Long-term orexigenic effects of AgRP-(83—132) involve mechanisms other than melanocortin receptor blockade

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Hagan, Mary M., Paul A. Rushing, Laurel M. Pritchard, Michael W. Schwartz, Alison M. Strack, Lex H. T. van der Ploeg, Stephen C. Woods, and Randy J. Seeley. Long-term orexigenic effects of AgRP-(83—132) involve mechanisms other than melanocortin receptor blockade. Am J Physiol Regulatory Integrative Comp Physiol 279: R47–R52, 2000.—Overexpression of agouti-related peptide (AgRP), an endogenous melanocortin (MC) 3 and 4 receptor antagonist (MC3/4-R), causes obesity. Exogenous AgRP-(83—132) increases food intake, but its duration and mode of action are unknown. We report herein that doses as low as 10 pmol can have a potent effect on food intake of rats over a 24-h period after intracerebroventricular injection. Additionally, a single third ventricular dose as low as 100 pmol in rats produces a robust increase in food intake that persists for an entire week. AgRP-(83—132) completely blocks the anorectic effect of MTII (MC3/4-R agonist), given simultaneously, consistent with a competitive antagonist action. However, when given 24 h prior to MTII, AgRP-(83—132) is ineffective at reversing the anorectic effects of the agonist. These results support a critical role of MC tone in limiting food intake and indicate that the orexigenic effects of AgRP-(83—132) are initially mediated by competitive antagonism at MC receptors but are sustained by alternate mechanisms.

MTII obesity; arcuate nucleus; hyperphagia; hypothalamus; α-melanocyte-stimulating hormone

CONSIDERABLE EVIDENCE IMPLICATES an important role for melanocortin (MC) in the regulation of food intake and body weight. MC peptides are cleaved from the precursor polypeptide proopiomelanocortin, which is synthesized in the arcuate nucleus of the hypothalamus and brainstem (2, 16, 28). α-Melanocyte-stimulating hormone (α-MSH) is an MC peptide that acts as an endogenous agonist of MC3 and 4 receptor subtypes (MC3/4-R). Through this action, α-MSH is hypothesized to provide a tonic inhibition of food intake that constrains body weight gain. This role for α-MSH is evidenced in its ability to reduce food intake for up to 48 h in rats when centrally injected (30, 37), a property shared with MTII (MC3/4-R agonist), a synthetic ligand of α-MSH (6, 9, 11, 25, 35). Dependency on the MC4-R for these effects is supported by the inability of MTII to reduce intake in MC4-R-deficient mice, which are hyperphagic and obese (22).

The actions of α-MSH on MC3/4-Rs and food intake are potently antagonized at these receptors by agouti protein (39) and the structurally related agouti-related peptide (AgRP), a 132-amino acid peptide (20, 27, 42) that is synthesized exclusively in the arcuate nucleus of the hypothalamus (1). These neurons project extensively to hypothalamic areas implicated in the control of food intake, including the paraventricular and dorsomedial nuclei, and lateral hypothalamic areas (5, 14). AgRP is extensively colocalized with the orexigenic peptide neuropeptide Y (NPY) throughout these hypothalamic nuclei (1, 13).

The importance of MC tone in energy homeostasis is strongly suggested by the ability of MC3/4-R antagonism to induce hyperphagia and obesity. Most notably, overexpression of either agouti (A’ mice) (20) or AgRP (10) results in phenotypes that include obesity, hyperphagia, and hyperinsulinemia, and this phenotype is recapitulated in mice deficient in MC4-R (15). In mouse models of obesity caused by leptin deficiency (ob/ob) or leptin receptor dysfunction (db/db), an 8- to 10-fold elevation in hypothalamic AgRP mRNA is found (27, 34). In normal but 48-h fasted mice, when leptin levels would predictably be decreased, AgRP mRNA is observed to reach 13- and 15-fold elevations (23, 40). As with AgRP, synthetic MC3/4-R antagonists such as SHU-9119 and HSO-14 also potently stimulate mRNA is observed to reach 13- and 15-fold elevations (23, 40). As with AgRP, synthetic MC3/4-R antagonists such as SHU-9119 and HSO-14 also potently stimulate food intake in sated rats (6, 12, 25) up to 96 h postfusion (11). Recently, we showed that MC3/4-R antagonists with central injection of SHU-9119, at a subthreshold dose for stimulating food intake, potently reverses the natural hypophagia displayed by animals involuntarily overfed to obesity (12). This further sup-

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ports the pivotal role of MC signaling in mediating anorectic signals meant to restore energy homeostasis after positive energy balance.

Aside from the number of in vitro studies characterizing binding properties of AgRP to MC receptors (8, 27, 31, 41), only one study other than the present has examined the effect of exogenous AgRP in vivo. In this study, Rossi and colleagues (32) tested the intraventricular effect of the amidated COOH-terminal AgRP fragment (83–132)-NH₂ (31) on food intake in rats and found it to increase intake up to 24 h as potently as the synthetic MC3/4-R antagonist SHU-9119. Coadministration of α-MSH with AgRP-(83–132) had no effect on this hyperphagic response when α-MSH was given either simultaneously or 9 h prior to AgRP. These results support the proposition raised by Rossi and colleagues (32) that the effect of AgRP on food intake may be due to persistent or irreversible antagonism of MC3/4-R or changes in these receptors such as desensitization or downregulation (32). To date, the exact mechanism by which AgRP induces food intake when given exogenously or by which it endogenously regulates food intake is an important unanswered question.

The purpose of the present study was to explore further the effect of MC antagonism on food intake and body weight by exogenous administration of AgRP-(83–132) and test in vivo whether these effects are mediated by continuous competitive antagonism of MC3/4-R. To this end, we first examined the effect of a wide range of AgRP-(83–132) doses on food intake and body weight and followed ensuing effects on feeding and body weight beyond 24 h. In two subsequent experiments, we characterized the actions of AgRP-(83–132) on MC3/4-R in vivo by observing changes in food intake induced by AgRP-(83–132) when the competing agonist MTII was centrally injected concomitant with AgRP and 24 h after AgRP-(83–132) administration, when its orexigenic effects were still potent.

MATERIALS AND METHODS

Animals. Long-Evans rats (n = 76 total), weighing 440–480 g at the onset of the experiments, housed in individual cages, and maintained on a 12:12-h light-dark cycle, were 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 (29). After a minimum 10-day recovery period, placement of the i3vt cannulas was confirmed by i3vt infusion of 10 ng angiotensin II in saline while animals were water replete. Only those animals that drank a minimum of 5 ml within 1 h were used in the study. Six animals were excluded by this criterion leaving 70 rats to be used in the experiments. This protocol was approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Peptides. AgRP-(83–132) was purchased from Phoenix Pharmaceuticals (Mountain View, CA). MTII was also purchased from Phoenix Pharmaceuticals. Peptides were dissolved in physiological saline, which also served as the control solution. All solutions were infused i3vt in a 2-μl volume.

Experiment 1: Dose response and time course of AgRP-(83–132) on food intake and body weight. After 1 wk following angiotensin II tests, the animals were matched into four groups (n = 10/group) on the basis of 24-h food intakes for 2–3 days prior to the AgRP-(83–132) dose-response tests. On three different days the animals were administered a single infusion of either saline or 0.001, 0.005, 0.01, 0.1, 1.0, or 5.0 nmol of AgRP-(83–132). At the end of the experiment, each animal had received on different days and in counterbalanced fashion an infusion of two different doses of AgRP-(83–132) and one saline infusion. Each of the 3 days of dose-response tests were separated by at least 3 days following return to baseline food intakes in all rats. AgRP-(83–132) was given 1 h prior to lights off (1300), at which time ad libitum rat chow was provided. Food intake was recorded for 1, 2, 4, and 24 h and every day after until the intakes normalized to that of the controls. Body weight was also recorded for the first and subsequent 24-h periods.

Experiment 2: Effect of AgRP-(83–132) on MTII-induced suppression of food intake when infused simultaneously with MTII. After a minimum of 2 wk without testing, the same animals used in experiment 1 were infused, counterbalanced for prior peptide infusions, with either 1 nmol AgRP-(83–132) or saline, followed (no more than 10 min later) by either 0.1 nmol MTII or saline. A dose of 0.1 nmol MTII i3vt was previously shown to significantly reduce food intake in rats (35). Food was presented, and intake and body weights were recorded as in experiment 1.

Experiment 3: Effect of AgRP-(83–132) on MTII-induced suppression of food intake when infused 24 h prior to MTII. A separate group of rats (n = 30) were surgically prepared and tested for cannula placement as in experiment 1. They were baseline-intake matched into four groups (n = 7–8/group) and infused with either 1 nmol AgRP-(83–132) or saline, followed this time, 24 h later, by an infusion of either 0.1 nmol MTII or saline. Food was presented and intake and body weights were recorded as in experiment 1.

Data analyses. Data from each experiment were analyzed with ANOVA and Bonferroni post hoc tests with alpha set at 0.05. Data are expressed as food intake or percent of body weight change (g) ± SE.

RESULTS

Experiment 1: Dose-response and time course of AgRP-(83–132) on food intake and body weight. Concentrations of AgRP-(83–132) as small as 0.01, 0.1, and 1 nmol increased food intake within 1 h (P < 0.001) and continued to increase it at 2 (Fig. 1A) and 4 h (not shown). At 2 h, the highest dose tested, 5 nmol did not significantly increase food intake. By 24 h rats infused with these four doses ate 42 ± 2.9 to 45 ± 2.2 g (Fig. 1B), a 58% increase over control values (P < 0.001). As shown in Fig. 1, A and B, the relationship between the dose of peptide and food intake is not curvilinear. The threshold concentration to affect 24-h food intake was 0.01 nmol, and doses in the range of 0.01–1.0 nmol were equally as effective. The highest concentration tested, 5 nmol, increased intake as effectively but not in the first 24 h. Therefore, maximal concentrations for food intake appear to lie between 1 and 5 nmol. The stimulation of food intake caused by these low doses resulted in increases of body weight up to 5.5% (P < 0.0001) (Fig. 1C). More remarkably, a single i3vt dose of AgRP-(83–132) continued to stimulate food intake well beyond the first 24 h. As shown in Fig. 2A, a single dose of 0.01 or 1 nmol, produced a significant increase in daily food intake that was still detected 7 days after infusion. Higher doses of 1 and 5 nmol produced the
same long-lasting effects with greater efficacy to stimulate food intake. In fact, a high of 44 ± 0.6 g intake, P < 0.05, was recorded for rats injected 3 days prior with 1 nmol, and those injected with 5 nmol consumed almost 50 g over a single 24-h period 3 days after AgRP injection (Fig. 2B). As with food intake, body weights continued to increase above the controls during the 3 days post-AgRP injection (P < 0.05, data not shown). The 24-h food intake response to 5 nmol appeared maximal and was significantly greater than the response to 1 nmol only at 144 h postinjection (P < 0.01).

Experiment 2: Effect of AgRP-(83–132) on MTII-induced suppression of food intake when infused simultaneously with MTII. At 2 h (Fig. 3A) MTII significantly suppressed food intake in rats coinfected with saline rather than AgRP (1.9 ± 0.3 vs. 5.5 ± 0.5 g saline, P < 0.01). In contrast, AgRP-(83–132) coinfected with saline increased feeding at this time point but not significantly (7.0 ± 1.9 vs. 5.5 ± 0.5 g saline, P < 0.09, not significant). However, AgRP-(83–132) completely inhibited the anorectic effect of MTII when the two were coinfected (7.0 ± 0.7 g AgRP + MTII vs. 1.9 ± 0.3 g saline + MTII, P < 0.001) so that no difference in intake was observed between rats given saline or MTII just after AgRP-(83–132). At 24 h (Fig. 3B) MTII continued to suppress food intake (6.9 ± 2.3 vs. 19.4 ± 3.3 g saline, P < 0.05), whereas AgRP-(83–132) significantly increased food intake in the group coinfected with saline (33.7 ± 3.1 vs. saline, P < 0.01). AgRP continued to potentially suppress the anorectic effect of MTII (33.7 ± 3.5 g AgRP + MTII vs. 19.4 ± 3.3 g saline + MTII, P < 0.001), so that the mean intake from AgRP followed by saline exactly matched that produced by AgRP followed by MTII. As in experiment 1, AgRP plus saline treatment continued to increase intake beyond 48 h and AgRP plus MTII continued to suppress the anorectic effect of MTII (P < 0.05, not shown).
Experiment 3: Effect of AgRP-(83–132) on MTII-induced suppression of food intake when infused 24 h prior to MTII. As seen in experiment 2 (Fig. 3) animals injected with saline followed by MTII 24 h later had suppressed food intake at 2 h (0.86 ± 0.4 vs. 2.4 ± 0.6 g saline, \( P < 0.05 \)) and at 24 h (8.5 ± 3.1 vs. 21.1 ± 4 g saline, \( P < 0.05 \); Fig. 4). In contrast, animals injected with AgRP-(83–132) followed 24 h later with saline increased their food intake at 2 h (4.2 ± 0.8 vs. 2.4 ± 0.6 g saline, \( P < 0.05 \)) and at 24 h (36.26 ± 2.4 vs. 21.1 ± 4 g saline, \( P < 0.01 \)). However, in sharp contrast to Experiment 1 (Fig. 1), at 1 h (not shown) and 2 h, when AgRP injection was followed 24 h later by an MTII injection, it failed to inhibit the anorectic effect of MTII (1.1 ± 0.5 g AgRP + MTII vs. 0.86 ± 0.4 g saline + MTII, not significant). At 24 h, although the intakes of animals treated with this spaced coinjection of AgRP plus MTII increased somewhat relative to the intakes of animals receiving saline instead of AgRP prior to MTII, they were not statistically different. The failure of AgRP-(83–132) to inhibit MTII-induced suppression of food intake is clearly noted in Fig. 4 where intakes from saline and MTII given 24 h after AgRP differ at 2 h (1.1 ± 0.5 g AgRP + MTII vs. 0.86 ± 0.4 g saline + MTII, \( P < 0.01 \)) and at 24 h (19.2 ± 4.6 g AgRP + MTII vs. 8.5 ± 3.1 g saline + MTII, \( P < 0.01 \)). As in experiment 1 animals treated with AgRP followed by saline continued to increase their intake beyond 48 h (not shown).

**DISCUSSION**

The results of this study extend the original description of the effects of exogenous administration of AgRP-(83–132) on food intake and body weight by describing the effect of a wider dose range and following this effect beyond 24 h (experiment 1). In addition, results from experiments 2 and 3 indicate that although the short-term hyperphagic effects of AgRP-(83–132) may be mediated by competitive antagonism at the MC3/4-R, other mechanisms appear to be activated to sustain the long-lasting effects of this peptide.

Experiment 1 showed that i3vt doses as low as 0.01 nmol AgRP-(83–132) can induce a potent hyperphagic response within 1 h of administration. The more remarkable finding here is that the stimulation of food intake by a single dose of AgRP-(83–132) lasts up to an entire week. We found the lowest effective dose to be 0.01 nmol, and 7 days after i3vt injection even this low dose caused animals to eat significantly more than controls. The most effective doses tested were 1 and 5 nmol. One nanomole produced the largest increases in food intake at most time points with 5 nmol more effective only at the 144-h postinfusion time point.
Along with these potent changes in food intake, body weight also was increased in accordance with the effects on food intake.

The potent ability of AgRP-(83–132) to change both short- and long-term food intake is consistent with the hypothesis that the central MC system provides critical tonic inhibition on food. The current data along with several genetic and transgenic studies support the conclusion that interruption of MC-derived tonic inhibition can lead to increased food intake and sustained periods of positive energy balance (10, 15, 20).

The duration of the feeding response appears to be unique to this peptide and, to our knowledge, is unparalleled by any other known orexigenic substance. These potent effects to increase food intake are in some ways similar to, but in other ways different from, those obtained by intracerebroventricular administration of NPY or peptide YY, both highly potent orexigenic agents (3, 24). On a molar basis, AgRP-(83–132) is less effective than NPY in its orexigenic effects during the first 24 h after administration. However, a single dose of NPY, even at very high doses, has little or no effect on food intake beyond 24 h (7). Similarly, other orexigenic neuropeptides such as melanin-concentrating hormone, orexin, galanin, or opioids (17, 19, 21, 33) do not show such long-term effects on food intake. Analysis of the efficacy of orexigenic central nervous system neuropeptides must therefore consider not only the peak feeding response but the duration of the effect as well. If the time frame is 24 h or longer, AgRP-(83–132) is clearly the most potent endogenous substance described to date.

One hypothesis to explain the remarkably potent and long-lasting effects of AgRP on food intake is that it persistently occupies MC3/4-R over time and thereby exerts its long-term effect by continued blockade of ongoing MC signaling. This rather unique mechanism was suggested by previous findings that AgRP-(83–132) blocked anorexia induced by the MC3/4-R agonist α-MSH delivered up to 9 h subsequently (32). Experiments 2 and 3 were designed to test this hypothesis.

In experiment 2 (Fig. 3) the effect of the α-MSH synthetic analog MTII was completely blocked by immediate pretreatment with AgRP-(83–132). Such behavioral data parallel the in vitro receptor binding profile of AgRP in its displacement of agonist activity (8, 27, 31, 41) as well as in vivo studies showing that coinjection of equal concentrations of SHU-9119 and MTII, a synthetic MC4/3-R antagonist and agonist, respectively, produced food intakes that were not different from controls (25). However, and as shown in experiment 3 (Fig. 4), when MTII was centrally injected 24 h following AgRP-(83–132) injection, the effect of MTII on food intake was intact, even though daily food intake remained elevated at this time point in rats receiving AgRP without MTII. In fact, at 24 h postinjection, MTII was as effective at suppressing the food intake of AgRP-(83–132)-treated animals as it was in saline-treated animals. The hyperphagia induced by AgRP-(83–132), however, was evident once again after the anorectic effect of MTII had dissipated (within 48 h). Such results are not consistent with the hypothesis that the long-term effects of AgRP-(83–132) are associated with continued occupancy of critical MC3/4-R. Rather, these results support a model in which the orexigenic effects of AgRP-(83–132) are mediated initially by competitive antagonism at MC4-Rs but are sustained via an alternate mechanism.

**Perspectives**

The mechanism of action of AgRP continues to be a puzzle. First, there are few examples of endogenous orexigenic molecules with such potency. Second, some data indicate that whereas the actions of AgRP on the MC3-R is that of a classic competitive antagonist, it may not fit that model for its effects on the MC4-R (27). Third, there are other proteins such as mahogany, which appear to modulate the ability of MC receptor antagonists to exert their biological effects via a mechanism that remains unclear (4, 22, 26). Finally, the current data demonstrate a time frame for the effects of AgRP that cannot be explained readily as a result of competitive antagonism. Such long-lasting effects are more likely to involve transcriptional changes and alterations in the synthesis of proteins by hypothalamic and extrahypothalamic neurons involved in the control of food intake. One possibility that is well recognized in other systems but not often considered in energy homeostasis is neural plasticity (18). We suggest here that AgRP-induced changes in protein synthesis may lead to altered synaptic efficacy analogous to what has been described in the amygdala and hippocampus for systems involved in aspects of learning and memory (36, 38). Given these complexities and the salient effects observed here, a complete understanding of this critical protein and its role in the control of energy homeostasis is vital and will rely on multidisciplinary levels of investigation.

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