AGOUTI-RELATED PEPTIDE (AgRP) is an endogenous antagonist of central nervous system (CNS) melanocortin receptors, and several lines of evidence point to this peptide as having potent influences on food intake and body weight. AgRP is a peptide synthesized in the arcuate nucleus and projects to other key feeding hypothalamic nuclei and sites throughout the brain (2, 19, 26). Transgenic mice that overexpress AgRP are obese and hyperphagic (13), and mice that are obese as a consequence of deficient circulating leptin (ob/ob) or dysfunctional leptin receptors (db/db) have significant elevations of hypothalamic AgRP mRNA (3, 29, 31, 38). Energy deficiency in animals is associated with increased hypothalamic mRNA (18, 29) and AgRP-like immunoreactivity (25), and central administration of the fragment AgRP-(83–132) (35) increases food intake (16, 36). Thus the hypothesized function of AgRP in the CNS is to promote feeding and weight gain when animals are in negative energy balance.

The mechanisms by which AgRP stimulates feeding are not entirely understood. One established method of AgRP action is competitive binding as an endogenous antagonist at melanocortin 3 and 4 receptors (MC3/4-R) where AgRP inhibits signaling of α-melanocyte-stimulating hormone, a proopiomelanocortin-derived hormone indicated to tonically limit food intake (4, 9, 14, 17, 39). However, closer examination of the orexigenic properties of AgRP suggests additional alternate mechanisms of action. For example, a unique property of AgRP's orexigenic effects is its unparalleled long-lasting stimulation of food intake (infusion of a single picomolar dose produces hyperphagia in mice lasting 7 days). Although the acute hyperphagia is suppressed by central MC3/4-R agonist treatment, the long-term hyperphagia is not (16). In addition, mice deficient of MC4-R respond normally to AgRP's orexigenic effects (28).

A recent comparison of c-Fos-like expression induced by AgRP-(83–132) and neuropeptide Y (NPY), another potent orexigenic peptide (6, 30), suggests that both acutely activate parallel neuroanatomic circuits (14a). Hence, unknown mechanisms involved in AgRP-induced feeding may involve those underlying NPY-induced feeding. This hypothesis is supported by evidence of common physiological links between NPY and AgRP. For example, all AgRP terminals detected contain NPY (5), and AgRP mRNA is extensively coexpressed in NPY neurons (18). NPY and AgRP neurons contain leptin-receptor mRNA, and much evidence points to their mutual regulation by leptin (3, 29, 40).

A key CNS mediator of NPY-induced feeding and energy regulation is the CNS opioid system (22, 37). In light of the aforementioned behavioral and morphological links between NPY and AgRP, we hypothesize that opioid receptors may also play a key role in mediating
AgRP’s potent effects on food intake. Therefore, we explored the involvement of opioid receptors in AgRP’s orexigenic effects by testing the effect of a μ-κ antagonist on the acute and long-term hyperphagia induced by AgRP infusion. Because opioid receptors have been hypothesized to be involved in determining not just caloric intake but aspects of food choice as well, we also compared effects of an opioid receptor antagonist and AgRP on two different food choice situations.

MATERIALS AND METHODS

Animals. A total of n = 85 male Long-Evans rats, weighing 380–440 g at the onset of the experiments, individually housed and maintained on a 12:12-h light-dark cycle was implanted with a cannula aimed at the third cerebral ventricle (i3vt). Coordinates for this site were midline, 2.2 mm posterior to bregma, and 7.5 mm ventral to dura (32). After a minimum 10 days of recovery, accuracy of the i3vt placement was verified by cannula infusion of 10 ng angiotensin in saline. Only animals that drank at least 5 ml in a 1-h period were used in the experiments. By this criteria, 10 animals were excluded leaving 75 rats of which 26 served in experiments 1, 2A, and 2B, 27 in experiments 3, 4, and 5, and 22 in experiment 6. Protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Chemicals. AgRP-(83–132) was purchased from Phoenix Pharmaceuticals (Mountain View, CA). Naloxone hydrochloride (NALX) was purchased from Sigma (St. Louis, MO). Both substances were dissolved in physiological saline, which served as the control solution. AgRP and control saline were administered i3vt in a 2-μl volume; NALX and control saline were injected intraperitoneally.

Diets. Animals were maintained on regular rat chow (Harland-Teklad, Indianapolis, IN) from the time of weaning. However, high-fat (HF) and low-fat (LF) pellets manufactured by Dyets (Bethlehem, PA) were used in experiments 3 and 4. The macronutrient composition of these diets compared with regular rat chow is given in Table 1.

Experiment 1: dose response of NALX on intake of chow. To determine a threshold and subthreshold dose of NALX to suppress intake, a group of 27 animals was weight and baseline-intake matched into one of four groups (n = 6–7/group), each to be injected intraperitoneally with either saline, 0.3, 1.0, or 3.0 mg/kg NALX. Food was taken away 2 h before injections that were administered 30 min before lights off, and at 30 min before lights off, animals were infused with either saline + saline, saline + 0.3 mg/kg NALX, 1 nmol AgRP + saline, or 1 nmol AgRP + 0.3 mg/kg NALX. This dose of AgRP elicits a reliable and long-lasting increase in food intake (16, 36). The first (i3vt) injection was followed 10 min later by the second intraperitoneal injection. Five minutes before lights off, premeasured rat chow was placed in the cages. Water was available at all times. Food intake was measured at 1, 2, 3, 4, and 24 h.

Experiment 2A: effect of NALX on AgRP-(83–132)-induced food intake of chow when injected simultaneously with AgRP. To test the involvement of opioid receptors in the acute phase of AgRP-induced intake 2 wk after experiment 1, the animals were counterbalanced for prior drug treatments and also weight and baseline-intake matched into one of four groups (n = 6–7/group). On the day of testing, all food was removed 2 h before lights off, and at 30 min before lights off, animals were infused with either saline + saline, saline + 0.3 mg/kg NALX, 1 nmol AgRP + saline, or 1 nmol AgRP + 0.3 mg/kg NALX. This dose of AgRP elicits a reliable and long-lasting increase in food intake (16, 36). The first (i3vt) injection was followed 10 min later by the second intraperitoneal injection. Five minutes before lights off, premeasured rat chow was placed in the cages. Water was available at all times. Food intake was measured at 1, 2, 3, 4, and 24 h.

Experiment 2B: effect of NALX on AgRP-(83–132)-induced food intake of chow when injected 24 h after AgRP-(83–132). To test the involvement of opioid receptors in the long-term phase of AgRP-induced intake 2 wk after experiment 2A, the animals were again reassigned to one of four groups (saline + saline, saline + NALX, AgRP + saline, or AgRP + NALX), counterbalanced for prior drug treatment. This time, however, the intraperitoneal NALX or control saline injection was administered 24 h after the i3vt AgRP or saline injection. Food presentation and measurements were as in experiment 2A.

Experiment 3: effect of NALX on the intake of an HF vs. an LF diet. To further characterize specific properties of food intake mediated by an AgRP opioid interaction, this experiment was conducted to first test the effect of opioid blockade on diets varying in fat composition. A different squad of animals (n = 26) was weight and baseline-intake matched into one of three groups each receiving either saline, 0.3 mg/kg NALX, or 5.0 mg/kg NALX (n = 8–9/group). It was determined from experiment 1 that 3.0 mg/kg acted as a threshold dose to suppress intake (Fig. 1). To evaluate the anorectic effect of NALX between an HF and an LF diet, we chose to inject a clearly suprathreshold dose, 5.0 mg/kg NALX. To avoid neophobia to the novel diets, the animals were given ad libitum amounts of HF and LF pellets in separate food hoppers alongside of each other in their cages for 2 days before the infusions. On the day of testing, procedures were as in experiments 2A and 2B, except that two

Table 1. Macronutrient composition of low-fat, high-fat, and regular chow diets by kilocalories per gram and by percent of total kilocalories

<table>
<thead>
<tr>
<th>Macronutrient</th>
<th>Regular Chow</th>
<th>Low-Fat Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, kcal/g</td>
<td>0.8</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>Carbohydrate, kcal/g</td>
<td>2.1</td>
<td>2.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Fat, kcal/g</td>
<td>0.5</td>
<td>0.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Total, kcal</td>
<td>3.4</td>
<td>3.6</td>
<td>4.4</td>
</tr>
<tr>
<td>Protein, % of total kcal</td>
<td>23</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Carbohydrate, % of total kcal</td>
<td>62</td>
<td>75</td>
<td>45</td>
</tr>
<tr>
<td>Fat, % of total kcal</td>
<td>15</td>
<td>11</td>
<td>41</td>
</tr>
</tbody>
</table>

Values are means ± SE.

Fig. 1. Dose-response effect of naloxone (NALX) on intake of chow. *P < 0.05.
hoppers were placed side by side in the cages, each containing premeasured HF and LF pellets. Food intake was measured at 1, 2, 3, 4, 24, 48, and 72 h after injection.

Experiment 5: effect of AgRP-(83–132) on the intake of a 20% sucrose solution vs. chow. This test was conducted to test whether AgRP-induced selective hyperphagia for the HF diet may have been influenced by an increased motivation toward intake of a preferred food. Two weeks after experiment 4, the animals were acclimated to a 20% sucrose solution for 2 days, and 4-h baseline intake of sucrose solution and chow was recorded on the second day. The sucrose concentration was chosen based on its hedonic properties in rats (20, 23). The animals were then counterbalanced for prior drug treatment and total chow + sucrose kilocalories consumed on the second day of acclimation and were assigned to two groups to receive either i3vt saline (n = 7) or 1 nmol AgRP (n = 8). Procedures were as in experiment 4, except the animals were presented with premeasured 20% sucrose solution, chow, and water. Amount of intake was recorded after 1, 2, 3, 4, 24, and 48 h.

Experiment 6: effect of AgRP-(83–132) on the intake of a 20% sucrose solution as the only available choice. This experiment was conducted to test whether AgRP might have induced hyperphagia of the 20% sucrose solution if chow had not been available as a choice. A different group of animals

Fig. 2. A: effect of intraperitoneal 0.3 mg/kg NALX or saline when simultaneously injected with the third cerebral ventricle (i3vt) injection of saline or 1 nmol agouti-related peptide (AgRP) on chow intake over 1 h. *Different from saline + saline, P < 0.05; #different from AgRP + saline, P < 0.05. B: effect of intraperitoneal 0.3 mg/kg NALX or saline when administered 24 h after i3vt injection of 1 nmol AgRP or saline. *Different from saline + saline, P < 0.05; AgRP + NALX different from saline + saline (P < 0.02) when analyzed with least significant differences but not Tukey’s honestly significant difference (HSD) post hoc test.

Fig. 3. A: effect of 5.0 mg/kg NALX ip vs. saline on intake of the high-fat diet over 4 h when presented with a choice of the high- or low-fat diet. *P < 0.05; **P < 0.01. B: effect of 5.0 mg/kg NALX ip vs. saline on intake of the low-fat diet over 4 h when presented with a choice of the high- or low-fat diet.
(n = 22) was surgically prepared and evaluated for cannula placement as in experiment 1. Also as previously described, they were assigned to two groups (n = 11/group) and injected i3vt with either 1 nmol AgRP or saline. For the first 4 h from dark onset, only ad libitum 20% sucrose solution was given, and for the following 20 h, chow was presented along with the sucrose. Water was available at all times. Sucrose intake was measured during the first 30 min, 1, 2, and 24 h of presentation, and chow was measured 24 h after presentation.

Data analysis. Data represent mean food intake (kcal) ± SE. Each experiment was analyzed with a one-way or a two-way ANOVA (for experiments where doses of both NALX and AgRP were administered). Significant main effects were analyzed for differences between groups using Tukey's HSD post hoc test with significance set at P < 0.05.

RESULTS

Experiment 1: dose response of NALX on intake of chow. As shown in Fig. 1, doses of intraperitoneal NALX from 0.3 to 3.0 mg/kg did not significantly suppress intake in the first hour, and only 3 mg/kg significantly reduced intake from 2 to 4 h after injection. Animals compensated for the early anorectic effect of NALX so that by 24 h, no difference in total intake was detected between groups that received AgRP + saline and AgRP + NALX (data not shown).

Experiment 2A: effect of NALX on AgRP-(83–132)-induced food intake when injected simultaneous with AgRP-(83–132). As shown in Fig. 2A, a dose of 0.3 mg/kg NALX did not in itself affect food intake. AgRP + saline produced intake 85% greater than saline + saline-treated animals and, although not shown, continued to stimulate intake at 24 h (23.6 ± 2 vs. 37 ± 4 g; P < 0.001). More importantly, NALX, at the subthreshold dose, significantly reduced AgRP-induced eating to the level of saline + saline intake as early as hour 1 and continued to suppress it for 3 h (P < 0.05; not shown). The anorectic effect of NALX on AgRP-induced eating disappeared by hour 4, and by 24 h, animals treated with AgRP + NALX compensated for their early decreased intake to match that of animals treated with saline + AgRP (44.2 ± 1 vs. 38.5 ± 4 g, not significant; P < 0.01 different from saline + saline intake).

Experiment 2B: effect of NALX on AgRP-(83–132)-induced food intake when injected 24 h after AgRP-(83–132). After 24 h, rats receiving AgRP were still hyperphagic as shown in Fig. 2B. However, and in...
contrast to experiment 2A, when NALX was injected 24 h after AgRP, it failed to reduce the sustained AgRP-induced hyperphagia at 1 h (Fig. 2B) or 2–4 h after NALX injection (not shown). This result indicates that the long-term effects of AgRP do not depend on continued activation of opioid receptors. Additionally, this result indicates that the subthreshold dose of NALX is truly subthreshold even when the baseline intake is higher than in an untreated rat and that the results of experiment 2A are not simply the result of NALX’s effects depending on the baseline to which it is compared.

Experiment 3: effect of NALX on the intake of an HF vs. an LF diet. Intake measures taken on the second day of acclimation to the novel diets and a day before injections showed that 100% of the animals ate more kilocalories of the HF than LF diet in a 24-h period, and 84 ± 2% of this intake was composed of the HF diet compared with 16 ± 2% of the LF diet. As shown in Fig. 4A, AgRP did not change the animals’ apparent preference for the HF diet but increased it to 77, 91, and 79% more than controls’ HF intake during hours 2, 3, and 4 postinjection, respectively. Interestingly, AgRP had no effect on the LF diet, with animals eating equally low amounts of this diet as controls (Fig. 4B). The long-lasting orexigenic effects of AgRP are illustrated in Fig. 5A. AgRP sustained hyperphagia of the HF diet with animals eating 55% more of the diet than controls, even 72 h after AgRP injection. LF intakes beyond 24 h remained low under both treatments, differing only at 72 h when controls ate more of the LF diet than AgRP-treated animals (6.5 ± 1.2 vs. 2 ± 0.5 intake of the LF diet and, in fact, as shown in Fig. 3B, the trend was for NALX to increase intake of the less-preferred LF diet, although this was not statistically significant.

Experiment 4: effect of AgRP-(83–132) on the intake of an HF vs. an LF diet. Measures taken on the second day of acclimation to the novel diets and a day before injections showed that 100% of the animals ate more kilocalories of the HF than LF diet in a 24-h period, and 84 ± 2% of this intake was composed of the HF diet compared with 16 ± 2% of the LF diet. As shown in Fig. 4A, AgRP did not change the animals’ apparent preference for the HF diet but increased it to 77, 91, and 79% more than controls’ HF intake during hours 2, 3, and 4 postinjection, respectively. Interestingly, AgRP had no effect on the LF diet, with animals eating equally low amounts of this diet as controls (Fig. 4B). The long-lasting orexigenic effects of AgRP are illustrated in Fig. 5A. AgRP sustained hyperphagia of the HF diet with animals eating 55% more of the diet than controls, even 72 h after AgRP injection. LF intakes beyond 24 h remained low under both treatments, differing only at 72 h when controls ate more of the LF diet than AgRP-treated animals (6.5 ± 1.2 vs. 2 ± 0.5 intake of the LF diet and, in fact, as shown in Fig. 3B, the trend was for NALX to increase intake of the less-preferred LF diet, although this was not statistically significant.

Fig. 6. A: effect of 1 nmol AgRP i3vt vs. saline on intake of chow over 4 h when given a choice of chow and 20% sucrose solution. **P < 0.01. B: effect of 1 nmol AgRP i3vt vs. saline on intake of 20% sucrose solution over 4 h when given a choice of chow and sucrose.

Fig. 7. A: sustained effect of a single i3vt injection of 1 nmol AgRP or saline on noncumulative 24-h intakes of chow over 2 days after injection when presented with a choice between chow and 20% sucrose solution. ***P < 0.001; *P < 0.05. B: effect of a single i3vt injection of 1 nmol AgRP or saline on noncumulative 24-h intakes of 20% sucrose solution over 2 days after injection when presented with a choice between chow and sucrose.
of NALX is not simply to lower intake from an elevated baseline. Instead, this rather specifically indicates that the short-term effects of AgRP are mediated by activity at opioid receptors but that the unique long-term effects of AgRP are not. Consistent with the notion that AgRP increases activity at opioid receptors, AgRP was also found to preferentially increase intake of an HF diet over an LF diet, and opioid antagonism preferentially was found to suppress the HF diet over the LF diet.

This suggests that a function of AgRP’s early interaction with opioid systems is to drive ingestion of foods with higher fat-to-carbohydrate ratios. Although opioid interactions may not be involved in AgRP’s sustained selection of HF food, the response may be dependent on the initial activity change at opioid receptors. Further data will be needed to test this hypothesis with longer-acting opioid antagonists.

Numerous studies support the contribution of opioids in mediating fat-selective intake (7, 15, 27, 41), but evidence also exists to attribute opioids not with stimulation of fat intake per se but with increased intake of an inherently preferred food, regardless of its macronutrient content (8, 12). Baseline intakes taken before our HF vs. LF diet experiment determined that 100% of the animals consumed significantly more of the HF over the LF diet, indicating a preference for the HF diet. Therefore, it was reasonable to argue that if AgRP engaged opioid mechanisms to increase intake of the HF diet, it might do so primarily by increasing the animals’ motivation for a preferred food rather than for dietary fat. However, our results showed that the rats greatly preferred the sucrose solution to the plain chow diet in terms of calories consumed. AgRP treatment uniformly stimulated intake of chow in all of the rats including ones with the highest and lowest sucrose preference. Admittedly, this preference is confounded by the physical states of the choices, but an AgRP-induced selective increase for a higher fat diet is consistent with the recent finding that agouti (Ay/a) mice with chronic melanocortin receptor antagonism consume a greater proportion of their intake and gain the most weight from fat when provided with three-choice macronutrient diets (21). Another possibility that must be considered is a function of AgRP on the selective

Central administration of AgRP-(83–132) produces a potent and unique long-lasting hyperphagic pattern in rats. We previously found that although the acute increase in food intake after AgRP injection involves antagonistic actions at the MC3/4-R, other CNS receptor systems are likely to be involved (16). The main goal of this study was to test the participation of opioid receptors in mediating AgRP’s effects on feeding. Results showed that a subthreshold dose of NALX suppressed AgRP’s acute stimulation of food intake but failed to suppress it when delivered 24 h after AgRP administration. Critically, this indicates that the effect of AgRP-(83–132) on the intake of a 20% sucrose solution as the only available choice. As shown in Fig. 8A, even when the only nutrient available to the rats was a palatable 20% sucrose solution, AgRP did not increase its intake relative to saline. However, when chow was also made available, AgRP produced hyperphagia, but only on the intake of chow (Fig. 8B), with AgRP-treated rats consuming 207% more chow kilocalories than controls, indicating that the AgRP infusion had been effective. Even at this time point, the intake of sucrose with AgRP treatment did not significantly differ from amounts consumed with saline treatment (45 vs. 35 ± 6 g, respectively; \( P > 0.05 \); not shown).

DISCUSSION

Numerous studies support the contribution of opioids in mediating fat-selective intake (7, 15, 27, 41), but evidence also exists to attribute opioids not with stimulation of fat intake per se but with increased intake of an inherently preferred food, regardless of its macronutrient content (8, 12). Baseline intakes taken before our HF vs. LF diet experiment determined that 100% of the animals consumed significantly more of the HF over the LF diet, indicating a preference for the HF diet. Therefore, it was reasonable to argue that if AgRP engaged opioid mechanisms to increase intake of the HF diet, it might do so primarily by increasing the animals’ motivation for a preferred food rather than for dietary fat. However, our results showed that the rats greatly preferred the sucrose solution to the plain chow diet in terms of calories consumed. AgRP treatment uniformly stimulated intake of chow in all of the rats including ones with the highest and lowest sucrose preference. Admittedly, this preference is confounded by the physical states of the choices, but an AgRP-induced selective increase for a higher fat diet is consistent with the recent finding that agouti (Ay/a) mice with chronic melanocortin receptor antagonism consume a greater proportion of their intake and gain the most weight from fat when provided with three-choice macronutrient diets (21). Another possibility that must be considered is a function of AgRP on the selective
intake of other macronutrients (e.g., carbohydrates). Close neuroanatomic links between AgRP and NPY (5, 18) suggest they may have common macronutrient-selective functions on food intake. Analogous to our results for AgRP, NPY has been shown to preferentially increase intake of chow in a choice test with access to a sucrose solution and rat chow (10).

As with food selection, neuroanatomic sites of AgRP opioid interaction on feeding may also be shared with NPY, specifically with sites implicated in NPY opioid interactions on feeding including the central amygdala, nucleus of the solitary tract, and paraventricular nucleus (11, 21, 34). We have observed increased c-Fos in these nuclei after i3vt injection of AgRP as well as in the nucleus accumbens, a site critical in opioid-mediated rewarding properties of food intake (33). Interestingly, chronic morphine is shown to downregulate MC4-R expression in the nucleus accumbens (1), and OLEFT rats, which are obese and hyperphagic, show normal MC-R binding in hypothalamic nuclei but significantly reduced MC-R binding in the nucleus accumbens shell (25). Further studies targeting discrete hypothalamic and extrahypothalamic sites should help determine the exact site of AgRP’s interaction with opioids and the precise nature of its effect on the macronutrient and hedonic properties of food selection.

Perspectives

The current data indicate significant cross talk between melanocortin and opioidergic signaling in the CNS. The function of this AgRP opioid circuit may be to motivate the organism to seek out and ingest calorically dense foods (e.g., fat-containing foods) possibly by also increasing the rewarding properties of these foods so as to favor their overconsumption. The question remains as to whether this functional circuit is important in mediating the increased intake and obesity that result from exposure to HF diets in humans and rats. Even more interesting is whether differences in this functional circuit could account for the dramatically different responses observed between individual rats or humans exposed to these diets.

This work was supported by several grants from National Institutes of Health (DK-54080, DK-54890, DK-17844) and funds from Procter & Gamble.

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