Learned Meal Initiation Attenuates the Anorexic Effects of the Melanocortin Agonist MTII

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The central melanocortin system is critically involved in the control of food intake and body weight. Administration of melanocortin agonists reduces food intake and adiposity, and the central melanocortin system is demonstrated to mediate the anorexic effects of both leptin and insulin. An important unanswered question has been whether melanocortin agonists would also reduce food intake that is driven by factors other than homeostatic mechanisms (e.g., conditioned eating). In the first experiment, we identified that long-term maintenance on a meal-feeding schedule attenuated rats' sensitivity to central administration of the melanocortin agonist MTII. The results from a second experiment demonstrate that the attenuation of the MTII-induced anorexia was due to learned schedules of food intake rather than food deprivation per se. Results from the final experiment suggest that this attenuation of MTII-induced anorexia may be independent of the decreased sensitivity caused by a high-fat diet. These results support the hypothesis that meal-feeding schedules can lead to anticipatory physiological responses that attenuate the anorexic effects of exogenous melanocortin agonists. *Diabetes* 52:2684–2688, 2003

Body weight (or more accurately body adiposity) is often considered a tightly regulated variable. To maintain body adiposity, caloric intake must equal caloric expenditure over time. Such an intricate process relies on the interactions of a number of physiological systems. In particular, there is considerable evidence that body adiposity is the product of a negative-feedback regulatory system where signals from adipose tissue inform the central nervous system (CNS) about the status of peripheral energy balance. On one side of the hypothesized feedback loop are signals from peripheral fat stores, with several hormonal candidates having been proposed to relay adipose content information to the CNS, including the pancreatic hormone insulin and the adipose hormone leptin (1,2).

On the receiving side of this regulatory system are one or more central effector systems that translate adipose-store information into appropriate subsequent behavior. In the presence of low adipose stores (e.g., low leptin and insulin), food intake is increased while energy expenditure is decreased. In the presence of high adipose stores, intake is reduced while energy expenditure is increased. Indeed numerous studies support the hypothesis that such a negative-feedback loop is a major controller of food intake and that disruptions in that loop (e.g., mutations that abolish leptin production or action) result in profound obesity. Myriad data have established the hypothalamic melanocortin (MC) system (3–9) as an important mediator of both insulin and leptin's effects on food intake and body weight. Importantly, exogenous administration of MC agonists results in decreased food intake and body weight in numerous animal models, including diet-induced obesity (10–15). Indeed, MC-like compounds are currently under intense scrutiny as potential therapeutic mechanisms for the treatment of obesity.

Mutations in the leptin or MC systems are believed to account for only a very small fraction of the staggering rise in the prevalence of obesity seen in most of the industrialized world. Given this fact, it is therefore important to elucidate other potential mechanisms that might result in a failure of this negative-feedback system. One possibility is that under certain circumstances, ingestive behavior is the result of systems that operate independently of these regulatory systems. Animals and humans do, for example, consume food for reasons other than discrepant energy signals or negative-feedback loops, including being sensitive to hedonic, motivational, social, and other factors. Weingarten (16) and others have demonstrated that rats will consume large meals in the face of positive energy stores simply because they had been trained to do so (17). This effect is analogous to human patterns of socially and temporally associated food intake (18–20).

An important question is whether potential targets of obesity therapeutics will be effective in the face of these nonregulatory controls of ingestion. For example, will melanocortin ligands, which reduce food intake and mediate leptin's effect on body weight, also affect ingestion that occurs as a result of nonhomeostatic mechanisms (e.g., hedonics or learning)? The purpose of the present experiments was to assess the effects of the MC agonist MTII on conditioned food intake. Toward this end, rats were maintained on either ad libitum or restricted schedules of food availability, and the efficacy of centrally administered MTII to reduce food intake was assessed under both ad libitum and fasting test conditions.

RESEARCH DESIGN AND METHODS

Animals. Male Long-Evans rats (Harlan, IN) weighing 380–440 g at the onset of the experiments were individually housed in Plexiglas tubs and maintained

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CNS, central nervous system; i3vt, third-cerebral ventricle; MC, melanocortin; POMC, proopiomelanocortin.

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

Macronutrients	Regular diet	Low-fat diet	High-fat diet
Protein (kcal/g)	0.8	0.5	0.6
Carbohydrate (kcal/g)	2.1	2.7	2.0
Fat (kcal/g)	0.5	0.4	1.8
Total (kcal/g)	3.4	3.6	4.4
Protein (% of total kcal)	23	14	14
Carbohydrate (% of total kcal)	62	75	45
Fat (% of total kcal)	15	11	41

on a 12:12-h light-dark cycle in a temperature-controlled, Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited vivarium. All procedures were approved by the Internal Animal Care and Use Committee at the University of Cincinnati. The rats were maintained on ad libitum pelleted food and tap water unless otherwise noted. Each rat was implanted with a cannula aimed at the third-cerebral ventricle (i3vt). Coordinates for cannula placement were on the midline, 2.2 mm posterior to bregma and 7.5 mm ventral to dura, with bregma and lambda at the same vertical coordinate (21). After 10 days of recovery, accuracy of cannula placement was verified by i3vt infusion of 10 ng angiotensin II in 1 μl physiological saline. Only animals that drank at least 5 ml of water within 1 h were used in the experiments.

Drugs. MTII (Phoenix Pharmaceuticals, Mountain View, CA) (0.1, 0.3, and 1.0 nmol) was dissolved in physiological saline, which also served as the control solution. All i3vt injections were delivered in a volume of 1 μl.

Diets. Animals were maintained on pelleted rat diet (Harlan-Teklad, Indianapolis, IN) in experiments 2 and 3. High-fat and low-fat pelleted diets prepared by Dyets (Bethlehem, PA) were also used in experiments 1 and 3. The macronutrient composition of the high-fat and low-fat diets is given in Table 1.

Experiment 1. We have previously demonstrated that a high-fat diet decreases rats' sensitivity to the anorexic effects of i3vt MTII (22). This experiment was designed and executed to assess the effects of MTII on rats maintained on restricted access to a high-fat or low-fat diet for 10 weeks (23). All rats in this experiment were given daily access to a restricted amount of either high-fat or low-fat diet to prevent the onset of obesity. Each rat was given 85% of the calories normally consumed by rats fed ad libitum on these diets. All rats received daily rations (85% of normal ad libitum high-fat or low-fat intake) of diet at the onset of dark each day over 10 weeks. The third-ventricular cannulas were implanted after the 10-week dietary regimen. After recovery from surgery and angiotensin testing, all rats were administered i3vt MTII (0.1, 0.3, or 1.0 nmol) or saline, and food intake was measured after 2, 4, and 24 h. Each rat received each dose of MTII in counterbalanced order. Seven days separated injections.

Experiment 2. The purpose of this experiment was to assess the effects of a restricted chow schedule to MTII-induced anorexia. Separate groups of i3vt-cannulated rats (n = 10 per group) were given ad libitum or restricted access to Purina 5001 pelleted chow. Rats in the restricted group received 3-h access to chow each day. To entrain the food hopper as the stimulus to eat, the time of this access was varied randomly between 6 h before and 6 h after the onset of dark. At all other times, the food hopper was removed from the cages. After 2 months of ad libitum or restricted chow access, all rats received a series of i3vt infusions of either saline or MTII (0.1 nmol), after which food intake was measured at 2 and 24 h. Two of these test infusions were administered after the rats had been without food for 21 h, and the remaining infusions were given after all rats had ad libitum access to chow for 7 days. Thus, each rat, regardless of previous schedule of food availability, was tested with both MTII and saline, under conditions of ad libitum feeding and food restriction. The order of test conditions followed a complete, counterbalanced Latin square design.

Experiment 3. Four groups of i3vt-cannulated rats (n = 10/group) were given ad libitum or restricted access to either high-fat or lab diet. Rats in the restricted groups (high-fat and chow) received 3-h access to food each day, but the time of access varied between 6 h before and 6 h after the onset of dark. After 2 months of this regimen, all rats received the same i3vt infusions of (0.1 nmol) MTII and saline, under the same conditions as described in experiment 2. The order of test conditions followed a complete, counterbalanced Latin square design.

Data analysis. The data were analyzed by overall ANOVA, followed by Tukey's Honestly Significant Difference post hoc tests. Learned meal initiation (ad libitum fed versus restricted) and diet (where appropriate) were included as between-subjects factors. Type of test (21-h restricted vs. 7-day ad libitum) was included as a repeated measures factor. Separate ANOVAs were use to analyze 2-h and 24-h food intake data. Significance was set at $P < 0.05$, two tailed.

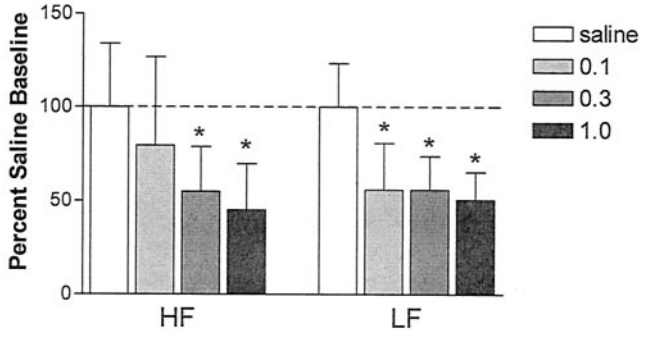

FIG. 1. Mean ± SE 2-h food intake (experiment 1). Data are expressed as percent of saline baseline. *$P < 0.05$, relative to saline baseline. HF, high fat; LF, low fat.

RESULTS

Experiment 1. Surprisingly, rats in both the high-fat and low-fat conditions spontaneously separated into two different groups based on their patterns of food intake. By the end of the 10 weeks with restricted access to food, half the rats in each group remained meal-fed or restricted, meaning that they consumed all of their daily food ration within 3 h of receiving it. The remaining half of the animals in each group did not consume their entire daily ration in a short period of time. Rather, food remained in the cages of these rats when the next day's allotment was made available. Thus, they became ad libitum fed. The principal difference between these two types of rats was the amount of food consumed when it became available each day, with one group of rats continuing to consume a large amount of food within 3 h.

MTII reduced food intake in both the high-fat and low-fat groups ($P < 0.05$). However, the effective dose for reducing intake was relatively higher in the high fat–fed rats. In low fat–fed rats, all doses reliably suppressed 2-h and 24-h food intake ($P < 0.05$, relative to saline). In high fat–fed rats, however, only 0.3 and 1.0 nmol/l reliably suppressed food intake (Fig. 1) ($P > 0.05$, relative to saline). Interestingly, the variability in this experiment was unusually high compared with our historical results (Fig. 1 represents mean percent saline intake [± SE]). Upon further inspection of the individual data points, we found that the responses to MTII were bimodally distributed and dependent on whether the rats had quickly consumed their entire daily ration of diet or whether food remained in the hopper when the next ration was delivered.

We then repeated the statistical analyses, accounting for whether rats consumed all of their food within 3 h or whether they had fed on an ad libitum basis. Figure 2 depicts the data in Fig. 1 with rats classified as being restricted or ad libitum rats. Restricted rats did not reduce their food intake following any dose of MTII ($P > 0.05$, NS). In contrast, ad libitum rats were quite sensitive to MTII ($P < 0.05$, relative to saline).

Experiment 2. MTII was relatively less efficacious in the 21-h restricted than in the 7-day ad libitum test conditions ($P < 0.05$). MTII was also less efficacious in rats that had

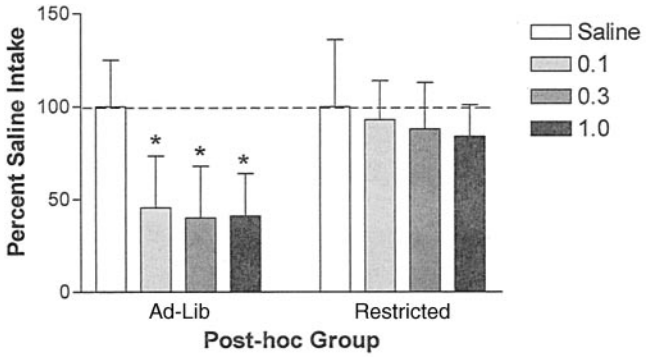

FIG. 2. Mean ± SE 2-h food intake (experiment 1) after 7 days of ad libitum feeding. Data are expressed as percent of saline baseline, collapsed across high-fat and low-fat diets. "Ad-lib" refers to rats that previously did not consume all of their daily ration of food within 3 h; "Restricted" refers to rats that previously consumed all of their daily food within 3 h. Saline baselines were 3.5 ± 0.8 g for Ad-Lib and 6.2 ± 1.1 g for Restricted rats. *$P < 0.05$ vs. saline baseline.

previously been maintained on a food-restriction schedule ($P < 0.05$, relative to the ad libitum condition). Importantly, rats in the restricted-food-access group had decreased sensitivity to i3vt MTII under both 7-day ad libitum and 21-h restricted test conditions. That is, regardless of the deprivation state at the time of the test, rats that had previously been trained to consume all of their daily calories within a short time had decreased sensitivity to MTII when assessed over 2 h (Fig. 3, left panel) ($P < 0.05$, relative to ad libitum condition). Figure 3 (right panel) depicts the 24-h data. This difference was apparent only during the time that the rats would normally have consumed their daily requirement of calories. When assessed over 24 h, MTII was equally effective under both 7-day ad libitum and 21-h restricted test conditions and in both groups of rats.

Experiment 3. Figure 4 depicts percent baseline food intake at 2 h. As depicted in the figure (left panel), ad libitum high fat–fed rats did not reliably reduce their food intake after i3vt MTII under either the 7-day ad libitum or 21-h restricted test conditions ($P > 0.05$ for both). Rats maintained on restricted access to the high-fat diet had a 50% reduction in food intake following MTII, when tested under ad libitum conditions, but not when tested after 21-h food restriction ($P < 0.05$ for both). Nonetheless, results from chow-fed rats were similar to that found in experiment 2. MTII was less effective to reduce food intake when rats were tested after 21-h restriction than after 7-day of ad libitum access to food (Fig. 4, right panel). Further, MTII was less efficacious in rats that had been previously maintained on food restriction ($P < 0.05$, relative to ad libitum conditions) even when tested after 7 days of ad libitum access to food.

Figure 5 depicts percent baseline food intake at 24 h. For rats on high-fat diet, MTII reliably reduced food intake in the ad libitum test condition (Fig. 5, left panel). Interestingly, MTII was more effective in previously restricted rats ($P < 0.05$). However, MTII was not effective to reduce food intake in restricted rats when tested after 21-h food restriction. Rats with ad libitum access to high-fat diet did reliably suppress food intake in the 21-h restricted test ($P < 0.05$). The effect of MTII in the ad libitum high fat–fed rats, however, was significantly attenuated, relative to ad libitum chow rats ($P < 0.05$). Thus, these data support our previous findings that maintenance on a high-fat diet disrupts baseline MC-induced anorexia (22). The right panel of Fig. 5 depicts data from rats maintained on chow. Unlike experiment 2, there were significant differences between 7-day ad libitum and 21-h restricted test conditions in both groups of chow-fed rats. Further, in the 21-h restricted test, MTII was ineffective to significantly reduce intake in either group of rats, although food intake was greater in previously restricted rats ($P < 0.05$).

DISCUSSION

A compelling body of evidence implicates the hypothalamic MC system as one of the central effectors controlling food intake and energy balance. First, expression of MC gene products is regulated by energy balance. During periods of negative energy balance (and consequently low levels of the adiposity hormones leptin and insulin), expression of Agouti-related protein (AgRP) mRNA is increased while expression of the MC precursor molecule, proopiomelanocortin (POMC), is decreased (24). Further, POMC-containing neurons also have receptors for both leptin and insulin (4–6,25). These findings suggest that the hypothalamic MC system is a likely central target of adipose signals and a mediator of their effects on food intake.

Additional evidence suggesting a role for the MCs in the control of food intake comes from experimental administrations of both naturally occurring and synthetic ligands of MC receptors. i3vt administration of α-melanocyte-stimulating hormone (MSH) decreases food intake (26,27), as does i3vt administration of synthetic agonists, including MTII and Ro27–3225 (13,28,29). Conversely, administra-

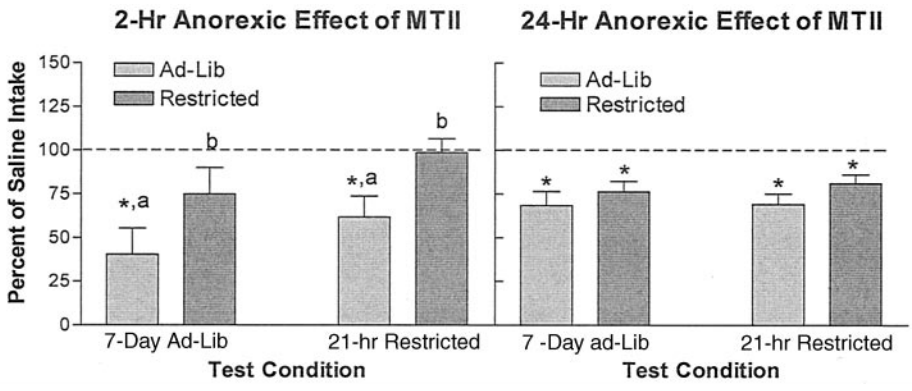

FIG. 3. Mean ± SE 2-h and 24-h food intakes (experiment 2) after 0.1 nmol/l MTII. Data are expressed as percent of saline baseline. Group bars: "Ad-Lib," previously fed ad-libitum; "Restricted," previously meal-fed. Test conditions: "7-Days Ad-Lib," tested after 7 days of ad libitum food intake; "21-h Restricted," tested when food deprived for 21 h. Different letters denote significant differences between bars. On the 7-day ad libitum test days, 2-h saline baselines were 2.8 ± 0.5 g for Ad-Lib and 5.3 ± 0.9 g for Restricted rats. On the 21-h restricted test days, 2-h saline baselines were 5.18 ± 0.6 g for Ad-Lib and 10.1 ± 1.1 g for Restricted rats. There were no differences in 24-h saline baselines between groups. *$P < 0.05$ vs. saline baseline.

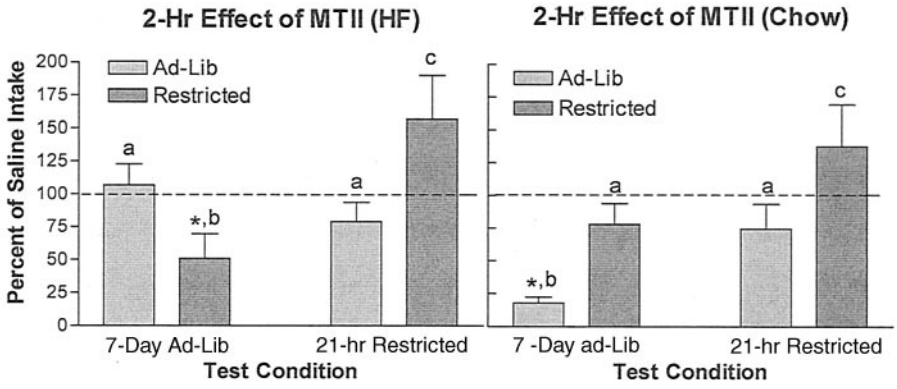

FIG. 4. Mean ± SE 2-h food intakes (experiment 3) after 0.1 nmol/l MTII. Data are expressed as percent of saline baseline. Group Bars: "Ad-Lib," previously fed ad-libitum; "Restricted," previously meal-fed; "HF," high fat; "LF," low fat. Test conditions: "7-days Ad-Lib," tested after 7 days of ad libitum food intake; "21-h Restricted," tested when food deprived for 21 h. Different letters denote significant differences between bars. *$P < 0.05$ vs. saline baseline. On the 7-day ad libitum test days, saline baselines were 3.4 ± 0.8 g for Ad-Lib and 6.1 ± 1.2 g for "Restricted" rats. On the 21-h restricted test days, saline baselines were 5.8 ± 0.7 g for Ad-Lib and 9.3 ± 1.3 g for "Restricted" rats.

tion of MC receptor antagonists, such as AgRP or the synthetic antagonist SHU-9119, elicits long-lasting increases in food intake (28,30–33). Collectively, these data suggest an important role for the MC system in the control of energy balance and as a potential target for the treatment of obesity.

Most previous experimental work on the anorexic effects of MC agonists has been conducted in subjects maintained ad libitum on low-fat diets. The present results indicate that the efficacy of i3vt MTII to reduce food intake in rats varies with the experimental paradigm. Specifically, the effect is attenuated by maintenance on a 21-h food restriction paradigm. That is, constraining rats to consume all of their daily energy within 3 h blunted the ability of MTII to reduce food intake. This could have been attributed either to the animals having learned to consume a day's requirement of calories in 3 h or to being food deprived for 21 h. However, the phenomenon persisted when the rats were allowed 7 days of ad libitum food access before MTII administration. Hence, deprivation per se is an unlikely explanation. Meal-fed rats are thought to acquire the ability to consume a large amount of food in a short period of time through learning (34,35). As an example, rats anticipating consuming a large meal secrete insulin, lower their blood glucose, and change their body temperature and metabolic rate at the time eating is imminent. The present results suggest that similar anticipation in the restricted rats results in a relative insensitivity to manipulation of the MC system.

The results of experiment 3 replicated our previous findings that maintenance on a high-fat diet also reduces sensitivity to i3vt MTII (22). The results further indicate that the reduced sensitivity is enhanced by maintenance on a restricted-feeding schedule, but only under negative energy balance. However, interpretation of the finding of greater MTII-induced anorexia in high-fat restricted than in ad libitum fed rats in experiment 3 is complicated by the fact that MTII was not less effective under the restricted test condition. Indeed, there may be an unidentified interaction between dietary fat content and meal-size on the ability of MC agonists to reduce food intake. Importantly, animals consume food for both regulatory (e.g., negative energy balance) and nonregulatory reasons (e.g., hedonics or learning). Data from these experiments support the hypothesis that MTII is effective to regulatory but not nonregulatory food intake. Thus, intake of high-fat diet under negative energy balance can be viewed as a combination of both controlling factors. Future work, including careful assessment of meal patterns, may help elucidate the specific controlling factors in these complex behaviors. Finally, in the present experiment, we found a significant effect of MTII to reduce food intake in high fat–fed rats. These data would appear at odds with our previous finding (22). However, when compared with rats maintained on ad libitum chow, the effect of MTII in rats maintained on ad libitum high-fat diet was attenuated in the present experiment. Further, rats in experiment 3 had only 2 months' access to high-fat diet, whereas we previously (22) assessed the effect of MTII in rats with access to high-fat diet for >3 months. Thus, the differences in sensitivity may be due to length of access to high-fat diet.

These results have important implications for the strategy of developing MC agonists to treat obesity. That is, many obese individuals are thought to consume diets with a relatively high fat content (36–39), implying that the drugs would be less efficacious in the very population targeted for therapy. Further, since most humans adopt regular feeding patterns, consuming food in scheduled or

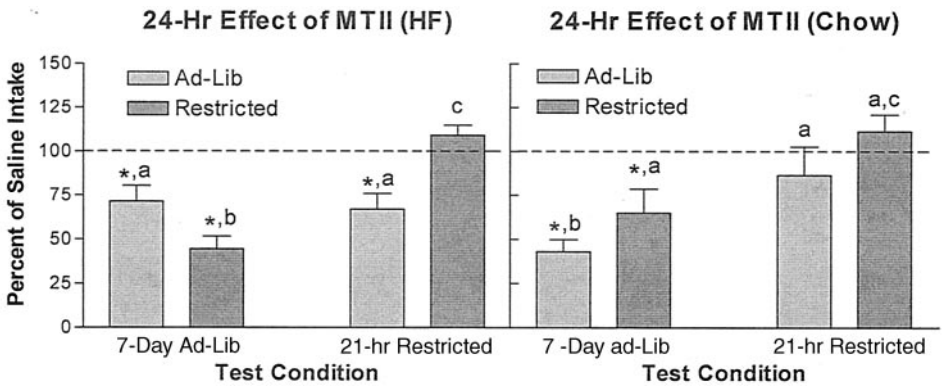

FIG. 5. Mean ± SE 24-h food intakes (experiment 3) after 0.1 nmol/l MTII. Data are expressed as percent of saline baseline. There were no significant differences between groups in the 24-h saline baselines. Symbol designations are identical to those in Fig. 4.

anticipated meals, the dose of MC agonist necessary to achieve significant reductions in body weight may be considerably higher and will therefore increase the potential for negative side effects.

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