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Food Reward in the Absence of Taste Receptor Signaling

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SUMMARY

Food palatability and hedonic value play central roles in nutrient intake. However, postingestive effects can influence food preferences independently of palatability, although the neurobiological bases of such mechanisms remain poorly understood. Of central interest is whether the same brain reward circuitry that is responsive to palatable rewards also encodes metabolic value independently of taste signaling. Here we show that *trpm5*$^{-/-}$ mice, which lack the cellular machinery required for sweet taste transduction, can develop a robust preference for sucrose solutions based solely on caloric content. Sucrose intake induced dopamine release in the ventral striatum of these sweet-blind mice, a pattern usually associated with receipt of palatable rewards. Furthermore, single neurons in this same ventral striatal region showed increased sensitivity to caloric intake even in the absence of gustatory inputs. Our findings suggest that calorie-rich nutrients can directly influence brain reward circuits that control food intake independently of palatability or functional taste transduction.

INTRODUCTION

It is commonly accepted that highly palatable foods, through their action on brain gustatory-reward systems, can override internal homeostatic mechanisms and eventually lead to overeating and obesity (Berthoud, 2004; Kelley et al., 2005; Pecina et al., 2006). However, little attention has been paid to a second pathway through which nutritive compounds can exert reinforcing influences on an organism, namely the postingestive controls on food intake (Sclafani, 2001; Sclafani and Ackroff, 2004). In fact, independent of the orosensory pleasure derived from eating calorie-dense foods, postingestive actions of nutrients have been shown to act as positive reinforcers on feeding behavior (Sclafani, 2001). However, the neural mechanisms controlling these purely metabolic-based behavioral adaptations remain largely unknown.

The role of brain dopamine systems in mediating food reward, and in encoding stimulus palatability, is well established. Dopamine antagonists attenuate the hedonic value of sweet-tasting nutrients; animals pretreated with either D1- or D2-type dopamine receptor antagonists show attenuated responses toward high concentrations of sucrose (Bailey et al., 1986; Geary and Smith, 1985; Wise, 2006; Xenakis and Sclafani, 1981). Conversely, tasting palatable foods elevates dopamine levels in the nucleus accumbens (NAcc) of the ventral striatum (Hernandez and Hoebel, 1988), a brain region largely implicated in food reinforcement (Kelley et al., 2005). In particular, NAcc dopamine levels increase in proportion to stimulus concentration even when no calories are absorbed (Hajnal et al., 2004). In humans, striatal dopamine release directly correlates with the perceived hedonic value of food stimuli (Small et al., 2003). Importantly, the palatability-induced dopamine release in the NAcc seems to effectively modulate brain reward processing: excitatory neurons in this region explicitly encode for hedonic value, including the relative salience of sucrose reinforcers as assessed by contrast paradigms (Taha and Fields, 2005). Finally, such neural sensitivity to oral rewards is independent of familiarity or learning (Roitman et al., 2005).

A central question that remains to be addressed is whether in the absence of taste receptor activation the brain dopamine-reward system is also sensitive to the metabolic value of nutrients. This would imply that physiological events starting in the gastro-intestinal tract have direct access to the central reward circuits controlling motivated consummatory behaviors, providing a neural substrate for postingestive controls on food intake. To investigate this hypothesis we performed behavioral, neurochemical, and electrophysiological experiments in mice lacking a functional transient receptor potential channel M5 (Zhang et al., 2003) (*trpm5*$^{-/-}$ mice). The TRPM5 ion channel is expressed in taste receptor cells (Perez et al., 2002) and is required for sweet, bitter, and amino acid taste signaling (Zhang

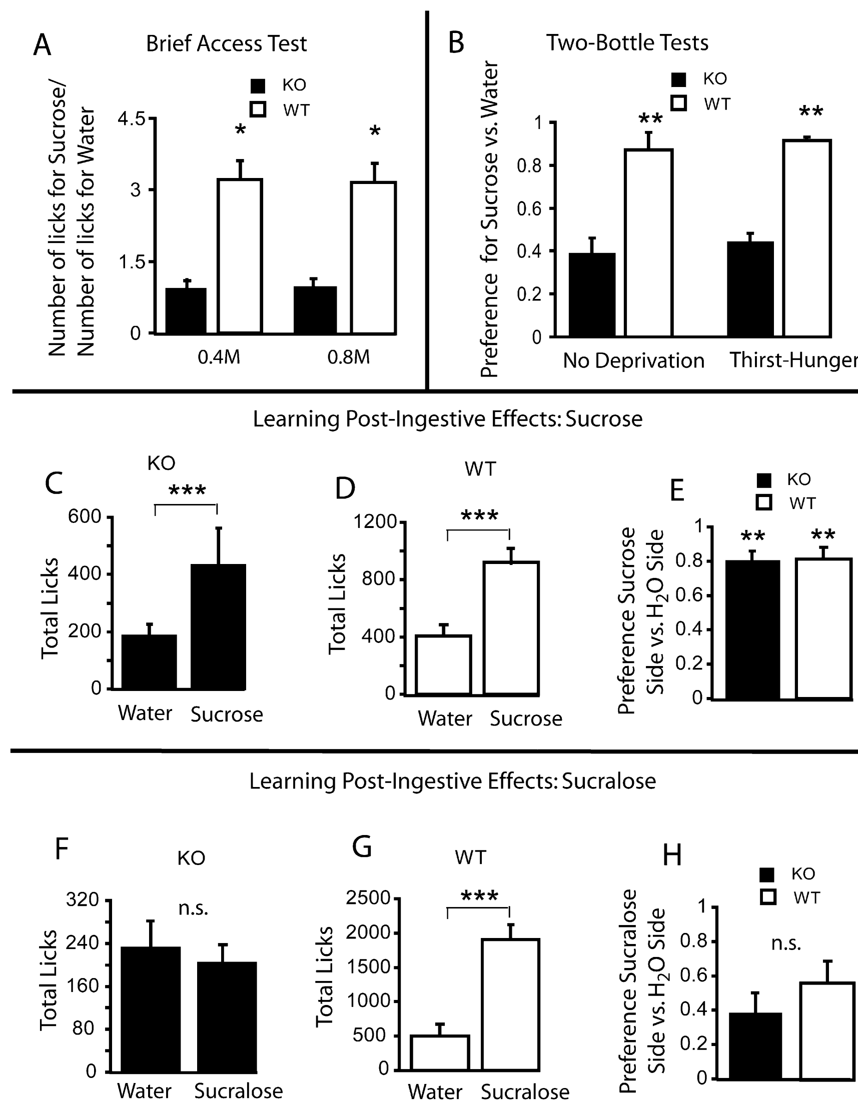

Figure 1. Sweet-Blind *trpm5*$^{-/-}$ Mice Can Develop a Preference for Sucrose, but Not Sucralose, through a Conditioning Protocol

Error bars in all figures indicate means ± SEM. A complete statistical summary can be found in the Supplemental Material section.

(A) In brief-access tests (Glendinning et al., 2002), WT mice displayed strong attraction for both 0.4 M and 0.8 M sucrose, with lick ratios (number of licks for sucrose/number of licks for water) significantly larger than 1.0. KO mice, however, did not produce increased lick responses to either sucrose solution compared with water, with lick ratios close to 1.0 (independent t test against 1.0, *$p < 0.005$).

(B) In two-bottle preference tests, while WT animals displayed strong attraction to 0.8 M sucrose against water with preference ratios significantly higher than 0.5, KO animals were indifferent to either choice. This result did not depend on whether the animals were water and food deprived (independent t test against 0.5, **$p < 0.02$).

(C and D) During 30-min conditioning sessions, animals were given alternated access to either water only (assigned to the sipper of initial side bias preference) or 0.8 M sucrose only (assigned to the opposite sipper) for 6 consecutive days. During these conditioning sessions that allowed KO animals to associate sipper side with postingestive effects, both KO (C) and WT (D) animals consumed significantly more sucrose than water (paired two-sample t test, ***$p < 0.05$).

(E) During 10-min two-bottle postconditioning test sessions where water was accessible from both sippers, a significant reversal of initial bias was observed in both WT and KO animals, as revealed by the measured preference ratios (independent t test against 0.5, **$p < 0.02$). Reversal of bias in KO mice indicates that animals successfully associated sipper side with its postingestive effects.

(F and G) The same conditioning protocol was then applied to a new group of animals with 30 mM sucralose (a noncaloric sweetener) replacing sucrose. Whereas WT animals consumed significantly more sucralose than water during the conditioning sessions (G), unlike the sucrose case, KO animals consumed the approximately the same amount of sucralose and water (F). (Paired two-sample t test, ***$p < 0.002$.)

(H) Unlike the sucrose case, no preference was displayed for the sipper previously associated with sucralose access.

et al., 2003). We first reasoned that sweet-blind knockout (KO) animals would develop a preference for spouts associated with the presentation of sucrose solutions when allowed to detect the solutions' rewarding postingestive effects. We then inquired whether such a behavioral pattern is associated with dopamine release in the ventral striatum of KO mice. Finally, we investigated whether populations of neurons in the same region of the ventral striatum, and in the anatomically associated orbitofrontal cortex (OFC), were modulated by metabolic changes independently of palatability.

RESULTS

Behavioral Tasks

We first confirmed that KO animals are insensitive to the orosensory rewarding properties of sucrose. In brief-access tests (Glendinning et al., 2002), water-deprived KO animals ($n = 5$) and their wild-type (WT) counterparts ($n = 5$) were presented with water, 0.4 M sucrose, or 0.8 M sucrose across successive 5-s trials, and standardized lick ratios defined as number of licks for sucrose solution/number of licks for water were calculated. Two-way repeated-measures ANOVA revealed a significant effect of genotype ($p < 0.0003$), but not of tastant ($p > 0.95$), on lick ratios. While WT animals displayed a strong attraction to both 0.4 M and 0.8 M sucrose, with lick ratios significantly larger than the indifference ratio of 1.0 (3.16 ± 0.38 and 3.22 ± 0.39, respectively, independent one-sample t tests, both $p < 0.005$), in KO animals these ratios approximately equaled 1.0 (0.98 ± 0.13 and 0.95 ± 0.16, respectively, both $p > 0.75$, Figure 1A; a complete statistical summary is included in Table S1, available online, and information on deprivation schedules/basal number of licks is included in Table S2). Using two-bottle preference

tests, we also measured preference ratios for 0.8 M sucrose against water in nondeprived animals. Whereas WT mice (n = 5) displayed an increased attraction for sucrose, as expressed by a preference ratio significantly larger than the indifference ratio of 50% (87% ± 8%, independent one-sample t test, $p < 0.02$), KO animals (n = 8) displayed preferences similar to 50%, with a nonsignificant bias toward water (39% ± 6%, $p > 0.15$). Results were unaltered when mice were food and water deprived (ratios 91% ± 1% for WT, $p < 0.0001$; 44% ± 3% for KO, $p > 0.18$; Figure 1B).

Once the insensitivity of KO mice to the orosensory reward value of sucrose was established, we inquired whether a preference for sippers associated with caloric sucrose solutions could develop in water- and food-deprived KO mice when they are allowed to form an association between a particular sipper in the test chamber and the postingestive effects produced by drinking from that sipper. This was accomplished in sweet-taste-naive animals by first determining the initial side-preferences using a series of preliminary two-bottle tests where both sippers contained water. The hungry and thirsty mice (n = 9 for KO and n = 5 for WT) were then exposed to a procedure, henceforth called the "conditioning protocol," where alternated access to either water or 0.8 M sucrose was given for 6 consecutive days. Conditioning sessions consisted of 30 min daily of free access to either water (assigned to the same side of initial bias) or 0.8 M sucrose (assigned to the opposite side) while access to the other sipper was blocked. Significant main effects on acceptance (i.e., total consumption measured in number of licks) were found for genotype (two-way repeated-measures ANOVA, $p < 0.007$) and taste stimulus ($p < 0.003$). However, no significant genotype × stimulus interaction was observed ($p > 0.29$; see Figure S1A available online), since during conditioning sessions both WT and KO animals consumed more sucrose than water (KO: 189 ± 35 licks for water and 440 ± 125 for sucrose, Figure 1C; WT: 407 ± 76 licks for water and 920 ± 107 for sucrose, Figure 1D; lick counts for each conditioning day are included in Table S2).

These conditioning sessions were then followed by two-bottle tests identical to those run to determine initial side biases. During test sessions, both WT and KO animals reversed their initial side-preference biases by drinking significantly more water from the sipper that, during conditioning sessions, had been associated with 0.8 M sucrose. In fact, the preference ratio for KO mice was 80% ± 5% ($p < 0.0006$, independent one-sample t test against 50%), and for WT mice, 81% ± 6% ($p < 0.02$, Figure 1E). Noticeably, in this two-bottle test no significant difference was observed between WT and KO preference ratios (unpaired two-sample t test, $p > 0.94$; Figure 1E). We ascribe these results to the ability of KO animals to detect the postingestive reinforcing properties of sucrose.

Conversely, we hypothesized that the effect described above should vanish when sucrose is replaced by sucralose, a noncaloric but highly palatable sucrose-derived sweetener. When the conditioning protocol was run on a group of naive animals (n = 5 KO and n = 6 WT) using 30 mM sucralose instead of sucrose, we observed significant main effects of genotype (two-way repeated-measures ANOVA, $p < 0.002$) and taste stimulus ($p < 0.0003$) on acceptance. However, unlike for the sucrose case, here we found a significant genotype × stimulus interaction ($p < 0.0003$; see Figure S1B). This prompted us to perform separate within-genotype paired t tests: whereas WT animals consumed significantly more sucralose than water during the conditioning sessions (1901 ± 505 and 226 ± 166 licks for sucralose and water, respectively, $p < 0.002$, Figure 1G), KO animals consumed approximately the same amount of both (204 ± 34 and 233 ± 49 licks for sucralose and water, respectively, $p > 0.3$, Figure 1F). The equivalent tests performed for the sucrose case show that, unlike for sucralose, both WT and KO animals consumed significantly more sucrose than water ($p < 0.05$ for both, Figures 1C and 1D). Furthermore, during the two-bottle test sessions, conducted after conditioning to sucralose, neither WT nor KO animals showed a preference for the sipper associated with the delivery of sucralose ($p > 0.4$ for both WT and KO mice, Figure 1H). We conclude that sipper-associated preferences in *trpm5*$^{-/-}$ mice depend exclusively on the compound's postabsorptive metabolic value.

Finally, we eliminate the possibility that KO mice might have acquired a preference for caloric sucrose via detection of nonsweet sensory cues possibly present in the solution by performing a control experiment where mice licked from a single central spout during conditioning sessions. Such design denies animals the occasion to associate caloric load with a particular drinking sipper. We did not find any increases in sucrose intake in KO mice during either postconditioning brief-access or two-bottle tests (see Figures S2 and S3).

Changes in Blood Glucose Levels during Caloric Intake

To verify whether KO mice would display increases in blood glucose levels after sucrose intake comparable to those observed in WT mice, an additional group of 22-hr food- and water-deprived animals (five KO, five WT animals) was exposed to one-bottle sucrose sessions and subsequently sampled for blood glucose levels at regular intervals. We found comparable glucose level increases between genotypes (Figure 2), implying that sweet-blind KO mice do not display any major alterations in metabolic function during carbohydrate intake.

Taste-Independent Increases in Dopamine Levels during Caloric Intake

We then inquired whether the mesolimbic dopamine system, known to be responsive to the receipt of palatable rewards, is associated with the behavioral patterns described above. We specifically inquired whether caloric intake per se, independently of palatability, was sufficient to increase extracellular dopamine levels in reward-processing regions. Naive KO (n = 5) and WT (n = 4) animals were implanted with microdialysis probes in NAcc, and dialysate samples were collected from 22-hr food- and water-deprived animals 60 min previous to and during 30-min, one-bottle free access to sucralose (day 1) or sucrose (day 2) solutions (see Figure S4A for intake data). Samples continued to be collected for an additional 30 min after completion of the licking task. By performing three-way ANOVA analyses (genotype × tastant × sampling time), we found a significant interaction between genotype and tastant ($p < 0.02$) on the measured relative (percent from baseline) increases in extracellular dopamine levels. We thus performed separate two-way

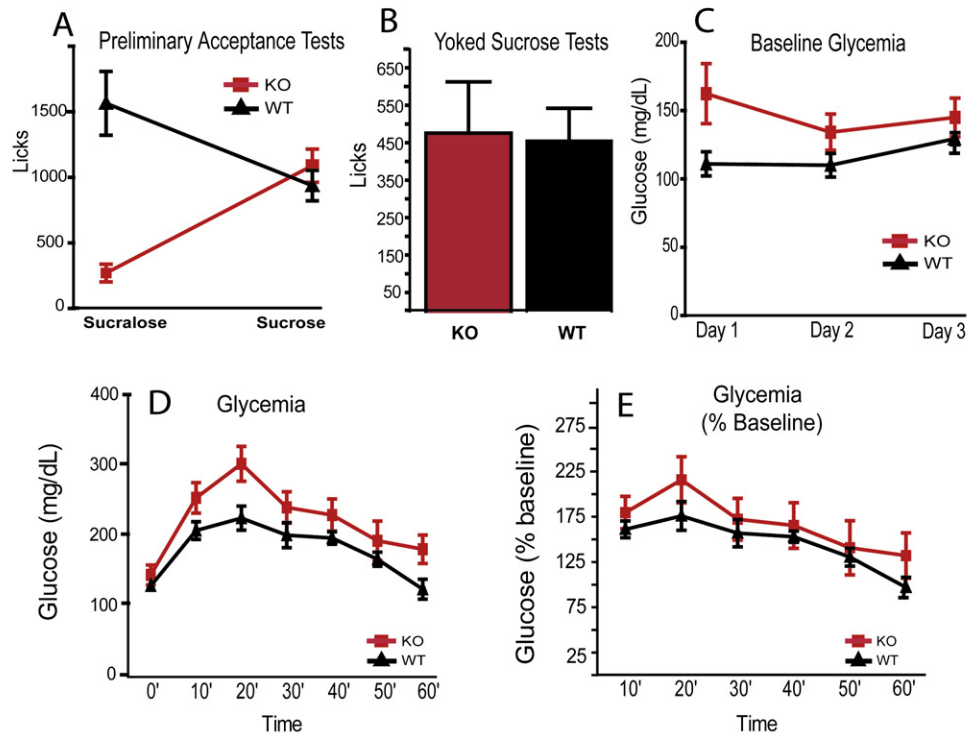

Figure 2. *trpm5*$^{-/-}$ Animals Display Normal Changes in Blood Glucose Levels during Sucrose Intake

(A) We first assessed the metabolic effects of sucrose through exposure to 30-min one-bottle sessions of sucralose (day 1) or sucrose (day 2). WT mice consumed significantly more sucralose, but not sucrose, than KO mice (sucralose consumption: 354 ± 67 and 1647 ± 243 licks for KO and WT, respectively, post hoc two-sample t test, $p < 0.01$; sucrose consumption: 1172 ± 127 and 1019 ± 116 licks for KO and WT, respectively, $p > 0.05$). This demonstrates a taste-independent sensitivity to the metabolic effects of sucrose in this group of naive KO animals.

(B) On day 3 mice were exposed to 10-min one-bottle 0.8 M sucrose sessions, preceded and followed by tail-blood sampling at 10 min intervals (0 min and 10 min to 60 min) for measurements of glycemia. To avoid large differences in total glucose absorption between genotypes, the maximum amount of sucrose allowed for each WT animal was yoked to the amount consumed by KO animals in a paired-subject design. This resulted in approximately the same total consumption between KO and WT mice (475 ± 137 and 455 ± 86 licks for KO and WT mice, respectively, two-sample t test, $p > 0.9$).

(C) Baseline measurements of blood glucose levels taken before animals entered the behavioral apparatus on test days 1–3 showed significantly higher glycemic levels in KO animals compared with WT under food and water deprivation (ANOVA, $p < 0.04$; average glycemia across days: 134 ± 8 and 110 ± 6 mg/dl for KO and WT, respectively). Effect of test days was not significant ($p > 0.3$).

(D) Two-way ANOVA (genotype × time) on glucose levels across time on the 10-min sucrose session day (0 to 60 min) shows that KO and WT animals displayed comparable rates of glucose absorption, with a strong main effect of time ($p < 0.0002$) since plasma glucose concentration tended to peak at approximately 20 min within session start in both KO and WT mice. A trend toward higher glucose levels was observed in KO mice, with a borderline significant genotype effect ($p = 0.075$).

(E) Since the trend likely results from the constitutively higher baseline glucose levels in KO animals described above, glycemia values were recalculated as percent changes with respect to baseline. The percent change data reveal that absorption rates were virtually the same between KO and WT mice, as shown by a nonsignificant effect of genotype ($p > 0.4$).

Error bars, means ± SEM.

ANOVAs (genotype × sampling time) for each tastant, and significant main effects of genotype were found on dopamine release during sucralose ($p < 0.0003$, Figure 3A), but not sucrose ($p > 0.9$, Figure 3B), sessions. Unpaired two-sample t tests performed subsequent to averaging over sampling time confirmed that sucralose, but not sucrose, intake produced significantly higher increases in dopamine levels in WT animals compared with KO animals (sucralose: 95.5% ± 5.6% for KO and 134.6% ± 4.1% for WT, $p < 0.0003$; sucrose: 129.4% ± 11.1% for KO and 127.9% ± 17.6% for WT, $p > 0.9$). Furthermore, paired t tests conducted separately for each genotype revealed that sucrose intake, compared with sucralose intake, produced significantly higher levels of dopamine release in KO (mean percent change 129.4% ± 11.0% for sucrose and 95.5% ± 5.5% for sucralose, $p < 0.03$, Figure 3C), but not WT, animals (134.6% ± 4.1% and 127.9% ± 17.5%, respectively, $p > 0.7$ Figure 3D). Furthermore, these response patterns were maintained when the analysis was restricted to the 30 min licking periods (KO: 102.66 ± 7.75 for sucralose and 153.88 ± 16.23 for sucrose, paired two-sample t test, $p < 0.03$, Figure 3E; values expressed in percent of baseline dopamine level; WT: 134.04 ± 8.82 for sucralose and 157.37 ± 23.33 for sucrose, $p > 0.3$, Figure 3F).

In addition, we found significant effects of sampling time during sucrose ($p < 0.002$), but not sucralose ($p > 0.8$), sessions. To further investigate tastant-specific temporal effects on transmitter release, we additionally made use of the sample values

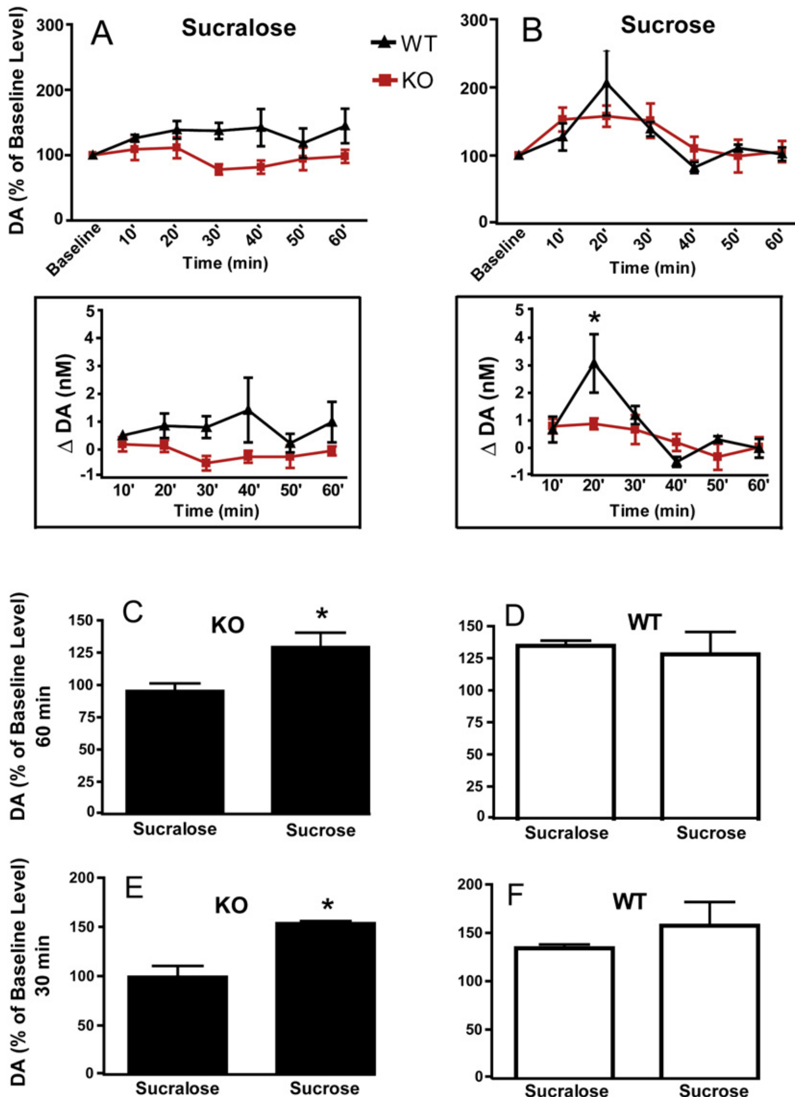

Figure 3. NAcc Dopamine Levels Increase in Response to Sucrose, but Not Sucralose, Intake in *trpm5*$^{-/-}$ Animals

(A) Time course of changes in accumbal dopamine levels during and after 30-min free access to sucralose, calculated as percent changes with respect to preingestion baseline measures. WT, but not KO, animals displayed significant increases in dopamine levels during sucralose consumption. Insert box: the data are depicted as sample dopamine concentrations in nM units (ΔDA corresponds to changes in sample values with respect to baseline values).

(B) Same as in (A), but with respect to sucrose intake. This time, even in the absence of taste input, dopamine release is evoked in KO animals at virtually the same levels as those observed in WT animals. Insert box: the data are depicted as sample dopamine concentrations in nM units (ΔDA corresponds to changes in sample values with respect to baseline values). Two-way ANOVAs on dopamine sample data revealed significant effects of sampling time and sampling time × tastant interactions during sucrose sessions (*p < 0.01, post hoc two-sample t test).

(C and D) Paired t tests performed separately for each genotype reveal significantly higher levels of dopamine release for sucrose, in comparison to sucralose, intake in KO animals (C) across the 60-min postbaseline session. No significant differences in dopamine levels were detected in WT animals between the sucralose and sucrose cases (D). (Paired two-sample t test, *p < 0.03.)

(E and F) The above pattern of changes in dopamine levels is also evident when the analysis was restricted to the initial 30 min within consumption. (Paired two-sample t test, *p < 0.03.)

Error bars, means ± SEM.

corresponding to the observed dopamine concentration (in nM units) for each time point. The related time courses are shown in Figures 3A and 3B. We performed two-way (sampling time × genotype) repeated-measures ANOVAs for each tastant separately. During sucralose sessions, as expected, we found a significant effect of genotype ($p < 0.0004$), but not of sampling time ($p > 0.69$) or interaction ($p > 0.56$), on dopamine levels. However, the same analyses applied to sucrose sessions revealed no effects of genotype ($p > 0.11$), but robust sampling time ($p < 0.0001$) and sampling time × genotype interaction effects ($p < 0.05$) on dopamine levels. Post hoc two-sample t tests then revealed significantly higher dopamine levels in WT mice compared with KO mice at 20 min sampling time ($p < 0.01$). It could be inferred from the above that the temporal patterns associated with accumbal dopamine release might reflect the nature of the inputs (i.e., caloric, gustatory, or both) eliciting transmitter release during feeding.

Finally, it could be argued that the above pattern of dopaminergic response was due to differential effects produced by licking activity. However, we found no correlation between licking responses and average dopamine release during each 30 min sucralose or sucrose licking period ($r^2 = 0.098$, $p > 0.2$, see Figure S4B).

Our results demonstrate that even in the absence of taste transduction or palatability, caloric intake produces measurable tonic increases in NAcc dopamine. Thus, both palatability and postingestive factors can independently increase dopamine levels in brain reward circuits.

Electrophysiological Measurements of Neural Activity in NAcc and OFC

We next inquired whether the pattern of dopamine release in KO animals described above was coupled to an effective modulation of the brain reward circuitry. We thus simultaneously recorded populations of neurons in the NAcc and associated OFC of naive KO mice (n = 4) under conditions of sucralose and sucrose intake similar to those used during the microdialysis experiments. We note that because we aimed at assessing the hypothesis that NAcc/OFC neurons are sensitive to the metabolic effects of sucrose independently of taste or palatability, only KO animals were recorded, since it is impossible to isolate taste-independent neuronal responses in WT animals by definition. Figures S5 and S6 provide details on our recording methodology.

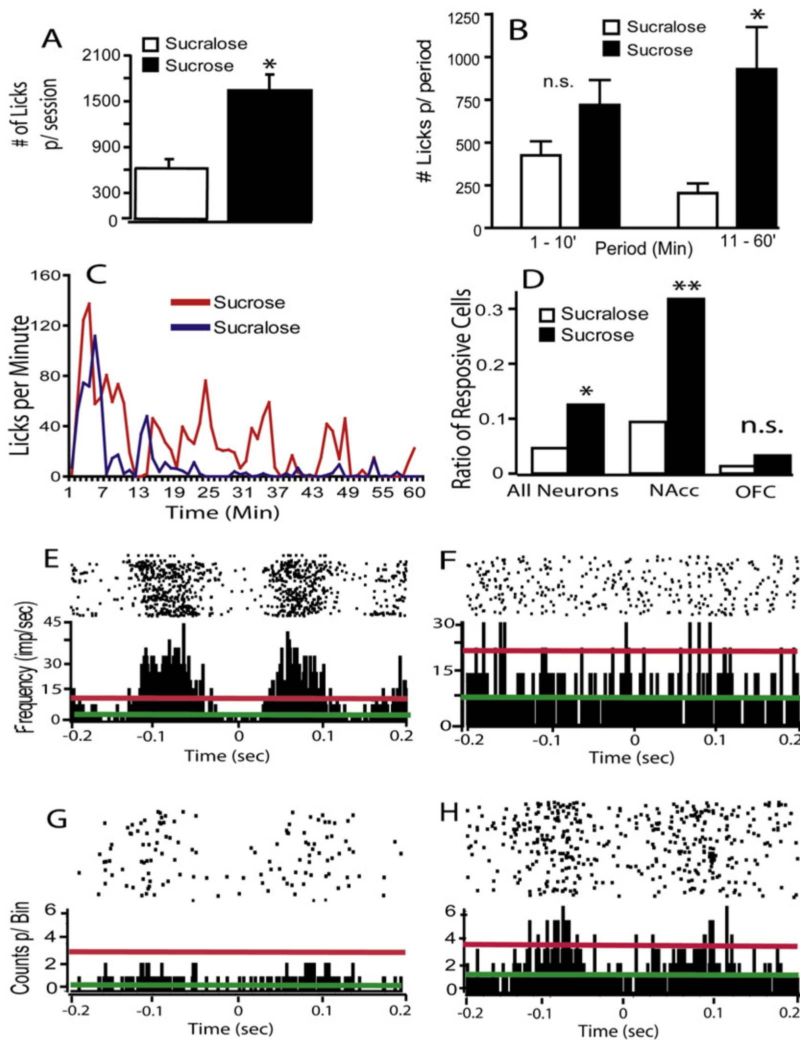

Figure 4. Single Neurons in NAcc, but Not OFC, Are Sensitive to Calorie Intake Independently of Taste Signaling

(A) Mean total consumption during the 1-hr sucralose and sucrose recording sessions. As expected, $trpm5^{-/-}$ mice consumed significantly more sucrose than sucralose (paired t test, *p < 0.03).

(B) The 1-hr sessions were divided into two periods of 10 and 50 min, and the mean total consumption for each period was calculated for each session. A significant effect of tastant, but not time, was found on intake (two-way ANOVA, p < 0.03 and p > 0.9, respectively). However, while there was no significant difference between consumption in sucrose and sucralose sessions during the first 10 min (post hoc paired two-sample t test, p > 0.05, Bonferroni corrected), as sessions progressed, a significant difference in consumption was observed during the 11 to 60 min period (post hoc paired two-sample t test, *p < 0.05, Bonferroni corrected). We attribute this behavioral pattern to the animal's ability to detect the sucrose solution's postingestive effects.

(C) Minute-by-minute time course of consumption per minute, averaged across all recorded animals separately for sucralose (blue) and sucrose (red) sessions. While consummatory patterns are initially similar, in sucralose sessions consumption decays rapidly, while in sucrose sessions it is maintained.

(D) Relative proportions of water-responsive single neurons recorded during sucralose and sucrose sessions. The ratios of neurons that were water responsive during sucralose (ratio = 0.0547) and sucrose (ratio = 0.1324) were significantly different (Fisher Exact test, *p < 0.04). When restricted to NAcc neurons, these ratios were 0.0943 and 0.3182 (sucralose and sucrose, respectively) and again, were verified to be significantly different (Fisher Exact test, **p < 0.03). However, effects observed exclusively in OFC neurons were not significant (ratios 0.0133 and 0.0435 for sucralose and sucrose, respectively, p > 0.38).

(E) Example of a NAcc neuron recorded during a sucrose session that responds significantly during licking to water.

(F) Example of a NAcc neuron recorded from the same electrode as in (E) during the corresponding preceding sucralose session. The graphs correspond to perievent histograms (PEHs) based on licks for water as the reference events. The green line indicates baseline level of activity, and red lines define the 99% confidence interval, assuming spike trains are Poisson distributed. Above each PEH is depicted the corresponding raster plot, where each dot represents a detected action potential from that particular unit. Each line of the raster plot corresponds to an individual trial sampled within the time limits of the PEH.

(G and H) PEH of an example NAcc neuron recorded during a sucrose session. This unit did not respond to water during the initial 10 min (G) but did develop a significant response during the final 50 min (H) of the session, the period when behavioral responses indicate that animals detected the solution's postingestive effects. Above the PEHs are depicted the corresponding raster plots, where each dot represents a detected action potential from that particular unit. Each line of the raster plot corresponds to an individual trial sampled within the time limits of the PEH.

Error bars, means ± SEM.

During recording day 1, food- and water-deprived KO animals were given free access for 1 hr to sucralose solutions, and on day 2, to sucrose solutions. Stimulus delivery was performed via the opening of solenoid valves upon lick detection such that in 25% of occurrences of sustained licking responses (randomly selected), water was delivered instead of sucralose (day 1) or sucrose (day 2) solution.

As expected, during these 1-hr sessions, KO mice consumed significantly more sucrose than sucralose (mean number of licks 627 ± 111 for sucralose and 1647 ± 205 for sucrose, paired two-sample t test, p < 0.03, Figure 4A). However, when each 1-hr session was divided into a period of 10 and 50 min, an interesting temporal pattern was revealed. Repeated-measures two-way ANOVA (taste × time) on the intake data showed a significant effect of tastant (p < 0.03), but not time (p > 0.9), on total consumption. Interestingly, during the first 10 min, there was no significant difference in consumption between sucrose and sucralose sessions (718 ± 146 versus 424 ± 82 licks, respectively, post hoc paired two-sample t test, p > 0.05, see Figure 4B). However, as sessions progressed, animals became increasingly sensitive to the postingestive effects of each solution and consequently returned more often to the sipper during sucrose sessions compared with sucralose sessions. Thus, a significant difference in consumption was observed during the last 50 min of each session (929 ± 245 and 203 ± 57 licks for sucrose and sucralose, respectively, p < 0.05). This pattern

can be observed in the minute-by-minute time courses of the licking behaviors across all recorded animals, depicted in Figure 4C.

We then inquired whether any of the two simultaneously recorded areas displayed an increased sensitivity to caloric intake by comparing the neuronal responses during licking obtained from sucralose versus sucrose sessions. Because single neurons in OFC are known to respond to stimulus attributes such as viscosity (Verhagen et al., 2003), we only analyzed water trials that were uniformly dispersed within the sucralose and sucrose trials (see above and Experimental Procedures).

A total of 128 single units were recorded during the sucralose sessions (53 neurons in NAcc and 75 in OFC) and 136 neurons during sucrose sessions (44 neurons in NAcc and 92 in OFC). No assumptions were made on whether or not a given unit was recorded during both sessions. For both the NAcc and OFC, we calculated the relative proportions of stimulus-responsive neurons during sucralose and sucrose sessions. We found that, overall, 7/128 neurons showed water sensitivity during sucralose sessions, whereas for sucrose sessions 18/136 neurons were water responsive. This difference in proportions is significant (Fisher Exact test, $p < 0.04$, see Figure 4D). This result demonstrates that the NAcc-OFC circuit is sensitive to caloric intake and metabolic cues even in the absence of taste signaling. However, when the same analysis was performed for each brain region separately, it was found that the difference in proportions was significant for NAcc (6/53 versus 14/44 neurons for sucralose and sucrose sessions, respectively, Fisher Exact test, $p < 0.03$), but not for OFC (1/75 versus 4/92 neurons, respectively, Fisher Exact test, $p > 0.38$, Figure 4D). As examples, Figure 4E shows a water-responsive NAcc neuron recorded during a sucrose session, whereas Figure 4F shows a neuron recorded from the same electrode during the corresponding sucralose session.

Overall, we observed that whereas the dopamine-targeted NAcc showed an increased sensitivity to caloric intake, taste-independent metabolic changes were insufficient to engage the OFC in stimulus encoding. We note that these results remained essentially unchanged when the same analyses were performed across sucrose and sucralose trials instead of water trials (see Figure S7).

Given the behavioral results showing that when compared with sucralose, sucrose consumption was different only 10 min after session start, we suspected that the stimulus sensitivity displayed by NAcc neurons could be accounted for mainly by stimulus response properties that developed during the last 50 min of sucrose sessions. We therefore counted, for all sucrose sessions, the number of neurons that were responsive to water during the sessions' final 50 min, but not during the initial 10 min. We found that, out of the 14 neurons that were found to be responsive to water during sucrose sessions, 8 responded to water only during the final 50 min of the session. This ratio differs significantly from the overall ratio of NAcc water-responsive cells during sucrose sessions (8/44 versus 14/44 neurons, respectively, McNemar's test for proportions with repeated observations, $p < 0.05$). Additionally, if the cells that responded only during the last 50 min of the sucrose session are not accounted for as water-responsive cells, the ratios between sucralose and sucrose sessions no longer differ (6/53 versus 6/44 neurons, respectively, Fisher Exact test, $p > 0.7$). Therefore, the effect observed in NAcc neurons was largely due to neuronal response properties that developed later (>10 min) in sucrose sessions. Figures 4G and 4H show an example of a NAcc neuron recorded during a sucrose session that did not respond to water during the initial 10 min (Figure 4G) but developed a significant response during the final 50 min (Figure 4H).

The above single-cell results suggest that once a caloric load was introduced, NAcc, but not OFC, neurons changed their overall response properties. We therefore investigated the changes in the overall response patterns of NAcc and OFC neurons by performing principal component analyses (PCAs) on the perievent histograms (PEHs) defined across the licking events for each single unit. We performed PCAs separately for PEHs produced during sucralose and sucrose sessions. Individual PEHs from both NAcc and OFC neurons were projected onto the space generated by the three first principal components derived from sucralose and sucrose sessions separately (Figure 5A). While NAcc (blue) and OFC (red) PEH response patterns displayed a low degree of separation during sucralose sessions, reflecting the low sensitivity of both regions to the noncaloric sweetener sucralose, in sucrose sessions, the equivalent projection (Figure 5B) depicts a much clearer degree of separation between NAcc and OFC response patterns. This separation reflects the abovementioned finding that single neurons in NAcc, but not OFC, showed increased sensitivity during licking for water during sucrose, but not sucralose, sessions.

In order to quantify the degree of separation between individual PEHs projected on 3D PCA spaces, we calculated the equivalent Mahalanobis distances between NAcc and OFC [the Mahalanobis distance is a scale-invariant multivariable metric that takes into account the degree of correlation between variables; see Mahalanobis (1936)]. As predicted from the single-unit studies, distances increased significantly during sucrose sessions when compared with sucralose sessions (mean distance 119.05 ± 58.91 for sucrose and 17.07 ± 5.88 for sucralose sessions, nonparametric Wilcoxon ranking test, $z = 3.92$, $p < 0.00009$, Figure 5C). Thus, differences between NAcc and OFC PEH response properties were significantly higher during sucrose sessions when compared with sucralose sessions.

The pattern above was maintained even when the analysis was restricted to PEHs obtained during licks for water only (mean distance 42.45 ± 16.79 for sucrose and 13.06 ± 5.65 for sucralose sessions, nonparametric Wilcoxon ranking test, $z = 3.12$, $p < 0.002$, Figure 5D). This finding on the water PEHs strongly supports our single-cell analyses. In addition, inspection of the first principal components for each area separately also confirms this overall pattern. Figures 5E and 5F depict, for one recorded animal, the first principal component, plotted against time, obtained from all NAcc (blue) and OFC (red) PEHs entered in the PCA. During sucralose sessions, a comparison between the first principal components of each area reveals an undifferentiated response pattern, reflecting low stimulus sensitivity (Figure 5E). However, during sucrose sessions, a stimulus-induced response pattern becomes apparent for NAcc, but not OFC, first principal components (Figure 5F).

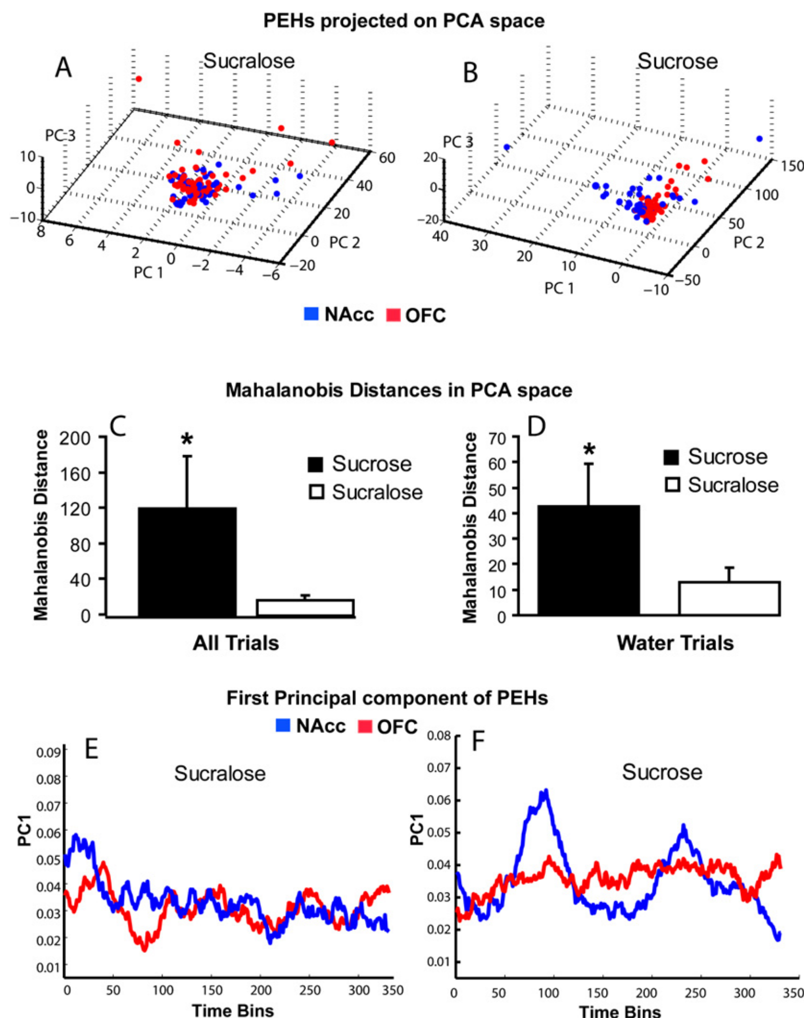

Figure 5. Principal Component Analysis, or PCA, on PEHs for NAcc and OFC Neurons

(A) Projection of PEHs obtained during sucralose sessions onto the space generated by the first three principal components. Principal components were obtained by applying PCAs on PEHs obtained from all recorded neurons in all animals. PCAs were performed separately for sucralose and sucrose sessions. Blue: PEHs projected in PCA space from NAcc neurons; red: from OFC neurons.

(B) Same as in (A), but for sucrose sessions. Note the decreased similarity between NAcc and OFC PEHs during sucrose sessions when compared with the sucralose sessions (shown in [A]). Blue, NAcc PEHs; red, OFC PEHs.

(C) Mean Mahalanobis distance calculated between NAcc and OFC PEHs projected on the 3D PCA space depicted in (A) and (B). Mean distance increased significantly for sucrose sessions when compared with sucralose sessions (nonparametric Wilcoxon ranking test, $*p < 0.00009$). Thus, NAcc PEH response properties differed significantly from those of OFC during sucrose sessions when compared with sucralose sessions.

(D) Same as in (C), but for the case where analysis was restricted to PEHs obtained during licks for water only (nonparametric Wilcoxon ranking test, $*p < 0.002$). This finding further supports single-cell analyses presented in Figure 4D.

(E and F) Inspection of the first principal components for each area separately also confirms the overall patterns described above; (E) depicts the first principal component, plotted against time, relative to all NAcc (blue) and OFC (red) PEHs entered in the PCA for one recorded animal. During sucralose sessions, comparison among the first principal components of each area reveals an undifferentiated response pattern, reflecting low stimulus sensitivity to noncaloric sweetener. During sucrose sessions, however, a stimulus-locked response pattern is observed for NAcc, but not OFC, first principal components.

Error bars, means ± SEM.

Overall, the electrophysiological data strongly support the microdialysis findings and implicate the NAcc in the neural circuitry that mediates the postingestive influences on feeding behavior.

DISCUSSION

We showed in this study that brain reward dopamine systems respond to the caloric value of sucrose, even in the absence of taste receptor signaling, palatability, or changes in flavor evaluation. Specifically, we have shown that sucrose, but not the noncaloric sweetener sucralose, induces dopamine release in the NAcc of mice lacking functional sweet taste transduction. In addition, NAcc, but not OFC, neurons increased their responsiveness during a caloric load. Overall, the similarity between NAcc and OFC response patterns decreased significantly upon the presence of caloric load. Consequently, dopamine-NAcc reward pathways do not seem to exclusively encode the hedonic impact of foods present in the oral cavity. Rather, physiological events starting in the gastro-intestinal tract do not necessarily require the mediation of gustatory inputs to convey information on the metabolic value of nutrients to these reward systems.

Previous behavioral studies showed that animals can develop preferences for arbitrary flavors that have been associated with positive postingestive effects (Sclafani, 2001; Sclafani and Ackroff, 2004). Our results showing that *trpm5*$^{-/-}$ mice condition to sipper tubes associated with sucrose intake generalize this observation to the case where no taste transduction is present. We infer that the development of preferences based on the solution's postingestive effects does not require associations with distinct flavors.

We note that two different strains of *trpm5*$^{-/-}$ mice have been described (cf. Damak et al., 2006; Zhang et al., 2003). For the animals used in this study, previous evaluations did not detect any residual sensitivity to sweet tastants during either behavioral or cranial nerve recording experiments (Zhang et al., 2003). With respect to the strain described by Damak et al. (2006), residual sensitivity to high-concentration sucrose solutions has been found at both behavioral and cranial nerve recordings. It should be noticed, however, that in their report the residual sensitivity to sucrose was observed during 24-hr two-bottle tests, but not brief-access tests, an effect possibly accounted for by postingestive factors. A recent study (Sclafani et al., 2007) using the same KO mice as in Damak et al. (2006) attributed the

development of preferences to the flavors of nonsweet carbohydrates and fat emulsions in *trpm5*$^{-/-}$ mice to postoral effects. However, because in that study experience with flavorful nutrients is confounded by exposure to increasing stimulus concentrations, it is not clear whether the observed effects were exclusively due to flavor-nutrient associations or were contributed by the residual taste sensitivity previously claimed for this particular strain. In any event, the central fact to be retained here is that the strain used in the present study does not display any residual sensitivity to sweet tastants, as was shown previously (Zhang et al., 2003) and in conformity with our own findings.

The behavioral data presented in this study contributes to the ongoing debate on whether nutrient-sensing by the gastro-intestinal system makes use of taste-like transduction pathways to detect luminal contents and regulate nutrient absorption (Dyer et al., 2005; Hofer et al., 1996). Indeed, the fact that sweet-blind KO animals developed a preference for sucrose, whereas WT animals did not condition to sucralose (a noncaloric substance that activates the same taste transduction pathways as sucrose), shows that the presence of the taste TRP channel M5 in the gastro-intestinal tract (Bezencon et al., 2007) is neither necessary nor sufficient for sweet nutrients to act centrally as reinforcers. This behavioral observation is strengthened by the fact that during sucrose intake, KO animals displayed changes in blood glucose levels comparable to those observed in WT.

One principal finding of this study concerns the ability of dopamine-related brain reward systems to detect internal physiological changes, even in the absence of taste input. We showed that changes in extracellular levels of dopamine in the NAcc could be induced via two independent pathways: one relating to calorie-independent palatability (i.e. during sucralose intake in WT animals), and a second one relating to taste-independent caloric load (i.e. during sucrose intake in *trpm5*$^{-/-}$ animals). In other words, besides confirming that dopamine release in NAcc reflects the hedonic value of sugars even if no calorie is absorbed (Hajnal et al., 2004), we also show that NAcc dopamine levels signal caloric intake through a taste-independent pathway. We thus reason that the putative role of dopamine transmission in overeating and obesity might not be restricted to oral hedonics; rather, dopamine signaling could influence behavior also by coding for the food's nutritive value (Figlewicz, 2003; Fulton et al., 2006; Hommel et al., 2006; Palmiter, 2007).

Our microdialysis data were consistent with our finding that NAcc neurons in *trpm5*$^{-/-}$ animals increased their sensitivity to oral stimuli upon the presence of caloric load. Furthermore, PCAs pointed out significant differences between the two regions in their overall response patterns to the stimuli (Figure 5). This suggests that during caloric intake dopamine release in NAcc is effectively coupled to changes in activity in NAcc reward circuits. Neural populations in NAcc are known to encode the palatability of sweet tastants (Roitman et al., 2005; Taha and Fields, 2005), and we now provide evidence that the same region also contains neurons that encode for changes in metabolic status. The extent to which these two populations overlap should be determined by future experiments combining electrophysiological recordings with pharmacological manipulations.

Simultaneous to recording in NAcc, we have also recorded neural populations in the OFC. This region is known to be generally involved in reward processing (Pais-Vieira et al., 2007; Schoenbaum and Roesch, 2005), more particularly in representing changes in internal states (de Araujo et al., 2006; Rolls, 2004). Interestingly, we did not observe alterations in taste-independent stimulus sensitivity in OFC neurons due to metabolic changes. Although the functional and anatomical heterogeneity of the OFC precludes any a priori explanations, our results suggest that gustatory inputs are required to engage the OFC in the explicit representation of physiological states. In fact, the presence of sensory-specific satiety neurons in OFC (Rolls et al., 1989) implicates this region in encoding interactions between the sensory and physiological properties of taste stimuli. Here we suggest that one function of the OFC in feeding specifically involves the utilization of orosensory cues to guide appropriate selection of calorie-rich nutrients.

In summary, we showed that dopamine-ventral striatum reward systems, previously associated with the detection and assignment of reward value to palatable compounds, respond to the caloric value of sucrose in the absence of taste receptor signaling. Thus, these brain pathways do not exclusively encode the sensory-related hedonic impact of foods, but might also perform previously unidentified functions that include the detection of gastro-intestinal and metabolic signals.

EXPERIMENTAL PROCEDURES

Subjects

A total of 103 male mice with a C57BL/6 background were used. At the time of experiments animals were 3 to 6 months old. Fifty-five of these animals were homozygous for a partial deletion of the *trpm5* gene (KO), as described previously (Zhang et al., 2003), and were bred from mice generously donated by C. S. Zuker (UCSD, San Diego, CA). Forty-eight mice were WT C57BL/6 animals that were obtained from the Jackson Laboratory (Bar Harbor, ME). Genotype was confirmed by PCR amplification of *trpm5*. Four KO mice were implanted with microelectrode arrays for neural recordings. Seven KO and seven WT mice were implanted with microdialysis probes. The remaining animals were used solely for behavioral experiments. All procedures were approved by the Duke University Institutional Animal Care and Use Committee.

Stimuli

All solutions [sucrose: 0.2 M, 0.4 M, and 0.8 M, Sigma; sucralose (1′,6′-dichloro-1′,6′-dideoxy-β-d-fructofuranosy-l-4′-chloro-4′-deoxy-α-d-galactopyranoside) 30 mM, McNeil Specialty] were made at room temperature in distilled water. Distilled water was also used as baseline stimulus (every mention of "water" in this paper refers to usage of distilled water). Solutions were prepared daily.

Behavioral Experiments

All behavioral experiments were conducted in either of two mouse behavior chambers enclosed in a ventilated and sound-attenuating cubicle (Med Associates Inc., St. Albans, VT). Each chamber was equipped with two slots for sipper tubing placement in symmetrical locations of one of the walls. Access to sipper tubes could be blocked by computer-controlled doors (allowing one or two-bottle testing to be performed in the same chamber), and all slots were equipped with licking detection devices with 10 ms resolution. In one chamber, contact lickometers (Med Associates Inc., St. Albans, VT) were used for licking detection. The alternate chamber was adapted with custom-made beam lickometers, where lick detection depends on the interruption of a photobeam sensor, to allow minimization of electrical artifacts in neural recordings during licking (Figure S5B). Behavioral testing by the brief-access test was conducted in the chamber with beam lickometers. All other behavioral tests were conducted in the chamber with contact lickometers.

Brief-Access Tests

Five KO and five WT mice were tested in a 30-min brief-access test, conducted similarly to a previous description (Glendinning et al., 2002), although in our case animals were not food deprived. Briefly, each animal had access to only one sipper, to which it was given intermittent access in sequential trials. Animals started each trial voluntarily, the structure of which is as follows. Following the animal's first lick to a solution, the sipper would deliver one tastant aliquot (~3 μl) for each detected lick response for a short period (5 s), after which access to the sipper was blocked for 7 s by sliding the door placed in front of the sipper. After this intertrial period animals were allowed to initiate a new trial. A computer-controlled and gravity-driven valve system (ALA Scientific, Westbury, NY) allowed water and two sucrose solutions with different concentrations (0.4 M and 0.8 M) to be presented randomly within blocks of three trials, with one tastant per trial. The cumulative number of licks for all trials of each tastant was recorded and used to calculate the respective lick ratio (see below). The animals were first habituated to the behavioral chamber and trained in the task by receiving water following 22-hr water deprivation. Once stable licking rates were achieved, the mice were tested under a schedule of 8- to 10-hr water deprivation. To reduce variability, average lick ratios from 2 consecutive testing days were calculated for each animal.

Two-Bottle Preference Tests

To further investigate preference for sucrose solutions, mice were tested in sucrose versus water two-bottle choice tests. Once habituated to the behavioral chamber, each animal was presented with two bottles, one of which contained water and the other a 0.8 M sucrose solution, to which they had constant free access during the duration of the experiment. The number of licks for each sipper was recorded and used to calculate the preference ratio for sucrose (see below). To reduce confounds produced by side biases, mice were tested in each condition for 4 consecutive days with daily inversion of sucrose and water bottle positions. The average preference ratio for sucrose across testing days was then calculated for each animal. Eight KO and five WT mice were used in 20-min tests under a nondeprived state. To investigate modulation of sucrose preference by hunger, seven KO and five WT mice were tested under a 20- to 22-hr food deprivation schedule. These animals were also water deprived in order to increase motivation in KO mice to perform the task. To minimize intrasession modulation of preference by postingestive factors, food-deprived animals were tested during 10-min sessions.

Conditioning to Postingestive Effects

To verify whether KO mice can detect the postingestive effects of sucrose, we designed a conditioning protocol that would allow the animal to manifest tastant-independent preferences. All conditioning experiments were conducted with naive animals under a 20- to 22-hr food and water deprivation schedule. In nine KO and five WT mice, side bias was first determined for each animal in a 10-min two-bottle water versus water test. Once a clear side bias was established, the animals were conditioned for 6 days with daily 30-min sessions of free access to either water or 0.8 M sucrose in one-bottle forced-choice training sessions. Water was presented on the initially biased side for 3 days that were intercalated with 3 other days where sucrose was presented on the opposite side of the chamber. After training, reversal of side bias was tested in 10-min two-bottle water versus water tests. As a control, this procedure was conducted in five KO and six WT animals, but 30 mM sucralose was substituted for sucrose during conditioning. This high concentration of sucralose is known to be highly attractive to mice (Bachmanov et al., 2001), and a two-bottle choice test revealed that WT mice did not significantly differ in their preference for 0.8 M sucrose versus 30 mM sucralose (Table S3).

Blood Glucose Measurements

Mice were presented with 30 mM sucralose on day 1 and 0.8 M sucrose on day 2, in order to confirm genotype-dependent differences in the consumption of these substances. After these two training days, the same animals were given access to 0.8 M sucrose during 10-min sessions. Experiments with KO animals were performed first, and the maximum amount of sucrose allowed for each WT animal was approximately yoked to KO mice in a paired-subject design, to avoid large differences in total sucrose availability between genotypes. In this third day of testing, glycemia was measured both before and immediately after exposure to the testing chamber (0 min and 10 min, respectively), and also at 10-min intervals after the animal was returned to his home cage, for up to 1 hr (20, 30, 40, 50, and 60 min.).

Stereotaxic Surgery for Implantation of Guide Cannulas for Microdialysis Probe or Multielectrode Microarrays

Seven WT and eleven KO mice were anesthetized using 5% halothane followed by intramuscular injection of xylazine (5 mg/kg) and ketamine (75 mg/kg). Supplemental doses were administered whenever necessary. In seven KO and seven WT mice, a small circular craniotomy was drilled at [AP = 1.2 mm, ML = ±0.6 mm], and a guide cannula (CMA-11; CMA Microdialysis, Solna, Sweden) was implanted above the NAcc [DV = −3.2 mm from the brain surface], for posterior insertion of a microdialysis probe (final probe tip position [DV = −4.2 mm]). In four KO mice two ~1 mm^2 craniotomies were drilled on the same side of the skull at [AP = 2.3 mm, ML = ± 0.8 mm] and [AP = 1.3 mm, ML = ± 0.8 mm] relative to bregma, to target the OFC and NAcc, respectively. The microarray design consisted of 32 (4*8) S-isonel-coated tungsten microwire electrodes (35 μm diameter), a printed circuit board (PCB) connected to the microwire electrodes, and a miniature connector attached to the opposite side of the PCB. Microwires were cut at several lengths to allow multiple depths of implantation (Figures S5C–S5F: ~1.7 and 2 mm for the OFC and ~4 mm for the NAcc; Paxinos and Franklin, 2001). The side of implantation was balanced between left and right hemispheres across animals.

Microdialysis

Microdialysate samples were collected, separated, and quantified by HPLC as previously described for freely moving mice (Sotnikova et al., 2004). Following recovery mice were put under a 22-hr food and water deprivation schedule and a microdialysis probe (1 mm membrane length, 0.24 mm outer diameter, Cuprophane, 6 kDa cutoff; CMA-11, CMA Microdialysis, Solna, Sweden) was inserted into the NAcc through the previously implanted guide cannula. Twenty-four and forty-eight hours after probe insertion, each animal was placed in the respective behavior chamber and the microdialysis probe was connected to a syringe pump (Ranzel, Stamford, CT) and perfused at 1 μl/min with artificial CSF (CMA Microdialysis, Solna, Sweden). After a 40–60 min washout period, perfusates were collected every 10 min. Once six samples were collected to establish baseline dopamine levels, the animal was given access to 30 mM sucralose (day 1) or 0.8 M sucrose (day 2) for 30 min in one-bottle forced-choice tests. This design was chosen to minimize reward expectation effects that could have been produced had sucrose been presented on day 1. Access to the sipper was then again blocked, and the animal was kept in the chamber for collection of a further six postlicking samples, in a total of 12 perfusate samples for each of two sessions conducted in each animal. Both tastants were presented on the same side of the same behavior chamber, across the two testing days. One KO mouse died between test sessions, and in another it was not possible to establish baseline dopamine level for day 2. In three WT animals probe tips were incorrectly placed outside the NAcc (Figure S8). Data from any of these animals were excluded, resulting in five KO and four WT animals being used.

Neuronal Recordings

All the experiments were performed in the chamber equipped with beam lickometers described above. Simultaneous neural activity was recorded from the 32 implanted microwires and processed by using a Multineuron Acquisition Processor (Plexon Inc., Dallas, TX). Timestamps of licking responses and neural activity were recorded simultaneously and recordings were synchronized under a single master clock. Only single neurons with action potentials of signal-to-noise ratios >3:1 were analyzed. The action potentials were isolated online by means of voltage-time threshold windows and a three principal components contour templates algorithm. A cluster of waveforms was assigned as a single unit only when both interspike intervals (ISIs) were larger than the refractory period (set to 1.5 ms) and when the 3D projection of the first three principal components formed a visible cloud with no overlapping points with a different unit cluster. Waveforms were resorted offline and waveform alignment inspected. Only timestamps from offline sorted waveforms were analyzed. Stability of waveform shape across a session was confirmed by using the Waveform Tracker software (Plexon Inc., Dallas, TX). Neural activities from NAcc and OFC, along with timestamps of licking responses, were recorded simultaneously from each animal during two 1-hr sessions, separated by 24 hr, where animals were given free access to either sucralose (day 1) or sucrose (day 2). This design was chosen to minimize reward expectation effects that could have been produced had sucrose been presented on

day 1. Sucrose and sucralose concentrations were the same ones used for behavioral and microdialysis experiments. Animals were 22-hr food and water deprived at the start of both sessions.

Stimulus Delivery during Recording Sessions

Water delivery during single-sipper sucrose and sucralose sessions was performed as follows. Stimuli were delivered by the opening of a solenoid valve, contingent upon lick detection by the beam lickometer (so that for each detected lick a small aliquot of liquid [$\sim$3 μl] was delivered to the animal's oral cavity). Four solenoid valves controlled stimulus delivery through a single sipper. Three of these contained either sucralose (day 1) or sucrose (day 2), while the fourth valve contained dH_2O. The device was programmed to open the same valve for each five consecutive licks. Following each five-lick block, a new valve would open for the next five licks. The order in which valves were opened was determined by a uniform distribution-based block design (no repetition within blocks), resulting in water being delivered for 25% of licks detected. This design provides an appropriate control for potentially confounding sensory properties of tastants such as texture or viscosity.

Behavioral Data Analysis

Results from data analyses were expressed as mean ± SEM. Analyses of behavioral data were performed with custom software written in Matlab (R14, MathWorks, Inc.) or with Prism (GraphPad, San Diego), and made use of three-way, two-way, or one-way ANOVAs and two-sample or independent one-sample t tests. Bonferroni corrections for multiple comparisons were performed whenever appropriate.

Lick Response and Preference Measures

For brief-access tests, lick ratios are defined as the amount of sucrose consumed with respect to water:

$$lick\ ratio = \frac{n(sucrose)}{n(water)}$$

where n(.) denotes the total number of licks for a given stimulus during a session. These values were entered in a two-way ANOVA genotype × sucrose concentration model and tested against 1.0, which is the reference value meaning indifference with respect to water.

All two-bottle preference tests were analyzed by calculating the preference ratios as

$$\text{Preference for sipper } 1 = \frac{n(sipper1)}{n(sipper1) + n(sipper2)}$$

The significance tests were based on one-sample t tests against 0.5, which is the reference value meaning indifference with respect to either sipper.

For conditioning and microdialysis sessions, numbers of licks during each session were entered in a two-way ANOVA genotype × taste stimulus model followed by post hoc Bonferroni-corrected two-sample t tests.

Microdialysis Data Analysis

Analyses of the microdialysis data were performed with custom software written in Matlab (R14, MathWorks, Inc.) or with Prism (GraphPad, San Diego).

Baseline dopamine was defined as the mean dopamine concentration (nM) from a minimum of three out of the six collected baseline samples. Dopamine levels for each of the six other samples collected in each session were expressed as percent change with respect to baseline dopamine levels. Dopamine levels were entered in a three-way ANOVA genotype × tastant × time (samples one to six) model. In the case of significant interaction between factors, post hoc two-way ANOVAs and paired t tests were conducted. To analyze time-related effects on dopamine release, dopamine concentration (nM) data, following subtraction of mean baseline values, were entered in sampling time × genotype two-way repeated ANOVA models (samples one to six) for each tastant. In the case of significant effects for a particular factor, post hoc paired t tests were conducted as appropriate.

Electrophysiology Data Analysis

All neuronal data analyses were performed with custom software written in Matlab (R14, MathWorks, Inc.) or with the Nex software (Nex Technologies, TX).

PEHs

PEHs show the conditional probability of observing a spike in the spike train at time t, on the condition that there is a reference event (detected licks for sucrose, sucralose, or water) at time zero. The time axis is divided into 1 ms bins. Spike counts were normalized as spikes/s, where bin counts are divided by number of spikes × bin size in seconds. Analyses of sucralose, sucrose, and water responses in single cells were performed by constructing 400 ms (±200 ms) PEHs with 1-ms bins using the licks to sucrose, sucralose, or water as defining events. Confidence intervals were obtained by assuming that spike trains are Poisson distributed, as described previously (Abeles, 1982). Responses were considered significant whenever $p < 0.01$. A given unit was considered to be stimulus responsive or stimulus sensitive if in the corresponding PEH the values of at least three consecutive bins were outside the 99% confidence interval. PEHs were constructed using the Peri-Event Histogram function of the Nex software (Nex Technologies, TX).

Multivariate Analyses on PEHs

Responses of each neuron around licking events were normalized and limited to a time window of 400 ms (±200 ms) and structured as a PEH as described above. PEHs across either sucralose or sucrose sessions were then combined as an n-by-p data matrix X, where rows of X correspond to different neurons and columns to time bins within a PEH. PCA (e.g., Maier et al., 2007) was used for visualization of the mean response vector space and for assessment of the multidimensional separation of the mean responses. Separation was computed as Mahalanobis distances defined as follows. For any two mean-subtracted vectors $\mathbf{x}^i$ and $\mathbf{x}^j$ in an N-dimensional space, the Mahalanobis distance d_M between $\mathbf{x}^i$ and $\mathbf{x}^j$ was calculated as

$$d_M = (\mathbf{x}^i - \mathbf{x}^j)\mathbf{C}^{-1}(\mathbf{x}^i - \mathbf{x}^j)^T$$

where $\mathbf{C}$ denotes the sample covariation matrix and $\mathbf{x}^T$ the transpose of vector $\mathbf{x}$. Pairwise statistical comparisons between distances were performed using nonparametric Wilcoxon ranking tests.

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/57/6/930/DC1/.

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