Anatomical dissociation of melanocortin receptor agonist effects on taste- and gut-sensitive feeding processes

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Baird JP, Palacios M, LaRiviere M, Grigg LA, Lim C, Matute E, Lord J. Anatomical dissociation of melanocortin receptor agonist effects on taste- and gut-sensitive feeding processes. Am J Physiol Regul Integr Comp Physiol 301: R1044–R1056, 2011. First published July 6, 2011; doi:10.1152/ajpregu.00577.2010.—Injections of the melanocortin 3/4 receptor (MCR) agonist melanotan II (MTII) to a variety of brain structures produces anorexia, suggesting distributed brain MCR control of food intake. We performed a detailed analysis of feeding behavior (licking microstructure analysis) after a range of MTII doses (0.005 nM to 1 nM) was targeted to the forebrain (third ventricle, 3V) or hindbrain (fourth ventricle, 4V) regions. MTII (0.1 nM and 1 nM) delivered to the 3V or 4V significantly reduced 0.8 M sucrose intake. The anorexia was mediated by reductions in the number of licking bursts in the meal, intrameal ingestion rate, and meal duration; these measures have been associated with postigestive feedback inhibition of feeding. Anorexia after 3V but not 4V MTII injection was also associated with a reduced rate of licking in the first minute (initial lick rate) and reduced mean duration of licking bursts; these measures have been associated with taste evaluation. MTII effects on taste evaluation were further explored: In experiments 2, 3V MTII (1 nM) significantly reduced intake of noncaloric 4 mM saccharin and 0.1 M and 1 M sucrose solutions, but not water. The anorexia was again associated with reduced number of licking bursts, ingestion rate, meal duration, initial lick rate, and mean burst duration. In experiments 3 and 4, brief access (20 s) licking responses for sweet sucrose (0.015 M to 0.25 M) and bitter quinine hydrochloride (0.01 mM to 1 mM) solutions were evaluated. Licking responses for sucrose were suppressed, whereas those for quinine solutions were increased after 3V MTII, but not after 4V MTII injections (0.1 nM and 1 nM). The results suggest that multiple brain MCR sites influence sensitivity to visceral feedback, whereas forebrain MCR stimulation is necessary to influence taste responsiveness, possibly through attenuation of the perceived intensity of taste stimuli.

MC4R; satiation; satiety; gustatory; food; consumption

IT IS WELL ESTABLISHED THAT brain melanocortins influence feeding behavior and energy balance as demonstrated, for example, by natural human and experimental animal mutations of melanocortin receptors (MCRs) that result in hyperphagic and obese phenotypes (16, 32). Although the MCRs involved in feeding behavior (particularly MC4R) are expressed in neurons throughout the brain (18, 19), the contributions of different anatomical MCR populations to feeding behavior require clarification. A distributed MCR influence on food intake originating from at least partially nonoverlapping MCR-expressing brain structures was first suggested by the observation that anorexic responses to various doses of the MC3/4R agonist melanotan II (MTII) were comparable whether MTII was infused to the forebrain lateral ventricle (LV) or to the hindbrain fourth ventricle (4V) (14). Two independent studies of 0.7 M glucose solution consumption in rats also reported comparable dose-response functions whether MTII was delivered to the forebrain third ventricle (3V) (1) or to the 4V (30). It was later suggested that hypothalamic MCRs are central for MCR-mediated anorexia, whereas the hindbrain MCRs may be more important for energy control (7); this notion was based on evidence that reexpression of MC4Rs in the paraventricular hypothalamus (PVN) and the amygdala of obese MC4R-knockout mice rescued a normophagic phenotype without improving energy balance. This hypothesis, however, is strained to accommodate reports that PVN lesions do not abolish anorexic responses to MTII (11) and that injections of MTII to the hindbrain dorsal vagal complex are sufficient to produce anorexia [(31); see (25) for further discussion]. MTII injections into several feeding-related nuclei in the hindbrain and the forebrain were recently shown to elicit comparable metabolic and feeding responses (25). The similarity of the anorexic responses resulting from stimulation of disparate injection sites supported the conclusion that the MCR system is distributed and possibly redundant with respect to MCR control of feeding behavior (25).

While these results raise important questions regarding the functional and anatomical organization of central MCR systems, it is also important to ensure that the apparently redundant anorexic responses observed to date are not rather due to a limited fidelity or construct validity of the measures employed. The mass of food consumed in a given time period is the result of several factors that influence how frequently and rapidly food is consumed across and within meals; these include learned, sensory, motor, and physiological state factors, such as foraging activity, learned preferences, taste reactivity, postigestive feedback (satiation), and oromotor coordination. Analyses of food intake over relatively long time periods may mask more subtle and potentially independent influences of different anatomical MCR populations on the different behavioral processes that codetermine overall consumption (5, 8–10). Therefore, we used a detailed analysis of feeding known as a licking microstructure analysis to identify potential differences in the contributions of different populations of MCR-expressing neurons to feeding behavior. The licking microstructure analysis method has the capacity to identify ingestion measures that are associated with taste evaluation, postigestive feedback inhibition of feeding (satiation), nausea, and oromotor coordination (5, 6, 8–10, 27). To further explore potential MCR influences on taste responsiveness, we also used brief-access testing, an established and complementary method for the evaluation of gustation in rodents (23, 26). We compared the responses of rats licking for a variety of taste solutions after a range of doses of MTII were applied to

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the 3V or to the 4V. Different behavioral outcomes across 3V and 4V injection conditions would suggest differential contributions of forebrain and hindbrain populations of MCR-expressing neurons to food intake control. Furthermore, because particular measures of feeding microstructure are associated with taste, postigestive, or oromotor responses, differences in those measures affected after 3V vs. 4V MTII could provide clues as to the specific contributions of forebrain and hindbrain MCR populations to the processing of the sensory and motor controls of feeding behavior.

METHODS

Animals

Adult albino male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 336 g and 489 g on the first day of the experiment were used. Rats were maintained individually in plastic tubes (48 × 25 × 15 cm) with wire lids on a 12:12 light-dark schedule in a temperature-controlled room. Food (Purina rat chow 5001, Lab Diets, St. Louis, MO) and tap water were available ad libitum in the home cage, except where noted below. Rats were tested at the same time each day, between 4 and 10 h after lights on (0700), in a separate test cage.

Surgery

Procedures were approved by the Amherst College Institutional Animal Care and Use Committee. Rats were anesthetized intraperitoneally with a mixture of ketamine HCl (66 mg/kg) and xylazine HCl (6 mg/kg). A 22-gauge guide cannula (Plastics One, Roanoke, VA) was stereotaxically implanted into either the 3V (from bregma: AP: −2.3 mm, ML: 0 mm, DV: −8.5 mm from skull surface) or 4V (from lambda: AP: −3.2 mm, ML: 0 mm, DV: −7.2 mm from skull surface), and fastened with dental acrylic and skull screws. The 28-g injection cannula extended 1 mm below the tip of the guide cannula, and a dummy cannula cut flush to the guide tip was maintained in the guide cannula at all other times. After surgical recovery, correct placement of the 4V cannula was confirmed by an increase in blood glucose after a cannula infusion of the antimetabolite, 5-thio-D-glucose [5-TG; 120 μg/2 μl; 2 μl/min; see (24)]. Tail blood was collected every 10 min starting 20 min prior to the 5-TG injection, using a glucometer and test strips. Rats that did not exhibit at least a 50% increase in blood glucose within 30 min of injection were removed from the study. Correct placement for the 3V cannula was confirmed by a minimum of 5 ml water consumption within 30 min of a 50 ng/5 μl 3V cannula injection of ANG II (3–5).

At the conclusion of behavioral experiments, cannula placements were again confirmed; India ink (2 μl) was injected immediately following a lethal overdose of pentobarbital sodium (100 mg/kg). Rats were then transcardially perfused with isotonic saline followed by 10% formalin. The brain was removed, and the whole brain was bisected midsagittally and inspected. Data for rats with no ink perfusion of either the 3V or 4V (depending on cannula placement) were discarded.

Apparatus

Experiments 1 and 2 (lickometer). Rats were taken from their home cages and tested in individual plastic tubes (48 × 25 × 15 cm). A drinking spout (3 mm orifice; Girton, Millville, PA) was introduced to the test chamber with the spout orifice positioned 4 cm from the floor and 0–1 mm behind a slat (8 × 28 mm) in a metal plate attached to the front of the cage. A lickometer (MS-108, DiLog Instruments, Tallahassee, FL) and PC computer were used to record licking: tongue contacts with the spout completed a circuit, which allowed the computer to record the time of each lick with 1-ms resolution. Files for each test session for each rat were saved for off-line analysis.

Experiments 3 and 4 (Davis rig). Rats were placed in an enclosed cage with a slit in the wall that was covered by a moveable shutter. Up to 16 bottles of taste solutions with sipper tubes (3-mm orifice) were mounted in a block on a motorized moveable rack outside the test chamber, and bottles were presented in random order via alignment of the rack. For each presentation, the shutter opened, allowing access to the licking spout of the aligned bottle, and the clock began with the first lick. At the end of the each trial (20 s from time of the first lick), the shutter closed for 7.5 s while the next sipper tube was positioned. If a rat did not initiate a trial within 20 s, the trial was terminated, and the next trial was initiated. Because of deprivation and conditioning, the 20-s window was sufficient for reliable sampling behavior, particularly in water-deprived rats (on average, 2–3 trials out of 24 were not sampled per test session). There was somewhat less sampling in nondeprived rats (on average, 8 trials were not sampled); therefore, each concentration of each taste solution was offered 3 or 4 times during testing, and when a given concentration was not sampled, it was reoffered until the animal did sample it. Stimulus bottles were weighed to the nearest 0.01 g before and after the session to monitor intake for each bottle.

Procedures

Experiment 1: dose-response analyses. Prior to testing, rats in all experiments were daily habituated in the test cage, where they were free to ingest 0.5 M sucrose solution for 90 min. Habituation training continued until session intakes stabilized and exceeded 5 ml per session (2–5 days).

For the experiment, rats (4V group n = 7; 3V group n = 13) were offered 0.8 M sucrose once daily for 90 min. On days 3, 6, 9, 12, and 15, rats also received a 2-μl cannula injection (1 μl/min) of either vehicle (artificial cerebrospinal fluid, aCSF) (Harvard Apparatus, Holliston, MA), or 0.005, 0.01, 0.1 or 1 nM of MTII (American Peptide, Sunnyvale, CA) in counterbalanced order 30 min prior to behavioral testing. These doses correspond to the range of doses previously tested for intracerebroventricular MTII responses for solid and liquid comestibles (e.g., 1, 14, 20, 30). The 2-min infusions were performed using a 10-μl Hamilton syringe in a programmable syringe pump (model #100; KD Scientific, Holliston, MA).

Experiment 2: taster response analysis. To further assess the influence of 3V injections of MTII on gustatory and postigestive sensitivity, naïve rats fitted with 3V cannulas (n = 9) were offered a range of taste solutions varied in caloric and gustatory intensity after vehicle or 1 nM MTII doses, the latter being the strongest anorexic dose identified in experiment 1. The training and testing procedures, and the apparatus, were identical to experiment 1, except as noted below.

Each taste solution was presented in single bottle 90-min tests in the lickometer over five consecutive days in counterbalanced order, to minimize contrast effects. Drug infusions were performed on test days 3 and 5 for each taste solution, also in counterbalanced order.

Rats were offered 0.1 M and 1 M sucrose, 4 mM saccharin, and water as test solutions. These solutions produce systematic differences in licking and intake responses. Water is noncaloric, less palatable, and usually less consumed than either sucrose solution (4, 5). The 0.1 M and 1 M sucrose solutions often produce distinct licking microstructure profiles, even though these differences can result in the same volume consumed (4, 5, 9). We have shown that rats exhibit robust licking responses to 1 M sucrose, as indicated by a rapid initial rate of licking, larger mean lick burst sizes/durations (reflecting taste evaluation), and a steep rate of decline in the ingestion rate due to the caloric load, which generates a meal of moderate duration (2). When offered the less caloric and less preferred 0.1 M sucrose solution, rats exhibit slower initial lick rates and smaller and shorter licking bursts (suggesting weaker ingestive taste reactivity), and a flatter rate of decline in ingestion rate, with a longer meal duration and more bursts of licking, reflecting the reduced caloric inhibition (see also Refs. 8.
and 9 for further discussion). Similarly, the 4-mM saccharin solution usually produces gustatory (initial lick rate and burst size/duration) responses greater than observed for water, but it provides no caloric benefit and presumably less postingestive feedback inhibition than caloric stimuli.

Experiment 3: 3V MTII effects on brief access licking for sucrose and QHCl solutions. To further evaluate the effects of 3V MTII on gustatory responses, we utilized a brief access testing paradigm (23, 26). In this paradigm, several concentrations of a taste stimulus are presented over very brief (5–30 s) trials, allowing a concentration–lick count function to be determined. The brief nature of the taste trials minimizes postingestive accumulation of taste stimuli, allowing the specific effects of MTII on orosensory processing, if any, to be revealed through curve shifts in the concentration–response function (e.g., 4, 12, 23). It is also informative to assess MTII effects on responses to normally avoided tastants; therefore, we evaluated brief access responses to quinine hydrochloride (QHCl) solutions. An effect of MTII on sucrose responses with no effect on QHCl responses would imply an effect specific to normally accepted/preferred stimuli. Comparable decreases in responses to QHCl and sucrose after MTII would suggest suppression of oromotor output independent of taste hedonics, whereas increased responses to QHCl would suggest a reduced sensitivity to the aversive properties of the taste solution.

TRAINING. Rats fitted with 3V cannulas were assigned to either a sucrose (n = 6) or a QHCl (n = 6) group and tested individually. The rats were habituated to the test apparatus over daily sessions. Rats were first offered access to a spout containing 0.5 M sucrose (sucrose group) or water (QHCl group) in 2 consecutive 8-min trials until they sampled (2 to 4 tests). Rats were then conditioned to sample during brief 20-s access trials. Rats in the sucrose group were offered 24 samples from 6 bottles containing 0.5 M sucrose (4 presentations per bottle). Rats in the QHCl group were offered 24 trials involving 8 bottles of dH2O with 3 presentations of each bottle. Training continued until the rats sampled on at least half of the trials (2 to 3 days). For the final phase of training, rats in the sucrose group received four 20-s presentations, each consisting of 6 doses of sucrose solutions (0, 0.015, 0.03, 0.0625, 0.125, and 0.25 M; 24 presentations total) in daily test sessions. To promote sampling, rats in this group were given light (4 h) water deprivation prior to testing. For the QHCl group, training proceeded as with the sucrose group, except that rats were exposed to three 20-s pre-saturations of each of six concentrations of QHCl (0, 0.01, 0.05, 0.1, 0.5, and 1 mM). To sustain sampling, an additional 6 water trials were included for a total of 24 trials. Rats in this group were water deprived throughout training and testing phases; thus, after testing each day, the rats were also offered water in the home cage for 15 min. Training continued until rats sampled each concentration of sucrose or QHCl at least once on 2 consecutive days (2 to 5 days).

TESTING. Concluding training, the rats received baseline test days the same as for the final phase of the training tests. On days 3, 6, and 9, they were injected with aCSF or MTII (0.1 nM or 1 nM) using parameters identical to experiment 1, in counterbalanced order. If a rat did not sample each concentration at least once, it was retested for the nonsampled concentration(s) at the end of its test session. Water deprivation and repletion schedules were maintained the same as they were for the training period.

Experiment 4: 4V MTII effects on brief access licking for sucrose and QHCl. Rats fitted with 4V cannulas (sucrose group n = 5; QHCl group n = 5) were trained and tested using parameters identical to experiment 3.

Data Analyses

Data were analyzed according to previously established analysis parameters, as follows (see Refs. 3, 4, and 27 for details). Meal analyses were limited to the first meal in the test session.

The temporal distribution of licking was analyzed using a variety of custom-made programs (3, 6, 17). Meal size (ml) was calculated as the number of licks in the meal [first lick of the first burst to last lick of the last burst; (3, 28)]. The end of the meal was defined by a pause in licking greater than or equal to 10 min (27). Meal duration (min) was defined as the session time of the last lick in the meal minus the session time of the first lick in the meal. Average ingestion rate (licks/min) was calculated as the number of licks in the meal divided by meal duration. To analyze ingestion rate over the course of the meal, we analyzed the rate of licking in 30-s epochs for the first 5 min after meal onset. Meals were also temporally divided into thirds based on the meal duration value and the rate of licking for each meal third was also calculated. A licking burst was defined as two or more consecutive licks with no inter-lick interval (ILI) exceeding 1 s. Thus, pauses greater than 1 s determined burst termination (28). Burst count, therefore, represents the number of lick bursts in the meal. A burst duration was calculated by subtracting the session time of the first lick in the burst from the time of the last lick in that burst. Mean burst size (lick count) was calculated as the cumulative number of licks in all bursts in the meal divided by the burst count. Mean burst duration (s) was calculated as the cumulative time of all bursts in the meal divided by the burst count in the meal. To minimize artifact registrations due to nonlingual spout contacts, meal onset was defined as the first lick of the first burst containing at least 3 licks. Initial lick rate was the number of licks in the first minute of the meal. The within-burst lick rate was determined by averaging all ILIs less than 1 s. Because more than 95% of all ILIs in a meal are less than 250 ms (in rats) and are normally distributed below this cutoff (3, 6, 28), the average duration of ILIs less than 250 ms was also determined. The ratio of longer ILIs within bursts (250 ms to 999 ms) relative to ILIs < 250 ms was also calculated. Increases in this ratio have been associated with aversive taste reactivity and nausea (6).

For experiment 1, results for the five drug levels (aCSF, 0.005, 0.01, 0.1, and 1 nM) were analyzed using a one-way repeated-measures ANOVA (SPSS 16.0; SPSS, Chicago, IL). Planned comparisons were used to evaluate MTII responses for each drug level, to inform any significant main effect. Two-way repeated-measures ANOVA (dose × time epoch) was used to analyze licking counts in 30-s epochs of the first 5 min of the intake test after meal onset and the rate of licking (licks/min) across temporal meal thirds. Differences between the 3V and 4V groups were evaluated using two-way mixed factors ANOVA and between-subject t-tests to compare individual effect sizes (drug minus vehicle conditions) across the two groups. Responses for each measure in experiment 2 across the two drug injection days (MTII/aCSF) for each of the taste solutions were compared with two-way (drug × tastant) repeated-measures ANOVAs. Three-way repeated-measures ANOVA (dose × tastant × time epoch) was used to analyze lick counts in 30-s epochs of the first 5 min of the meal and the rate of licking (licks/min) across temporal meal thirds. In experiments 1 and 2, planned t-tests were used to compare drug responses for each taste solution or drug dose. T-tests were corrected for family-wise comparison error using Tukey tests (α = 0.05). For each group in experiments 3 and 4, the mean number of licks for each concentration of each taste solution that was sampled (defined by at least 3 licks during the stimulus presentation) was determined for each rat. QHCl concentration responses were then expressed as a ratio of the responses to water (12). Data were analyzed using a two-way repeated-measures ANOVA (drug condition × tastant concentration). Water responses were also separately evaluated using a 1-way repeated-measures ANOVA. The frequency of nonsampled trials for sucrose or QHCl was also assessed using a two-way mixed-factors ANOVA (ventricle group × dose).
RESULTS

Experiment 1

4V MTII dose-response effects. MTII significantly decreased meal size across doses \[F(4,24) = 6.96; \text{ } P < 0.01; \text{ Fig. 1}\]. Planned comparisons indicated that 0.1 nM and 1 nM MTII significantly reduced meal size \([P \text{ values} < 0.05; \text{ } Q \text{ values} > 4.23]\).

Burst count was significantly decreased across doses \([F(4,24) = 6.43; \text{ } P < 0.01; \text{ Fig. 2}\], with comparisons indicating that the 0.1 nM and 1 nM MTII dose responses were significantly lower than vehicle \([P \text{ values} < 0.05; \text{ } Q \text{ values} > 4.29]\). MTII also significantly decreased meal duration \([F(4,24) = 4.44; \text{ } P < 0.01; \text{ Fig. 2}\], an effect carried by the 1 nM dose \([P < 0.05; \text{ } Q = 3.67]\), with a comparable but nonsignificant reduction observed at the 0.1 nM dose of MTII. There was no significant effect of MTII on the overall average ingestion rate \([P < 0.05; \text{ } Q = 2.84, \text{ } P < 0.04]\).

3V MTII dose-response effects. MTII significantly decreased meal size across doses \([F(4,48) = 8.46, \text{ } P < 0.001]\). As with the 4V group, only the 0.1 nM and 1 nM doses significantly reduced meal size \([P \text{ values} < 0.05; \text{ } Q \text{ values} > 4.52; \text{ Fig. 1}\].

The burst count was significantly different across MTII dose conditions \([F(4,48) = 3.21; \text{ } P < 0.02; \text{ Fig. 2}\]. MTII significantly reduced meal duration \([F(4,48) = 2.70; \text{ } P < 0.05]\), and the comparison for the 1 nM MTII dose was significant \([P < 0.05; \text{ } Q = 4.04; \text{ Fig. 2}\]. There was no significant effect of MTII on the overall average ingestion rate \([F(4,48) = 0.71, \text{ } P < 0.05]\). However, the rate of licking over the first 5 min of the meal was significantly reduced by MTII \([F(4,48) = 6.02; \text{ } P < 0.001]\) at the 0.1 nM and 1 nM doses \([P \text{ values} < 0.003]\). There was no significant interaction \([F(36,432) = 0.91, \text{ } NS; \text{ Fig. 6}\]. Analysis of lick rate across meal thirds also indicated a significant meal-third \(\times\) dose interaction \([F(8,48) = 3.52, \text{ } P < 0.003; \text{ Fig. 4}\].

3V MTII produced a dose-dependent reduction of the initial lick rate (the number of licks in the first minute) \([F(4,48) = 5.71, \text{ } P < 0.001; \text{ Fig. 5}\]. with a statistically significant reduction at the 1 nM dose \([P < 0.05; \text{ } Q = 6.34]\). The mean burst duration was also dose dependently reduced by MTII \([F(4,48) = 5.73, \text{ } P < 0.05; \text{ Fig. 5}\] with a marginal but nonsignificant reduction at the 1 M sucrose dose \([P < 0.06; \text{ } Q = 2.93]\). Although the pattern of responses was similar, the mean burst size did not reach a similar statistical result \([F(4,48) = 1.96, \text{ } P = 0.12; \text{ data not shown}\].

Analysis of licking within bursts indicated that MTII had no statistically significant effect on the proportion of longer ILIs within bursts, but MTII significantly increased the rate of licking within bursts \([F(4,48) = 4.68, \text{ } P = 0.003]\) by 8% for the 0.1 nM and 1 nM doses \([P \text{ values} < 0.05; \text{ } Q \text{ values} > 5.31]\).

4V-3V group comparisons. A two-way ANOVA (ventricle group \(\times\) MTII dose) of meal size responses across the 4V and 3V test groups indicated no differential effects of MTII on meal size across the two ventricle groups, as the ventricle group \(\times\) MTII dose interaction term was not statistically significant \([F(4,72) = 0.95, \text{ } NS]\). Between-subjects t-tests of the individual difference scores (drug minus vehicle) revealed no significant difference in the meal size reduction for either the 0.1 nM \([t(18) = -0.11, \text{ } NS]\) or the 1 nM \([t(18) = -0.21, \text{ } NS]\) dose conditions. There was also no significant difference between ventricle groups in the difference scores for the size of the MTII reduction of meal duration at either the 0.1 nM \([t(18) = 0.91, \text{ } NS]\) or the 1 nM \([t(18) = 0.58, \text{ } NS]\) doses. There was no significant difference in effect size for the reduction of the burst count at the 1 nM MTII dose \([t(18) = 1.32, \text{ } NS]\). A difference at the 0.1 nM dose was not statistically supported after correction for family-wise comparison error \([t(18) = 2.13, \text{ } P = 0.08; \text{ Fig. 6}\].

There were no significant differences in mean burst duration \([F(4,48) = 0.79; \text{ } NS; \text{ Fig. 5}\], mean burst size \([F(4,48) = 0.97; \text{ } NS; \text{ not shown}\], or initial lick rate across doses \([F(4,48) = 0.43; \text{ NS; Fig. 5}\].

Analysis of the rate of licking within bursts indicated that MTII produced a small but statistically significant change in the rate of licking \([F(4,48) = 2.88, \text{ } P < 0.05]\). However, the effect was carried by diverging responses at doses that did not affect food intake \([0.005 nM \text{ MTII: } 6\% \text{ reduction of lick rate}; 0.01 nM \text{ MTII: } 7\% \text{ increase in lick rate}]\). MTII also significantly reduced the proportion of longer ILIs within bursts \([F(4,48) = 2.84, \text{ } P < 0.04]\).
Q = 3.01]. Consistent with this finding, the 2-way ANOVA (ventricle group × MTII dose) for burst count was only marginally significant for a main effect of ventricle group \[F(1,18) = 4.22, P = 0.06\] and a ventricle group × dose interaction \[F(4,72) = 2.28, P = 0.07\], although there was a robust main effect of MTII, indicating suppression of burst count in both ventricle groups \[F(1,18) = 19.98, P < 0.001\].

**Experiment 2**

As expected, there were significantly different responses across the taste solutions, as indicated by statistically significant ANOVA main effects for the tastant term for all of the microstructure measures presented below [all P values < 0.05].

Fig. 2. A: mean (plus standard error) meal duration (min) values for aCSF (open bar) and four doses of MTII (hatched and filled bars) in 4V-cannula fitted rats \((n = 7)\) ingesting 0.8 M sucrose solution (*\(P < 0.05\)). B: mean (+ SE) burst count for the same conditions as in A (*\(P < 0.05\)). C and D: same conditions as in A and B, respectively, in rats fitted with 3V cannulas \((n = 13; *P < 0.05)\).
MTII significantly suppressed meal size [drug main effect: $F(1,8) = 45.01, P < 0.001$] for a subset of the taste solutions, as indicated by a significant drug $\times$ taste solution interaction term [$F(3,24) = 4.82, P = 0.009$]. Comparisons indicated that meal size was significantly suppressed for the saccharin, 0.1 M and 1 M sucrose solutions, but not for water [$P$ values $< 0.05$; $Q$ values $> 5.03$, Fig. 8].

MTII significantly suppressed the burst count as indicated by a main effect of drug [$F(1,8) = 5.76, P = 0.04$], but there was no significant interaction term [$F(3,24) = 0.61, NS$; Fig. 9]. There was no significant effect of MTII on meal duration [$F(1,8) = 3.21, NS$] and no significant interaction term [$F(3,24) = 0.43, NS$], though planned comparisons indicated that MTII significantly reduced burst count and meal duration.

Fig. 4. Mean (± SE) ingestion rate (licks/min) across meal thirds in rats ingesting 0.8 M sucrose after 4V injection of aCSF (open symbols) or four doses melanotan II (MTII; solid symbols): 0.005 nM (A, ▼), 0.01 (B, ▲), 0.1 nM (C, ◆), and 1 nM (D, ■). Meals for each rat were temporally divided into thirds, and the mean ingestion rate (licks/min) associated with each meal third was calculated. The mean lick rate for each meal third is plotted at the temporal midpoint of each meal third (i.e., at 1/6th, 3/6ths, and 5/6ths of the average meal duration). The same rats were tested in each dose condition ($n = 7$). The aCSF results are replotted in each figure to provide a baseline for comparison with each MTII dose.

Fig. 5. A: mean (± SE) initial lick rate (number of licks in the first minute of the meal) values for aCSF (open bar) and four doses of MTII (hatched and filled bars) in 4V-cannula fitted rats ($n = 7$) ingesting 0.8 M sucrose solution. B: mean (± SE) burst duration for the meal for the same conditions as in A. C and D: same conditions as for A and B, respectively, in rats fitted with 3V cannulas ($n = 13$; *$P < 0.05$; +$P < 0.06$).
for the 1 M sucrose solution (P values < 0.05; Q values > 4.11). There was no significant main effect of drug or interaction term for the average rate of ingestion [drug: F(1,8) = 0.21, NS; interaction: F(3,24) = 0.17, NS]. There were statistically significant main effects of drug [F(1,8) = 131.67, P < 0.001], tastant [F(3,24) = 39.23, P < 0.001], and time epoch [F(9,72) = 7.48, P < 0.001] on the rate of licking during the first 5 min. There was also a statistically significant drug × tastant interaction [F(3,24) = 5.95, P < 0.004], but no other interactions were statistically significant. The interaction was likely due to a suppressive

Fig. 6. Mean ingestion rates (mean lick count ± SE) for each 30-s epoch of the first 5 min of the intake test after meal onset in rats ingesting 0.8 M sucrose after 3V injection of aCSF (open symbols) or four doses MTII (solid symbols): 0.005 nM (A), 0.01 (B), 0.1 nM (C), and 1 nM (D). The same rats were tested in each dose condition (n = 13). The aCSF results are replotted in each figure to provide a baseline for comparison with each MTII dose.

Fig. 7. Mean (∓ SE) ingestion rate (licks/min) across meal thirds in rats ingesting 0.8 M sucrose after 3V injection of aCSF (open symbols) or four doses MTII (filled symbols): 0.005 nM (A), 0.01 (B), 0.1 nM (C), and 1 nM (D). Meals for each rat were temporally divided into thirds, and the mean ingestion rates (licks/min) associated with each meal third was calculated. The mean lick rate for each meal third is plotted at the temporal midpoint of each meal third (i.e., at 1/6th, 3/6ths, and 5/6ths of the average meal duration). The same rats were tested in each dose condition (n = 13). The aCSF results are replotted in each figure to provide a baseline for comparison with each MTII dose.
effect of MTII on lick rates for the saccharin and sucrose solutions but not for water because a 2-way ANOVA (drug × time epoch) of licking for the water conditions was not significant [MTII: F(1,8) = 0.86, NS; interaction: F(9,72) = 0.41, NS], whereas two-way ANOVAs for saccharin and the two sucrose solutions all revealed significant main effects of drug (P values < 0.01; see Fig. 10). Analysis of meal thirds indicated significant main effects of tastant [F(3,24) = 3.02, P < 0.05] and meal third [F(2,16) = 16.42, P < 0.001], and a statistically significant three-way interaction [F(6,48) = 2.4, P < 0.04]. Follow-up two-way ANOVAs suggested that MTII suppressed licking in the first and second third of the meal for 1 M sucrose [drug × third interaction: F(2,16) = 5.67, P < 0.02; data not shown].

MTII significantly reduced the initial rate of licking in the meal [F(1,8) = 11.51, P < 0.01; Fig. 11]. The interaction term was not significant [F(3,24) = 2.02, NS]. The initial lick rate reduction was significant for saccharin [t(8) = 2.71, P < 0.05, Q = 3.93] and marginally significant for the two sucrose solutions [P values < 0.07; Q values > 2.91], however, there was no support for an MTII effect on licking for water [t(8) = −0.5, Q = 0.7, NS]. There was no main effect of MTII on the mean burst duration [F(3,24) = 3.92, P = 0.08], but the interaction term was marginally significant [F(3,24) = 2.93, P = 0.052; Fig. 11]. Comparisons indicated a significant reduction for the 0.1 M sucrose solution [P < 0.05; Q = 4.23] and a notable but nonsignificant reduction for the 1 M sucrose solution [t(8) = 2.05, P = 0.07, Q = 2.90, NS]. The pattern of results was similar for the mean burst size: there was no significant overall effect of MTII on the mean burst size [drug: F(1,8) = 2.12, NS; interaction: F(3,24) = 2.21, NS], although the planned comparison for 0.1 M sucrose did reach statistical significance [P < 0.05; Q = 3.41], and mean burst size for 1 M sucrose was reduced by 50% [Q = 2.44, NS; data not shown].

Analysis of licking within bursts indicated that MTII had no statistically significant effect on the rate of licking within bursts [F(1,8) = 2.95, NS] or on the proportion of longer ILIs within bursts [F(1,8) = 0.26, NS], and none of the interaction terms were significant [F values < 1.5].

**Experiment 3**

**Group 1:** brief-access sucrose responses after 3V MTII. Licking for sucrose during the 20-s brief-access tests increased with concentration, as indicated by a main effect of concentration in a two-way (drug × concentration) ANOVA [F(5,25) = 36.01, P < 0.001; Fig. 12]. There was a significant main effect of the drug term [F(2,10) = 8.50, P = 0.007], but the drug × concentration interaction term was not significant [F(10,50) = 1.62, NS]. Comparisons for the main drug term indicated that lick responses overall after 1 nM (P = 0.04) but not after 0.1 nM MTII were significantly smaller than responses after aCSF treatment. A separate one-way ANOVA confirmed that MTII did not affect licking for water [F(2,10) = 0.36, NS]. The results overall support the conclusion that 1 nM MTII right-shifted the sucrose concentration-response function.

**Group 2:** brief-access quinine HCl responses after 3V MTII. Licking for water during the 20-s brief-access tests was not significantly affected by MTII [F(2,10) = 2.51, NS; range of means = 127 to 137 licks/20 s]. The QHCl/water lick ratio significantly decreased as concentration increased, as indicated by a main effect of concentration [F(4,20) = 77.02, P < 0.001; see Fig. 12B]. Contrary to the effect for sucrose, MTII significantly increased licking for QHCl [F(2,10) = 5.96, P = 0.02], but the drug × concentration interaction term was not significant [F(8,40) = 1.80, NS]. Comparisons for the main drug term indicated that overall responses at both 0.1 nM and 1 nM
doses were significantly greater than responses under aCSF conditions \((P\) values \(< 0.05)\). The results support the conclusion that MTII right-shifted the QHCl concentration-response function.

**Experiment 4**

**Group 1: brief-access sucrose responses after 4V MTII.** Licking for sucrose during the 20-s brief-access tests increased with concentration, as indicated by a main effect of concentration in a two-way (drug \(\times\) concentration) ANOVA \([F(5,20) = 41.96, P < 0.001; \text{Fig. 13}]\). There was no significant main effect of MTII \([F(2,8) = 0.69, \text{NS}]\), and no significant MTII \(\times\) concentration interaction \([F(10,40) = 0.99, \text{NS}]\). A separate one-way ANOVA confirmed that 4V MTII did not affect licking for water \([F(2,8) = 0.12, \text{NS}]\).

**Group 2: brief-access quinine HCl responses after 4V MTII.** Licking for water during the 20-s brief-access tests was not significantly affected by MTII \([F(2,8) = 3.1, \text{NS}; \text{range of means} = 127 \text{ to } 137 \text{ licks/20 s}]\). The QHCl/water lick ratio significantly decreased as concentration increased, as indicated by a main effect of concentration \([F(4,16) = 57.15, P < 0.001; \text{see Fig. 13}]\). 4V MTII had no significant effect on licking for QHCl \([F(2,8) = 0.03, \text{NS}]\), and the drug \(\times\) concentration interaction term was not significant \([F(8,32) = 0.78, \text{NS}]\).

**SAMPLING BEHAVIOR.** Analyses in experiments 3 and 4 included lick responses only if the tastants were sampled (trials with 0 to 2 licks were excluded). MTII also increased the number of unsampled trials. Two-way ANOVAs (ventricle group \(\times\) dose) indicated significant main effects of MTII in both the sucrose \([F(2,18) = 12.14, P < 0.001]\) and the QHCl \([F(2,18) = 4.49, P < 0.03]\) test groups. There was no difference in the number of unsampled trials across ventricle groups for either tastant [sucrose: \(F(1,9) = 0.11, \text{NS}; \text{QHCl: } F(1,9) = 0.25, \text{NS}]\). Although MTII appeared to have little influence on sampling behavior in the 4V QHCl group, no ventricle group \(\times\) dose interaction terms were significant [sucrose: \(F(2,18) = 1.82, \text{NS}; \text{QHCl: } F(2,18) = 2.81, P < 0.09, \text{NS}; \text{see Fig. 14}]\).

**DISCUSSION**

Many studies have identified melanocortin influences on feeding after MCR ligand injection into the forebrain, while fewer have evaluated the contributions of hindbrain MCR stimulation to food intake control \((13, 14, 20–22, 30, 33)\). The first study to evaluate hindbrain injections of MCR ligands on feeding observed that chow intake was suppressed at the same MTII dose levels (0.1 nM and 1 nM, but not 0.01 nM) whether MTII was injected to the forebrain LV or to the hindbrain 4V \((14)\). The authors noted that the superimposition of the dose-response curves for MTII anorexia after either LV or 4V injection suggested that at least two populations of MCRs influenced food intake: If a single forebrain or brain stem site of action were responsible for MTII anorexia, one would expect a shift in the dose-response curve from one injection site relative to the other due to dilution of the MTII from the distal site. We observed a similar outcome as the effective anorexic doses in this study were the same (also 0.1 nM and 1 nM) after MTII was injected to either the 3V or the 4V, even though different foodstuffs (0.8 M sucrose vs. chow) and forebrain sites of injection (3V vs. LV) were tested relative to the prior work. The extent of intake suppression was also comparable across the 3V and 4V ventricle groups tested in this study. A distributed network of MCR-sensitive sites that contribute to feeding control is also implicated by reports that lesions of the PVN, or lesions of the area postrema and portions of the underlying nucleus of the solitary tract (NST), do not abolish anorexic responses to intracerebroventricular MTII injections.

![Fig. 10. Mean ingestion rates (mean lick count \(\pm\) SE) for each 30-s epoch of the first 5 min of the intake test after meal onset in rats ingesting water (A), 4 mM saccharin (B), 0.1 M sucrose (C) and 1 M sucrose (D) after 3V injection of aCSF (open symbols) or MTII (1 nM; solid symbols). The same rats were tested in each dose and tastant condition (\(n = 9\)).](http://ajpregu.physiology.org/doi/10.1001/jama.2011.1453)
Further, anorexia has been reported after intraparenchymal injections of MCR ligands to different hindbrain and forebrain nuclei (25, 31, 33). A recent study reported that injections of MTII (5 or 10 pM) to forebrain (PVN and retrochiasmatic area) and hindbrain (NST, rostroventral lateral medulla, and parabrachial nucleus) nuclei all reduced 18 h chow intake to about the same extent (10–15% intake reduction), suggesting a distributed and possibly redundant anatomical organization of the MCRs that influence food intake (25). Nevertheless, the aforementioned studies (including the meal size results of the current study) do not distinguish whether these anorexic responses emerged as a result of MTII effects on one or several of the behavioral processes that contribute to overall consumption. The results of this study suggest independent contributions of forebrain and hindbrain MCR substrates to feeding behavior.

MTII injected into the 4V did not affect the initial rate of licking, the mean burst size or duration, or brief-access licking for sucrose or QHCl solutions, suggesting that there was no 4V MTII influence on gustatory responsiveness (8–10, 23, 26, 27). This result is consistent with our prior report that 4V MTII did not reduce the initial rate of licking or burst size in the beginning of the meal in rats consuming 0.7 M glucose (30). We also confirmed that MTII did not suppress food intake by an impairment of oromotor coordination, as the rate of licking within bursts was not slowed by anorexic doses of MTII, and the proportion of longer pauses within bursts was not increased after either 4V or 3V MTII.

In both the current study and our prior work, 4V MTII suppressed intake by reducing the burst count, ingestion rate in later phases of the meal, and the duration of the meal, a result consistent with the interpretation that MTII enhanced inhibitory postigestive feedback (4–6, 8–10, 23, 26, 27). The primary effect of MTII in this regard appears to be an early termination of the meal because the average ingestion rate, in which meal size is divided by meal duration, was not affected. When meal duration differences were controlled through analysis by meal thirds, effects of MTII on ingestion rate were observed, suggesting that 4V MTII injection influences at least two processes, one affecting the rate of ingestion and another influencing the decision to terminate the meal. This hypothesis is consistent with reports that MCRs are located.
ever, 3V MTII additionally reduced the initial rate of lick-

burst count, ingestion rate, and meal duration, suggesting a
directed to the forebrain via 3V injections also reduced the
afferent input (29).

postsynaptic currents of NST neurons in response to vagal
that MCR ligands presynaptically modulate the excitatory
neurons MCR ligand) are in close proximity to NST neurons
active for alpha-melanocyte stimulating hormone (endoge-
lateral parabrachial nucleus (18, 19); that fibers immunore-
in hindbrain visceral-sensory processing nuclei, including
in that 3V MTII reduced the initial lick rate and the mean burst duration for saccharin
and/or sucrose solutions, but not for water. The suppression of saccharin consumption
suggests an effect potentially mediated by an influence on
taste evaluation. In support, we replicated and extended our
observations from experiment 1 in that 3V MTII reduced
the first minute of the meal of nondeprived rats, where
baseline licking was sufficient to observe a potential reduc-
tion also increased licking for concentrations of QHCl that
were generally avoided under control conditions. The increase
in licking for QHCl suggests that MTII may have reduced the
perceived intensity of taste stimuli. This result, coupled with
the findings that MTII did not affect licking for water in any
group tested, reduce the within-burst lick rate, or increase the
proportion of longer ILIs within bursts, further discounts the
notion that MTII suppressed licking for the sucrose solutions
due to impaired motor function or general malaise.

We found no effects of MTII on licking for water in three
experiments. An important concern is that intake values for
water in nondeprived rats were low, raising the possibility of
a floor effect that could mask potential MTII influences.
Two of our measures, however, did not suffer from this
limitation. In experiment 2 we analyzed licking for water in
the first minute of the meal of nondeprived rats, where
baseline licking was sufficient to observe a potential reduc-
tion. Second, we analyzed water licking in the two water-
deprived groups of rats tested for QHCl responses, where
licking for water was robust. Murphy et al. (20) also
analyzed 3V MTII effects on longer-term (17 h) food and
water intake and observed food intake reduction at doses
within the range tested in our study (0.05 nM and 0.5 nM).
However, a suppression of water intake was only observed
at doses at least 50-fold greater (50 nM and 500 nM) than
the strongest dose tested in our study (1 nM). The authors
suggested that the water drinking effect was possibly a side
effect due to low affinity (or perhaps nonspecific) binding of
MTII at other receptors, or other nonspecific behavioral
effects of the very high doses of MTII.

To our knowledge, only one prior study has evaluated
potential MCR influences on taste-related behavior. Con-
trasting with the current results, Eylam et al. (12) reported
that MC4R-knockout mice exhibited no differences in taste
responses relative to wild-type mice in brief access tests
similar to those used in experiments 3 and 4. The discrep-
ancy with the current data could be due to species differ-
ences in MCR expression, compensatory developmental

As observed after 4V MTII injection, we found that MTII
directed to the forebrain via 3V injections also reduced the
burst count, ingestion rate, and meal duration, suggesting a
similar influence on postigestive feedback inhibition. How-
ever, 3V MTII additionally reduced the initial rate of lick-

ing, and it moderately reduced the mean burst duration,
suggesting a possible MTII effect on behavioral processes
related to taste evaluation (8–10, 27). Therefore, we tested
MTII effects on licking for a range of taste solutions that
varied in caloric and gustatory intensity (experiment 2) and
for a range of concentrations of sucrose and QHCl solutions
using brief access taste-response tests (experiments 3 and 4).
MTII injected into the 3V depressed intake of noncaloric 4
mM saccharin, as well as 0.1 M and 1 M sucrose solutions,
but not water. The suppression of saccharin consumption
suggests an effect potentially mediated by an influence on
taste evaluation. In support, we replicated and extended our
observations from experiment 1 in that 3V MTII reduced
the initial lick rate and the mean burst duration for saccharin
and/or sucrose solutions, but not for water. In experiment 3,
3V MTII at its most anorexic dose (1 nM) also significantly
suppressed brief access licking responses for sucrose solu-
tions (Fig. 12A), again with no effect on responses to water.
Importantly, 4V MTII had no effect on brief access licking
for sucrose or QHCl solutions, even though both 3V and 4V
MTII injections influenced appetitive motivation to sample
the tastants.

Surprisingly, MTII delivered to the forebrain via 3V injec-
tion also increased licking for concentrations of QHCl that
were generally avoided under control conditions. The increase
in licking for QHCl suggests that MTII may have reduced the
perceived intensity of taste stimuli. This result, coupled with
the findings that MTII did not affect licking for water in any
group tested, reduce the within-burst lick rate, or increase the
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![Graph](http://ajpregu.physiology.org/DownloadedFrom/by/10.2202/32.247)
responses to the null mutation, or other factors. It is also important to note that licking microstructure measures are not strictly fixed to sensory or state variables (e.g., 17), thus microstructure analysis has exploratory value to the extent that the effects can be reliably observed and replicated. To this end, we replicated our findings of 3V MTII influences on taste measures across three experiments using two complementary methods of taste analysis in rodents. Our conclusion of an MTII effect on taste evaluation could be further supported by evaluating MTII effects on orofacial taste reactivity (15).

Perspectives and Significance

We have determined that divergent behavioral feeding responses underlie what previously appeared to be similar anorexic responses to MTII after MTII was applied to different neuroanatomical structures. In these studies, the intake measures were limited to the mass of food consumed, which may have masked differences in the behaviors contributing to the intake responses. In this study, either forebrain (3V) or hindbrain (4V) delivery of MTII influenced behavioral measures associated with inhibitory gut feedback, whereas measures associated with gustatory evaluation were only affected after forebrain (3V) MTII delivery. One limitation of this study is that 3V and 4V injections produce a partially nonoverlapping rather than segregated pattern of brain stimulation, as the caudal flow of CSF distributes infusates to the forebrain and hindbrain after a 3V injection, whereas 4V injections are restricted to the hindbrain (as we confirmed through ink injections after testing). This logic strongly implicates forebrain MCR stimulation to be necessary for the MTII effect on taste-related measures, but it does not clarify whether a single hindbrain or multiple MCR sites contributed to the observed MTII effects on measures related to postigestive inhibition. Consistent with prior studies, we did not identify any significant differences in the potency of 3V vs. 4V MTII on intake reduction or behavioral measures associated with postigestive (gut) feedback inhibition, and this lack of difference in effect size disfavors a single hindbrain site of MCR influence on visceral sensitivity. It will be important for future work to determine whether taste, gut-related, or other feeding-relevant responses measured both behaviorally and neurophysiologically are selectively affected when MCR ligand injections are specifically targeted to individual MCR-expressing brain nuclei implicated in food intake control.

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Fig. 14. Mean (+ SE) number of unsampled trials (trials with 0 to 2 licks) in rats tested for brief-access responses to sucrose (top) or quinine hydrochloride (QHCl; bottom) in experiments 3 and 4. Rats received 3V (left; n = 6 per tastant) or 4V; (right; n = 5 per tastant) injections of aCSF, 0.1 nM and 1 nM MTII. Each tastant concentration was presented at least 3 times per test session. When a tastant was not sampled at least once, additional trials were conducted until all concentrations were sampled once or more. Rats ingesting sucrose were 4 h water-deprived prior to testing, and rats ingesting QHCl were 23 h water-deprived prior to testing. Unsampled trial data were not included in the calculation of licking responses to sucrose or QHCl (see Figs. 12 and 13).
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