Flavor-Independent Maintenance, Extinction and Reinstatement of Fat Self-Administration in Mice

Supplemental Information

Supplemental Methods and Materials

Subjects

A total of 35 C57BL/6J (purchased from Jackson Laboratory Bar Harbor, ME) male mice were used. At the time of the experiments animals were 10 to 16 weeks old. All experiments were conducted in accordance with the J.B. Pierce Laboratory and Yale University regulations on usage of animals in research.

Surgical Procedure for Implantation of Gastric Catheters and Microdialysis Guiding Cannulae

Once animals had been anesthetized with an intraperitoneal injection of ketamine/xylazine (100/15 mg/kg), a midline incision was made into the abdomen. The stomach was exteriorized through the midline incision and a purse string suture was placed in its non-glandular region, into which the tip of MicroRenathane tubing (Braintree Scientific Inc., Braintree, MA) was inserted. The purse string was tightened around the tubing, which was then tunneled subcutaneously to the dorsum via a small hole made into the abdominal muscle; a small incision to the dorsum between the shoulder plates was then made to allow for catheter exteriorization. Incisions were sutured and thoroughly disinfected and the exterior end of the catheter plugged. Immediately after the above procedure, the animal was placed on a stereotaxic apparatus (David Kopf) under constant flow of ~1% isoflurane anesthesia (1.5 L/min) and a

circular craniotomy was drilled at AP = 1.3 mm, ML = ±1.3 mm implantation of a guide cannulae [DV = -0.5 mm from brain surface] for posterior insertion of a microdialysis probe into the dorsal aspect of the striatum (final probe tip positions [DV = -2.5 mm from brain surface]).

Stimuli and Calculation of Caloric Densities

Mice were trained to obtain intra-gastric infusions of a fat emulsion (30% IntraLipid®, Baxter Healthcare, Deerfield, IL). The emulsion contains as main components 30% soybean oil, 1.2% egg yolk phospholipids, 1.7% glycerin, and water. The caloric density of 30% Intralipid® is 3.0 Kcal/mL, with 2.7 Kcal/mL accounted for by soybean oil and 0.3 Kcal/mL by phospholipids + glycerin. The original 30% emulsion was diluted into an emulsifying control solution (1.2% phospholipids + 1.7% glycerine, in water) in order to prepare the 7.5% and 15% dilutions. The resulting caloric densities of the infusions were as follows. For 30% emulsions: 0.18 Kcal/intra-gastric infusion (see below details on behavioral protocol). For 15% emulsions: 0.099 Kcal/infusion. For 7.5% emulsions: 0.059 Kcal/infusion.

Behavioral Apparatus

Behavioral experiments were conducted in either one of three identical mouse behavior chambers enclosed in a ventilated and sound-attenuating cubicle (Med Associates Inc., St. Albans, VT). Each chamber is equipped with two slots for sipper tubing placements, at symmetrical locations on one of the cage walls. All sippers are connected to a contact-based licking detection device allowing for measurements of licking responses with 10 ms resolution. All lick timestamps were saved in a computer file for posterior analysis. Software-controlled

infusion pumps equipped with TTL input devices were connected to the behavioral chambers and programmed to automatically trigger infusions in response to the detection of licks.

Behavioral Protocol

Mice were trained to produce licks to a dry metallic spout in order to receive intra-gastric infusions of the fat emulsions. The exterior part of the gastric catheter was connected to a segment of MicroRenathane tubing secured to the tip of a 3 mL standard syringe containing the solutions to be infused and mounted on the syringe pump. The syringe pump was placed near a small hole made on the superior part of the sound attenuating box in such a way that mice could move freely inside the behavioral chambers. During the task, a detected dry lick triggered an intra-gastric infusion of the fat emulsion that lasted for 6 seconds at a rate of 0.6 mL/min. However, licks detected while an infusion was taking place had no programmed consequences (i.e. did not result in additional infusions). Experimental tests typically lasted for 1 hour, although 3 hour-long additional tests were performed in a subgroup of animals subjected to microdialysis measurements (see below). Animals were tested once a day in their responses to one single concentration chosen from a pre-determined random distribution, with all animals being tested in all concentrations across days. Specifically, the concentration of the emulsion being infused during any given experimental session (i.e. 7.5, 15 or 30%) was randomly assigned (so that the actual concentration of the infused solution in any given session could not be predicted by the animal), with the assignment sequence being counterbalanced across animals. In order to train the animals in this task, once mice had recovered from surgery and been habituated to the behavioral chambers, a small amount of standard chow was placed behind the spout's orifice (so that they could be smelled but not reached) to prime naive animals to dry lick and

obtain intra-gastric infusions. Training sessions lasted for 1 h and were performed daily under food (20 h) deprivation. After 4 priming sessions, the spouts containing chow were replaced by clean odorless ones. Animals were considered trained to perform the experiments once they showed less than 10% between-session variability, a criterion reached within 10 consecutive sessions.

Extinction and Progressive Ratio Schedules of Reinforcement

The above procedure (1 dry lick triggers one intra-gastric infusion) is referred to in the manuscript as the fixed-ratio schedule of reinforcement. Animals trained in this procedure were also exposed to extinction sessions that were essentially as described above, except that no emulsions were infused into the stomach ("sham infusions"), although pumps were activated as described above (so that animals were able to hear any distinctive noises associated with pumps activation). For progressive ratio schedules of reinforcement, in order to obtain one single infusion trial *t*, animals were required to produce *x* dry licks according to the equation:

Number of dry licks (x) required for reinforcement in trial t: $x(t=1)=1$; $x(t>1)=3\times(t-1)$

The above equation corresponds to a prototypical progressive ratio schedule of reinforcement in which the number of responses required for obtaining one reward unit increased by a fixed increment on each consecutive trial (1, 2). Specifically, after the detection of the first dry lick in the session (and the triggering of one infusion), the requirement for obtaining one intra-gastric infusion increased by 3 dry licks on each consecutive trial. "Break points", defined as the number of dry licks required for the completion of the last rewarded trial (2), was the outcome variable of the progressive ratio experiments.

The progressive ratio schedule of reinforcement protocol described above was adapted to oral self-administration of the same lipid emulsion. A separate group of mice was habituated to orally obtain either 7.5% or 30% IntraLipid by licking the same metallic spouts used during the dry lick tests. For each detected lick, one IntraLipid drop (~20 μL) was delivered via a solenoid valve. To minimize the action of post-ingestive factors on oral intake of IntraLipid, habituation sessions consisted of a fixed-ratio 1 brief-access trial lasting 2 minutes per day (3), performed for 6 consecutive days. After the habituation sessions, the oral progressive ratio task was performed. In the oral progressive ratio task, the number of dry licks required for obtaining the oral delivery of one drop of IntraLipid increased by a fixed increment on each consecutive trial, exactly as specified for the dry lick task. Break points were also computed exactly as in the dry lick progressive ratio task.

Two-dry Sipper Task and Cue Reinstatement

In two additional experiments, the dry lick task was once again used except that two symmetrically positioned dry sippers were presented to a new group of naïve mice. In both cases one of the sippers was set as the active sipper (i.e. detected dry licks triggered intra-gastric infusions) and the other was set as the inactive sipper (i.e. detected dry licks had no programmed consequences). In all cases the first three daily sessions were associated with the placement of chow behind the sippers as described above, in order to prime mice for dry licking. In one experiment, the only cue associated with intra-gastric infusions was the side of the cage to which the active dry sipper was attached. In the second experiment, a 1 sec auditory cue was triggered concomitantly to activation of the infusion pump upon the detection of dry licks to the active sipper. Mice were arbitrarily divided in two groups; one group of mice (named “Cue+/+”) was

presented with the auditory cue during both training and extinction phases, while a second group ("Cue+/-") was presented with the auditory cue during the training, but not during the extinction, phase. Therefore, during 10 training sessions, all mice detected the auditory cue upon infusion pump activation. Extinction tests were immediately performed after the 10 daily training sessions. After three daily extinction sessions, the auditory cue was reinstated for the Cue+/- mice (cue reinstatement was tested in extinction, i.e. in the absence of intra-gastric infusions as above).

Dopamine Measurements during Behavioral Performance

To assess the potential role of the neurotransmitter dopamine in mediating intra-gastric fat self-administration, a subgroup of mice performed the behavioral task as described above concomitantly to collecting microdialysate samples from dorsal striatum. Specifically, during the experimental sessions, microdialysate samples from the freely-moving mice were collected, separated and quantified by high-performance liquid chromatography (HPLC) coupled to electro-chemical detection methods ("HPLC-ECD"). Briefly, after recovery from surgery and behavioral training as above, a microdialysis probe (2 mm CMA-7, cut off 6 kDa, CMA Microdialysis, Stockholm, Sweden) was inserted into the striatum through the guide cannula (the corresponding CMA-7 model). All probes were initially cleaned overnight by perfusion with ultra-pure water. On the day of testing the probes were placed approximately two hours previous to the beginning of baseline sampling, and during this period the brain was perfused with artificial cerebrospinal fluid (CSF). After insertion, probes were connected to a syringe pump and perfused at 1.2 µl/min with artificial CSF (Harvard Apparatus). After a ~120 min washout period, dialysate samples were collected every 6' and immediately manually injected into a

HTEC-500 HPLC unit (Eicom, Japan). Analytes were then separated via an affinity column (PP-ODS, Eicom), and compounds subjected to redox reactions within an electro-chemical detection unit (amperometric DC mode, applied potential range from 0 to ~2000 mV, 1 mV steps). Resulting chromatograms were analyzed using the software EPC-300 (Eicom, Japan), and actual sample concentrations were computed based on peak areas obtained from 0.5 pg/µl dopamine + serotonin standards (Sigma) and expressed as % changes with respect to the mean dopamine concentration associated with baseline (i.e. behavioral task) samples. Baseline level was determined as follows: After the washout period, five samples were collected while the animals were still in their home cages, i.e. previous to insertion into the behavioral boxes. These samples were used to calculate the baseline level for each animal. Then, three additional samples were collected after placement of the animals into the behavioral cage, but before the dry sipper was made available, to confirm that moving the animal from the home cage to the behavioral box did not produce significant fluctuations in baseline levels. These three pre-licking samples correspond to the first three time points shown in the graphics. Fluctuations from baseline were analyzed as within-subject measurements. Locations of microdialysis probes were confirmed histologically.

Statistical Analyses

Data analyses were performed using SPSS (PASW Statistics Release 18.0.0) and made use of linear mixed regression analyses as well as two- and one-way repeated measures analyses of variance. Data are reported as mean ± SEM unless stated otherwise. Kcal and number of dry licks data associated with the learning sessions were fitted with a Boltzmann model $y = A_2 + [(A_1-A_2)/(1 + \exp((x-x_0)/dx))]$ and the corresponding R^2 and EC_{50} values reported.

Supplemental References

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