Central infusion of melanocortin agonist MTII in rats: assessment of c-Fos expression and taste aversion

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Thiele, Todd E., Gertjan van Dijk, Keith A. Yagaloff, Stewart L. Fisher, Michael Schwartz, Paul Burn, and Randy J. Seeley. Central infusion of melanocortin agonist MTII in rats: assessment of c-Fos expression and taste aversion. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R248–R254, 1998.—Like leptin (OB protein), central infusion of the nonspecific melanocortin agonist MTII reduces food intake for relatively long periods of time (i.e., 12 h; W. Fan, B. A. Boston, R. A. Kesterson, V. J. Hruby, and R. D. Cone, Nature 385: 165–168, 1997). To test the hypothesis that MTII may influence ingestive behavior via mechanisms similar to those that mediate the effects of leptin, we infused a single dose of MTII into the third ventricle (i3vt) of Long-Evans rats and examined three dependent measures that have been studied following i3vt infusion of leptin: 1) effects on long-term food intake and body weight (48 h), 2) patterns of c-Fos expression in the brain, and 3) conditioned taste aversion learning. Similar to leptin, MTII reduced 48-h food intake (1.0 nmol dose), reduced body weight at 24 and 48 h (0.1 and 1.0 nmol doses, respectively), and induced c-Fos expression in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala. In contrast to leptin, MTII was found to produce conditioned taste aversions. These results are consistent with the hypothesis that MTII may influence regulatory behavior via mechanisms similar to those that mediate the effects of leptin.

A GROWING BODY OF EVIDENCE points to an important role for the hypothalamic melanocortin (MC) system in the regulation of food intake and body weight (4, 5, 11, 15). The MC system is composed of peptides that are cleaved from the polypeptide precursor, proopiomelanocortin (POMC), which is produced by neurons within the hypothalamic arcuate nucleus (6). One posttranslational product of POMC is α-melanocyte-stimulating hormone (α-MSH). Central infusion of α-MSH has been shown to reduce food intake in rats (10). Furthermore, because targeted disruption of the MC4 receptor system results in mice with elevated food intake and body weight (5), anorexia produced by central infusion of α-MSH may involve activation of MC4 receptors. Additionally, it has been shown that synthetic ligands of this receptor, such as MTII, also cause anorexia (4).

Recent evidence suggests that the effects of MTII on food intake may involve pathways similar to those that mediate the anorectic actions of leptin (OB protein; see Ref. 2 for review) and that leptin may reduce food intake and body weight by stimulating the hypothalamic MC system (Seeley, unpublished observations). Leptin, a peptide hormone secreted from white adipose tissue (9, 23), has been shown to reduce food intake and body weight in rats and mice following both central and peripheral administration (3, 17, 19). Unlike most peptides that are considered satiety factors and which appear to have short-term effects on food intake (e.g., 16, 18, 20–22), a single central administration of leptin has been found to reduce eating and body weight for periods of 24 h or greater (14). Similar to leptin, central infusion of MTII produces relatively long-term effects on feeding (i.e., greater than 12 h; Ref. 4). Recently, we showed that the MC receptor antagonist SHU-9119 blocked the ability of leptin to reduce long-term food intake and body weight at doses that had no effect on food intake by themselves. Furthermore, this antagonist attenuated leptin-induced c-Fos expression in the paraventricular nucleus of the hypothalamus (PVN). Additionally, expression of the POMC gene in the rostral portion of the arcuate nucleus is stimulated by central infusion of leptin (13).

The goal of the present research was to test the hypothesis that the MC agonist MTII influences ingestive behavior via mechanisms similar to those that mediate the effects of leptin. To address this possibility, we infused MTII into the third ventricle (i3vt) of rats and examined three dependent measures that have been studied following i3vt infusion of leptin: 1) effects on long-term food intake and body weight (48 h), 2) c-Fos expression in the brain to determine patterns of neuronal activation, and 3) conditioned taste aversion (CTA) learning to determine whether administration of the peptide is associated with nonspecific aversive side effects. Leptin reduces long-term food intake and body weight without producing taste aversions (17) and produces a pattern of c-Fos activation in the brain that is distinguishable from many anorexia-producing peptides (19). If MTII and leptin act via similar central mechanisms, then we would expect to find similar results with each of these dependent measures following i3vt infusion of MTII to those that have been observed following i3vt infusion of leptin.
FOS ACTIVATION AND TASTE AVERSION BY I3VT MTII

MATERIALS AND METHODS

Animal preparation. Subjects were male Long-Evans rats weighing between 300 and 400 g at the onset of the experiments. They were individually housed in hanging wire stainless-steel cages and maintained on a 12:12-h light-dark cycle. Laboratory rat chow and water were provided ad libitum (except where noted). Under equithesin (3.3 ml/kg ip) anesthesia, rats were implanted with 21-gauge stainless-steel cannulas (Plastics One, Roanoke, VA) aimed at the third ventricle. With bregma and lambda at the same vertical coordinate, the sagittal venous sinus was carefully displaced laterally with a metal probe, and the cannulas were placed directly on the midline, 2.2 mm posterior to bregma and 7.4 mm ventral to dura, and fixed to the skull with anchor screws and dental acrylic. The cannulas were fitted with removable obturators that extended 0.5 mm beyond the tip of the guide cannula. Rats that received CTA training were also implanted unilaterally with intraoral cannulas constructed of PE-100 tubing. The cannulas were placed anterior to the first maxillary molar and threaded subcutaneously to exit the top of the head. Each rat was given 0.15 ml each of chloromycetin (100 mg/ml sc) and gentamicin (40 mg/ml ip) prophylactically. When rats regained their preoperative body weights after surgery (~3 wk), placements of i3vt cannulas were confirmed by administration of 10 ng angiotensin II in saline while the animals were water replete. Animals that did not drink at least 5 ml of water within 60 min were not used in the study.

Experiment 1: Food consumption and body weight. Rats were weighed and their food hoppers were removed from the cages 2 h before the beginning of the dark phase (6:00 PM). Rats were assigned to one of four body weight-matched groups. Approximately 1 h before the beginning of the dark phase, rats were given i3vt infusions (3.5-μl volume manually infused with a Hamilton syringe over 60 s; see Ref. 19 for details) of either synthetic cerebrospinal fluid (s-CSF; n = 8), 0.01 nmol MTII (dissolved in s-CSF; n = 5), 0.1 nmol MTII (n = 6), or 1.0 nmol MTII (n = 6). At the onset of the dark phase, food hoppers were weighed and returned to the rats’ cages; they were again weighed 4, 24, and 48 h later. Rats were weighed at the same time each day for the remainder of the experiment.

Experiment 2: CTA. Rats were initially habituated to a Plexiglas observation chamber and intraoral fluid infusion by being placed in the chamber for 20 min/day over 4 days. On the last 2 days of habituation, rats received an intraoral infusion (via the intraoral cannula) of 5 ml distilled water (0.5 ml/min) during the first 10 min in the chamber. On the conditioning day, rats were placed in the observation chamber for 5 min and intraorally infused with 2.5 ml of 0.15% saccharin delivered by infusion pump at a rate of 0.5 ml/min. During the infusion, rats were videotaped, and the amount of time (s) that passed before the rat rejected fluid (passive thirsting) was scored. Immediately after taste exposure, rats were given i3vt infusion of either 1.0 nmol MTII (n = 5), 0.1 nmol MTII (n = 6), or s-CSF (n = 3); these doses of MTII were the lowest doses to attenuate body weight and/or food intake in experiment 1 (see below). As a positive control for taste aversion conditioning, some rats were given intraperitoneal injection of lithium chloride (LiCl; 127 mg/kg, 0.15 M solution dissolved in distilled water; n = 5) or an equal volume of isotonic saline (n = 3). LiCl in this dose has been found to produce strong CTAs (17). After the conditioning day, rats that had received drug (MTII or LiCl) were administered the appropriate vehicle (s-CSF or saline, respectively), whereas those that had received vehicle on the conditioning day received the appropriate drug. Animals were treated with the drug or the vehicle, without exposure to the taste stimulus, and immediately returned to their home cages. With this experimental design, each treatment condition had similar exposure to drugs and to saccharin; however, in the groups that received drugs on the conditioning day, the drug was presented immediately after saccharin exposure, whereas the control groups received the taste and the drug on separate days.

Two independent measures were used to examine CTA expression. First, on the test day, rats were again given intraoral infusion of the saccharin (2.5 ml over 5 min), without exposure to the drug, and the amount of time that passed before the animal rejected the saccharin (active or passive thirsting) was used as an index of CTA learning. Immediately after the intraoral infusion test, a second measure of CTA learning was assessed by measuring rats’ 24-h consumption from two available bottles, one containing 0.15% saccharin and one containing distilled water. The saccharine preference ratio was calculated as the amount of saccharin consumed divided by total consumption of both fluids.

Experiment 3: c-Fos immunohistochemistry. To prevent stress-induced c-Fos expression on the test day, rats were regularly handled during recovery from surgery. As with the CTA study, we chose to examine the two lowest doses of MTII that reduced food intake and body weight. On the test day, rats were given i3vt infusion of 1.0 nmol MTII (n = 7), 0.1 nmol MTII (n = 6), or s-CSF (n = 6). Two hours after injection, rats were anesthetized with equithesin (3.3 ml/kg ip) and transcardially perfused with isotonic phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed for 24 h, cryoprotected in 20% sucrose in 0.1 M phosphate buffer, and sectioned in 30-μm coronal planes on a cryostat (100–120 μm). Tissues were rinsed (3× in PBS, 3× in 3% hydrogen peroxide, 3× in 0.1% Triton X-100, 3× in PBS, 3× in 0.05% Triton X-100, 3× in PBS, 3× in phosphate buffer), and developed with diaminobenzidine substrate (30 min). Sections were rinsed with PBS, incubated for 20 min in 0.3% H2O2 in absolute methanol to quench endogenous peroxidase, rinsed (3× PBS), and incubated 1 h in 1% gelatin, 5% normal goat serum in PBS. Sections were then washed 3× for 10 min in PBS, incubated with the primary antibody solution, consisting of 0.005 g/ml polyclonal rabbit anti-c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA), which recognizes residues 3–16 of the c-Fos protein. After ~24 h of incubation on ice, slides were rinsed (10× PBS) and processed with the ABC method (Vector Laboratories, Burlingame, CA). Slides were transferred to biotinylated goat anti-rabbit antibody for 1 h, rinsed (6× PBS), transferred to avidin-biotinylated peroxidase for 1 h, rinsed (3× in PBS, 3× in phosphate buffer), and developed with diaminobenzidine substrate (6 min). Sections were rinsed (10× PBS), mounted on slides, and covered slipped with Permount. Camera lucida drawings of c-Fos-positive brain structures were prepared by an experimenter naïve to group treatments. Care was taken so that structures were scored in approximately the same plane. Drawings were scored by blinded raters who recorded the number and location of c-Fos-positive nuclei. Scores across raters were averaged for statistical analyses.

Data analyses. Data from each study were analyzed using one-way analyses of variance (ANOVA). Because there were no statistical differences between the control groups treated with s-CSF or saline in experiment 2, data from these rats...
were collapsed to form one control group. For data from experiment 1, drug-treated groups were compared with the control group on food intake and change in body weight (relative to pretreatment baseline body weights). In experiment 2, rats that received paired exposure of saccharin with drug were compared with the unpaired control group on the latency to reject the saccharin and on 24-h saccharin preference ratios (saccharin intake divided by total fluid (saccharin plus water) intake). For experiment 3, drug-treated groups were compared with the vehicle-treated group on c-FLI; data from each brain region were analyzed separately. When significant differences were found, post hoc analyses were conducted using t-tests. In all cases, P < 0.05 (two-tailed) indicated statistical significance.

RESULTS

Experiment 1: Food consumption and body weight. Food intake data are shown in Fig. 1 and reveal that i3vt infusion of MTII caused a dose-dependent decrease in food intake. Four hours after infusion, the 0.1 and 1.0 nmol doses of MTII significantly reduced food intake relative to the control treatment by ~45 and 65%, respectively (Fig. 1A). However, when measures were collected at 24 and 48 h after drug infusion, only the 1.0 nmol dose of MTII was found to significantly reduce food consumption by ~47 and 50% at each time interval, respectively (Fig. 1, B and C). ANOVAs performed on the 4-h [F(3,21) = 9.90], 24-h [F(3,21) = 8.81], and 48-h [F(3,21) = 9.65] food intake data were significant, and post hoc tests confirmed the above conclusions (see Fig. 1).

Figure 2 shows the effect of MTII on body weight 24 h (Fig. 2A) and 48 h (Fig. 2B) after drug administration. Again, MTII produced a dose-dependent effect, with both the 0.1 and 1.0 nmol doses significantly reducing body weight relative to the control condition 24 h after infusion. However, only the 1.0 nmol dose reduced body weight at 48 h postinfusion. ANOVAs run on the 24-h data [F(3,18) = 4.87] and the 48-h data [F(3,21) = 5.05] each attained statistical significance, and post hoc tests confirmed group differences (see Fig. 2).

Experiment 2: CTA. On the conditioning day, saccharin was ingested throughout all or most of the 5-min infusion period, and there were no significant differences between the groups. Fluid rejection data collected on the test day are presented in Fig. 3A, and saccharin preference data collected during the two-bottle test are presented in Fig. 3B. Relative to rats that received saccharin paired with the control stimulus (and thus unpaired exposure to saccharin and drug), rats that received saccharin paired with either i3vt infusion of MTII (0.1 and 1.0 nmol) or intraperitoneal injection of LiCl rejected saccharin earlier in the test session (Fig. 3A). Additionally, rats had a decreased preference for saccharin if it was paired with infusion of 1.0 nmol MTII or injection of LiCl (Fig. 3B). ANOVAs performed on the fluid rejection data [F(3,18) = 9.27] and saccharin preference data [F(3,18) = 5.34] were both found to be significant, and tests confirmed group differences.

Experiment 3: c-Fos immunohistochemistry. Data representing numbers of nuclei positive for c-FLI in forebrain regions and brain stem regions are presented in Fig. 4, A and B, respectively, and representative photomicrographs of these brain regions are presented in Fig. 5. In the forebrain, MTII caused significant dose-dependent induction of c-FLI in the PVN (0.1 and 1.0 nmol doses), the SON (0.1 and 1.0 nmol doses), and the CEA (1.0 nmol dose). ANOVAs performed on data collected from the PVN [F(2,16) = 5.28], SON [F(2,16) = 10.39], and CEA [F(2,16) = 7.01] each attained statistical significance, and post hoc tests confirmed the differences between groups.

In the brain stem, relative to s-CSF infusion, central infusion of MTII caused significant c-FLI in the AP and
the PBN, at both the 0.1 and 1.0 nmol doses in each case; however, significant induction of c-FLI was not observed in the NTS. ANOVAs performed on c-FLI data obtained from the AP \[F (2,16) = 7.69\] and PBN \[F (2,16) = 7.41\] each attained statistical significance, and differences between groups were confirmed by post hoc tests.

**DISCUSSION**

Consistent with previous findings in mice (4), central administration of the MC receptor agonist MTII was found to attenuate food intake in a dose-dependent manner; both the 1.0 and 0.1 nmol doses caused reductions of short-term (4 h) food intake, and the 1.0 nmol dose reduced long-term (48 h) feeding (see Fig. 1). Relative to the previous report with mice, the dose of MTII required to reduce food intake was much smaller in the present experiments. This discrepancy may reflect either species differences (rat vs. mouse) or the route of MTII administration (third vs. lateral ventricle). i3vt administration of 3.0 nmol of MTII consistently produced barrel rolling, a sign of toxicity not reported in mice (4).

In addition to its effects on food intake, we demonstrated that MTII causes robust reductions in body weight, with the 0.1 nmol dose reducing weight 24 h after drug infusion and the 1.0 nmol dose reducing weight at both 24 and 48 h after drug infusion (see Fig. 2). Interestingly, the observation that the 0.1 nmol dose attenuated 24-h body weight but did not significantly reduce 24-h food intake raises the possibility that stimulation of the brain MC system may increase energy expenditure in addition to its effect on food intake. Alternatively, the 0.1 nmol dose of MTII may have reduced fluid intake (hypodipsia) up to 24 h after drug infusion, which could account for reduced body weight. Future studies are required to specifically address these possibilities.

The observation that MTII caused long-term reductions in food intake and body weight is consistent with the hypothesis that MTII and leptin may produce their effects via similar regulatory mechanisms. We have previously shown that reduced food intake associated with leptin administration cannot easily be explained by potential nonspecific aversive side effects produced by leptin; in doses that caused long-term reductions in food intake (16 h) and body weight (24 h), leptin did not support a CTA (17). In the present experiment, the lowest dose of MTII that reduced eating also produced CTAs, although the taste aversion generated by the 0.1 nmol dose was detectable only by means of the more sensitive intraoral infusion technique and was not seen with the two-bottle consumption test (see Fig. 3). These data indicate that reduced eating and body weight after i3vt infusion of MTII may be explained by nonspecific aversive side effects.
body weight by acting on hypothalamic MC4 receptors (13), this peptide may influence eating and an agonist that acts nonspecifically on several MC parabrachial nucleus (PBN). *Significant at area postrema (AP), nucleus of the solitary tract (NTS), and lateral doses) or s-CSF.

Thus i3vt infusion of MTII produces some effects that are both similar to and distinguishable from those produced by i3vt infusion of leptin. Both peptides produce long-term reductions in food intake and body weight, and both peptides produce c-FLI in the PVN and the CEA. As previously mentioned, we have found that blockade of the MC4 receptor with the antagonist SHU-9119 prevented i3vt infusion of leptin from reducing long-term food intake and body weight and attenuated leptin-induced c-FLI in the PVN. Furthermore, central infusion of leptin increases POMC mRNA expression in the arcuate nucleus of the hypothalamus (13), whereas food restriction and food deprivation have been found to decrease POMC mRNA in the arcuate nucleus (1). These data suggest that leptin may stimulate the hypothalamic MC system, and this may be a mechanism by which leptin reduces eating and body weight. The overlap of c-FLI produced by MTII and leptin (the PVN and the CEA) may be an indication of brain regions that are activated by both leptin and the melanocortin system. Alternatively, leptin activation of the MC pathways in the brain may lead to induction of c-FLI in these areas. On the other hand, activation of the DMH by leptin may precede activation of the MC system or be the result of changes in other neurochemical systems.

The observation that MTII causes CTAs, unlike leptin, which does not produce this effect, does not preclude the possibility that the MC system mediates the anorectic actions of leptin. It is likely that leptin activates the components of the MC system that are specific to reducing food intake and body weight in the hypothalamus. MTII administered i3vt, on the other hand, may activate a variety of MC receptors in multiple brain regions producing aversive effects in addition to reduced eating and body weight. The development of specific MC4 agonists, and the administration of these drugs directly in the hypothalamus, may resolve this issue.

Perspectives

Recently, several peptides have been proposed as agents that may be endogenous regulators of food intake and body weight. One such peptide, GLP-1, is similar to MTII in that both peptides produce reductions in food intake and both peptides are capable of producing CTAs. Additionally, for both the MC and GLP-1 systems, administration of a receptor antagonist produces increases in food intake (4, 18). Despite these similarities between the effects of agonists for the
MC and GLP-1 receptors, the potential efficacies of such compounds as therapeutics for obesity are quite different. i3vt administration of GLP-1, unlike MTII, in a number of different paradigms does not produce long-term changes in food intake or body weight (i.e., 24 h or greater; Ref. 14). Additionally, targeted disruption of the GLP-1 receptor has no effect on food intake and body weight (12), whereas MC₄ receptor deficiency causes hyperphagia and a profound obesity syndrome (5). Consequently, it would appear that the hypo-
lamic MC system, but not the GLP-1 system, is an endogenous system critical to the regulation of body weight and hence a potential target for pharmacologi-
ca}l intervention in the treatment of obesity. Endog-
enous GLP-1, on the other hand, more likely functions as a short-term satiety agent. These current comp-
ounds highlight the complexity that is associated with
ascribing a role to a peptide as an endogenous regulator of food intake and/or body weight. In addition to short-term effects on feeding, we suggest that long-
term effects on food intake and body weight need to be demonstrated with receptor agonists, antagonists, and/or alterations in peptide or receptor expression before a peptide should be considered an endogenous regulator of body weight.

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