Inhibition of Dopamine and Norepinephrine Reuptake Produces Additive Effects on Energy Balance in Lean and Obese Mice

Sonja K Billes[1] and Michael A Cowley*,[1]

[1]*Division of Neuroscience, Oregon National Primate Research Center, Oregon Health and Science University, Beaverton, OR, USA*

Although originally developed as an antidepressant, long-term bupropion (BUP) treatment was recently shown to cause 5–8% weight loss over placebo in clinical trials with obese adults. BUP's antidepressant properties probably stem from its ability to increase extracellular brain dopamine (DA) and norepinephrine (NE) levels by inhibiting their reuptake, although the mechanism of BUP-induced weight loss is unknown. Consequently, the acute effects of DA and NE reuptake inhibition on energy homeostasis were determined by measuring food intake and body weight in mice following peripheral (intraperitoneal (i.p.)) administration of either BUP, a selective DA (GBR12783), or a selective NE (nisoxetine (NIS)) reuptake inhibitor. BUP, GBR12783, and NIS all dose-dependently decreased acute food intake in fasted lean mice. The ability of BUP to decrease food intake was independent of its ability to cause a temporary increase in locomotor activity. The inhibitory effects of acute GBR12783 and NIS on short-term food intake were additive. Subchronic (via mini-osmotic pump) administration of GBR12783 and NIS produced a transient nonadditive effect on food intake, but produced an additive reduction in body weight (8–10%). Because obesity can affect catecholaminergic signaling, we determined the effects of i.p. BUP, GBR12783, and NIS on short-term food intake in obese mice. Acute BUP, GBR12783, and NIS dose-dependently reduced acute food intake, and the additive effect of GBR12783 and NIS on acute food intake was preserved in obese mice. These results demonstrate that combined DA and NE reuptake inhibition produces additive effects on energy balance in lean and obese mice on both standard and high-fat diet, providing a foundation for further research on the effects of BUP and similar compounds on energy balance in mice.
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INTRODUCTION

Bupropion (BUP) is an atypical antidepressant currently approved for the treatment of depression (Wellbutrin®) and as an aid in smoking cessation (Zyban®) (Ascher *et al*, 1995; Hughes *et al*, 2004). BUP has also recently been demonstrated to produce a significant 5–8% weight loss in clinical trials with obese patients (Gadde *et al*, 2001; Anderson *et al*, 2002; Jain *et al*, 2002); however, the mechanism of BUP-induced weight loss is unknown.

BUP inhibits reuptake of the catecholamines dopamine (DA) and norepinephrine (NE) through blockade of the dopamine (DAT) and norepinephrine reuptake transporters (NET) (Ascher *et al*, 1995; Hoffman, 2001; Stahl *et al*, 2004). These transporters are generally located on the presynaptic cell membrane and serve as the primary mechanism for clearance of these catecholamines (Eisenhofer, 2001). By

blocking the removal of synaptic DA and NE, acute peripheral treatment with BUP produces transient changes in extracellular DA and NE concentrations in the brain (Nomikos *et al*, 1992), and may also alter the activity of the neurons that release DA and NE (Ascher *et al*, 1995; Paladini *et al*, 2003).

DA is essential for normal feeding behavior; DA-deficient mice do not eat in spite of hypothalamic changes that indicate they sense a nutritional deficit (Zhou and Palmiter, 1995). In contrast, NE-deficient mice are slightly hyperphagic (Thomas and Palmiter, 1997). Hypothalamic infusion of DA or NE can either increase or decrease food intake, depending on the location of infusion (Yang *et al*, 1997; Wellman, 2005). As both DA and NE are involved in the regulation of energy homeostasis (Wellman, 2005), BUP's unique effect on body weight may be owing to its combined effects on both dopaminergic and noradrenergic signaling.

The therapeutic potential for treatments that affect dopaminergic and noradrenergic signaling is apparent in that DA receptor agonists reduce elevated levels of the orexigenic neuropeptide, neuropeptide Y (NPY), in the hypothalamus and normalizes the hyperphagia, body weight gain, and hyperglycemia observed in obese *ob/ob* mice (Bina and Cincotta, 2000). In addition, the anorectic

effects of the only centrally acting Food and Drug Administration (FDA)-approved weight-loss drug, sibutramine (an NE and serotonin reuptake inhibitor), are abolished when rats are pretreated with an α_1-adrenoceptor antagonist (Jackson et al, 1997). Combined DA and NE reuptake inhibition (as with BUP) may therefore have an increased effect on energy homeostasis, owing to an additive or synergistic effect on these two systems.

Acute BUP increases energy expenditure in rodents (by increasing thermogenesis and VO_2 consumption) and appears to decrease food intake; however, the efficacy with which BUP inhibits food intake in rats is variable (Zarrindast and Hosseini-Nia, 1988; Liu et al, 2004). Reports that rats do not make significant quantities of hydroxybupropion, a BUP metabolite that possesses affinity for both the DA and NE reuptake transporters (Welch et al, 1987; Bondarev et al, 2003), suggest that rats may not be an optimal model for investigating the mechanism of BUP-induced weight loss. Although mice make the same three major bioactive BUP metabolites made by humans (Welch et al, 1987), the effects of BUP on energy balance in mice have not yet been reported.

The aim of this study was to investigate how DA and NE reuptake inhibition affects energy balance in lean and obese mice. First, we examined the effects of acute administration of BUP on short-term food intake and locomotor activity in lean mice, and examined the effects of selective DA and NE reuptake inhibitors on food intake. We then assessed how acute and subchronic coadministration of selective DA and NE reuptake inhibitors affects food intake and body weight in lean mice. Finally, we compared the effects of acute DA and NE reuptake inhibition on energy balance in lean and obese mice on a high-fat diet.

MATERIALS AND METHODS

Animal Care and Housing

All animal procedures were approved by the Oregon National Primate Research Center Institutional Animal Care and Use Committee. Adult male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were individually housed under a 12 h light/dark cycle and constant temperature (23°C). Food and water were available ad libitum, except where noted. Surgical procedures were performed under isoflurane anesthesia and using aseptic surgical procedures. Mice were maintained on standard chow, except where noted (Purina Lab Chow #5001; 3.34 kcal/g of energy, 59.8% carbohydrate, 28.0% protein, and 12.1% fat).

Drugs

All drugs were freshly prepared on the day of use. BUP (Sigma, St Louis, MO) was dissolved in sterile nonpyrogenic 0.9% NaCl. Nisoxetine (NIS) (Tocris, Ellisville, MO) was also dissolved in sterile saline, except during coadministration studies, when both NIS and GBR12783 (GBR; Tocris) were dissolved in 10% dimethyl sulfoxide and sterile saline (vehicle). Drugs were administered intraperitoneally (i.p.) in a volume of 0.1 ± 0.02 ml (according to body weight) for feeding tests. Control mice received vehicle in a corresponding volume.

Behavioral Testing Procedure

Individually housed mice were habituated to the behavioral testing procedure by daily i.p. injection of 0.1 ml sterile saline and 16 h (overnight) fasting every third day for at least 1 week before treatment. All mice were randomly distributed (according to body weight) to the treatment groups. On the morning of the testing day, fasted mice were injected with drug or vehicle, and given six previously weighed food pellets. Food was weighed at 1, 2, 4, 8, and 24 h postinjection. One week was allowed between testing sessions. Because BUP can cause seizures at high doses and dopaminergic drugs can affect locomotor activity, animals were closely monitored for the first few hours following drug injection and any adverse side effects were noted.

Telemetric Transponder Implantation and Activity Measurement

To explore the specific effect of acute BUP administration on locomotor activity, mice were implanted interscapularly with a PDT-4000 E-mitter sensor (MiniMitter, Sun River, OR). Mice were allowed 1-week recovery before behavioral feeding studies commenced. Three separate feeding experiments were performed (as described above), each with a different dose of BUP. For each experiment, 16 mice were divided into two weight-matched groups, one receiving vehicle injection and the treatment group receiving 10, 20, or 40 mg/kg BUP. Locomotor activity measurements were taken every 6 min using VitalView and Actiview software (MiniMitter).

Subchronic Drug Administration Studies

Adult male mice were divided into four weight-matched groups. Subcutaneous Alzet® minipumps (model 1007D and 2001; Durect Corporation, Cupertino, CA) containing drug or vehicle were implanted via a small incision in the skin behind the scapulae. The incision was closed with wound clips and mice were monitored daily. To minimize possible drug interaction effects, all mice were implanted with two minipumps. One minipump contained either NIS or NIS vehicle (90% sterile nonpyrogenic saline + 10% dimethyl sulfoxide), and the other contained GBR or GBR vehicle (50% sterile H_2O + 50% dimethyl sulfoxide). Minipumps delivered the following doses of drug: NIS (1.5 mg/kg/h), GBR (2.5 mg/kg/h), NIS (1.5 mg/kg/h) + GBR (2.5 mg/kg/h), or vehicle. These doses were based on a pilot experiment ($n = 2$/group), where the same dose of NIS was administered in combination with a higher dose of GBR (3.75 mg/kg/h). Mice that received GBR (3.75 mg/kg) exhibited stereotypy (by severely overgrooming) and were killed. Because stereotypy is a side effect of dopaminergic drugs, the dose of GBR was decreased to 2.5 mg/kg/h and no stereotypy was observed at this dose. Food intake and body weight were recorded daily.

Generation of Obese (Diet-Induced Obese, DIO) Mice

Eight-week-old male mice were kept on a high-fat diet (Rodent Chow #D12451, Research Diets Inc., New Brunswick, NJ; 4.75 kcal/g of energy, 35.0% carbohydrate, 20.0% protein, and 45.0% fat) for 20 weeks. After 20 weeks, DIO

mice are characterized by increased body weight and fat deposits, and reduced central leptin sensitivity (Lin *et al*, 2000). Age-matched lean mice were used as a control. Because all behavioral studies on obese mice were conducted by measuring intake of high-fat diet, lean age-matched (control) mice were also put on a high-fat diet just before feeding tests. The novel high-fat diet causes a temporary increase in food intake, so feeding tests were not begun until daily food intake stabilized (about 1 week).

Statistical Analysis

Data sets were analyzed using Prism Software (Graph-Pad Software, San Diego, CA) to generate sigmoidal dose–response curves, ID_{50}s (the estimated doses that result in 50% inhibition of food intake), area under the curve (AUC), and to determine statistical significance. One-way analysis of variance (ANOVA) and Tukey's multiple comparison post-tests were used to determine significant differences between treatment groups. Activity measurements were analyzed by comparing AUC values for vehicle and treatment groups and data were analyzed using one-way ANOVA (as above). For drug coadministration studies, two-way ANOVA was used to identify significant drug interactions (additivity/synergism) and independent main effects. A *P*-value < 0.05 was considered significant. Data are expressed as mean $\pm$ SEM.

Analysis of Drug Additivity/Synergy

Drug additivity occurs when the response to both drugs equals the sum of the individual responses to each drug.

Synergism occurs when the response to both drugs is significantly greater than the sum of their individual responses and the null hypothesis for synergism is an additive effect or the lack of a drug interaction. Isobolographic analysis and two-way ANOVA were both used to analyze feeding data in lean mice; two-way ANOVA was used to determine the effects of NIS and GBR for all other coadministration studies. An isobologram is constructed by plotting different doses of drug that produce the same effect (in this case, the doses of drug that produced the same fractional reduction in food intake) (Wessinger, 1986). For isobolographic analysis, doses of NIS and GBR that reduced food intake by about 50% were plotted and compared to the combined doses of these drugs that reduced food intake by 50%. For two-way ANOVA analysis, data (food intake or body weight) were plotted using Prism Software (Graph-Pad Software). The F-statistic for an interaction effect in a two-way ANOVA is mathematically comparable to testing the null hypothesis (for a review, see Slinker, 1998); therefore, a significant interaction between drug-treated animals ($P < 0.05$) was considered indicative of a synergistic interaction (Slinker, 1998).

RESULTS

Acute Inhibition of DA and NE Reuptake by BUP Causes a Dose-Dependent Reduction in Food Intake in Lean Mice

BUP (5, 10, 20, 40, or 80 mg/kg i.p.) dose-dependently inhibited 1-h food intake in 16 h food-deprived mice (Figure 1a; $n = 7$/group). The ID_{50} for BUP was 50 mg/kg
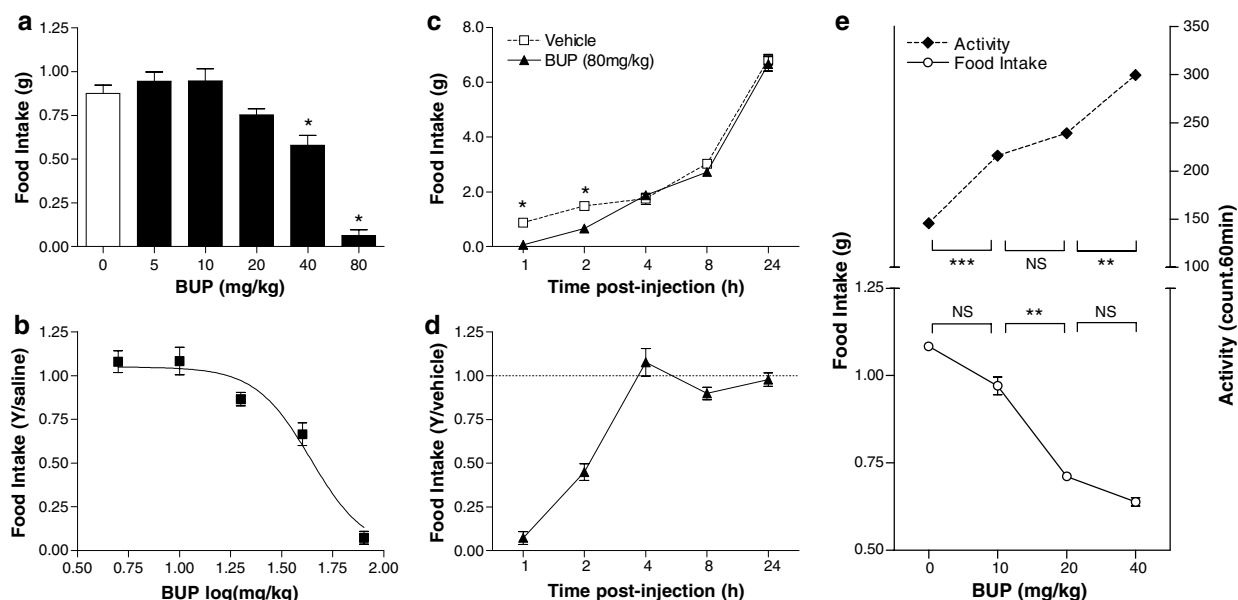
Figure 1 Acute combined inhibition of DA and NE reuptake by BUP produces a dose-dependent reduction in 1 h food intake in lean mice. Overnight food-deprived mice were treated with BUP (5, 10, 20, 40, or 80 mg/kg i.p.) or vehicle and their subsequent food intake was recorded. (a) BUP (40 and 80 mg/kg) significantly reduced 1 h food intake compared to vehicle (*$P < 0.05$). (b) Dose–response curve for reduction in 1 h food intake by BUP ($R^2 = 0.87$). (c) Time course for 80 mg/kg BUP and vehicle. Food intake differed significantly from vehicle at 1 and 2 h postinjection (**$P < 0.01$). (d) Time course for 80 mg/kg normalized to vehicle ($n = 7$/group). (e) BUP caused a dose-dependent increase in locomotor activity that was independent of food intake ($n = 8$/group). Larger doses of BUP that caused relative increases in activity (0–10 and 20–40 mg/kg) did not produce a comparable decrease in intake. Similarly, increasing the BUP dose from 10 to 20 mg/kg caused a significant reduction in food intake from 10 mg/kg, but no significant change in activity (**$P < 0.01$ and ***$P < 0.001$).

(Figure 1b). The effect of BUP on feeding was transient; cumulative intake of BUP-treated mice was similar to vehicle-treated animals by 4 h postinjection (and all subsequent time points) for all doses of BUP (Figure 1c and d). In pilot studies, a dose of 160 mg/kg appeared to produce seizures in some animals, as has been reported for high doses of this drug (Davidson, 1989). Therefore, 80 mg/kg was chosen for the highest dose to be tested; this dose did not produce seizures in any animals.

## BUP Produces Independent Effects on Food Intake and Locomotor Activity

Food intake decreased significantly from control only with the 20 and 40 mg/kg doses and only at 1 h postinjection (Figure 1e; $n = 8$/group). All doses of BUP (10, 20, and 40 mg/kg) significantly increased activity from control at 1 h postinjection (Figure 1e; $n = 8$/group) and activity returned to baseline 2–4 h postinjection. The increase in activity caused by peripheral BUP was dose-dependent. The effects of BUP on food intake and locomotor activity are independent, such that increasing doses of BUP that caused an incremental increase in activity did not also significantly decrease food intake (0–10 and 20–40 mg/kg). Likewise, the incremental decrease in food intake between the 10 and 20 mg/kg doses was not correlated with a simultaneous increase in activity.

Selective Inhibition of DA Reuptake by GBR12783 Dose-Dependently Reduces Food Intake in Lean Mice
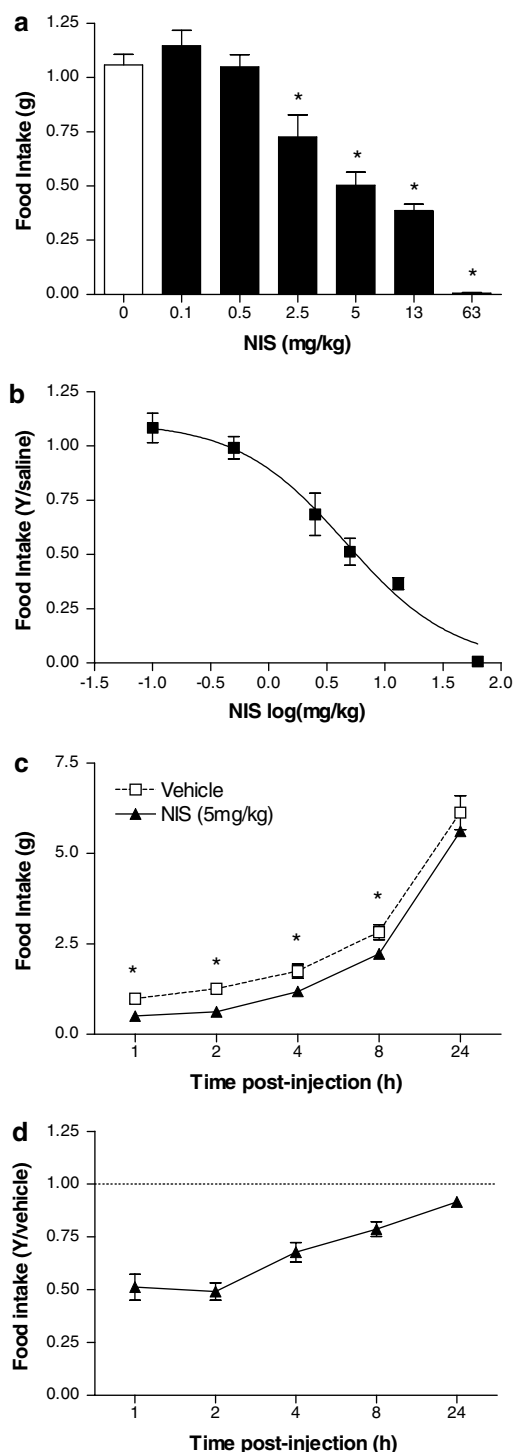
To determine whether DA reuptake inhibition contributes to the hypophagic response to BUP, the effect of a selective DA reuptake inhibitor, GBR12783 (GBR), on food intake was tested (Bonnet and Costentin, 1986). GBR (1, 2.5, 6, 16, or 40 mg/kg) or vehicle was administered i.p. to overnight (16 h) food-deprived mice. GBR reduced food intake in a dose-dependent manner (Figure 2a; $n = 6$/group). The extrapolated ID_{50} for GBR at 1 h was 14 mg/kg (Figure 2b). Acute administration of GBR had a transient effect on feeding; the reduction in food intake by GBR was absent by 4 h postinjection (Figure 2c and d). Anecdotal observation revealed that large doses of GBR (40 mg/kg) increased locomotor activity but no stereotopy or abnormal locomotor behaviors were observed.

Selective Inhibition of NE Reuptake by NIS Dose-Dependently Reduces Food Intake in Lean Mice

To determine whether inhibition of NE reuptake contributed to the reduction in feeding caused by BUP, the effect of NIS, a selective NE reuptake inhibitor (Cheetham et al, 1996), was determined by monitoring food intake in NIS- or vehicle-treated animals. Peripheral NIS (0.1, 0.5, 2.5, 13, and 63 mg/kg) produced a dose-dependent decrease in 1-h food intake (Figure 3a; $n = 6$/group). The extrapolated ID_{50} was 5 mg/kg at 1 h (Figure 3b). The reduction in feeding caused by peripheral NIS persisted longer than 8 h, but was absent 24 h postinjection (Figure 3c and d).
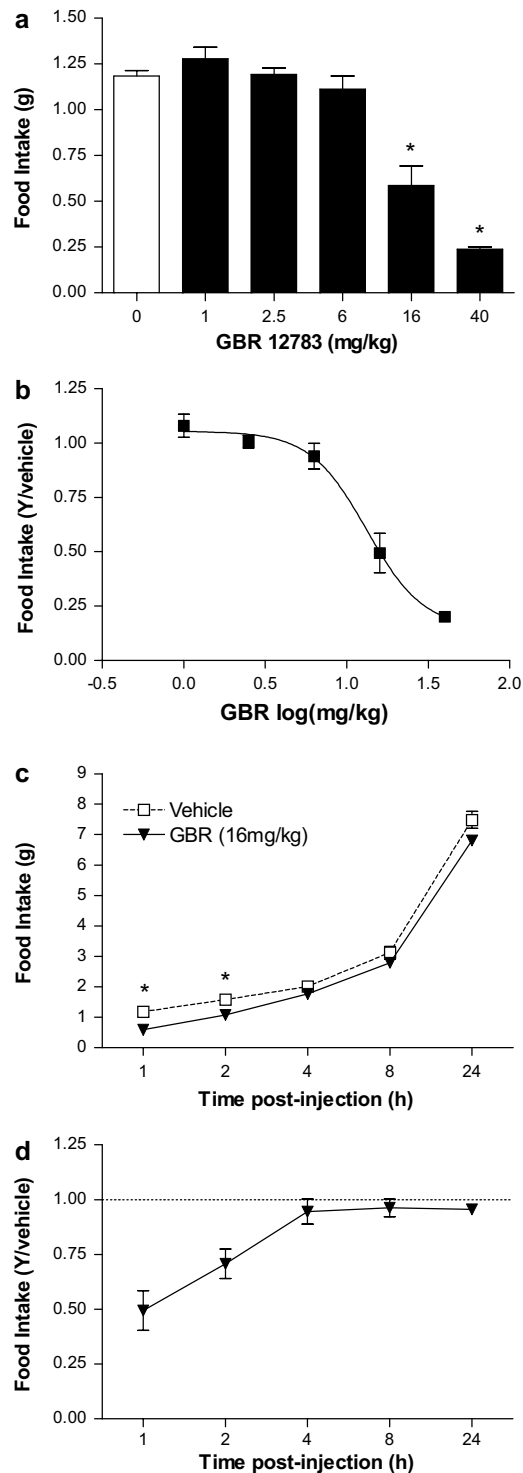
Figure 2 Acute selective inhibition of DA reuptake with GBR12783 produces a dose-dependent reduction in 1 h food intake in lean mice. Sixteen-hour food-deprived mice were treated with GBR (1, 2.5, 6.3, 16, and 40 mg/kg i.p.) or vehicle and their subsequent food intake was recorded. (a) GBR (16 and 40 mg/kg) significantly reduced 1 h food intake ($P < 0.05$). (b) Dose–response curve for reduction in 1 h food intake by GBR ($R^2 = 0.85$). (c) Time course for 16 mg/kg GBR and vehicle. Food intake differed significantly from vehicle at 1 h postinjection ($P < 0.05$). (d) Time course for GBR (16 mg/kg) normalized to vehicle ($n = 6$/group).

Acute Coadministration of DA and NE Reuptake Inhibitors has an Additive Effect on Food Intake in Lean Mice

Because acute inhibition of DA and NE reuptake both reduced short-term food intake, we tested whether simultaneous administration of GBR and NIS would produce an additive or synergistic effect on food intake. Combined administration of low drug doses that have minimal independent effects on food intake enables detection of a larger effect caused by a drug interaction. Thus, low doses of NIS and GBR were administered for coadministration studies. To determine how slight variations in dose might affect possible drug interactions, we administered two doses of GBR. The ID_{15} dose of NIS (3.5 mg/kg), and the ID_{15} (GBR1 = 4.5 mg/kg) and ID_{25} (GBR2 = 7.5 mg/kg) doses of GBR were extrapolated from the dose–response curves for each drug (Figures 2b and 3b). The ID_{15} dose of NIS was administered alone or in combination with the ID_{15} or ID_{25} doses of GBR (Figure 4a–f).

At 1 h postinjection, NIS (3.5 mg/kg) and GBR1 (4.5 mg/kg) independently reduced short-term food intake and caused a significantly greater decrease in food intake when administered together (Figure 4a; $n = 21$/group). NIS (3.5 mg/kg) reduced 1 h food intake by 17% and GBR1 (4.5 mg/kg) reduced 1 h food intake by 13% relative to control (Figure 4b). The predicted additive effect of combined administration of these drugs would be 30% inhibition. The actual reduction in food intake by coadministration of NIS and GBR1 was 34%. The comparable predicted additive and actual effects implies that NIS and GBR1 have an additive effect on food intake. NIS + GBR2 also produced a greater reduction in food intake than NIS or GBR2 alone that was absent by 4 h (Figure 4d and f). The predicted additive effect for NIS and GBR2 would be 41% inhibition of 1 h food intake (Figure 4e). The actual effect of combined administration of NIS and GBR2 was a 51% inhibition of food intake, suggesting that NIS and GBR2 might interact in a synergistic manner.

To further explore this possibility, isobolographic analysis was performed for NIS and GBR2 (Figure 5a). The combined doses of NIS and GBR that produced a 50% reduction in food intake fell on the additivity line, suggesting that acute NIS and GBR2 had only an additive effect on food intake. Two-way ANOVA analysis of mean 1 h food intake for NIS and GBR2 supported this finding (Figure 5b; $P = 0.205$, no significant interaction). NIS and GBR2 had significant independent effects on food intake that were additive ($P < 0.001$ for both drugs at 1 and 2 h postinjection). As expected, two-way ANOVA analysis of 1 h food intake following acute NIS and GBR1 also revealed no significant interaction (Figure 5c; $P = 0.490$), but a significant main effect of NIS and GBR1 at 1 h ($P < 0.001$ for both drugs). This analysis also revealed no significant interaction for NIS and GBR1 and for NIS and GBR2 at any other time points ($P > 0.05$; data not shown). Because two-way ANOVA provides both a visual and statistical interpretation of the data, this was the primary method we used for interpreting future data obtained from drug coadministration.
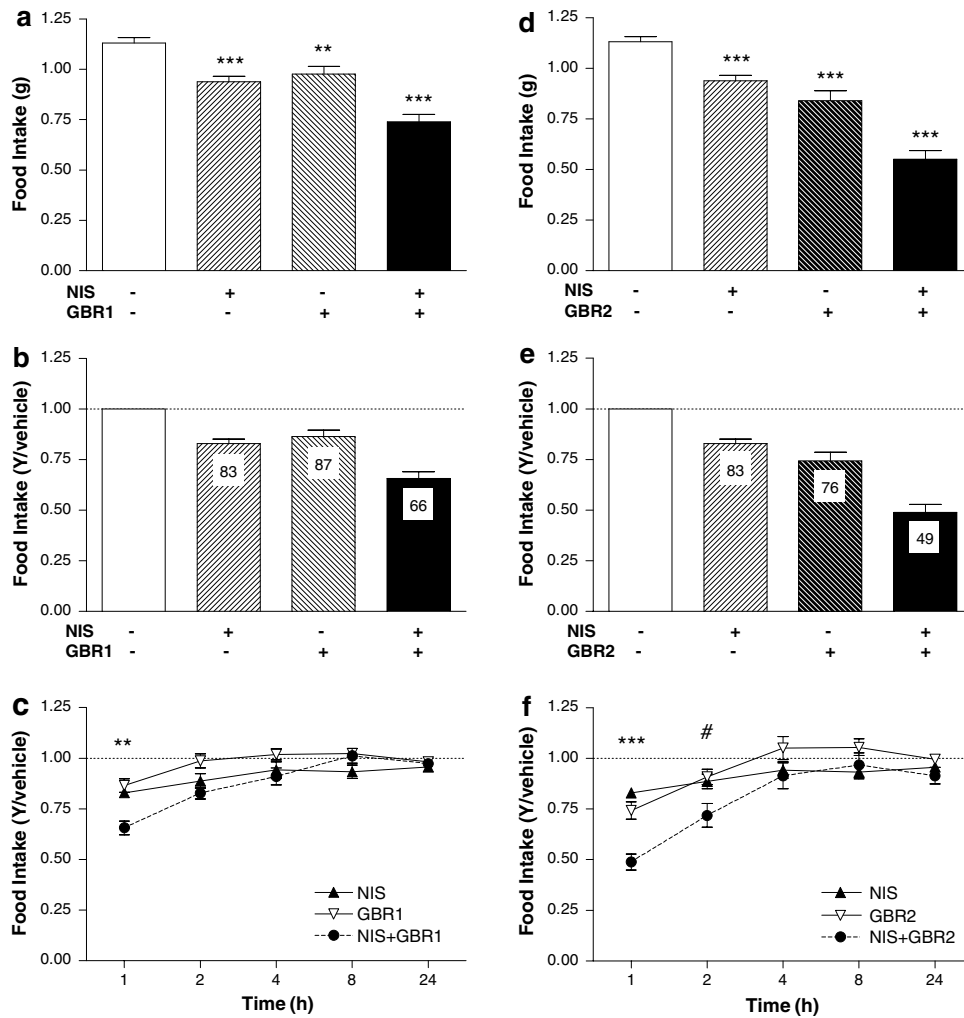
Figure 3 Acute selective inhibition of NE reuptake by NIS produces a dose-dependent reduction in 1 h food intake in lean mice. Sixteen-hour food-deprived mice were treated with NIS (0.1, 0.5, 2.5, 5, 13, and 63 mg/kg i.p.) or vehicle and their subsequent food intake was recorded. (a) NIS (2.5, 5, 13, and 63 mg/kg) significantly reduced 1 h food intake compared to vehicle ($P < 0.05$). (b) Dose–response curve for reduction in 1 h food intake by NIS ($R^2 = 0.89$). (c) Time course for 5 mg/kg NIS and vehicle. Food intake differed significantly from vehicle at 1, 2, 4, and 8 h postinjection ($P < 0.05$). (d) Time course for NIS (5 mg/kg) normalized to vehicle ($n = 6$/group).
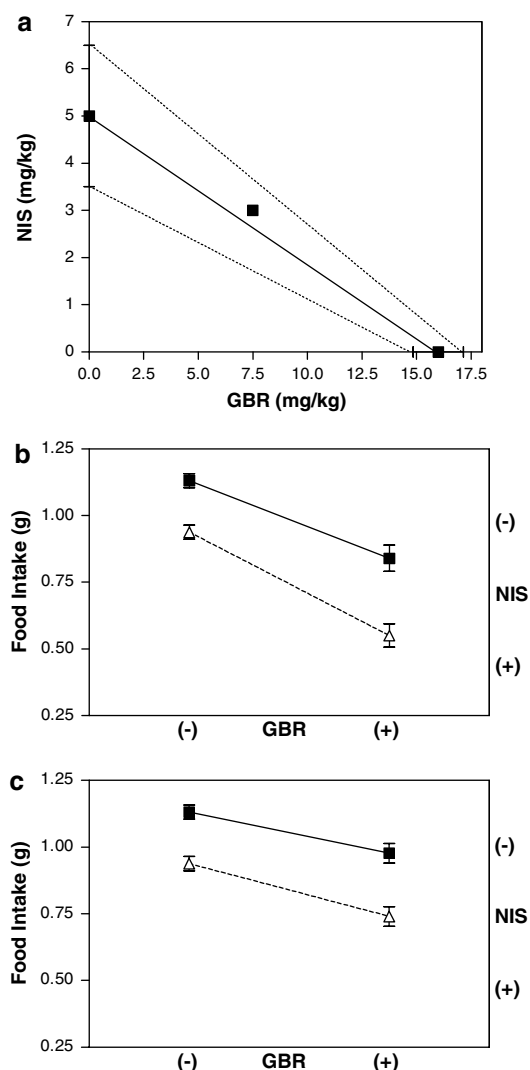
Figure 4 Acute inhibition of DA and NE reuptake reduces food intake in lean mice. (a–c) Sixteen-hour food-deprived mice were treated with NIS (3.5 mg/kg i.p.) or GBR1 (4.5 mg/kg i.p.). (a) NIS, GBR1, and NIS + GBR1 all produced a significant reduction in cumulative 1 h food intake compared to vehicle ($n = 21$/group). (b) One-hour food intake normalized to vehicle. Numbers in the column represent % of control intake. (c) Time course for NIS, GBR1, and NIS + GBR1 (normalized to vehicle). The reduction in food intake caused by NIS, GBR1, and NIS + GBR1 was absent by 2 h postinjection (**$P < 0.01$). (d–f) Food intake for mice treated with NIS (3.5 mg/kg i.p.) or GBR2 (7.5 mg/kg i.p.). (d) NIS, GBR2, and NIS + GBR2 all significantly reduced food intake relative to vehicle ($n = 21$/group). (e) One-hour food intake normalized to vehicle. Numbers in the column represent % of control intake. (f) Time course for NIS, GBR2, and NIS + GBR2 (normalized to vehicle). NIS or GBR2 caused a reduction in 1 h food intake only (***$P < 0.001$), but NIS + GBR2 caused a reduction in 1 and 2 h food intake (#$P < 0.01$).

Subchronic DA and NE Reuptake Inhibition Produces an Additive Decrease in Body Weight in Lean Mice

To determine how the effects of acute DA and NE reuptake inhibition on energy balance compare with the effects of continuous subchronic administration, NIS and GBR were administered for 1 week via mini-osmotic pump. Food intake differed significantly from vehicle ($n = 4$) in all three drug treatment groups (GBR, $n = 4$; NIS, $n = 4$; GBR + NIS, $n = 3$) on the first day of drug infusion, but returned to control for the duration of the infusion (Figure 6a). Subchronic inhibition of DA or NE reuptake caused a small reduction in body weight for the duration of the infusion that returned to control levels after drug infusion (Figure 6b). Of the three drug treatments given subchronically (NIS, GBR, and NIS + GBR), only NIS + GBR caused significant weight loss compared to

control on days 2–7 of drug infusion. On day 5, body weight of animals that received GBR + NIS was significantly reduced compared to animals that received only GBR or NIS (Figure 6c). By a loose definition, this could be interpreted as a synergistic interaction between the DA and NE reuptake inhibitors because neither the GBR- nor the NIS-treated animals differed significantly from control for the duration of the drug infusion. However, two-way ANOVA demonstrated that there was no statistically significant interaction between GBR and NIS on body weight on day 5 (Figure 6d; $P = 0.483$) or any other day (data not shown). Instead, two-way ANOVA revealed that NIS and GBR both produced significant main effects on days 4 and 5 ($P < 0.05$ for both NIS and GBR), indicating that NIS and GBR had significant independent effects on body weight that were additive.

Figure 5 Acute combined inhibition of DA and NE reuptake produces an additive reduction in food intake in lean mice. (a) Isobologram depicting doses of NIS (5 mg/kg; $n = 6$), GBR (16 mg/kg; $n = 6$), and NIS + GBR2 (NIS = 3.5 mg/kg; GBR2 = 7.5 mg/kg; $n = 21$) that reduced food intake by approximately 50% from control. (b) Two-way ANOVA of 1 h food intake in lean mice treated with vehicle, NIS (3.5 mg/kg), GBR2 (7.5 mg/kg), or NIS + GBR2 revealed no significant interaction between NIS and GBR2 ($P > 0.05$; $n = 21$/group). (c) Two-way ANOVA of 1 h food intake in lean mice treated with vehicle NIS (3.5 mg/kg), GBR1 (4.5 mg/kg), or NIS + GBR1 revealed no significant interaction between NIS and GBR1 ($P > 0.05$; $n = 21$/group).

Inhibition of DA and NE Reuptake Causes a Dose-Dependent Reduction in Food Intake in Obese Mice

To determine whether DA and NE reuptake inhibitors decrease food intake in an obese rodent model, BUP, GBR, and NIS were administered to lean (control; $n = 5$/group) and obese (DIO; $n = 7$/group) mice on a high-fat diet. Inhibition of DA and NE reuptake by acute (i.p.) injection of BUP (10, 50, and 80 mg/kg) caused a transient and dose-dependent reduction in 1 h food intake in lean and obese mice with an ID_{50} of 41 mg/kg for both lean and obese mice (Figure 7a and d). Acute inhibition of DA reuptake by GBR

(1, 2.5, 6.3, 14, and 40 mg/kg) transiently reduced intake of a high-fat diet in lean ($ID_{50} = 8$ mg/kg) and obese ($ID_{50} = 7$ mg/kg) mice (Figure 7b and e). Inhibition of NE reuptake by NIS (0.5, 2.5, 5, 13, and 30 mg/kg) also caused a dose-dependent reduction in short-term food intake in lean and obese mice (Figure 7c and f). The ID_{50} for NIS in obese mice was 5 mg/kg, about the same as for lean mice on a standard diet; however, NIS was slightly less efficacious at reducing intake of a high-fat diet in lean mice ($ID_{50} = 9$ mg/kg).

Acute Coadministration of DA and NE Reuptake Inhibitors has an Additive Effect on Food Intake in Obese Mice

To determine whether DA and NE reuptake inhibitors have an additive effect on food intake in obese mice, NIS (3.5 mg/kg), GBR (7.5 mg/kg), NIS + GBR, or vehicle were administered i.p. to 16 h food-deprived lean ($n = 9$/group) and obese ($n = 15$/group) mice on a high-fat diet. Although GBR and NIS + GBR significantly reduced 1 h intake of high-fat diet in lean mice, NIS (3.5 mg/kg) had no effect at 1 h (Figure 8a) or any of the time points measured (data not shown). GBR (7.5 mg/kg) produced a 49% reduction in intake of a high-fat diet at 1 h, and coadministration of NIS + GBR caused considerable (85%) inhibition of 1 h food intake (Figure 8b). However, two-way ANOVA revealed no significant interaction between NIS and GBR on intake of a high-fat diet in lean mice at 1 h postinjection (Figure 8c; $P = 0.116$) or at any other time points (data not shown), but instead indicated a significant effect of GBR on food intake ($P < 0.001$ for 1, 2, 4, and 8 h postinjection) but not for NIS ($P > 0.05$; Figure 8d).

NIS (3.5 mg/kg), GBR (7.5 mg/kg), and NIS + GBR all significantly reduced 1 h food intake in obese mice (Figure 8d). Two-way ANOVA of food intake for obese mice indicated that there was no significant interaction between NIS and GBR treatments at 1 h (Figure 8g; $P = 0.782$) or at any other time points postinjection (data not shown). However, NIS and GBR independently produced significant reductions in food intake up to 8 h postinjection ($P < 0.001$ for both drugs at 1, 2, 4, and 8 h; Figure 8h). Food intake returned to baseline 24 h following drug administrations for all treatment groups in both lean and obese mice.

DISCUSSION

Here, we have demonstrated that acute treatment with a dual DA and NE reuptake inhibitor (BUP) caused a dose-dependent reduction in short-term food intake in hungry mice that was independent of its effects on locomotor activity. Moreover, selective inhibition of either the DA (GBR12783) or NE (NIS) transporter also produced a dose-dependent reduction in short-term food intake. These independent effects of NIS and GBR were found to be completely additive when both drugs were coadministered. During subchronic drug administration, GBR and NIS only reduced food intake on day 1 of infusion and the acute additive effect of these compounds on feeding was absent. Instead, subchronic coadministration of NIS and GBR produced an additive reduction in body weight, with an
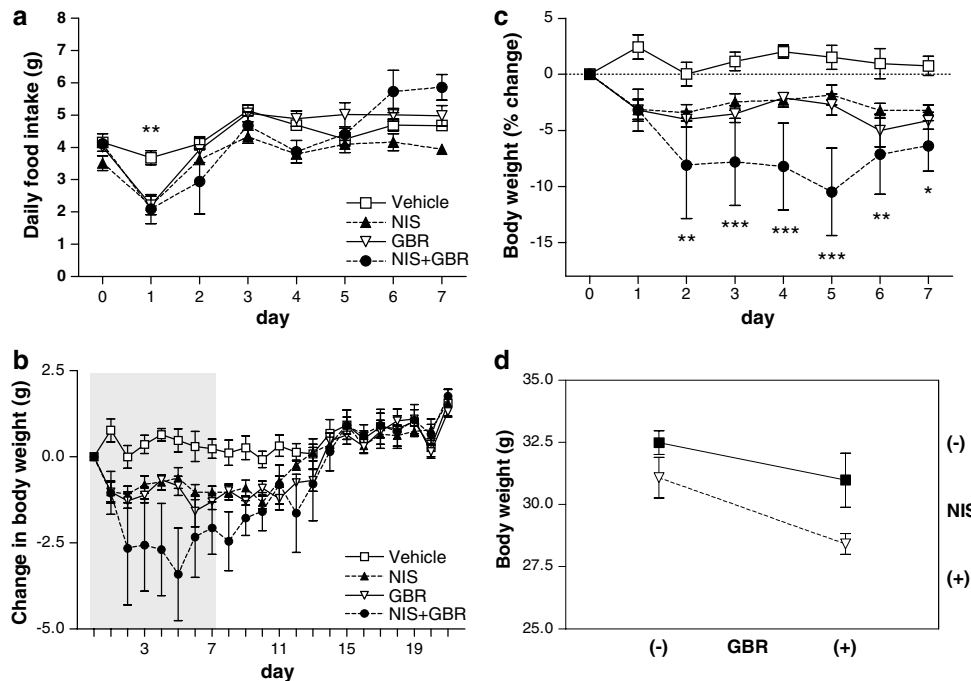
Figure 6 Subchronic DA and NE reuptake inhibition in lean mice produces an additive reduction in body weight without reducing food intake. NIS (1.5 mg/kg/h), GBR (2.5 mg/kg/h), or vehicle was infused via mini-osmotic pump that lasted for 1 week. (a) Daily food intake was significantly reduced from vehicle for all treatment groups only on day 1. (b) Change in body weight from preinfusion (average body weight on day 0 = 32 g ± SEM). Shaded region indicates duration of drug infusion. (c) % Change in body weight from day 0 for each animal. NIS + GBR caused a significant reduction in body weight compared to vehicle-treated animals. (d) Two-way ANOVA of body weight on day 5 of infusion reveals no significant interaction ($P > 0.05$). (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; vehicle, $n = 4$; NIS, $n = 4$; GBR, $n = 4$; NIS + GBR, $n = 3$).

average weight loss of 8–10% from control. Finally, the hypophagic effects of BUP, GBR, and NIS on energy balance were preserved in obese mice, as were the additive effects of acute NIS and GBR. These results demonstrate that both DA and NE may contribute to the anorectic effects of BUP and that the hypophagic efficacy of DA and NE reuptake inhibitors is not reduced in obese mice.

A complete isobolographic analysis requires the acquisition of multiple data points using several drug proportions that produce the exact same effect on energy balance, followed by regression analysis (Tallarida, 2001). Two-way ANOVA is a superior (and somewhat more rigorous) method for analysis of drug interactions in behavioral pharmacology because it does not require acquisition of multiple data points and has a simple visual and statistical interpretation of data that can be used to identify significant synergistic or additive effects (Slinker, 1998).

Isobolographic analysis and two-way ANOVA both indicated that GBR and NIS have additive effects on energy balance. The significance of an additive effect is better understood when presented in the context of other drugs that are associated with weight loss. The only FDA-approved centrally acting weight loss drug, sibutramine, inhibits reuptake of both serotonin and NE (Halford, 2006). Sibutramine's efficacy at causing weight loss is probably owing to an additive effect of serotonin and NE on energy balance (Rowland *et al*, 2001). Other drug combinations have been studied, but failed to produce an additive, let alone synergistic, effect on energy balance (Kaya *et al*, 2004).

The acute hypophagic effect of BUP presented here is seemingly in contrast to a previous observation in rats where acute oral BUP administration (10, 20, and 30 mg/kg *per os*) did not affect food intake (Liu *et al*, 2004). There are several explanations for this disparity. First, high doses of BUP are required to produce a reduction in 1 h food intake. The oral route of administration and low doses administered in the aforementioned experiment were likely insufficient to produce a measurable reduction in food intake. Second, the first feeding measurements were taken 4.5 h following drug administration. Here, we demonstrate that the inhibitory effect of BUP on feeding is transient——even moderate to high doses of BUP had no effect on food intake 4 h postinjection. Therefore, feeding measurements taken 4.5 h following drug administration may have missed a short-term effect of BUP on feeding. Additionally, BUP is metabolized differently in rats and mice; rats do not make significant quantities of the bioactive BUP metabolite, hydroxybupropion (Welch *et al*, 1987). Hydroxybupropion has a longer half-life than BUP, inhibits both the NE and DA transporters, and also has a functional role as an antidepressant (Martin *et al*, 1990; Ascher *et al*, 1995; Bondarev *et al*, 2003), implying that it may contribute to BUP-induced hypophagia; however, any effects of hydroxybupropion on appetite would not be observed in rats.

In contrast to the anorexigenic effect observed with acute administration, continuous infusion of catecholamine reuptake inhibitors produced a temporary reduction in 24 h food intake that was absent 48 h after drug infusion. Despite this short-term effect on feeding, NIS and GBR
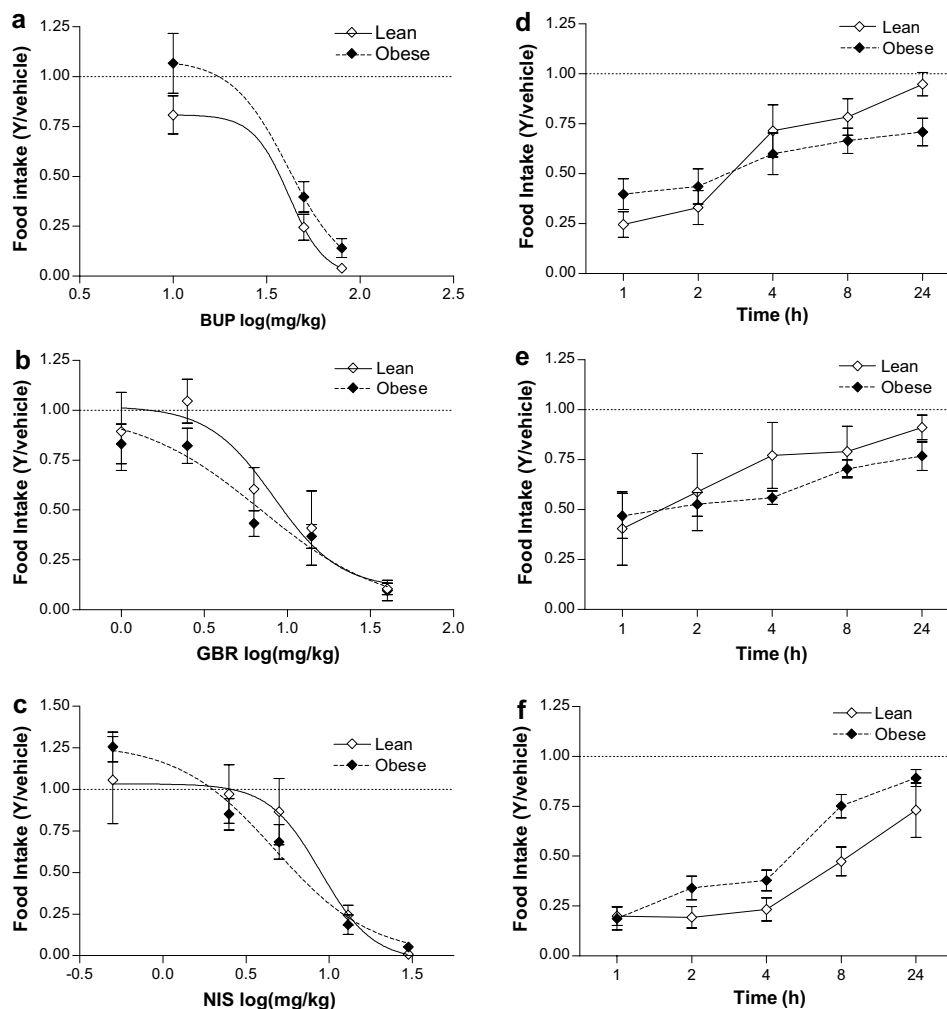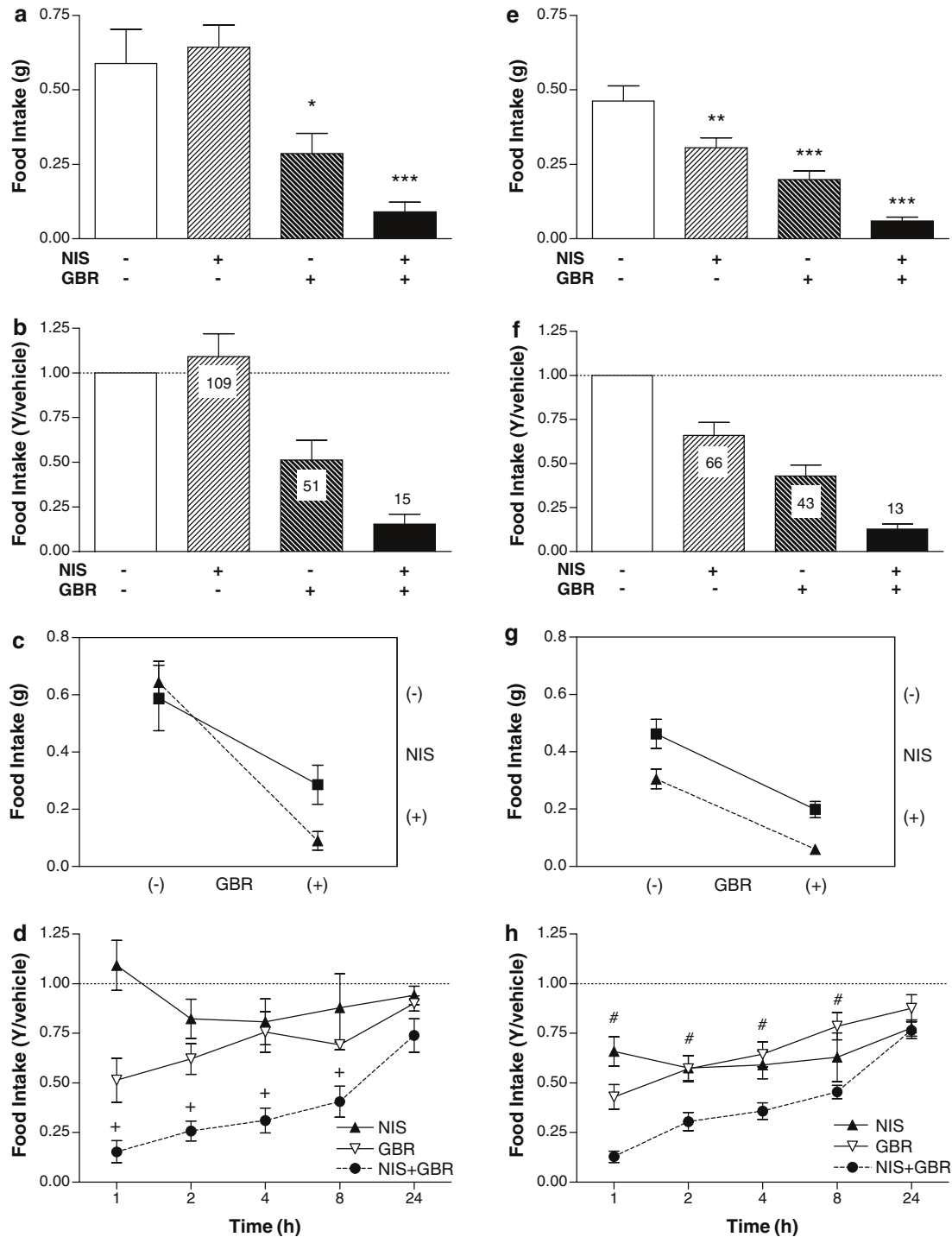
Figure 7 Acute inhibition of DA and NE reuptake produces a dose-dependent reduction in 1 h food intake in lean and obese mice on high-fat diet. (a–c) Dose–response curves for reduction in 1 h food intake in lean and obese mice on a high-fat diet. Data are normalized to vehicle-treated animals. (a) BUP: 10, 50, and 80 mg/kg i.p. ($R^2 = 0.86$ for lean and 0.72 for obese mice); (b) GBR: 1, 2.5, 6.3, 14, and 40 mg/kg i.p. ($R^2 = 0.55$ for lean and 0.53 for obese mice); (c) NIS: 0.5, 2.5, 5, 13, and 30 mg/kg i.p. ($R^2 = 0.56$ for lean and 0.78 for obese mice); and (d–f) Time course for reduced food intake. Data are normalized to vehicle-treated animals. (d) BUP (50 mg/kg), (e) GBR (14 mg/kg), and (f) NIS (13 mg/kg). (Lean mice, $n = 5$/group; obese mice, $n = 7$/group.)

produced significant independent effects on body weight on days 4 and 5 that were additive and resulted in 2.5–3 g or 8–10% weight loss. This finding is consistent with the observation that BUP fails to decrease appetite or daily caloric intake in humans (Harto-Truax et al, 1983), despite its significant effect on body weight. Because daily food intake never differed significantly across treatment groups, the decrease in body weight by NIS and GBR infusion was probably owing to increased energy expenditure. There is substantial evidence that BUP increases energy expenditure in rodents (Liu et al, 2004; Hasegawa et al, 2005) and, consistent with the acute effect of BUP on locomotor activity, this effect may not require activation of peripheral β_3-adrenoceptors in brown adipose tissue (Liu et al, 2004). Whether weight loss in lean mice was owing to a loss in lean or fat mass was not studied, but merits further investigation. If inhibition of DA and NE reuptake causes increased loss of fat mass, then greater weight loss might be expected in obese mice.

In addition to decreasing food intake, acute BUP also dose-dependently increased locomotor activity. This result is consistent with a previous finding that BUP increases locomotor behavior in rodents (Zarrindast and Hosseini-Nia, 1988) and is similar to the acute effects of psychostimulants, which also cause hyperactivity and hypophagia (Cooper and van der Hoek, 1993). A concurrent effect of BUP on activity and feeding could be owing to interference between hyperactivity and food intake, in which case decreased intake might not be owing to decreased appetite. However, the parallel activity and feeding measurements presented here do not suggest that hypophagia is a byproduct of increased activity because incremental increases in BUP produced independent changes in activity and intake. For example, increasing the BUP dose to 20 mg/kg significantly decreased food intake from 10 mg/kg, but caused no significant incremental increase in locomotor activity. Likewise, increasing the dose of BUP from 0 to 10 and 20 to 40 mg/kg increased hyperactivity without

Figure 8 Acute combined inhibition of DA and NE reuptake produces an additive reduction in food intake in lean and obese mice on high-fat diet. (a–d) Sixteen-hour food-deprived lean mice on high-fat diet were injected with NIS (3.5 mg/kg), GBR (7.5 mg/kg), NIS + GBR, or vehicle, and subsequent intake of high-fat diet was recorded ($n = 9$/group). (a) One-hour cumulative food intake was reduced significantly compared to vehicle-treated animals in GBR- and NIS + GBR-treated animals. (b) One-hour food intake normalized to control. Numbers indicate % reduction from vehicle-treated animals. (c) Two-way ANOVA of 1 h food intake revealed no significant interaction for NIS and GBR at 1 h ($P > 0.05$), but a significant main effect for GBR ($P < 0.001$). (d) Time course for food intake in drug-treated animals. GBR produced a significant independent reduction in food intake that lasted up to 8 h ([+]$P < 0.001$ for 1, 2, 4, and 8 h postinjection). (e–h) Sixteen-hour food-deprived obese mice on high-fat diet were injected with NIS (3.5 mg/kg), GBR (7.5 mg/kg), NIS + GBR, or vehicle, and subsequent intake of high-fat diet was recorded ($n = 15$/group). (e) NIS, GBR, and NIS + GBR all decreased 1 h food intake compared to vehicle. (f) One-hour food intake normalized to control. Numbers indicate % reduction from vehicle-treated animals. (g) Two-way ANOVA of 1 h food intake revealed no significant interaction for NIS and GBR ($P > 0.05$), but a significant main effect for both NIS and GBR ($P < 0.001$). (h) Time course for food intake in drug-treated animals. At 1, 2, 4, and 8 h postinjection, GBR and NIS produced significant independent reductions in food intake that were additive ([#]$P < 0.001$ for both NIS and GBR).

significantly increasing hypophagia (Figure 1e). These data suggest a discrete effect of BUP on activity and reinforces an independent effect of BUP on energy balance.

DA and NE are involved in many brain signaling pathways; however, considerable evidence suggests that altering catecholaminergic signaling will affect food intake and body weight through classic hypothalamic systems involved in the regulation of energy balance. The arcuate nucleus of the hypothalamus (ARH) is the primary forebrain sensor of peripheral cues that relay information about energy availability and storage and contains two types of cells that have opposing effects on energy balance. Activation of NPY-expressing cells causes hyperphagia and energy conservation, whereas activation of cells that express pro-opiomelanocortin (POMC) is associated with hypophagia and increased energy expenditure (Cone, 2005). NPY and NE are colocalized in select neurons in the brain and periphery, and ascending neurons from the brainstem corelease NPY and NE (Inenaga et al, 1986). An NE reuptake inhibitor like NIS could potentially cause anorexia by decreasing activity of cells that express NPY and NE. DA receptor activation is also highly correlated with ARH neuropeptide expression. Selective activation of the D2 receptor reduces ARH NPY mRNA and increases POMC mRNA expression, whereas antagonism of the D2 receptor causes the opposite effect (Pelletier and Simard, 1991; Tong and Pelletier, 1992). Increased extracellular DA (caused by inhibition of the DA reuptake transporter) could account for part of the hypophagic and thermogenic effects of BUP. Such an idea is supported by evidence that D1/D2 receptor blockade significantly attenuates BUP-induced thermogenesis (Liu et al, 2004).

Instead of reducing appetite, DA and/or NE reuptake inhibition could induce nausea and supersede hunger caused by short-term food deprivation. We have not directly assessed the ability of BUP, GBR, or NIS to produce conditioned taste aversion, but considerable evidence suggests that these drugs do not induce illness. Although nausea is one of the adverse events associated with BUP treatment in humans (occurring in more than 5% of subjects), the low percentage of subjects that discontinue treatment (7–11%, depending on dosing, vs 4% for placebo) supports favorable tolerability of BUP in humans (Settle et al, 1999). Additionally, DA, NE, and/or serotonin reuptake inhibitors are commonly prescribed in the treatment of mood disorders and are generally well tolerated (Scates and Doraiswamy, 2000; Simpson and Plosker, 2004), and psychostimulants that affect NE and DA reuptake (like amphetamine and cocaine) also suppress appetite without inducing illness (Wellman, 2005). Finally, results from subchronic NIS and GBR infusions suggest that continuous inhibition of DA and NE reuptake has a prolonged effect on body weight, but only a transient effect on appetite. The lack of a sustained effect of NE and DA reuptake inhibitors on daily food intake during subchronic drug administration is inconsistent with the notion that these drugs reduce food intake by causing nausea.

There are reports that chronic obesity can lead to changes in catecholamine turnover (Levin et al, 1986; Wang et al, 2002) and several studies have demonstrated that expression of brain D1 and D2 receptors is altered in obese humans (Wang et al, 2002) and obese mice and rats (Fetissov et al, 2002; Huang et al, 2005). However, these changes do not appear to reduce the hypophagic effects of catecholamine reuptake inhibitors—our results indicate that BUP, GBR, and NIS all cause dose-dependent hypophagia in obese mice. These findings are supported by the efficacy of BUP in reducing body weight in obese humans (Gadde et al, 2001; Anderson et al, 2002; Jain et al, 2002) and validate the need for further research on the effects of catecholamine reuptake inhibitors on energy balance in both lean and obese animal models.

The availability of highly palatable food increases reward-based or hedonic feeding in humans and animals (Saper et al, 2002). Although NIS did not independently reduce food intake in lean mice on a high-fat diet, it appeared to potentiate the significant inhibitory effect of GBR. This result implies that catecholamine reuptake inhibitors not only decrease standard chow intake but also decrease intake of a more palatable and rewarding high-fat diet. This idea is consistent with the involvement of DA in mediating both incentive motivation and the rewarding effects of reinforcing stimuli (such as palatable food) (Wise, 2004). Considering the vast availability of highly palatable food in developed countries, the ability of a DA and NE reuptake inhibitors to over-ride potential hedonically driven aspects of feeding behavior is especially applicable to humans (Wang et al, 2002).

Successful obesity treatments will likely require combination therapy that targets multiple systems that contribute to regulation of energy homeostasis (Horvath, 2005). Evidence that the NET is able to take up DA (Millan et al, 2000) and that adrenergic receptors are able to modulate dopaminergic cell firing rate (Paladini and Williams, 2004) suggests that there is a significant amount of interplay between catecholaminergic systems. Because DA and NE are two predominant neurotransmitters in the brain that are intimately involved in the regulation of energy balance (Wellman, 2005), the sustained effect of BUP on body weight is likely owing to BUP's action as a dual DA and NE reuptake inhibitor. This study demonstrates that selective and combined DA and NE reuptake inhibition reduces food intake and body weight in lean and obese mice. Furthermore, we provide evidence that DA and NE reuptake inhibitors produce independent additive effects on energy balance that may contribute to the anorectic effect of BUP.

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