Inventory of Supplementary Information

Figure S1 – A diagram summarizing some of the many factors known to affect food intake in laboratory rodents: This figure provides an illustration of the factors that affect food intake in laboratory mice and the relationships between them.

Supplemental Assay Protocols

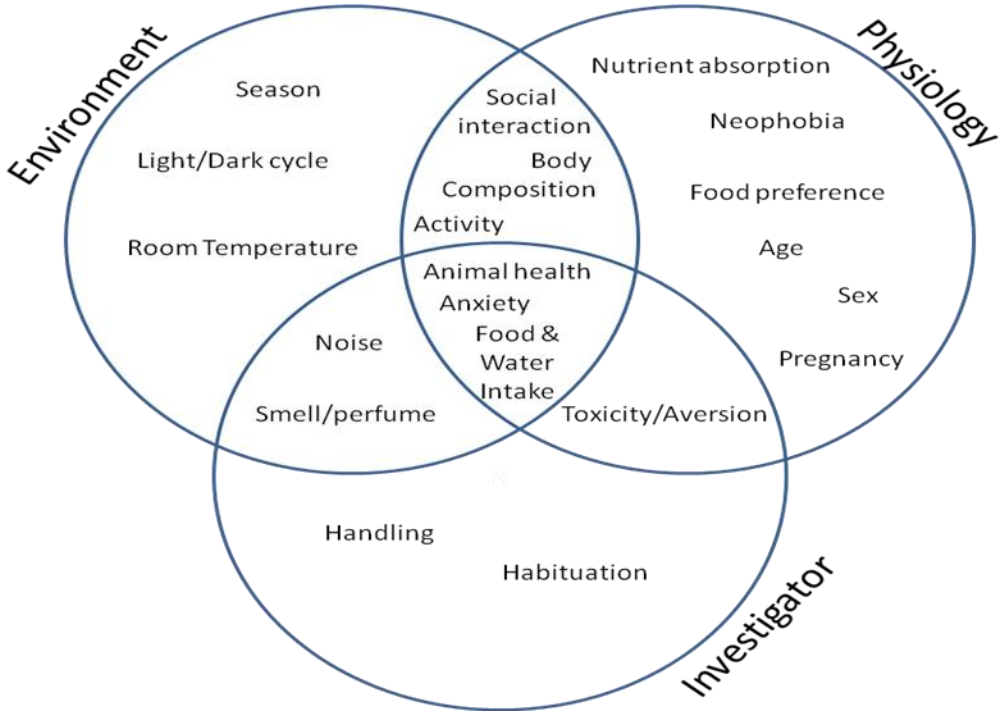
These protocols are intended to provide more detailed methodological information for techniques discussed in the main text. More detail is provided than in traditional "materials and methods" sections to help investigators who do not have experience with these protocols design and carry out these tests.

1. **Types of food intake measurement, accuracy and potential errors**
2. **Creating diet-induced obese (DIO) mice**
3. **Pair-Feeding**
4. **Identifying and reducing sources of experimental stress**
5. **Conditioned Taste Aversion (Supplemental Table 1. A sample two-bottle conditioned taste aversion paradigm)**
6. **Pica (kaolin/clay consumption – Supplementary Table 2. An example of a pica test paradigm).**

Supplementary References

SUPPLEMENTAL DATA

Figure S1. A diagram summarizing some of the many factors known to affect food intake in laboratory rodents – All of the aspects of physiology are also influenced by genetics including variation between strains.



Supplemental Assay Protocols

Types of food intake measurement, accuracy and potential errors

The easiest way to measure food intake in mice is to give them a known (i.e., pre-weighed) amount of food in their home cage hopper and weigh the remaining food once per day, or at shorter intervals if needed for the questions being asked. Errors that affect this measure included spillage and hoarding, but is the easiest approach, requires no specialized equipment, and the associated errors can be reduced by averaging values over time. As a general rule, food intake should be assessed in mice that are housed individually due to the influence of social behavior on feeding, and because mice that are group-housed tend to huddle together to stay warm and thus are able to maintain their body temperature without expending as much energy. While food intake can also be assessed in group-housed animals, results can be greatly influenced by social hierarchy (Tamashiro et al., 2007)

To avoid issues of spillage and/or hoarding, mice may be housed in wire-bottomed cages where spilled food can be collected and corrected for, but such cages are not suitable for long-term housing. Using powdered rather than pelleted chow can minimize spillage and/or hoarding, but this requires an enclosed food hopper that allows the animal to eat without removing food. Liquid or gel-like diets can also increase accuracy of intake assessment, but these may introduce other confounds in terms of palatability or water balance. Accuracy and reproducibility in measurement of food intake are critical considerations, as a difference of 500 mg of measured intake can amount to >10% of daily food consumption in a typical mouse.

The time(s) that food intake is manually recorded should be planned beforehand. Such measures provide information only about average food consumption over the interval selected and thus miss smaller windows of time when important changes occur. For example, intake of a HFD is often greatest during the first few days of diet exposure and returns towards normal values thereafter. Hence, if intake is not measured immediately following the switch in diet, a significant period of hyperphagia may be missed. Manual intake measures also provide no insight into changes of meal size, meal frequency or intermeal interval, parameters important in the control of overall energy homeostasis and which can be altered even in the absence of changes of total intake. The time-consuming nature of manual measures and the need for inconvenient night-time/dark-phase measurements (see below) are other notable limitations.

There are a number of commercially available systems for automated monitoring of food intake and meal-patterning of mice. These allow real-time assessment of food intake and many are constructed so as to reduce spillage and hoarding. Operant chambers in which mice must make a specific response to obtain a small amount of food can also be used for analysis of meal patterns, but they require that the animals be trained to make the response (e.g., nose-poking to receive food).

Creating diet-induced obese (DIO) mice

DIO is produced in mice by providing *ad libitum* access to a palatable, energy-rich diet (usually high in fat content, HFD). Ideally, mice are housed individually, but if space and cost are limiting, they can be group-housed. In the latter case, food intake of individuals cannot be obtained; it may take longer for animals to become obese; and variance in weight gain may increase due to the influence of a social hierarchy among cage-mates. The starting age varies depending upon the questions being asked. Waiting until the mice are mature (7-8 weeks of age) may result in more rapid development of obesity than starting animals at weaning, as more calories can be stored as fat as opposed to used to fuel growth. Sex is also an important consideration since female mice are often more resistant to develop DIO than males (Nishikawa et al., 2007). Finally, background strain is a key consideration, and relevant information is available on many of the sites listed in **Table 1**.

Numerous obesity-inducing diets are commercially available that vary in the proportion and source of ingredients (e.g., saturated vs. unsaturated fats). Alternatively, mice can be offered "cafeteria diets," with access to a variety of palatable, energy-dense foods (e.g., (Gault et al., 2007)), and energy-rich liquid supplements such as sucrose solutions or nutritionally complete liquid diets may also be used. One advantage of commercially-available purified diets is that micronutrient and vitamin levels are matched between the experimental HFD and the corresponding purified low-fat control diet. Cafeteria diets have the advantage of more closely mirroring human diets. Standard laboratory chow is often used as a control diet for commercially available or in-house generated HFDs, and while such diets are usually nutritionally balanced, they may not be matched to an experimental diet with respect to micronutrient or vitamin levels. Standard laboratory chow is not purified and consequently susceptible to batch-to-batch variation both micro- and macronutrient composition. Further, lab chow typically has more plant-based fiber than purified diets, which are often rich in simple carbohydrates such as sucrose. Using matched HF and low-fat diets (LFD) is particularly important when assessing parameters of glucose homeostasis since different dietary carbohydrates may affect the pattern of insulin secretion. Using matched diets is more costly, however, and mice often gain weight more rapidly on purified LFDs than on standard chow, thus prolonging the time for differences of body weight between HFD and LFD groups to become apparent. A final consideration is that unpurified diets are often made with soybean meal, a source of phytoestrogens and isoflavones which may affect reproductive or other endocrine systems (Brown and Setchell, 2001).

Pair-Feeding

When designing a pair-feeding study, the timing of measures obtained from animals in different groups often has to be staggered such that the pair-fed group is offered the amount of food eaten by the comparison group on the previous day. An important consideration in designing such studies is that if the pair-fed animals are provided a daily amount of food that is well below what they would voluntarily consume, they often consume all of the provided food soon after it is made available and then are effectively fasted until the next day's allotment is provided; such animals are said to be meal-fed. Thus, it is important to consider that if body weight or metabolic measurements are taken just prior to the allocation of food, these may be significantly

confounded by the extended fasting period in pair-fed, relative to experimental mice. Similarly, if the same measures are made immediately after feeding, the results may be confounded by the recent consumption of a relatively large nutrient load. These consequences become progressive over time, in parallel with the progressive deficit in energy balance (due to restricted access to food). Efforts to minimize these effects include dividing the amount of food the pair-fed mice are to receive on a given day into two or more allotments dispersed at different times; e.g., part at the onset of the dark cycle and another at the onset of the light cycle. Another important consideration is that pair-fed/meal-fed rodents typically become entrained to their feeding times, and this in turn can shift the secretion of metabolic hormones (Drazen et al., 2006). Ideally, the best way to pair-feed mice is with "yoke" feeding, where pair-fed mice receive food at the same times that experimental mice actually eat. Consequently, pair-fed mice are able to consume meals in a more physiological manner and time and are not susceptible to abnormal periods of fasting. However, yoked feeding requires the use of automated feeding systems which are not available to most investigators.

It is essential that food restriction studies be performed in animals that are individually housed to avoid confounding issues related to social hierarchy within a cage. Otherwise, a dominant animal will consume the majority of the food available, and the less dominant animals will effectively be starved. Furthermore, as with fasting, issues relating to stress are important confounders to be aware of. A detailed discussion of the physiological and welfare issues relating to food restriction in rodents can be found in (Rowland, 2007).

Identifying and reducing sources of experimental stress

Since individual housing can also be stressful, animals should be exposed to this environment well in advance of the start of an experiment so that they can adapt to both the new cage as well as to the associated social isolation. Animals that come from distant institutions and vendors need a minimum of one week to recover from the stress of shipping and to acclimatize to the new facility prior to any other manipulations. Additionally, habituation to handling is critical for studies investigating the effect of an experimental intervention in which mice are removed from their home environment (e.g., for drug administration, or determination of energy expenditure) to reduce the possibility that the effect of the intervention is masked by a stress response (Abbott et al., 2006; Halatchev et al., 2004). We recommend daily handling of mice; i.e., several minutes per day for at least 4-5 days prior to the start of the experiment, using conditions that best mimic the experimental procedure, and performed consistently by a single member of the research team. For example, if mice are to receive intraperitoneal (i.p.) injections as a part of the experimental protocol, the habituation period would ideally include injecting each mouse with sterile isotonic saline i.p. on several consecutive pre-experimental days. During this habituation period body weight and food intake should also be recorded daily as means to monitor stress levels (food intake can only be assessed if the mice are housed individually). Early in the habituation period, for example, food intake and body weight may decrease, but as the animals become more accustomed to daily handling their food intake and body weight will stabilize, and this new steady-state should optimally be established for several days prior to the start of the experiment. The length of the habituation period needed may vary depending on the level of innate stress in different strains or genotypes of mice, and stabilization of body weight and food

intake are relevant and easily obtained indicators of the length of the habituation period required. Finally, as even a clean home cage can represent a novel environment, cage changing should be avoided whenever possible during experiments; alternatively, their effects should be anticipated in chronic experiments where cage changing is necessary for husbandry purposes, and key manipulations should be avoided on those days. Ideally, experimental animals should be housed in a room entered only by a single investigator, since general housing rooms are associated with noise during cage changes or experimental manipulations of other animals that can be a source of stress.

Conditioned Taste Aversion

In a typical CTA paradigm, the administration of a test compound is "conditioned" or temporally associated with the consumption of a solution containing a novel palatable flavor (often a weak saccharin solution) during a training session in water-deprived mice. After the mouse has consumed the flavored solution for 15 or 30 min, either the test compound or its vehicle is then administered. On a subsequent test day (typically one or two days later), the animal is presented with two bottles, one that contains water and the other containing the novel flavor. The ratio of the consumption of the flavored solution to total fluid consumption (water plus flavored water) is calculated and compared between the test compound and vehicle groups. If mice administered the test compound consume less of the flavored water, this outcome is interpreted as evidence of a CTA to the novel flavor as a consequence of receiving the test compound, implying that the compound caused them to be ill.

There are several accepted variations of the paradigm. In one, several pairings of the novel flavor with the test compound (or with the vehicle in control mice) can occur prior to the two-bottle test. In another, the mouse receives the test compound after drinking one flavor (called Flavor A) on one day, and receives the vehicle after consuming a second distinct novel flavor (Flavor B) on a separate day. On the test day the animal is presented with both novel flavors and the ratio of the volume of flavor A (linked to treatment or vehicle) to flavor B is calculated. The experimental paradigm is summarized in **Table S1**. If a drug treatment induces nausea or a sickness-like feeling following its administration, the animal associates this with novel flavor A and avoids consuming it again on the test day, instead preferring to consume flavor B. As with the previously described paradigm, the ratio of the flavored solution A to flavored solution B is calculated and compared between the test compound and vehicle groups to determine if a CTA developed to flavor A due to the test compound. An advantage of this paradigm over the single-flavor paradigm described above is that both the vehicle and the test compound are associated with novel flavors and the investigator is able to condition different mice to either flavor A or B within the experiment thereby reducing any potential confounding effect that bias or neophobia (fear of novelty) might have on the response to a particular flavor.

In both paradigms, the animals are water-deprived overnight prior to presentation of the novel palatable flavors during the training sessions as well as on the test day to stimulate fluid consumption. Numerous and varied flavors have been used for these studies, including saccharin or 20% sucrose. It is preferable to use non-caloric distinct flavors, such as saccharin-sweetened Kool-Aid®, to avoid the potential impact of differences of energy intake on study outcomes. On

test days, the two bottles are randomly assigned to the left and right-hand sides of the cage. Training and testing are preferably not conducted on consecutive days to give the animal an opportunity to rest and to minimize stress caused by individual housing, handling and injection as described above. During this period the animals should have two water bottles available in their cage and daily food intake and body weight should be recorded. Examples of studies using taste-aversion paradigms in mice can be found in (Halatchev and Cone, 2005; Lachey et al., 2005). Intraperitoneal lithium chloride (LiCl; 65mg/kg) administration is known to both reduce food intake and produce an aversive response (and a CTA) and is often used as a positive control.

Supplemental Table 1. A sample two-bottle conditioned taste aversion paradigm

	Habi-tuation	*Training*								*Test*
Day	**1-5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Bottle 1	H_2O	Flavor A	H_2O	H_2O	H_2O	H_2O	H_2O	Flavor B	H_2O	Flavor A
Bottle 2	H_2O	H_2O	H_2O	Flavor B	H_2O	Flavor A	H_2O	H_2O	H_2O	Flavor B
Treatment	Vehicle	Vehicle Or drug	Vehicle	Vehicle Or drug	Vehicle	Vehicle Or drug	Vehicle	Vehicle Or drug	Vehicle	None

Pica (kaolin/clay consumption)

Increased kaolin (clay) consumption is a form of pica that can be used as a surrogate measure for nausea in rodents, and while it has been used extensively to assess the effects of chemotherapeutic agents, its utility as a true measure of "sickness-like" behavior has been questioned (Liu et al., 2005). As with all feeding studies, animals should first be habituated to single housing and to handling/injections prior to testing. During the habitation and test periods animals should have access to both their standard diet as well as to kaolin pellets, and their daily intake of food, kaolin and water should be recorded. Kaolin pellets are available from commercial vendors and should be similar in size to the chow pellets. After mice establish a stable baseline of kaolin intake for at least 3 days, testing can be performed with a dark-phase or fasting-induced feeding paradigm except that both chow and kaolin pellets are available to the animals. Chronic studies should only be performed using the nocturnal paradigm as repeated fasting on consecutive days is not recommended. Examples of pica test paradigms are presented in Supplemental **Table 2**. As with CTA paradigms, LiCl can be administered as a positive control, although the chemotherapeutic agent cisplatin (5 mg/kg i.p.) has been more extensively characterized in this assay as a positive control. Studies that illustrate the use of pica to measure sickness are available (Sun et al., 2007; Yamamoto et al., 2004)

Supplemental Table 2. An example of a pica test paradigm

	Habituation	*Acute Test*	*Chronic test*		
Day	**1-5**	**6**	**7**	**8**	**9**
Hopper 1 contents	Chow	Kaolin	Chow	Kaolin	Chow
Hopper 2 contents	Kaolin	Chow	Kaolin	Chow	Kaolin
Treatment	Vehicle	Vehicle or drug	Vehicle or drug	Vehicle or drug	Vehicle or drug

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