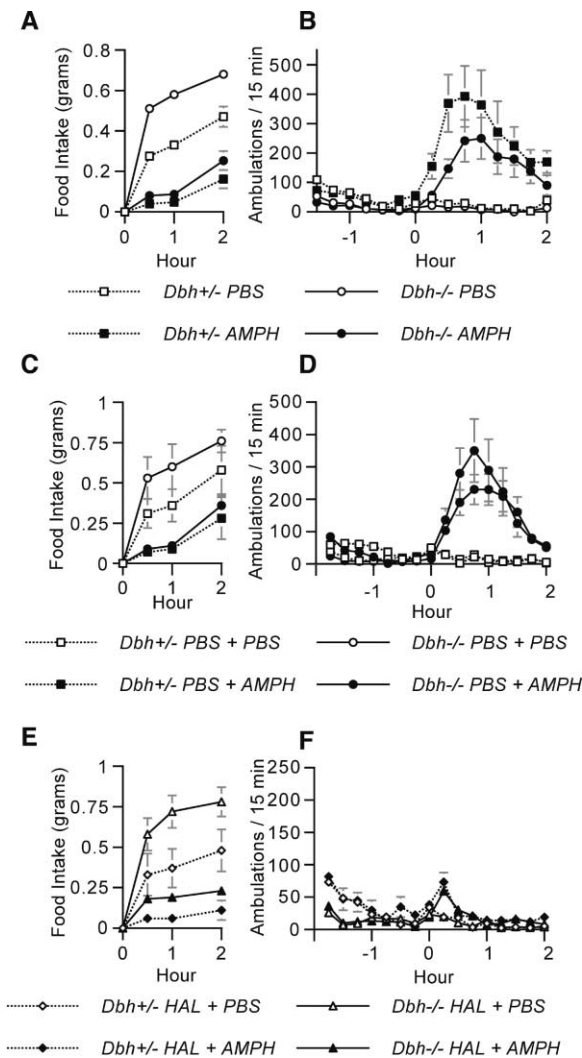
(C) As a percentage of the activity before and after PBS injection, both 2 and 5 µg/g AMPH resulted in increased ambulations. Lower doses did not reliably increase ambulations in all mice. For clarity, some of the lower doses are not shown. A separate study confirmed no difference between the ambulatory response of male and female mice to AMPH in this protocol (data not shown).

for the synthesis of the  $\beta$ -adrenergic ligands NE and Epi. Female *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice reduced food intake equally after AMPH (Figure 2A), as did male *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice (data not shown). Basal ambulations by *Dbh*<sup>-/-</sup> mice were slightly less than that of *Dbh*<sup>+/-</sup> controls, as was their ambulatory response to AMPH (Figure 2B). While the hypophagic response of *Dbh*<sup>-/-</sup> mice to AMPH suggests that activation of  $\beta$ -adrenergic receptors is not required for AMPH-induced hypophagia, we sought to exclude the possibility that activation of  $D_2$  receptors compensated for the loss of NE and Epi in these mice by blocking  $D_2$  receptors with haloperidol (HAL). The dose of HAL (0.25 µg/g) was chosen because it did not cause catalepsy or increase feeding by mice within the first 2 hr of administration, whereas higher doses (0.5 and 1.0 µg/g) did increase feeding (Kaur and Kulkarni, 2002). Despite blocking striatal dopaminergic activation after AMPH, as evidenced by the absence of AMPH-induced hyperactivity (Figures 2D and 2F), HAL did not counter AMPH-induced hypophagia in *Dbh*<sup>+/-</sup> or *Dbh*<sup>-/-</sup> mice (Figures 2C and 2E), in contrast to the “behavioral competition” hypothesis (Blundell and Rogers, 1980; Joyce and Iversen, 1984). A trend at 30 min after injection ( $F[1, 14] = 1.7, p = 0.22$ ) toward increased intake following HAL and AMPH in the *Dbh*<sup>-/-</sup> mice as compared to the controls treated in the same way may suggest enhanced sensitivity to the effects of HAL or merely the increased basal intake of the knockouts. In a previous report (Leibowitz, 1975b), the large amount of HAL injected into the pLH (3.8 µg/side) may have had nonspecific effects; moreover, the effects of this dose of HAL alone were not reported. We conclude

that NE and Epi are not required for AMPH-induced hypophagia even in the presence of DA receptor blockade.

Mice lacking dopamine  $D_2$  receptors (*D2r*<sup>-/-</sup>) also responded to AMPH with hypophagia equivalent to that of their littermate controls (*D2r*<sup>+/-</sup>). Female *D2r*<sup>-/-</sup> and *D2r*<sup>+/-</sup> mice reduced their food intake (Figures 3A and 3C) and increased their activity (Figures 3B and 3D) after AMPH, as do males (data not shown). The  $\beta$ -adrenergic receptor antagonist propranolol (PROP, 1.25 µg/g) did not block AMPH-induced hypophagia (Figure 3E) or hyperactivity (Figure 3F) in female *D2r*<sup>+/-</sup> or *D2r*<sup>-/-</sup> mice. This dose of PROP was chosen because it maintains relative specificity and reversed restraint stress-induced behavior in mice (Gorman and Dunn, 1993). Even higher doses of PROP (4–16 µg/g) fail to block AMPH-induced hypophagia in mice (Dobrzanski and Doggett, 1979) and rats (Chen et al., 2001; Lazareno, 1979; Willner and Towell, 1982). We conclude that signaling via  $\beta$ -adrenergic receptors does not compensate for the lack of  $D_2$  receptor signaling during AMPH-induced hypophagia.

Although there are few  $D_1$  receptors present in the pLH and HAL is selective for  $D_2$ -like receptors at low doses, it is possible that the high dose of HAL given directly into the pLH inhibited  $D_1$  receptors (Leibowitz, 1975b). This interpretation might explain why systemic administration of a selective  $D_1$ -type receptor antagonist (SCH23390) blocked hypophagia in response to a low dose of AMPH, while the  $D_2$ -type antagonist sulpiride did not (Gilbert and Cooper, 1985; Lutz et al., 2001). Therefore, we also sought to evaluate AMPH-induced hypophagia in mice lacking dopamine  $D_1$  receptors



**Figure 2.** *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> Mice Reduced Fasting-Induced Intake after AMPH, but Did Not Increase Locomotion during D<sub>2</sub> Receptor Blockade

(A) Naive 2-month-old female mice (8 *Dbh*<sup>+/-</sup> and 8 *Dbh*<sup>-/-</sup>) demonstrated significant hypophagia in response to AMPH (2 μg/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$  by Tukey's post hoc analysis). The *Dbh*<sup>-/-</sup> mice consumed more food during the first 0.5 and 1 hr after PBS injection (values shown are the average of three PBS trials,  $p < 0.01$ ), which may reflect aberrant satiety mechanisms

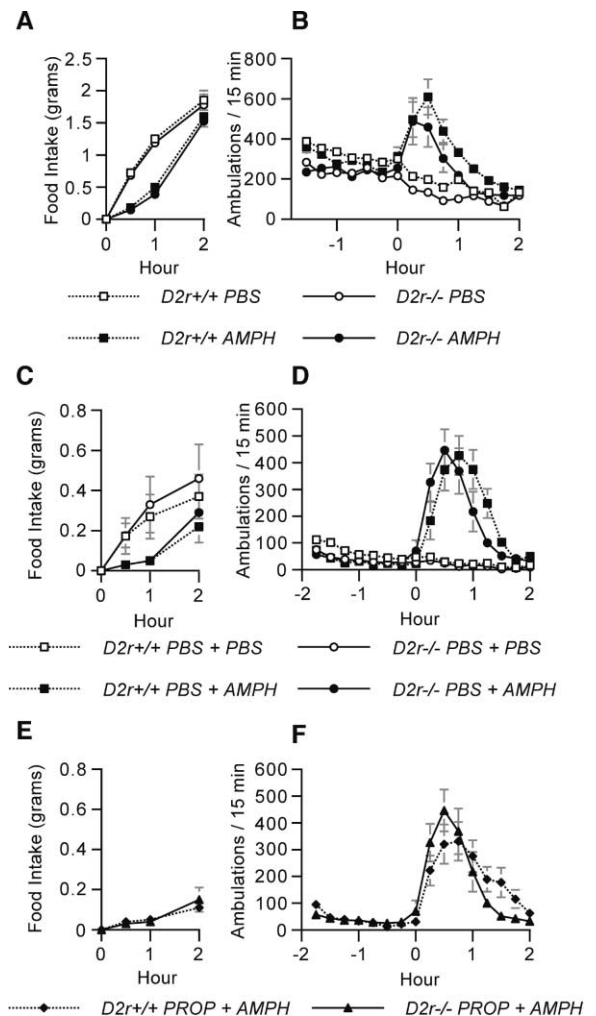
(B) Both *Dbh*<sup>+/-</sup> controls and *Dbh*<sup>-/-</sup> mice responded to AMPH with hyperactivity, and neither group demonstrated stereotypy. The activity of *Dbh*<sup>-/-</sup> mice was slightly less than that of the controls,  $F(1,14) = 5$ ,  $p < 0.05$ .

(C) After a break of 2 months, the same mice were tested again to determine the effect of the D<sub>2</sub> receptor antagonist HAL on AMPH-induced hypophagia and locomotion. When PBS was given 15 min prior to PBS or AMPH, female mice of both genotypes were significantly hypophagic in response to AMPH (2 μg/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ).

(D) AMPH induced hyperlocomotion, and neither group demonstrated stereotypy.

(E) All mice received the DA receptor antagonist haloperidol (HAL; 0.25 μg/g IP) 15 min prior to injection of PBS or AMPH and return of food. HAL did not affect hyperphagia following a fast or AMPH-induced hypophagia in *Dbh*<sup>+/-</sup> controls or *Dbh*<sup>-/-</sup> mice ( $p = 0.87$ ).

(F) Prior treatment with HAL completely counteracted the locomotor effect of AMPH in both *Dbh*<sup>+/-</sup> controls and *Dbh*<sup>-/-</sup> mice ( $p < 0.01$ ).



**Figure 3.** *D2r*<sup>-/-</sup> Mice Reduced Fasting-Induced Food Intake and Increased Locomotion after AMPH, Regardless of β-Adrenergic Blockade

(A) Naive 2-month-old female mice (12 *D2r*<sup>+/-</sup> and 12 *D2r*<sup>-/-</sup>) were significantly hypophagic in response to AMPH (2 μg/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ).

(B) The activity of female *D2r*<sup>-/-</sup> mice after AMPH was comparable to that of the *D2r*<sup>+/-</sup> controls, and neither group demonstrated stereotypy.

(C) Naive 4-month-old female mice (8 *D2r*<sup>+/-</sup> and 7 *D2r*<sup>-/-</sup>) were pretreated with PBS 15 min prior to either PBS or AMPH. Female mice of both genotypes were significantly hypophagic in response to AMPH (2 μg/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ).

(D) Both *D2r*<sup>-/-</sup> and *D2r*<sup>+/-</sup> mice became hyperactive in response to AMPH.

(E) Propranolol (PROP; 1.25 μg/g) did not significantly affect AMPH-induced hypophagia in *D2r*<sup>+/-</sup> controls or *D2r*<sup>-/-</sup> mice ( $p = 0.91$ ).

(F) There was no effect of prior PROP on activity after AMPH, and neither group demonstrated stereotypy.

(*D1r*<sup>-/-</sup> mice). *D1r*<sup>-/-</sup> mice reduced food intake after AMPH (Figure 4A) and became hyperactive (Figure 4B). Although there was a trend toward reduced ambulations after AMPH by the *D1r*<sup>-/-</sup> mice, this did not reach significance. However, the trend toward increased basal activity in the fasted *D1r*<sup>-/-</sup> mice was significant ( $p < 0.05$ ). We were unable to test mice lacking both D<sub>1</sub> and D<sub>2</sub>

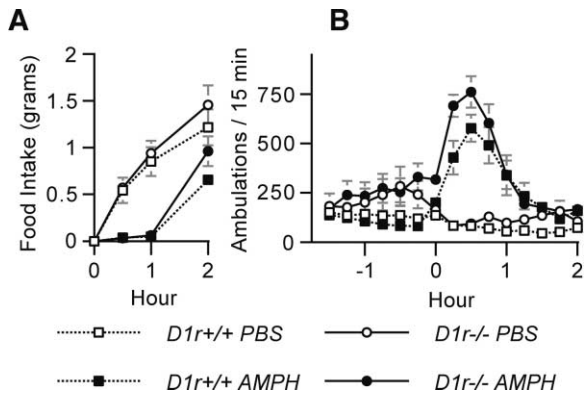


Figure 4. *D1r*<sup>-/-</sup> Mice Reduced Fasting-Induced Food Intake and Increased Locomotion after AMPH

(A) Naive 4-month-old male and female mice (7 *D1r*<sup>+/+</sup> and 6 *D1r*<sup>-/-</sup>) were significantly hypophagic in response to AMPH (2 μg/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ).

(B) The activity of the fasted *D1r*<sup>-/-</sup> mice during the baseline period was significantly greater than that of the controls ( $p < 0.05$ ). After AMPH, both groups demonstrated comparable hyperactivity ( $p < 0.01$ ).

receptors, as these mice die perinatally (Kobayashi et al., 2004), and we cannot eliminate the possibility that remaining DA receptors may mediate AMPH-induced hypophagia. However, the present results contradict the prevailing explanation for hypophagia in response to systemic AMPH by demonstrating that activation of *D*<sub>1</sub>-, *D*<sub>2</sub>-, and β- (or any other) adrenergic receptors are not required for AMPH-induced hypophagia.

#### 5-HT<sub>2C</sub> Receptors, Melanocortin Receptor Signaling, and NPY Are Not Required for AMPH-Induced Hypophagia

Obese mice with impaired 5-HT<sub>2C</sub> or MC4R receptor signaling (*Htr2c*<sup>-/-</sup> and *A<sup>y</sup>*, respectively) are insensitive to the anorectic effects of serotonin reuptake inhibitors (Heisler et al., 2003; Heisler et al., 2002; Vickers et al., 1999), and pharmacological studies have implicated a role for serotonin in AMPH-induced hypophagia (Parada et al., 1988a, 1992). Because activation of several different serotonin receptors may suppress food intake, we also tested AMPH-induced hypophagia in mice deficient in melanocortin 4 receptor (MC4R) activation, because signaling via MC4R may be necessary for serotonin's effects on food intake. For example, mice that express the MC4R antagonist agouti ectopically (*A<sup>y</sup>*) are hyperphagic, obese, and (like 5-HT<sub>2C</sub> and 5-HT<sub>1B</sub> knockout mice) insensitive to the anorectic effects of serotonin reuptake inhibitors such as d-fenfluramine and m-chlorophenylpiperazine (Butler and Cone, 2002; Heisler et al., 2003; Heisler et al., 2002; Tecott and Abdallah, 2003; Vickers et al., 1999). Thus, *Htr2c*<sup>-/-</sup> and *A<sup>y</sup>* mice might be expected to demonstrate comparable insensitivity to AMPH, if AMPH-induced hypophagia is mediated via this hypothalamic circuit. However, both *Htr2c*<sup>-/-</sup> and *A<sup>y</sup>* mice demonstrated equivalent AMPH-induced hypophagia (Figures 5A and 5C) and hyperactivity (Figures 5B and 5D). The obese *A<sup>y</sup>* mice (27.2 ± 1.1 g) were not

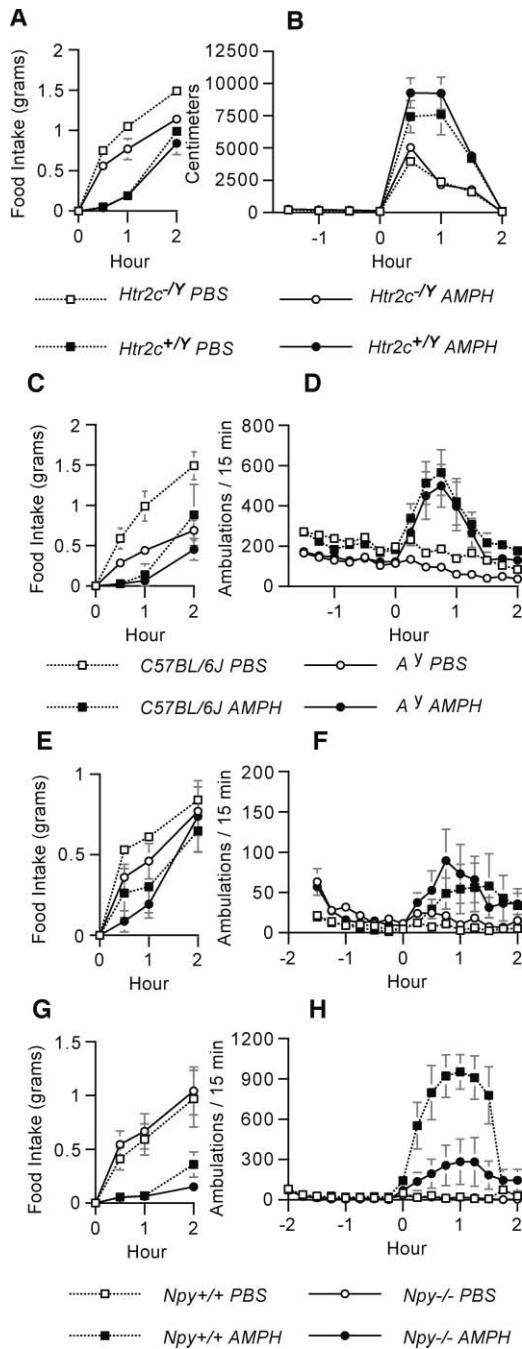
as hyperphagic after a fast as were the lean (20.5 ± 0.6) controls (Figure 5C).

The release of NPY and AgRP by arcuate neurons is thought to stimulate feeding. DA exerts tonic inhibitory control over NPY expression in the arcuate nucleus (Li and Pelletier, 1986; Obuchowicz, 1996; Pelletier and Sirmard, 1991), and it has been hypothesized that NPY participates in the hypophagic response to AMPH (Gillard et al., 1993; Kuo, 2003; Kuo et al., 2001). At a dose of 2 μg/g AMPH, *Npy*<sup>-/-</sup> and *Npy*<sup>+/+</sup> mice displayed equivalent AMPH-induced hypophagia and hyperactivity (Figures 5E and 5F). However, the locomotor response of these mice, maintained on the 129/SvCPJ background, was markedly less than that of mice maintained on the C57BL/6 strain. Therefore, an additional experiment was conducted with a higher (4 μg/g) dose of AMPH. We again observed no difference between the *Npy*<sup>-/-</sup> and *Npy*<sup>+/+</sup> mice in their hypophagic response to AMPH. While a small trend appeared during the second hour, there was no difference between the intake of the knockouts and that of the controls during the last hour of the testing interval ( $p = 0.99$ , Figure 5G). In contrast, *Npy*<sup>-/-</sup> mice were more sensitive to the locomotor effects of AMPH and quickly entered stereotypy (Figure 5H and the Supplemental Data [http://www.neuron.org/cgi/content/full/44/3/509/DC1]).

Mice lacking serotonin 2<sub>C</sub> receptors or signaling via melanocortin 4 receptors (*A<sup>y</sup>*) or NPY are equally hypophagic in response to AMPH, suggesting that these monoamine and neuropeptide feeding effectors are not necessary for hypophagia in response to a moderate systemic dose of AMPH. Given these results and our prior observations that NE, Epi, *D*<sub>1</sub>-, and *D*<sub>2</sub>-receptors are likewise not required for AMPH-induced hypophagia, we hypothesized that the effects of AMPH on feeding do not depend upon monoamines acting primarily at hypothalamic sites associated with the control of feeding. To test this hypothesis, we used mice that completely lack DA and mice that lack DA in all areas (including the hypothalamus) outside the nigrostriatal terminal fields of the caudate-putamen (CPU).

#### DA Is Necessary and Striatal DA Dysregulation Is Sufficient for AMPH-Induced Hypophagia

DA is required for life-sustaining feeding behavior, as evidenced by the profound hypophagia of DD mice, which starve unless maintained by daily injection of the precursor L-dopa (Zhou and Palmiter, 1995). However, DD mice do manifest some ingestive behavior in the absence of releasable DA (Cannon and Palmiter, 2003). We observed their response to AMPH using computerized lickometer cages, which record each individual lick (Figure 6). To obtain similar levels of intake in the DD mice and controls in response to PBS, the mice were not fasted, and basal licking (Figure 7A) and activity (Figure 7D) in the 2 hr preceding injection were low for both genotypes. Both DD and control mice initiated intake in response to injection with PBS (Figure 7B), presumably due to the mild stress of being handled and injected. In response to AMPH 24 hr after L-dopa, control mice reduced intake, but DD mice did not (Figure 7B). This is consistent with the response of reserpinized rats to AMPH, in which a single injection of AMPH stimu-



**Figure 5.** *A<sup>y</sup>*, *Htr2c*<sup>-/-</sup>, and *Npy*<sup>-/-</sup> Mice Demonstrate Robust AMPH-Induced Hypophagia

(A) Naive 3- to 5-month-old male mice (7 *Htr2c*<sup>-/-</sup> and 8 *Htr2c*<sup>+/-</sup>) reduced fasting-induced intake in response to AMPH ( $p < 0.01$ ). (B) Hyperactivity in response to AMPH was observed in both *Htr2c*<sup>-/-</sup> and *Htr2c*<sup>+/-</sup> mice ( $p < 0.01$ ). *Htr2c*<sup>-/-</sup> and *Htr2c*<sup>+/-</sup> mice were tested in a separate animal facility using the same protocol. (C) Naive 2-month-old female mice (6 C57BL/6J controls and 6 A<sup>y</sup>) responded with robust hypophagia after systemic AMPH ( $p < 0.01$ ), although the hyperphagic response to fasting of A<sup>y</sup> mice was less than that of controls ( $p < 0.01$ ). (D) Both control and A<sup>y</sup> mice demonstrated comparable hyperactivity in response to AMPH ( $p < 0.01$ ), although the A<sup>y</sup> mice were less active prior to injection ( $p < 0.05$ ). (E) Naive 3-month-old male mice (7 *Npy*<sup>+/+</sup> and 8 *Npy*<sup>-/-</sup>) were significantly hypophagic in response to AMPH (2  $\mu$ g/g) as compared to

deprivation-induced intake after PBS injection ( $p < 0.01$ ). In this experiment, the *Npy*<sup>-/-</sup> mice tended to manifest less robust fasting-induced hyperphagia in the first 30 min after PBS, although this trend was not significant.

(F) *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice maintained on the 129/SvCpJ background demonstrated significant hyperactivity after injection with AMPH (2  $\mu$ g/g) ( $p < 0.05$ ), although their response at this dose was not as robust as that of mice maintained on the C57BL/6J or mixed backgrounds. (G) Naive 4-month-old male mice (6 *Npy*<sup>+/+</sup> and 7 *Npy*<sup>-/-</sup>) were significantly hypophagic in response to AMPH (4  $\mu$ g/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ). (H) *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice demonstrated significant hyperactivity after injection with AMPH (4  $\mu$ g/g) ( $p < 0.05$ ), although the ambulatory response of the *Npy*<sup>-/-</sup> mice was limited as they quickly entered a stereotypic behavioral pattern. Increased anxiety may have contributed to their reduced hyperlocomotion (a movie documenting the behavioral responses of *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice to AMPH is included with the Supplemental Data [http://www.neuron.org/cgi/content/full/44/3/509/DC1]).

lated feeding (Neill and Grossman, 1971). In fact, DD mice given AMPH drank more than when given PBS ( $p < 0.01$ ). Some residual DA remains 24 hr after L-dopa, and this DA is liberated by AMPH, which accounts for the locomotor activation of DD mice following the first dose of AMPH (Figure 7E). However, after purging the residual DA by prior AMPH treatment, DD mice demonstrated no locomotor activation in response to subsequent AMPH (Figure 7F), in agreement with previous results (Cannon and Palmiter, 2003; Heusner et al., 2003; Szczypka et al., 1999). DD mice responded to the second injection of AMPH with increased feeding (Figure 7C). Thus, injection of AMPH failed to suppress the intake of liquid diet by DD mice in the absence of any releasable DA or locomotor hyperactivity. Restoration of DA to the dorsal striatum (caudate putamen [CPu]) using recombinant adeno-associated viruses expressing tyrosine hydroxylase (TH) and GTP cyclohydrolase 1 (GTPCH1) rescues feeding behavior in DD mice, such that they can sustain themselves without L-dopa injections (Szczypka et al., 2001). In the present study, virally rescued DD mice (vrDD) respond to a fast with increased food intake (Figure 7G). In response to AMPH, vrDD mice reduced food intake similar to control mice (Figure 7G). Thus, AMPH-induced hypophagia is intact in the complete absence of hypothalamic DA. Taken together with our prior results, that NE and Epi are not required, we conclude that hypothalamic catecholamines are not necessary for hypophagia in response to a moderate systemic dose of AMPH. The vrDD mice were more active during the basal, fasting interval relative to controls; however, vrDD mice actually decreased their locomotor behavior once food was presented (Figure 7H). The locomotor response of vrDD mice to AMPH was blunted (Figure 7H) because DA action in the NAc is required for hyperactivity after AMPH (Heusner et al., 2003; Kelly et al., 1975). However, the vrDD mice did manifest some behavioral signs of AMPH treatment not observed after injection with PBS, including sniffing and occasional sprints down the length of the cage. They were not in stereotypy.

In conclusion, mice lacking DA are resistant to the

deprivation-induced intake after PBS injection ( $p < 0.01$ ). In this experiment, the *Npy*<sup>-/-</sup> mice tended to manifest less robust fasting-induced hyperphagia in the first 30 min after PBS, although this trend was not significant. (F) *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice maintained on the 129/SvCpJ background demonstrated significant hyperactivity after injection with AMPH (2  $\mu$ g/g) ( $p < 0.05$ ), although their response at this dose was not as robust as that of mice maintained on the C57BL/6J or mixed backgrounds. (G) Naive 4-month-old male mice (6 *Npy*<sup>+/+</sup> and 7 *Npy*<sup>-/-</sup>) were significantly hypophagic in response to AMPH (4  $\mu$ g/g) as compared to deprivation-induced intake after PBS injection ( $p < 0.01$ ). (H) *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice demonstrated significant hyperactivity after injection with AMPH (4  $\mu$ g/g) ( $p < 0.05$ ), although the ambulatory response of the *Npy*<sup>-/-</sup> mice was limited as they quickly entered a stereotypic behavioral pattern. Increased anxiety may have contributed to their reduced hyperlocomotion (a movie documenting the behavioral responses of *Npy*<sup>+/+</sup> and *Npy*<sup>-/-</sup> mice to AMPH is included with the Supplemental Data [http://www.neuron.org/cgi/content/full/44/3/509/DC1]).

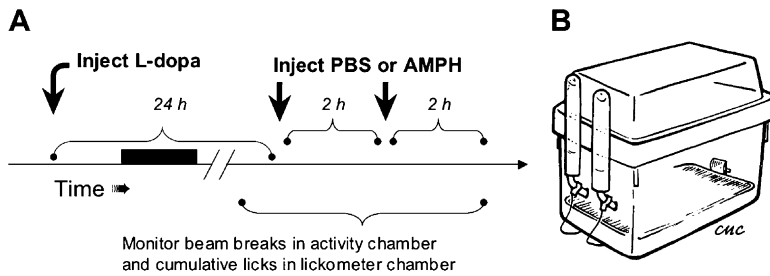


Figure 6. Procedure for Tests of AMPH-Induced Hypophagia in DD Mice

(A) L-dopa was given daily, except on the day of testing. Twenty-four hours after L-dopa injection, an initial dose of AMPH (2  $\mu$ g/g) was administered to eliminate any residual DA in dopaminergic terminals from prior L-dopa treatment. Two hours later, a second dose of AMPH was administered to determine the response of DD mice to AMPH in the absence of releasable DA.

(B) Because DD mice are profoundly hypo-

phagic and may, in addition, have difficulty in grasping and manipulating solid food pellets, we tested these mice for their response to AMPH in computerized lickometer chambers that were modified for mice as described (Cannon and Palmiter, 2003). Mice lived in the chambers during the duration of the experiment, and were given access to the palatable liquid diet LD-101 and water at all times.

hypophagic effects of AMPH and will, in fact, increase food intake after AMPH injection. Restoration of DA signaling to only the CPU restored the hypophagic response to AMPH. These data support the hypothesis that AMPH-induced hypophagia can be caused by release of DA only in the striatum and are able to explain observations that were inconsistent with previous accounts of AMPH-induced hypophagia. For example, it has been suggested that hypothalamic catecholamine receptors were not required, because excitotoxic lesions of the LH did not attenuate AMPH-induced hypophagia (Clark et al., 1992; Winn et al., 1984). Our results also agree with the observation that AMPH does not reduce the voluntary intake of a diet infused directly into the mouth, but does reduce the intake of the same diet when available from a bottle in the cage (Wolgin et al., 1988), suggesting that AMPH reduces food intake without necessarily reducing hunger.

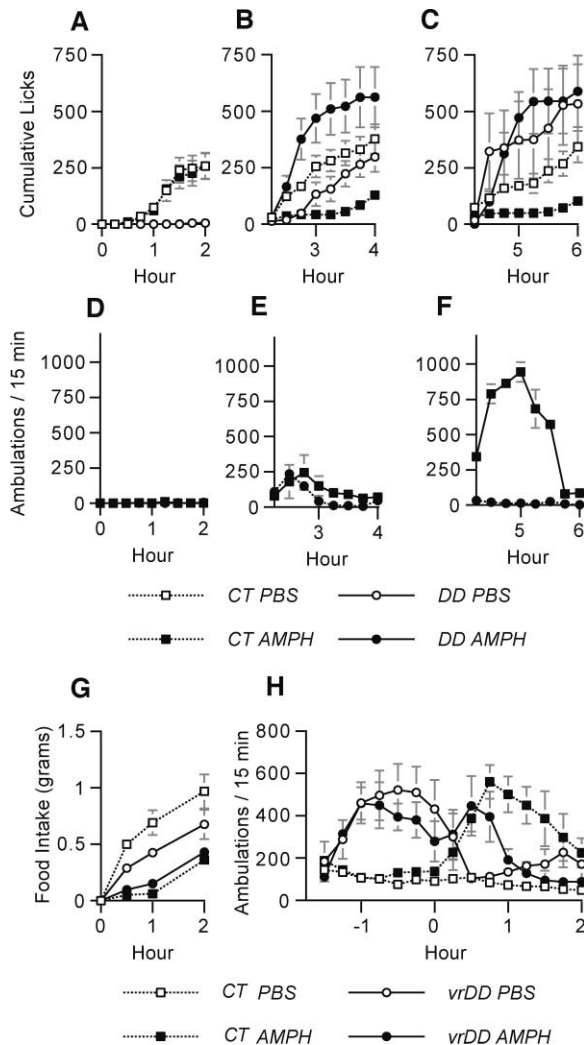
### General Discussion

We suggest that the effects of DA in the CPU on feeding are “downstream” of identified pathways controlling food intake in other brain areas, including the hypothalamus, NAc, cortex, and brainstem. This is predicated upon the number and variety of feeding effectors that fail to affect feeding in the absence of regulated striatal DA signaling. Mice lacking both DA and leptin (DD  $\times$  *ob/ob*) are as hypophagic as DD mice, despite greater levels of locomotor activity (Szczytko et al., 2000), and weight loss and aphagia are observed following severe 6-OHDA lesions in all obese models studied (e.g., Bailey and Flatt, 1990; Pellemounter and Lorden, 1983). Furthermore, insufficient DA or blockade of DA receptors abolishes the hyperphagic effects of diverse orexigenic stimuli, including NPY (Levine and Morley, 1984), insulin (Breese et al., 1973; Marshall and Teitelbaum, 1973), the glucose antimetabolite 2-deoxyglucose (Lu and Rowland, 1993), the fatty acid oxidation inhibitor mercaptoacetate (Garosi et al., 1995), serotonin 1A receptor agonists such as 8-OH-DPAT and buspirone (Fletcher, 1991; Fletcher and Davies, 1990), the benzodiazepine diazepam (Naruse et al., 1991), the cholinergic agonist carbachol (Parker et al., 1991), intraaccumbens blockade of glutamate receptors (Maldonado-Irizarry et al., 1995), local injection of muscimol into the nucleus raphe dorsalis (Borsini et al., 1983), morphine-conditioned feeding (Kelley et al., 2000), sham feeding (Schneider, 1989), tail pinch stress (Antelman and Szechtman, 1975;

Morley et al., 1982; Samarghandian et al., 2003), and electrical brain stimulation (Phillips and Nikaido, 1975). Animals lacking striatal DA remain sensitive to homeostatic drives such as hunger (Papp and Bal, 1987) and demonstrate intact responses to natural rewards such as sucrose (Cannon and Bseikri, 2004; Cannon and Palmiter, 2003) but are not capable of adequate feeding behavior.

Separate and fundamentally different controls of feeding are exerted by catecholamines in different brain areas (Hanlon et al., 2003; Hoebel et al., 1989; Kaplan and Sodersten, 1994; Sederholm et al., 2002). That striatal DA is “downstream” of hypothalamic DA is consistent with the ability of  $D_2$  and  $\beta$ -adrenergic antagonists given locally into the pLH to attenuate the hypophagic effects of low doses (0.5–1  $\mu$ g/g) (Leibowitz, 1975a) but not moderate or high doses of AMPH (Gilbert and Cooper, 1985; Sanghvi et al., 1975). DA, NE, and Epi are released in the pLH during feeding (Yang and Meguid, 1995; Yang et al., 1996) and suppress feeding at  $D_2$  and  $\beta$ -adrenergic receptors, respectively (Yang et al., 1997), while  $D_2$  and  $\beta$ -adrenergic receptor antagonists stimulate feeding in the pLH (Leibowitz and Rossakis, 1979; Parada et al., 1988b). Thus, after a low dose of AMPH that did not result in excessive striatal DA release, the hyperphagic effect of DA blockade in the hypothalamus was revealed (Leibowitz, 1975a). However, after higher doses of AMPH, blockade of DA receptors in the pLH could not increase feeding (Gilbert and Cooper, 1985; Sanghvi et al., 1975). We suggest that this is because excessive DA release in the striatum interfered with feeding behavior.

In addition to our viral rescue experiments, substantial evidence suggests that DA signaling in the CPU is important for the feeding behavior of normal individuals. The CPU receives topographic, glutamatergic projections from feeding-associated areas of the cortex where lesions produce aphagia (Kolb and Nonneman, 1975; Kolb et al., 1977). Food, and food-related stimuli, such as the smell and sight of food, enhance activity in the caudate and putamen in humans (Gautier et al., 1999) and increase release of DA in the CPU of animals (Itoh et al., 1990; Joseph and Hodges, 1990). Although excessive release of DA causes hypophagia, modest increases in DA signaling in the CPU can elicit feeding in sated animals (Inoue et al., 1997; Pal and Thombre, 1993). In addition, obesity and striatal DA receptor signaling and dysfunction have been associated in humans (Chen, 2001; Noble, 2003; Wang et al., 2001). Thus, acute changes in the regulation of striatal DA signaling affect



**Figure 7. DD Mice Are Resistant to the Hypophagic Effect of AMPH, but Viral Restoration of DA to the CPu Restores AMPH-Induced Hypophagia**

(A) Nonfasted mice (control, or CT, PBS,  $n = 19$ ; CT AMPH,  $n = 20$ ; DD PBS,  $n = 21$ ; DD AMPH,  $n = 19$ ) demonstrated minimal licking behavior prior to handling, although CT mice demonstrated more licking late in the baseline period,  $F(1,75) = 27.6$ ,  $p < 0.01$ , perhaps in response to the entry of the experimenter into the testing room. (B) Injection ( $T = 2$ ) of AMPH inhibited PBS-injection-induced licking behavior in CT but not DD mice, such that there was a significant interaction between dose and genotype  $F(1,75) = 18.8$ ,  $p < 0.01$ . The intake of DD mice following AMPH was greater than that following PBS ( $p < 0.01$ ). (C) After a second injection ( $T = 4$ ), there was a significant effect of genotype,  $F(1,75) = 9.5$ ,  $p < 0.01$ . The consumption by CT mice following AMPH was significantly suppressed as compared to the intake of DD mice after AMPH,  $p < 0.01$ . (D) Basal activity of nonfasted male and female CT ( $n = 6$ ) and DD ( $n = 8$ ) mice was low prior to handling. (E) The first injection of AMPH ( $T = 2$ ) 24 hr after L-dopa stimulated locomotion in CT and DD mice. (F) A second injection of AMPH ( $T = 4$ ) stimulated locomotion in CT but not DD mice. The effect of PBS on CT and DD mice in this protocol has been published previously (Heusner et al., 2003). (G) Male and female mice (7 vrDD and 6 CT) were significantly hypophagic in response to AMPH (2  $\mu\text{g/g}$ ) as compared to deprivation-induced intake after PBS injection ( $p < 0.05$ ). For histology of a representative viral injection site, see the Supplemental Data (<http://www.neuron.org/cgi/content/full/44/3/509/DC1>).

feeding behavior in otherwise normal individuals, and enduring changes may ultimately influence body weight and obesity.

### The Importance of Regulated DA Release for Feeding

It has been suggested that striatal DA must remain at an "optimal level" for feeding behavior to occur (Heffner et al., 1977). Acute systemic administration of all drugs that prolong normal DA signaling, including AMPH, methamphetamine (Kraeuchi et al., 1985), cocaine (Balopole et al., 1979; Morley and Flood, 1987; Wellman et al., 2002), DA reuptake transporter blockers (van der Hoek and Cooper, 1994), monoamine oxidase inhibitors (Banchelli et al., 2001; Durcan et al., 1988), and DA receptor agonists (Chen et al., 2001; Cincotta et al., 1997; Al-Naser and Cooper, 1994; Kuo, 2002; Rahminiwati and Nishimura, 1999; Scislawski et al., 1999), can also produce hypophagia. Too little DA is equally disruptive to normal feeding, as demonstrated by the profound hypophagia of animals that lack DA (Szczycka et al., 1999; Ungerstedt, 1971; Zhou and Palmiter, 1995; Zigmond and Stricker, 1972). Injection of the DA precursor L-dopa is sufficient to restore adequate voluntary food intake in DD mice, demonstrating that the lack of DA is indeed the primary cause of their hypophagia (Szczycka et al., 1999; Zhou and Palmiter, 1995). The presence of DA alone, however, cannot normalize striatal function, particularly with respect to feeding behavior. For example, DA-releasing grafts of nigral tissue implanted into the striatum did not reverse the aphagia of rats after bilateral nigrostriatal lesion by 6-OHDA, despite improvement in their motor behaviors (Bjorklund et al., 1980). In fact, aphagia was exacerbated by the DA-releasing grafts and improved by their subsequent removal, perhaps because graft-derived DA reduced postsynaptic hypersensitivity to DA released from intact terminals. Likewise, DA receptor agonists fail to promote sustained feeding in DD mice, perhaps because the agonists do not mimic the normal dynamics of dopaminergic signaling (Kim and Palmiter, 2003). These results suggest that mere activation of DA receptors, by pharmacological agonists or DA itself, is inadequate for normal striatal function with respect to feeding behavior and that the dynamics of dopaminergic signaling may be critical.

Midbrain dopaminergic neurons become activated by a variety of salient environmental stimuli via neural inputs from the amygdala, lateral hypothalamus, accumbens, pedunculo-pontine nucleus, and feedback from the striatum and pars reticulata, resulting in bursts of action potentials and increased release of DA in the striatum. Bursts of impulses result in much higher tran-

(H) Activity of vrDD mice during the baseline period was significantly greater than that of the CT mice ( $p < 0.01$ ). Female and male vrDD mice were not significantly more or less hyperactive after AMPH as compared to their fasted, baseline activity, although there was a trend toward less activity in the 2 hr following AMPH ( $p = 0.07$ ). Fasting may have resulted in the basal hyperactivity of the vrDD mice, as consumption of food following PBS injection reduced their activity. However, after AMPH, neither group demonstrated stereotypy, and unfasted vrDD mice do not respond to AMPH with hyperactivity (Heusner et al., 2003).

sient extracellular concentrations of DA than the same number of more evenly spaced impulses (Grace and Bunney, 1984; Nissbrandt et al., 1994). As a consequence, D<sub>1</sub> receptors, which have a lower affinity for DA than D<sub>2</sub> receptors, are activated preferentially during bursts (Venton et al., 2003). Because mice lacking either D<sub>1</sub> or D<sub>2</sub> receptor signaling are able to eat, we suggest that neither receptor is required for regulated signaling to occur. Rather, the temporal pattern of regulated DA release in the striatum may modulate coincident cortical and thalamic inputs and amplify the difference between strong and weak signals (Nicola et al., 2000).

Our working hypothesis is that appropriate striatal integration of these diverse inputs may be essential for the ability to respond to salient environmental stimuli and homeostatic drives such as hunger and thirst with appropriate goal-directed behaviors, mediating the translation of homeostatic integration of metabolic and lipostatic signals into behavior. Striatal DA is not necessary for motor behavior per se but is necessary to direct motor behavior toward appropriate goals. For example, depletion of DA from the caudate results in profound hypophagia and hypoactivity, while surgical removal of the entire striatum, which also results in profound and irreversible hypophagia, is instead accompanied by intense hyperactivity (Sorenson and Ellison, 1970; Whittier and Orr, 1962). It is clear that excessive and/or unregulated striatal DA does not disrupt locomotion, because AMPH, cocaine, and other dopaminergic drugs cause hyperactivity. And while manipulation of the CPU affects other goal-oriented tasks in addition to feeding behavior, regulated release of striatal DA may not be similarly required for all goal-oriented behaviors. The consumption of freely available foods, operant responding for food reward, and intracranial self-stimulation all depend upon DA in the CPU (Baunez et al., 1995; Bittner et al., 1981; Fray et al., 1983; Inoue et al., 1995; Neill et al., 1975), but self-stimulation and operant responding are increased by the same doses of systemic AMPH that reduce the consumption of freely available food in fasted animals (Brady, 1956; Phillips et al., 1991; Skinner and Heron, 1937; Zhang et al., 2003), suggesting that regulated release of striatal DA is particularly important for feeding. The present data, taken together with an extensive literature, suggest that after moderate doses of AMPH, hypophagia is not caused primarily by loss of appetite but that the hypophagia resulting from either too much or too little DA is instead caused by “an altered brain state in which animals cannot respond selectively” (Heffner et al., 1977), particularly to internal homeostatic and motivational drives.

In conclusion, our current conception of the role of striatal DA in feeding suggests that the output of the striatum, which is modulated by regulated DA release, is essential for adequate feeding behavior. As a consequence, interference with transient changes in DA signaling in the striatum, which result from burst firing of dopaminergic neurons, inhibits voluntary food intake. AMPH-induced release of monoamines, including DA, in other brain regions may contribute to the overall effect. However, this view is fundamentally different than a favored alternative, which suggested that activation of hypothalamic dopaminergic nuclei, for example, by AMPH-induced release of catecholamines, inhibits feed-

ing, perhaps acting directly on other hypothalamic nuclei (Leibowitz, 1970a, 1975b). Though hypothalamic catecholamines may play a role in the control of feeding, the present results support the hypothesis that regulated striatal dopamine signaling is required for adequate feeding. A major hurdle at this stage is to understand where and how the CPU interacts with brainstem, hypothalamic, limbic, cortical, and thalamic pathways to organize behavioral responses that satisfy hedonic and homeostatic drives.

## Experimental Procedures

### Subjects

All experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee. All mice were bred and housed in a specific pathogen-free facility. Rooms were temperature and humidity controlled with a 12:12 light-dark cycle, with lights on at 0700 or 0800 hr. Standard polycarbonate home cages contained 1/8 inch BED-O-COB bedding (Animal Specialties) and a cotton nestlet block (Ancare Corp., N. Belmore, NY). Water was available ad libitum. Mice congenitally deficient in dopamine  $\beta$ -hydroxylase (*Dbh*<sup>-/-</sup>) were produced as described (Thomas et al., 1995) and maintained on a mixed 129/SvCPJ and C57BL/6J hybrid background. Heterozygous littermates (*Dbh*<sup>+/-</sup>) were used as controls for the *Dbh*<sup>-/-</sup> mice because they have normal levels of norepinephrine and epinephrine (Thomas et al., 1998). Neuropeptide Y knockout mice and controls (*Npy*<sup>-/-</sup> and *Npy*<sup>+/+</sup>) (Erickson et al., 1996) were maintained on a 129/SvCPJ background. Mice congenitally deficient in dopamine D<sub>2</sub> receptors (*D2r*<sup>-/-</sup>) (Kelly et al., 1997) or D<sub>1</sub> receptors (*D1r*<sup>-/-</sup>) (Drago et al., 1994) were maintained on a C57BL/6J background. Heterozygous male and female mice were bred to generate homozygous knockout and wild-type progeny. Mice were genotyped by PCR. Agouti obese (*A*<sup>y</sup>) mice and age-matched C57BL/6J controls were obtained from Jackson Labs (Bar Harbor, ME). Male hemizygous (the *Htr2c* gene is X-linked [Mlatovich et al., 1992]) 5-HT<sub>2c</sub> receptor mutant (*Htr2c*<sup>-y</sup>) and wild-type mice (*Htr2c*<sup>+y</sup>) were bred as described on a congenic C57BL/6J background (Nonogaki et al., 1998). Mice lacking tyrosine hydroxylase (TH) in dopaminergic neurons (DD or *Th*<sup>-/-</sup>; *Dbh*<sup>TH/+</sup>) were bred as described and maintained on a mixed C57BL/6  $\times$  129/SvEv genetic background (Zhou and Palmiter, 1995). Littermate controls (CT) had at least one intact *Th* and one intact *Dbh* allele, which are sufficient for production of nearly normal levels of DA and NE (Rios et al., 1999; Thomas et al., 1998). DD mice were injected daily with 3,4 dihydroxyphenylalanine (L-dopa, 50  $\mu$ g/g IP), a DA precursor. Mice were maintained on and tested with pelleted higher energy (11% fat) breeder diet (5LJ5, PMI Nutritional Inc., Brentwood, MO); the higher energy diet was necessary for some of the knockout strains to maintain weight during repeated fasting.

### Viral Restoration of DA

DA production was restored in the CPU by local injection of two recombinant adeno-associated viruses (pAAV-1) expressing tyrosine hydroxylase (pAAV2-CBA-rTH-CMV-DsRed2, at a titer of  $3.6 \times 10^{12}$  genomic particles/ml) and GTP cyclohydrolase 1 (1-pseudotyped AAV2-CBA-hGTPCH1-CMV-DsRed2,  $5 \times 10^{12}$ ). We injected 0.8  $\mu$ l of these viruses combined in a 1:1 mixture bilaterally into the dorsal striatum (caudate putamen), using the coordinates 0.8 mm rostral and 2.19 mm lateral to bregma and 3.59 mm beneath the skull as described (Heusner et al., 2003; Szczypka et al., 2001). Transduced cells produced the precursor L-dopa, which was taken up by dopaminergic terminals and converted to DA. Because the vrDD mice were used in additional experiments subsequent (but not prior) to the present studies, placement could not be verified by histology in every mouse (although see the Supplemental Data for a representative injection site [<http://www.neuron.org/cgi/content/full/44/3/509/DC1/>]). Instead, the “virally rescued” (vrDD) mice were identified by their ability to maintain their body weight for more than 30 days without L-dopa treatment and respond to a fast with increased food intake.

## Drugs

All drugs were administered in a volume of 10  $\mu$ l/g body weight by intraperitoneal (IP) injection using a 30 gauge needle. AMPH (d-Amphetamine sulfate, Sigma Chemical Co., St. Louis, MO) and the  $\beta_1/\beta_2$ -adrenergic and 5-HT<sub>1</sub>/5-HT<sub>2</sub> receptor blocker ( $\pm$ )-propranolol HCl (RBI, Natick, MA) were dissolved in sterile 10 mM sodium phosphate, 150 mM NaCl, pH 7.5 (PBS). The DA receptor antagonist haloperidol (Sigma) was dissolved in a minimal volume of 10 N HCl and then diluted with PBS and neutralized to pH 7 with NaOH.

## Tests of Amphetamine-Induced Hypophagia

All mice were drug naive and tested as described in Figure 1A, with the exception of the DD and vrDD mice as described below. Ambulations were measured in polycarbonate cages (20  $\times$  20  $\times$  40 cm) equipped with four infrared photobeams, spaced at 8.8 cm apart along the long axis (San Diego Instruments, San Diego, CA), and a water spout inserted 3 cm above the cage floor, in the center of one of the short walls. Each chamber had 1–2 cups of 1/8 inch cobb bedding placed roughly in the center. Because DD mice are profoundly hypophagic and may, in addition, have difficulty in grasping and manipulating solid food pellets, we tested these mice for their response to AMPH in computerized lickometer chambers (Columbus Instruments, Columbus, OH) that were modified for mice as described (Cannon and Palmiter, 2003). Mice lived in the chambers during the duration of the experiment and were given access to a palatable liquid diet (LD-101; Test Diet, Richmond, IN) and water at all times. L-dopa was given daily, except on the day of testing. Twenty-four hours after the prior L-dopa injection, an initial dose of AMPH (2  $\mu$ g/g) was administered to eliminate any residual DA in dopaminergic terminals from prior L-dopa treatment. Two hours later, a second dose of AMPH was administered to determine the response of DD mice to AMPH in the absence of releasable DA. Virally injected (vrDD) mice and controls were tested for AMPH-induced hypophagia under the standard protocol (Figure 1A), with the following exceptions. Mice were tested twice, in counterbalanced order for AMPH or PBS. The vrDD and control mice were allowed access to food and water ad libitum for 5–7 days before the protocol was repeated with the treatments reversed. Some of the rescued mice lost weight and stopped eating after AMPH. These mice were given a single dose of L-dopa 48 hr after AMPH, which reversed the aphagia.

## Statistics

Data were analyzed by repeated measures ANOVA, followed by Tukey's post hoc analyses with the program STATISTICA 6.0 (Stat-Soft, Tulsa, OK). Where results refer to an ANOVA analysis for either main effect or the effect of genotype, the F value is given. In all other instances, the p value refers to the Tukey's comparison.

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