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Rats acquire stronger preference for flavors consumed towards the end of a high-fat meal

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HIGHLIGHTS

- Rats learn to prefer cue flavors followed by the postingestive effects of fat.
- In a high-fat meal consisting of two flavors, rats learn stronger preference for the flavor occurring later in the meal.
- This differs from prior patterns seen with glucose.
- This suggests the postingestive reward generated by fat is of relatively slow onset, and potentially different from glucose.

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ABSTRACT

Rats learn to prefer flavors associated with postingestive effects of nutrients. The physiological signals underlying this postingestive reward are unknown. We have previously shown that rats readily learn to prefer a flavor that was consumed early in a multi-flavored meal when glucose is infused intragastrically (IG), suggesting rapid postingestive reward onset. The present experiments investigate the timing of postingestive fat reward, by providing distinctive flavors in the first and second halves of meals accompanied by IG fat infusion. Learning stronger preference for the earlier or later flavor would indicate when the rewarding postingestive effects are sensed. Rats consumed sweetened, calorically-dilute flavored solutions accompanied by IG high-fat infusion (+ sessions) or water (– sessions). Each session included an “Early” flavor for 8 min followed by a “Late” flavor for 8 min. Learned preferences were then assessed in two-bottle tests (no IG infusion) between Early(+) vs. Early(–), Late(+) vs. Late(–), Early(+) vs. Late(+), and Early(–) vs. Late(–). Rats only preferred Late(+), not Early(+), relative to their respective (–) flavors. In a second experiment rats trained with a higher fat concentration learned to prefer Early(+) but more strongly preferred Late(+). Learned preferences were evident when rats were tested deprived or recently satiated. Unlike with glucose, ingested fat appears to produce a slower-onset rewarding signal, detected later in a meal or after its termination, becoming more strongly associated with flavors towards the end of the meal. This potentially contributes to enhanced liking for dessert foods, which persists even when satiated.

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1. Introduction

Food intake is guided by the various taste and flavor properties of foods. Some food constituents, particularly sugars and fats, promote intake because of predominantly unlearned positive responses to their sensory properties. But the much broader range of complex flavors and odors in food become liked or disliked primarily through experience. Perception of a food's flavor always precedes its postingestive consequences, allowing animals to learn when particular flavors reliably predict either beneficial or aversive postingestive events and then use these associations to guide subsequent food selection and meal size.

One influential type of associative flavor–postingestive consequence learning is flavor–nutrient conditioning. In this Pavlovian-like process, a taste or flavor (CS) of a food is followed by the post-oral physiological effects of macronutrients (US) contained in the food (see reviews [1–4]). This flavor–nutrient association can powerfully alter subsequent reactions to the CS flavor. If an initially-neutral flavor is followed by caloric consequences (especially of glucose but also other carbohydrates, fats, proteins, or even ethanol) rats can learn to preferentially select that flavor and consume larger amounts of it, often treating the CS flavor itself as if it has become more hedonically positive [5–7].

Procedurally, flavor–nutrient learning can be measured in experiments where two distinctive flavors that are similar in initial attractiveness are given to subjects in different training sessions, but with one flavor providing nutritional consequences and the other not. With experience, increased intake of the nutrient-paired flavor relative to the unpaired flavor, and preferential intake of the former in a direct choice

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between the two (even when the nutrient is no longer present) are typical results showing that flavor evaluation is based on learned associations with nutrient consequences, rather than mere familiarity [8]. Experiments in which flavor consumption is accompanied by direct intragastric or intraduodenal nutrient infusion (versus water infusion) show that subjects are associating the cue flavor with the nutrient's postingestive properties rather than its inherently rewarding taste (e.g., sweetness). Animals may also associate cue flavors with a meal's satiating effects, which sometimes results in a net decrease in subsequent intake when that effect opposes the intake-promoting effects of learning [9,10]. This is still consistent with the idea that flavor evaluation is altered by associative experience with postingestive nutrient consequences.

Preference learning can be acquired in as little as a single flavor–nutrient pairing [11,12], and learned preferences are especially persistent once they are acquired [11,13]. In this manner, food selection is steered towards nutrient-rich foods not only by unlearned positive responses to nutrient tastes themselves but also by learned responses to whatever flavors in an animal's food environment tend to co-occur with nutrients. Thus in humans' modern environment of flavor-enhanced, energy-dense, processed foods, Pavlovian conditioned responses to flavors associated with caloric density may maladaptively encourage selection and overconsumption of obesity-promoting foods.

The present experiments are concerned with the psychobiological mechanisms of flavor–nutrient learning with high-fat food. Although rats learn to alter their preference and intake of flavors associated with different macronutrients, a variety of evidence shows that fats are somewhat less effective than carbohydrates as a postingestive US, even when equated for caloric density. First, preferences for fat-paired flavors are learned more slowly. Preference is learned with as little as a single flavor + nutrient pairing for glucose but requires several pairings for corn oil [12]. Second, even after extensive experience, fat-based preferences often remain weaker than carbohydrate-based preferences. Rats trained with one CS flavor paired with IG carbohydrate and a different CS flavor with equicaloric IG corn oil learn to prefer both flavors versus an unpaired control flavor, but typically still prefer the carbohydrate CS flavor over the fat CS flavor in a direct choice [14,15]. Third, in studies of conditioned meal size effects, flavor + fat learning requires more training exposures than flavor + carbohydrate learning when equated for caloric density [16], and flavor + fat learning requires a more calorically dense fat stimulus to be effective [10]. Thus the postingestive US effects of fat are generally considered less effective than carbohydrates in producing learned responses to cue flavors.

Since the precise physiological identity of the postingestive events acting as the US for flavor–nutrient conditioning is unknown (see reviews [4,17,18]), investigating these cross-nutrient differences may help identify the underlying physiological signals involved. There are a number of potential explanations for why fat is a less effective US than carbohydrate. First, in the course of a meal fat may be slower to produce the relevant postingestive reinforcing stimuli. As a form of Pavlovian conditioning, flavor–nutrient learning should be sensitive to the temporal contiguity between CS (flavor) and US (postingestive events), so a slower-onset postingestive US should be more difficult to associate with the preceding flavor. Second, there actually may be a number of distinct physiological signals generated at different time points during and after a meal, arising from different sites of action, that have additive or synergistic effects at reinforcing preference for the CS flavor. Fat may stimulate a smaller subset of these reward pathways than carbohydrates. Third, analysis of flavor–nutrient learning should also consider the possible general influence of the prandial rise in circulating glucose on immediate attention and information processing (e.g., [19,20]). A meal high in glucose or rapidly digested glucose-containing saccharides should be most effective at stimulating this attentional effect, making the flavors of a glucose meal more perceptually salient and memorable when delayed postingestive effects of nutrients subsequently arise. It is important to note the three potential explanations outlined above are not mutually exclusive.

Our recent work has been focused on the timing of onset of the critical postingestive US events, using a behavioral paradigm to determine at what point after meal initiation rats detect the onset of nutrient reward acting post-orally. This strategy involves training rats in sessions wherein they consume 'meals' of distinct CS flavor cues paired with intragastric (IG) nutrient infusion, except that some CS flavors are always encountered early in the meal and other flavors only late in the meal. For comparison purposes each rat also experiences meals with other flavors paired with IG water. If, as has been an implicit assumption in much flavor–nutrient research (e.g., [1]) the postingestive US effects of a meal are only detected after a relatively long delay, the strongest preference learning should accrue for the flavor routinely encountered towards the end of the meal, because of the closer temporal contiguity between that CS flavor and the US onset. Similarly, retroactive interference would further inhibit learning about the early flavor. But contrary to this prediction, we have shown [21] that when flavors are paired with IG glucose, rats acquire a strong preference for the early-occurring flavor in addition to the late flavor. Moreover, rats' learned responses to the early flavor were qualitatively different from their responses to the late flavor. Learned responses to the early flavor were expressed only when rats were hungry, whereas learning about the late flavor was not state dependent, and was exhibited regardless of whether rats were hungry or recently satiated.

We have argued [21] that this reveals two fundamental facts about the mechanisms of flavor–nutrient conditioning, at least when glucose is the US. First, the strong preference for the early flavor shows that some rewarding effects of the US are detected rapidly, within the first several minutes of the meal in progress. If postingestive reward arose only late in a meal or afterwards, temporal contiguity effects (e.g., trace decay and retroactive interference) should minimize or prevent learning about the early flavor. This conclusion is also consistent with the recent findings by Sclafani's lab that mice that are accustomed to drinking saccharin while being infused IG with water will accelerate their intake within minutes the very first time the IG infusion is switched to glucose or Intralipid [22]. Thus a fairly immediate effect of intragastrically infused nutrient can enhance appetitive motivation. We have recently replicated this "immediate appetite" effect in our lab using rats in a somewhat different protocol (Myers, Taddeo, and Richards, submitted).

Second, our prior results suggest that nutrients in a meal generate multiple, distinct US signals at different time points, supporting qualitatively different learned responses to the early and late flavors. For instance, there may be a rapid-onset signal generated by preabsorptive chemosensation in the proximal intestines, and a late-onset signal generated by metabolic byproducts or satiation factors later in the prandial sequence. Ordinarily in a meal of only one food, that flavor would come to be associated with both the early- and late-onset USs. Our recent findings suggest that a putative rapid-onset US produces state-dependent conditioning, and a separate delayed-onset US produces state-independent preference, potentially helping explain why "dessert" foods remain attractive when encountered during satiety.

The notion of multiple USs is consistent with several facts about the behavioral organization of flavor–nutrient conditioning. The conditioned response pattern has several dissociable behavioral/motivational components. Conditioning can separately influence appetitive and consummatory phases of meal patterning [23,24], acceptance and preference responses [6,13,25], and hedonic and non-hedonic aspects of flavor evaluation [7,26–29]. Postingestive nutrient effects can act to separately condition both intake-promoting preference responses and intake-suppressing satiation responses [9,10]. There is some evidence that the rewarding effectiveness of post-absorptive infusion sites (e.g., hepatic portal) may be modulated by whether or not there is also pre-absorptive nutrient stimulation (see [4]). Some neural and pharmacological manipulations (such as PBN lesions or surgical deafferentation at the celiac-superior mesenteric ganglia [30,31]) can significantly attenuate the strength of conditioning without blocking it altogether.

Thus there may be more than one site where the postingestive 'reward value' of a food is transduced and associated with the preceding flavor. Multiple, dissociable signals could each influence conditioned responses to the flavor, perhaps separately or synergistically.

The purpose of the present experiments is to apply the early flavor/late flavor conditioning paradigm to flavor–nutrient conditioning with fat, which may produce different results from our recent studies with glucose [21]. This paradigm investigates when rats are detecting the critical postingestive US events that support conditioned preference. As stated previously, rats can learn to prefer CS flavor cues paired with the postingestive consequences of fat, but less readily than they do for carbohydrate. If this is because, mechanistically, fat works similarly to glucose in flavor–nutrient conditioning but simply generates a weaker US, then we would expect to see a similar overall pattern of preferences as in our recent study, but perhaps requiring more training, or a more calorically dense US. The possibility of a rapid-onset US effect of fat similar to what we've seen with glucose is suggested by the "appetition" studies by Zukerman, Ackroff, and Sclafani mentioned above [22], wherein mice accelerated licking within minutes of the start of their first IG nutrient infusion. Those experiments found an effect of fat (Intralipid) infusion that was roughly similar in magnitude and time course to the effect of glucose. On the other hand, if fat is slower to generate a postingestive reward during/after a meal, or if it does so by activating a smaller subset of putative US signals, then a different pattern of preference learning may be seen in the early/late paradigm. A predominance of slowly-arising US effects should bias rats towards learning stronger preferences for flavors encountered later in a high-fat meal. Finally, in conditioning with glucose, preferences based on early- vs. late-onset USs appeared to be differently sensitive to deprivation-state manipulations [21]. This difference provides another way of inferring the involvement of at least two general categories of postingestive US effects, and is investigated in these experiments.

2. Experiment 1

In this experiment rats with intragastric (IG) catheters were trained in daily drinking sessions during which they consumed sweetened, low-calorie solutions with distinctive flavors added while also receiving concurrent IG infusion. Some flavors were always accompanied by IG infusion of a high-fat dairy product ("+" sessions), whereas different flavors always signaled IG water ("–" sessions). In both (+) and (–) sessions there was always an "Early" flavor provided for the first 8 min of the session, which was then removed and replaced with the "Late" flavor for the last 8 min. Thus each rat repeatedly experienced four different flavors throughout the training phase: Early(+) and Late(+) were consistently given in the first and second halves, respectively, of a meal with high-fat postingestive effects, while Early(–) and Late(–) were similarly always in the first and second halves of a session with IG water.

After the training phase consisting of eight (+) and (–) sessions, it was of interest to determine how this experience altered rats' preferences for each of the (+) flavors. In a series of two-bottle choice tests (without concurrent IG infusion) preference for the Early and Late (+) flavors were assessed relative to their (–) counterparts [i.e., Early(+) vs Early(–); Late(+) vs Late(–)]. Also, relative preference between the two (+) flavors was tested, as was preference between the two (–) flavors [i.e., Early(+) vs. Late(+); Early(–) vs. Late(–)]. Again, the main focus of this experiment is the time course of the rewarding, postingestive effects of fat. A relatively slow onset would be expected to produce a stronger preference for the Late(+) flavor than the Early(+) flavor, since Late(+) would be more closely associated in time with the experience of postingestive reward.

Moreover, each of these preferences was tested under both hungry and recently satiated conditions. This is because during training the Early(+) and Late(+) flavors were essentially always experienced in

different states, and therefore expression of any learned preference may also be state dependent.

2.1. Methods

2.1.1. Subjects

All animal procedures were approved by the university IACUC and were consistent with the NIH Guide for the Care and Use of Laboratory Animals. Sixteen experimentally naive, adult female Sprague–Dawley CD rats were used. Subjects were bred in our laboratory from stock originally obtained from Ace Animals (Allentown, PA). Rats were approximately 100 days old and weighed 257 ± 12.6 g (mean $\pm$ SD) at the outset. They were housed individually in $8 \times 16 \times 10.5$ " plastic tub cages with corn cob bedding. Each rat had an intragastric (IG) Silastic catheter (1.02 mm ID, 2.16 mm OD) surgically installed under ketamine/xylazine anesthesia (65 and 10 mg/kg) as described in [32]. The catheter was routed from the peritoneum subcutaneously to exit between the shoulders, where it was attached to a capped Luer-Loc connector on a backpack-style harness worn by the rat. A postoperative recovery period of at least ten days was allowed before proceeding. Beginning at that time rats were restricted to a ration of 14–15 g of chow daily. Tap water was available ad libitum in the rats' home cages.

2.1.2. Apparatus

Conditioning was conducted in eight identical cylindrical test chambers, 35 cm high $\times$ 25 cm diameter, made of opaque plastic with a wire grid floor. When a rat was placed in its test chamber, the Luer-Loc connector to the IG catheter was connected to infusion tubing extending from a fluid swivel held above the chamber on a counterbalanced lever arm. This was connected to tubing from a 30 ml syringe containing the fluid to be intragastrically infused, which was mounted on a syringe pump. This arrangement allows the rat unrestricted movement inside the apparatus and prevents damage to the infusion tubing and stress on the IG catheter.

The front of each chamber had two small apertures approximately 3 cm apart, through which the rat could access the sipper tubes of bottles carried on a motorized bottle retractor mounted on the exterior of the chamber. The bottle retractors (modified Med Associates ENV-252) could hold two 50 ml drinking tubes side by side, but throughout training the rats were actually only given one bottle at a time. Therefore the left–right position used on the bottle retractor was systematically varied across sessions to discourage rats from developing side preferences.

Drinking from the sipper tubes was monitored by electronic contact lickometers interfaced to a computer. This computer, which also controlled the bottle retractors, counted each rat's licks and in turn individually controlled the infusion pumps. A rat's infusion pump was activated whenever the rat was licking, delivering the IG infusate at a rate of 0.02 ml/s. This method approximately matches the rate and total volume of a rat's IG infusion to its oral consumption.

2.1.3. Procedure

2.1.3.1. Flavor preference conditioning. Session parameters were devised to replicate previous experiments on this topic in this lab [21]. Sessions were run in squads of eight rats at a time, with each rat always placed in the same chamber across sessions.

After rats had been on daily food rationing for at least six days, but before training began, rats were familiarized with the conditioning chambers in a series of twice-daily, 20-min long acclimation sessions in which they consumed a 2% fructose + 0.2% saccharin solution, which would become the vehicle for the CS flavors in the conditioning phase. This mixture is highly palatable but minimally satiating, and elicits fairly high drinking rates in brief sessions, which is appropriate for this paradigm to encourage substantial consumption of both the early and late flavors. Although the vehicle solution contained 2%

fructose and therefore provided some calories, several studies have shown that fructose does not generate strong postingestive rewarding stimulation to produce flavor–nutrient conditioning in such short sessions (reviewed in [17]). During the first four acclimation sessions rats were connected to the IG infusion tubing but not infused as they drank. In the next six they were infused with water IG as they drank, as described in the apparatus section.

The flavor preference conditioning phase consisted of two types of sessions in alternation. In (+) sessions consumption of both an Early and Late flavor were paired with IG infusion of a high-fat solution, and in (−) sessions an Early and Late flavor were paired with IG water. The fat solution was dairy heavy cream (Weis Markets brand, Sunbury, PA) diluted with tap water to a caloric density of 0.48 kcal/g (94.5% kcal from fat, 3.2% from carbohydrate, 2.3% from protein), which matches the caloric density of the glucose infusion (12% w/w) we have used in previous studies on this topic. The lipid profile consisted of ~65% saturated fatty acids (chiefly long-chain palmitic, stearic, and myristic acids), ~30% monounsaturated (chiefly oleic acid), and <5% polyunsaturated fatty acids. This profile differs from the corn oil and Intralipid stimuli more commonly used in fat conditioning, and the potential significance of that is addressed in the General Discussion. A practical advantage of the cream/water mixture is that it remains homogenous in the time frame of a session, so there is minimal physical separation inside the syringe pump that would change the actual fat concentration delivered across the session. This was verified with pilot measurements. The fat solution was always prepared immediately before use.

Each session began with the presentation of the Early flavor for 8 min. Then the bottles automatically retracted for a 4-min pause, during which the Early bottle was switched with the Late bottle. When the 4-min pause elapsed the Late flavor was inserted and was available for 8 min. Throughout the session, consumption of both the Early flavor and the Late flavor was accompanied by IG infusion as described above. Intakes were measured by weighing bottles before and after sessions.

For each rat, each particular flavor consistently occurred either early or late in either the (+) or the (−) sessions. Thus each rat was trained with four CS flavors: Early(+) and Late(+) were always paired with IG fat, whereas Early(−) and Late(−) were not. The flavors used as CSs were cherry, grape, lemon-lime, and orange Kool-Aid (Kraft Foods Inc., Glenview, IL; powdered unsweetened Kool-Aid mix was dissolved in the fructose + saccharin vehicle solution at a concentration of 0.05% Kool-Aid powder by weight). Flavor contingency assignments were completely counterbalanced. Sessions occurred twice daily, in the morning and mid-afternoon, separated by ~6 h. There was always one (+) and one (−) session per day, with order alternating across days in a double-alternation sequence. The entire training phase was eight consecutive days, consisting of eight (+) and eight (−) sessions. Chow rations were given 2 h after the end of the afternoon training session daily.

2.1.3.2. Two-bottle tests. Beginning two days after completion of the conditioning phase, rats' learned preferences for the flavors were assessed in a series of two-bottle choice tests, conducted in both deprived and satiated states. Tests were 30 min long each, and testing occurred twice daily on the same approximate schedule as the training sessions in the conditioning phase. But in this phase rats received their daily chow rations at mid-day, so that each day the morning two-bottle test occurred after overnight deprivation ("hungry" test) and the second occurred approximately ~90 min after feeding ("fed" test). Tests were arranged so that an afternoon (fed) test never included either of the flavors that were given in that morning's (hungry) test. Two-bottle tests occurred in the home cages, rather than the conditioning apparatus. This was primarily for convenience so that all 16 rats could be tested simultaneously instead of in squads. Thus there were no IG infusions during testing, but this is consistent with our goal of measuring conditioned changes in flavor evaluation/preference established by prior experience. Again, this mimics the procedure of our previous studies on this topic.

To begin the testing phase, rats were first acclimated to the home cage test schedule and two-bottle choice with two days of twice-daily practice tests. One bottle contained unflavored 1% fructose + 0.1% saccharin and the other contained 3% fructose + 0.3% saccharin, with the left–right positions of these reversed across tests. This encouraged rats to sample each bottle and allowed us to verify rats were choosing based on bottle contents and not position. During all two-bottle tests the bottles were placed simultaneously onto the wire lids of the cages so that the sipper tubes protruded into the cage, centered approximately 4 cm apart. Intake was measured by weighing each bottle before and after the test.

In the two-bottle test phase, the four critical preference tests compared Early(+) vs. Early(−), Late(+) vs. Late(−), Early(+) vs. Late(+), and Early(−) vs. Late(−). Testing occurred over eight days, with each of these tests repeated twice under each deprivation-state condition, with the left–right position of the flavors reversed for each repetition. The order of the four different tests across days was counterbalanced across rats, and for each rat the order was reversed in the first and second replications of the test series. The two repetitions of each test in a particular deprivation state were averaged for analysis of the results. This series of tests necessarily involves several exposures across days to each (+) flavor now unaccompanied by nutrient but any extinction during testing would favor a null result, so this is an inherently conservative measure of learned preference strength. Complete counterbalancing of test order further makes it unlikely that any extinction accruing during testing could systematically produce a Type-I error.

2.2. Results

2.2.1. Intakes during conditioning sessions

The most obvious pattern in training was that rats consumed similar amounts in (+) sessions as they did in (−) sessions, and in both session types they consumed less Late flavor than Early flavor. Intakes during the (+) and (−) conditioning sessions are depicted in Fig. 1. For analysis, the eight sessions of each type were condensed to four two-session blocks (each two consecutive sessions per type averaged) for a 2 (Session Type: + vs. −) X 2 (Early vs. Late flavor) X 4 (Trial Block) repeated measures ANOVA. Intakes were nearly identical in (+) and (−) sessions (no main effect of Session Type, $F[1,15]=0.01$, $p=0.98$) and remained consistent across sessions (no main effect of Trial Block, $F[3,13]=1.74$, $p=0.21$). Nor did those two variables significantly interact ($F[3,45]=1.92$, $p=0.14$). Rats typically consumed slightly but significantly more of the Early flavors than the Late flavors (grand Mean $\pm$ SEM intakes for all sessions, 6.0 ± 0.14 Early flavor, 4.6 ± 0.13 Late flavor; main effect of Early vs. Late, $F[1,15]=35.8$, $p<0.01$). This is not likely due to a satiating effect of the IG fat infusion in (+) sessions because this tendency was equally evident in (−) sessions (no Early vs. Late X Session Type interaction, $F[1,15]=0.39$, $p=0.54$).

2.2.2. Two-bottle tests for learned flavor preferences

Strength of any learned preference for the Early(+) and/or the Late(+) flavors was evaluated in choice tests between each and its corresponding (−) flavor. Also, relative preference strength for the Early(+) and Late(+) was assessed in direct choice between the two. Finally a choice between Early(−) vs. Late(−) would indicate any possible biasing effect of previous experience with flavor timing *per se* independent of flavor–nutrient conditioning. Each of these tests was repeated twice under both hungry and fed test conditions to assess the affect of deprivation of expression of learned preference. For analysis the two repetitions of each test in each deprivation condition were averaged, and intake of the two flavors within a two-bottle test were contrasted in a paired-sample *t*-test.

2.2.2.1. Two-bottle tests: hungry. In two-bottle choice tests when hungry (see Fig. 2), rats significantly preferred the flavor from the latter half, but not the early half, of high-fat meals. Rats preferred Late(+) and

Fig. 1. Intakes across the series of (+) training sessions (top panel) and (–) sessions (bottom panel) for Experiment 1. In (+) sessions IG infusion was fat (0.48 kcal/g), and in (–) sessions it was water. Training alternated between the two types of sessions, with one session of each type per day. Each bar is the average of two consecutive sessions of that type. The lower portion of each bar is intake (Mean $\pm$ SEM) of the Early flavor during the first 8 min of the session, and the top portion is intake (Mean $\pm$ SEM) of the Late flavor during the last 8 min.

over Late(–) (Fig. 2b, $t[15]=2.42$, $p<0.02$) but did not prefer Early(+) over Early(–) (Fig. 2a, $t[15]=0.88$, $p=0.20$). However, inconsistent with this apparent preference for Late(+) relative to Late(–), rats did not significantly prefer Late(+) to Early(+) in a direct choice between the two (Fig. 2c, $t[15]=1.58$, $p=0.13$).

A prior history of simply encountering flavors early or late in a meal independently of flavor–nutrient conditioning does not appear to significantly influence preference in this paradigm, since (consistent with expectations and our previous observations) rats exhibited no preference in a choice between Early(–) and Late(–) (Fig. 2d, $t[15]=0.81$, $p=0.43$).

2.2.2.2. Two-bottle tests: fed. Except for somewhat lower intakes overall (as would be expected, and which serves as a validity check for the pre-feeding manipulation) the tests conducted after recent feeding paralleled the hungry tests in all other respects, as shown in Fig. 3. Rats still significantly preferred Late(+) over Late(–) (Fig. 3b, $t[15]=3.86$, $p<0.01$) but did not prefer Early(+) over Early(–) (Fig. 3a, $t[15]=1.61$, $p=.13$). But again this preference for Late(+) was not clearly evident in a choice against Early(+) as there was only a non-significant tendency to prefer Late(+) over Early(+) (Fig. 3c, $t[15]=2.11$, $p=0.053$). Finally, again there was no preference between the two (–) flavors (Fig. 3d, $t[15]=1.16$, $p=0.27$).

2.3. Discussion

These results provide initial support for the conclusion that when fat is the main macronutrient rats learn to prefer a flavor occurring towards the end of the meal. This pattern suggests that the rewarding effects of ingested fat acting postingestively have a fairly slow onset, and are not sensed until the latter half of a meal or perhaps after its termination.

That closer temporal contiguity between the postingestive reward and the late flavor and would account for its enhanced preference.

This result stands in contrast to our prior report [21] showing that rapid detection of glucose postingestive reward early in the meal produces strong preference for an Early(+) flavor (as well as preference for the Late(+) flavor). In the present study, using the same training paradigm but with fat as the main macronutrient, we see no indication that rats increased their preference for the flavor encountered early in high-fat meals any more than their evaluation of the control flavors in the non-nutritive meals. Given that the training and test protocols were virtually identical with our previous glucose studies, the difference between fat and glucose as the nutrient is likely the chief factor in the different result.

This points to underlying differences in the time course of physiological signals involved in carbohydrate vs. fat postingestive reward, and adds to prior work supporting the general conclusion that the underlying mechanisms are not entirely overlapping. Other researchers have shown that the postingestive reward generated by fat may be weaker and less effective at supporting preference, e.g. [12,14,15]. But this present result adds to this specifically by pointing to a difference in the *timing of onset* of the postingestive reward signal(s) for glucose vs. fat.

However there is a notable ambiguity that makes it difficult to argue conclusively that these rats only learned to associate the late flavor with the postingestive fat reward. Namely, Late(+) was not preferred to Early(+) in a direct choice. It's unclear why that preference would not manifest if postingestive fat reward improved evaluation of Late(+) only. This will warrant additional investigation in future studies, since corresponding patterns were seen in some tests in our prior studies with glucose reinforcement, yet the psychological causes for this pattern may be complex. It may be that the two flavors cue relative, rather than absolute, differences in postingestive consequences, since there may have been some learning about the Early(+) that was not enough to produce significant preference over Early(–). This is unlikely to be a complete explanation in its own, since rats can learn strong preferences for a CS + flavor over a CS – flavor when the CS + is paired with a fairly dilute nutrient infusion (e.g., 1% (w/w) Polycose in [33], although that experiment did give rats 22 h/day access to the training solutions).

Direct choice between Early(+) and Late(+) is presumably complicated by the fact that they were (by definition) always experienced in different physiological states during training. Yet, so too were Late(+) and Late(–) and this choice yielded the strongest preference. Retroactive interference by the Late flavor could lead to a lower quality memory for the Early flavor during (+) training sessions, but that would likely weaken Early(+) preference relative to Late(+) in that test, rather than weakening it in the test vs. Early(–). In any case, this pattern suggests that choice between the Early(+) and Late(+) flavors is psychologically complex, but is consistent with the conclusion that with high fat meals, stronger learning occurs for the flavor late in the meal. There was a strong preference for Late(+) over Late(–), and no such preference for Early(+) over Early(–).

The IG fat infusion used in Experiment 1 was specifically chosen to match the caloric density of the glucose infusion we have used in prior experiments on this topic [21]. The next Experiment uses a more calorically-dense fat infusion. Some prior work suggests fat may have a higher minimum threshold to be effective as a US in flavor–nutrient learning [10]. Also, Zukerman, Ackroff, and Sclafani's recent work showed evidence for a rapid positive feedback signal generated by IG fat (Intralipid) that was roughly comparable to glucose [22], but those experiments used higher energy nutrient infusions than used here. Although there are also likely to be species and strain differences in pre-absorptive nutrient sensing in the gut, energy density is an obvious factor to explore. Therefore Experiment 2 was designed to pursue the possibility that rapid-onset US effects may exist in flavor–nutrient conditioning with fat in rats and would become evident in this Early-Late paradigm with a more calorically-dense fat infusion.

Fig. 2. Preference tests: Hungry. Average (Mean $\pm$ SEM) intakes during two-bottle preference tests conducted following overnight food deprivation without concurrent IG infusions. The top two panels (a, b) depict choices between a previously fat-paired (+) flavor versus its corresponding water-paired (–) flavor. The bottom two panels depict the choice between the two fat-paired (+) flavors versus one another (c), and the between the two water-paired (–) flavors (d). Each 30-min long test was conducted on two separate occasions and the figures are the average of the two repetitions. ** indicates $p < .01$.

Fig. 3. Preference tests: Fed. Average (Mean $\pm$ SEM) intakes during two-bottle preference tests conducted following recent feeding. Tests are as described for Fig. 2, except these tests were conducted beginning ~90 mins after rats were given their daily chow rations. ** indicates $p < .01$.

3. Experiment 2

3.1. Methods

The sixteen rats from Experiment 1 were kept for retraining in Experiment 2. Seven days elapsed after the end of the two-bottle test phase of Experiment 1 before proceeding. Then they were re-familiarized with the training apparatus in a series of four, 20-min long drinking/infusion sessions conducted across two days, in which they drank unflavored 2% fructose + 0.2% saccharin and were infused with water. Throughout Experiment 2 rats were maintained on 14–15 g/day chow rations delivered ~2 h after the second daily training session.

In all respects the day to day schedule and the procedure for conditioning and then two-bottle testing replicated experiment 1, except that the caloric density of the fat solution infused IG in (+) sessions was increased by 50% over that used in Experiment 1 (.74 kcal/g in Experiment 2 vs. .48 kcal/g in Experiment 1). Also, since rats were already trained with four Kool-Aid flavors, Experiment 2 instead used artificial flavor extracts (McCormick brand, Sparks, MD; almond, coconut, cinnamon, and vanilla flavors, 0.4 ml of extract per 100 ml of fructose + saccharin vehicle) to minimize any carry-over or generalization of learned responses in Experiment 1. Flavors were again assigned as Early(+), Late(+), Early(–) and Late(–) in a counterbalanced fashion, but care was taken to make these assignments orthogonal to Kool-Aid flavor assignments from Experiment 1.

3.2. Results

3.2.1. Intakes during training

The general pattern in training closely resembled Experiment 1 except that total intakes were noticeably higher throughout Experiment 2 than Experiment 1. This is presumably a non-specific effect not due to increased fat concentration, since it was evident from the outset of training and occurred for both (+) and (–) sessions equally. It may simply reflect a slightly increased initial attractiveness of the extract flavors in Experiment 2 relative to the Kool-Aid flavors in Experiment 1. Intakes during training are shown in Fig. 4, and were analyzed with a 2 (Session Type: + vs. –) X 2 (Early vs. Late flavor) X 4 (Trial Block) repeated measures ANOVA. Intakes were similar in (+) vs. (–) sessions (no main effect of Session Type, $F[1,15]=1.0$, $p=0.33$) and did not change significantly across training (no main effect of Trial Block, $F[3,13]=0.30$, $p=0.83$). Rats again consumed more of the Early flavors than the Late flavors (main effect of Early vs Late, $F[1,15]=47.2$, $p<0.01$), and this was equally true for (+) and (–) (no Early vs Late X Session Type interaction, $F[1,15]=0.07$, $p=0.80$). Thus, the increased caloric density did not produce a satiating effect that limited intake of the (+) flavors, nor did it obviously promote increased acceptance during training itself.

3.2.2. Two-bottle preference tests: hungry

Unlike Experiment 1, in Experiment 2 rats exhibited learned preference for both of the flavors they had encountered with fat. Yet again there is evidence the Late flavor became more strongly preferred. Intakes in the four types of two-bottle tests conducted while hungry are shown in Fig. 5. Rats consumed more of the Early(+) flavor than Early(–) (Fig. 5a, $t[15]=2.23$, $p<0.05$) and consumed more Late(+) than Late(–) (Fig. 5b, $t[15]=2.55$, $p<0.01$) showing a learned preference for both of these flavors relative to non-paired control flavors. But in a direct choice between the two, Late(+) was significantly preferred over Early(+) (Fig. 5c, $t[15]=2.72$, $p<0.05$). This is unlikely to be due to any inherent preference-biasing effect of simple history as an early or late flavor irrespective of nutrient-conditioning, or of any minor familiarity differences between flavor intakes in training, because no such preference was seen in the choice between the non-paired Early(–) vs Late(–) flavors (Fig. 5d, $t[15]=0.32$, $p=.75$).

Fig. 4. Training sessions, Experiment 2. Intakes across the series of (+) training sessions (top panel) and (–) sessions (bottom panel) for Experiment 2. In this experiment, the caloric density of the IG fat infusion in (+) sessions was increased to 0.74 kcal/g. Presentation of the data is as described for Fig. 1.

3.2.3. Two-bottle preference tests: fed

Overall intakes were slightly lower when rats were tested after recent feeding, but in all other ways the results paralleled the hungry tests, with rats preferring both Early(+) and Late(+) flavors over their corresponding (–) flavors, but also preferring Late(+) over Early(+). Intakes in fed tests state are shown in Fig. 6. Rats consumed more Early(+) than Early(–) (Fig. 6a, $t[15]=3.20$, $p<0.01$) and more Late(+) than Late(–) (Fig. 6b, $t[15]=2.91$, $p<0.01$), and more Late(+) in a choice versus Early(+) (Fig. 6c, $t[15]=2.60$, $p<0.05$). They were again indifferent in a choice between the two (–) flavors (Fig. 6d, $t[15]=0.18$, $p<0.86$), therefore preference can be attributed to learning based on postingestive nutrient.

3.3. Discussion

Increasing the caloric density of the IG fat infusion altered rats' pattern of learned flavor preferences. The main difference from Experiment 1 regards the Early(+) flavor, which did become preferred over the Early(–). Yet these tests also continue to support the overall conclusion that for high-fat meals learning is biased towards flavors later in the meal, as the Late(+) was significantly preferred over Early(+). So, comparing these results to Experiment 1, it appears that increased energy density enabled rats to learn an association between the Early flavor and the postingestive effects of fat, but it also apparently promoted learning about the Late flavor as well, such that Late(+) became more strongly preferred.

It should also be noted that rats consumed more overall during the training sessions than they did in Experiment 1, for unknown reasons. This was a general increase for both flavors in both types of session, but bears considering because this may have subtly influenced learning about the Early(+) flavor by influencing CS and/or US processing. Because rats consumed more of the Early flavor than they did in

Fig. 5. Experiment 2 Preference tests: Hungry. Average (Mean $\pm$ SEM) intakes during two-bottle preference tests in Experiment 2, conducted following overnight food deprivation. * indicates $p < .05$, ** indicates $p < .01$.

Experiment 1, this would presumably have helped create a stronger memory trace for it, improving its associability with a delayed US and making it somewhat less susceptible to retroactive interference by consumption of the Late(+) flavor. Also, because IG infusion was

yoked to and controlled by the rats' licking, the fat infused was not only more calorically dense, as intended, but also greater in volume in the first 8 min segment compared to Experiment 1. There is some circumstantial evidence that faster intake rate of fat may increase its

Fig. 6. Experiment 2 Preference tests: Fed. Average (Mean $\pm$ SEM) intakes during two-bottle preference tests in Experiment 2, conducted beginning ~90 mins after daily chow rations. * indicates $p < .05$, ** indicates $p < .01$.

postingestive conditioning effects [10] although presumably there should be a limit to this effect once a threshold is reached for triggering negative feedback control of gastric emptying. The likeliest consequence of the increased infusion rate would be that the earliest-detected US signals would arise somewhat sooner, consistent with the improved learning about Early(+). Regardless of whether this is due to the absolute density of the IG infusion or a larger bolus delivered in the early half of the session, it underscores the point of the experiment that bias towards learning about Early vs Late flavors is sensitive to the postingestive effects of the nutritive infusion.

In both Experiments 1 and 2 rats consumed much more Early(+) than Late(+) flavor yet learned stronger preference for the Late(+) flavor, pointing to the importance of the CS–US contiguity, rather than amount of experience with the CS *per se*. And just like in Experiment 1 and our prior experiments, rats were indifferent to the two (–) flavors relative to one another, showing that relative preferences between the (+) flavors are not artifacts of mere flavor order alone, but rather due to differences in flavor–nutrient associations between the two (+) flavors and the postingestive effects.

4. General discussion and conclusions

The current experiments consistently showed stronger preferences for a flavor routinely encountered towards the end of a high-fat meal. In Experiment 1 with a lower energy density infusion, rats learned to prefer the Late(+) but not the Early(+) flavor. In Experiment 2 with a higher energy density infusion rats learned to prefer both flavors, but more strongly preferred Late(+). This bias towards learning about the Late flavor was not seen in our previous experiments using glucose infusion [21], so by comparison the current results further contribute to the evidence that the postingestive US effects of fat differ from glucose. But more specifically, the Early/Late flavor learning paradigm adds to previous work on fat by showing more conclusively that this is at least partly due to *slower* onset (rather than simply ‘weaker’ or less salient potency) of relevant postingestive US signals.

We have also argued previously, as outlined in the Introduction, that the Early/Late paradigm using glucose infusion has revealed additional evidence for multiple, dissociable postingestive US events acting at different time points in flavor–nutrient learning. One goal here was to apply that analysis to conditioning with fat. Given that in Experiment 2, rats did learn to associate the Early flavor with postingestive effects of fat, we can consider whether this reflects (as we have argued is the case with glucose) the involvement of a rapid-onset US detected in the initial minutes of the meal. Such a possibility is suggested by rapid increased licking responses shown by mice in the first several minutes of an infusion of either Intralipid or glucose documented in [22], but it is still unclear what role that response plays in flavor–nutrient conditioning. Alternatively, both the Early(+) and Late(+) flavors could be remembered during the delay until slower-arising postingestive events occur later during or after the meal. While admittedly still speculative, the evidence in the current experiments generally seems more consistent with the latter alternative. Specifically, there are three fundamental differences between the present results with fat versus our previous results with glucose that argue against the involvement of a rapid-onset US in the early minutes of a high-fat meal.

First, both of the present experiments showed a clear bias towards learning about the Late flavor over the Early flavor when fat is infused, which was not the case with glucose [21]. This pattern would be expected if US onset occurs only sometime after rats begin consuming the Late flavor.

Second, the key evidence that conditioning of Early- and Late-flavor preferences with IG glucose may be based on different reward pathways was that they were differently state-dependent [21]. When glucose was the US, rats preferred the Early flavor when tested hungry (i.e., the state they would have been in when encountering the Early

flavor in training) but were indifferent to it when tested after recent feeding. But the Late flavor preference was expressed robustly regardless of whether rats were tested hungry or sated, thus suggesting a separate psychobiological mechanism underlying the conditioning. In the present experiment with fat, no indication of state dependence was seen. Learned preference for the Late flavor in Experiment 1 and for both Early and Late flavors in Experiment 2 was unaffected by hunger state at testing, which may reflect the involvement of only the putative slower onset, late-acting postingestive US events. Therefore nothing in the present results suggests that separate US events are acting to reinforce preference for the Early and Late flavors.

Third, in previous work with the Early/Late flavor paradigm with glucose, a distinctive pattern was noted during training sessions: during the course of training, rats began to significantly suppress their intake of the Late flavor in (+) sessions but not (–) sessions, despite subsequently preferring Late(+) strongly in two-bottle tests [21]. Rats often increase their acceptance of nutrient-paired flavors progressively over several flavor–nutrient conditioning sessions, so this suggests a demotivational response to the flavor removal/switching in (+) sessions. In other words, it is suggestive that rats detect a rapid-onset postingestive effect of glucose and ‘attribute’ it the flavor they are consuming within the early minutes of the meal. However, that pattern is clearly absent in the present experiments with fat, even with the more energy-dense infusion in Experiment 2. Rats consumed the same amount of Late(+) in training as Late(–). Since they still came to strongly prefer Late(+) over Late(–) but were not obviously reactive to the Early(+)/Late(+) flavor switch, it implies little or no motivational significance to any immediate postingestive consequences of the fat infusion.

Taken together, these several observations lead to the preliminary conclusion that flavor–nutrient conditioning with fat does not involve the putative rapid-onset US (presumably pre-absorptive, intra-luminal) that has been proposed for glucose. However, there are still relatively few experiments using this Early/Late flavor paradigm, so this conclusion may be considered tentative pending additional convergent evidence using other strategies for dissociating the complex psychological and behavioral correlates of the physiological cascade stimulated by nutrients in the gut. Nonetheless, this conclusion is highly consistent with existing evidence that flavor–nutrient conditioning with fat is weaker and less effective, specifically indicating that a high-fat meal not only stimulates postingestive rewarding signals more slowly than glucose, but also that it effectively stimulates a smaller subset of distinct reward pathways. Therefore learned liking and preferences for CS flavors signaling fat calories may be qualitatively different, behaviorally and psychologically, than preferences for flavors signaling carbohydrate.

To some degree the current results may reflect the specific form of fat used. Studies of flavor–nutrient conditioning with fats have found consistent effects with corn oil and soy oil (Intralipid) e.g., [22,34–36]. Dairy cream (from bovine milk) used in the present experiments has a different fatty acid profile. A series of experiments that compared conditioning using different fats showed that a wide variety of dietary fat sources can produce learned preference, but they do differ in effectiveness [37]. A rough rank ordering suggested by those experiments is: corn oil $\approx$ safflower oil > vegetable shortening $\approx$ beef tallow > MCT oil. Thus the reinforcing effectiveness of a lipid appears to positively correlate with the proportion of long-chain fatty acids and negatively correlate with fatty acid saturation. Compared to the most effective fats dairy cream has a lower proportion of long chain fatty acids (~81% compared to 100% for corn oil), and includes some short chains (~5%). It also has a substantially higher percentage of saturated fat (>60%, compared to ~13% for corn oil) and less polyunsaturated fat (<5%, compared to ~55% for corn oil). While it's reasonable to expect corn oil would be more effective than cream in the standard conditioning paradigm, it remains to be explored whether corn oil and other fats would produce the same bias towards learning about a late-meal flavor in this paradigm. Again, the similar licking stimulation caused by glucose and Intralipid infusions shown in [22] implies that a fat source like Intralipid could

perhaps produce more rapid (early meal) learning. On the other hand, this apparent mismatch between the conclusions drawn here and the glucose/fat similarities documented in [22] could also reflect a species difference. There is some evidence B6 mice may be especially sensitive to fat detected postingestively, making it as effective as carbohydrates for conditioning preferences in that strain [36]. Additional studies using this early/late paradigm could help elucidate how different mechanisms for postingestive actions of fats vary in time course.

In general, cross-lipid comparisons may help illuminate the mechanisms of action in fat conditioning. There are several effects of dietary lipids on appetite, metabolism, and weight balance that vary with fat form. In general, saturated fats contribute more to adiposity and weight gain than unsaturated fats, e.g. [38–41]. In part this is due to chronic effects of habitual saturated fat intake on postprandial sympathetic tone and gene expression regulating adipocyte metabolism [38,39,42,43] which may be unrelated to the rapid, acute consequences of fat ingestion involved in conditioning. But in the short term, too, saturation influences the handling and metabolic fate of ingested fat. Unsaturated fats produce a more rapid rise in postprandial lipidemia [44] and more postprandial thermogenesis than saturated fats [45–47] which leaves saturated fats more prone to storage.

The superiority of unsaturated fats with respect to conditioning may apply to both mono- and polyunsaturates, since beef tallow (which is effective but less so than corn oil [37]) is very low in polyunsaturates. The least effective fat in the comparisons by Ackroff, et al., [37] was MCT oil, which is entirely saturated fat. However it was still moderately effective and learning improved after extended training. Thus the presence of unsaturated fatty acids may promote postingestive conditioning without being entirely necessary. Yet MCT oil's poor effectiveness is in spite of the fact that medium (and short) chain fatty acids have a more rapid route to circulation and hepatic oxidation since they can diffuse across the intestinal wall without lymphatic transport. Together these facts are consistent with the suggestion of multiple pathways for postingestive reinforcement, and show that US potency does not depend only on the speed of postingestive handling. Yet it is puzzling that polyunsaturated fats are the most effective at conditioning food preference (thus potentially hyperphagia) but seem to contribute less to obesity in the long term.

In regards to fatty acid chain length, as mentioned above, the proportion of long chains positively affects preference learning [37], but the mechanism for this is unknown. Studies of the acute effects of the chain length composition of high-fat meals have primarily focused on satiety. Long chain fatty acids are often found to promote satiety and relaxed gastric tone (slower emptying), which is related to their effectiveness at stimulating CCK release [48,49]. Both short and long chain fatty acids produce characteristic patterns of vagal afferent firing, but apparently through different mechanisms. Vagal response to long chains is CCK-mediated but the response to short chains is not [50]. Such differences may or may not play a role in their differential conditioning effects, since satiation processes are largely dissociable from postingestive reinforcement. Evidence for the involvement of CCK in flavor–nutrient conditioning is mixed [51,52] but the negative evidence comes from experiments with carbohydrate reinforcement. Any conditioning effect of CCK would more likely be non-vagal since neither subdiaphragmatic vagotomy nor capsaicin deafferentation substantially interfere with conditioning, even with fat [30,53]. Again based on the idea of multiple pathways for postingestive reinforcement, it is conceivable some other effect of CCK (or another factor sensitive to fatty acid chain length, like GLP-1) is involved in the increased effectiveness of long chain fatty acids while being unnecessary for all instances of flavor–nutrient reinforcement. Of course other physiological mechanisms remain to be explored.

A potential practical application of the effects shown here follows from the long-standing suggestion that flavor–nutrient conditioning may play a role in endowing “dessert” flavors with special significance [1,54]. In many global cuisines, meals typically consist of multiple courses, with particular categories of foods and flavors consistently

occurring early in the meal and others as desserts. If there are multiple reward pathways stimulated by postingestive events, the longer-delayed US effects of the entire meal could become most strongly associated with the last food consumed, making flavors of dessert foods the disproportionate target of flavor–nutrient learning. The current results indicate that this indeed is likely true if the meal is high in fat, or at least saturated fat. This then poses an additional challenge for weight control efforts, since our prior and current work shows that when flavor–nutrient conditioning produces a preference for a late-occurring flavor (which, practically by definition is consumed while in or approaching a satiated state) that flavor remains preferred if later encountered during either hunger or satiety. In addition to their inherent palatability, sweets like cakes and candies, and confectionary flavors like vanilla and cocoa, may attain their status as tempting between-meal snacks as a result of this learning. It could then be feasible to attempt to increase the desirability and preference of lower energy dense alternatives snacks, like fruits, by habitually serving them as desserts when a high-fat meal is consumed.

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