2. Experimental Protocols

2.1. BAT Taste-Induced Odor Preference

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22007 IACUC Protocol

**Surgical procedures are as follows:**

• Adult rats will be anesthetized by intraperitoneal injections of a ketamine/xylazine mix (25 mg/mL ketamine, 1.33 mg/ml Xylazine in a 7.1/.4/22.5 Ket/Xyl/Saline mixture, [dosage titrated by weight to 100 and 5 mg/kg respectively](https://docs.google.com/spreadsheets/d/1VCzmohddc2-16-rrBvW4RZAsH7gLgklSeU_WLBJ-hE4/edit?usp=sharing)).

Isoflurane gas will be administered through a nose cone with oxygen so that the animal remains deeply sedated (2.5-3% for maintenance following induction). Depth of anesthesia will be monitored using frequent (4/hr) toe pinch—if any response is observed, isoflurane concentration will be increased. Temperature will be monitored (via rectal thermometer); any substantial changes will be compensated for via update of anesthesia or change of temperature of a heating pad placed under the animal through the duration of the surgery. Temperature will be kept at approximately 37C.

• During the surgery, sterile normal saline will be administered subcutaneously (2.75 ml/kg for each hour of surgery) as follows: 1) estimation of surgery length, on the basis of individual surgeon’s experience, 2) calculation of overall fluid dose; 3) delivery of 1/3 total dose at the start of surgery; 4) delivery of 2nd third of dose at the approximate halfway point; and 5) delivery of last third of dose, plus or minus correction, at surgery’s end.

• Alloxate (MELOXICAM) (0.05mL) will be administered subcutaneously to the anesthetized rat prior to incision. The animal will be placed on a standard stereotaxic frame.

• Its scalp will then be shaved and aseptically prepared (3 sequences of a betadine scrub followed by an isopropyl alcohol scrub) for surgery.

• Line blocks at the surgical incision sites will be done with approximately 0.2 mL lidocaine (0.5%) 5 minutes before making the incision. The scalp will then be incised.

• Up to 8 holes (1 mm diameter) will be bored in the skull for ground screws

• Screws will be placed in the skull, a fiber optic probe for delivery of light for optogenetics experiments, which will then be lowered into the brain, guided by stereotaxic measurements. Once in position, the assemblies will be cemented to the skull with dental acrylic.

• The entire surgical procedure takes 4-6 hours. [A log is kept of each surgery- rat #, anesthesia used (type and amount), length of procedure, etc. A separate log devoted to tracking of drug use (type and amount) is also kept with the drugs themselves (i.e., in a locked drawer within a 24-hour locked room).](https://drive.google.com/file/d/1elps5iF3SjYiAKCNg0H3o7N2rdJuNW9W/view?usp=drive_link)

**Virus surgery (optogenetics) for Rats**

Replication-incompetent virus (lentivirus or AAV) will be injected into gustatory cortex, by CITI-trained, Brandeis biosafety- trained lab personnel wearing lab coats, sterile gloves, and masks.

Surgeries will proceed identically to those described above, with the following exceptions. Two small, bilateral craniotomies will be made in the skull over the target region using a dental drill (location identified using cranial markers and stereotaxic measurements), and a glass micropipette containing 500 nL of virus will be then lowered into the brain (depth again guided by stereotaxic measurements). Ejection of virus will be made over the course of 2 min using a microinjector, which will then remain in place for 2 additional minutes (to ensure diffusion away from the pipette tip). Once the pipette is removed, sterile silicone will be used to fill the craniotomies. 5ul of virus over 3 injection sites in GC: D/V: -4.8, 4.6 and 4.4mm

Waste material from the procedure will be immediately placed in a biohazard bag and disposed of. Only persons trained to handle viral constructs or injected animals will be allowed to come in contact with substances or animals. If a cage change is performed in the 3 days during which an animal is considered a BSL-1, then the cage will be autoclaved or disinfected by laboratory personnel when they are empty, and contaminated bedding will be treated as biohazardous waste. Decontaminated cages will be removed immediately from the autoclave when the cycle is finished by trained laboratory personnel.

Trained  personnel will wear appropriate PPE when handling cages or animal bedding and hands will be washed after these procedures. Glass capillaries used for the injection procedure will be placed in a biohazard sharps container immediately after inoculation. Sharps containers will then be autoclaved once two-thirds filled. Gloves and other supplies used during injection will be disposed of into an autoclavable bag within a leak-proof, hard-sided autoclavable container and autoclaved. Laboratory personnel will be responsible for autoclaving all waste, and waste will be removed from the autoclave by personnel as soon as the autoclave cycle is finished.

**Post-operative care/recovery**

Immediately following surgery, adult animals (both rats and mice) will be carefully monitored for signs of postoperative pain (lethargy, ruffled fur, hypersensitivity to touch or noise, hunched back, failure to eat). The Alloxate dose will be repeated once a day for 48 hours. Following surgery, animals will be placed in a recovery cage heated from underneath by a heating pad, with access to water.

The animal will be returned to its home cage only when the anesthesia has worn off (i.e., the animal is awake and has begun moving around the cage). The adult rat will be housed singly: this is an absolute necessity for the chemosensory studies related below, both because (as described below) social interactions can cause confounds in any experiment designed to test taste preferences (as ALL of our studies do), and because group housing would make it nearly impossible to confidently relate our results to those of other labs (all of which single-house their adult rats). Veterinarian- approved environmental enrichment will be placed in home cages.

The animal will be monitored and weighed each of the following 6 days.

**Water Restriction**

Rats will be placed on a water restriction regime while undergoing behavior procedures (both during training and testing phases), to ensure they are motivated to drink during the experiment. In addition to the fluid they receive during daily experimental sessions (between 5 and 8 ml), rats will also receive access to an additional 15 ml of water per day in their home cage.

Note, this restriction will never amount to an entire day without water. This restriction is absolutely standard in behavioral neuroscience studies, daily evaluation and recording of weight will allow monitoring of health; in the unlikely event of weight loss that is larger than 15% normal body weight (as determined by Charles River, the breeder), that particular rat will be removed from the protocol.

**Preference Test**

This paradigm allows us to determine our animals’ preferences to different tastes and concentration ranges, which helps us in accessing the palatability relationship between tastants.

Length of training/ testing: Approximately 7 days.

Animals will be adapted to a variant of the “Davis Rig,” which allows an examination of taste preferences for a small set of odors in a single session. The rig is nominally a 1-lick spout chamber, but the spout is behind a sliding panel in the chamber. A computer- controlled conveyor belt allows any one of 12 actual lick spouts to be positioned behind the panel, which then opens for a period of 10 sec at a time (one “trial”). In adaptation sessions, the animal will learn to wait for the panel to slide out of the way, and then to drink from the proffered spout. Once the animal has learned to approach and lick water, sessions will ensue in which each trial is a randomly selected 1 of 2 odors is offered. An infrared detector, very similar to the beams that signal approach to the doors of many stores, will allow us to know exactly when the animal’s tongue extends to reach the lick spout. At the end of a 60- 90 min session, the animal will have consumed between 5 and 10 ml of fluid, and the resultant data- number of licks for each fluid- will provide us with more information as to the animal’s particular odor preferences.

**Taste-potentiated odor association (TPOA)**

This test investigates how taste and odors interact with one another, which is important when looking at the mechanisms behind taste preferences.

Length of training/ testing: Approximately 6 days.

1) in training sessions, tastes will be presented accompanied by the smell of either Carvone or Cis-3-hexenal diluted to 0.01% in water, a specialized lick spout will be used that emerges from a variant of the “Davis Rig” in a pseudorandom order and 2) a second testing session, in which water is available in the odorized lick spouts, will evaluate any learned aversions for the odor itself.

In a subset of these animals for 3 days prior to the BAT preference test the animal will receive 0.01% of Ethyl Butyrate and Methyl Valerate in their home cage water (50mL of solution per 36 hours).

Handling and care

Per protocol animals must be in vivarium for 1 week prior to any experimental procedures/surgeries.

These can be done days in a row, or with a couple days in between.

Animals should be handled for ~10 min each unless otherwise stated 3-4 times a week.

At least once a week (during cage changes if in early stages of handling) rats should be weighed and logged on the cage card.

If you are not logging your handling sessions by weighing them, you should be writing them in your notebook (good to do both)

After surgery or when on water dep animals must be weighed daily

* For the the first couple days once they arrive don’t handle them
* Handling day 1: first day place them in room they will be used in for 30 min with cage top off, but metal grate on to acclimate to sounds and smells.
* Handling day 2: After adjusting to the room with the lid off you can take off the cage and wait in the room with them. Go on phone, clean up around, do work on laptop etc.
* Handling day 3: Next day can start putting your hand in the cage, near the edge, just letting them get used to you. I like to stroke my thumb along my fingers to make a sound. I always make that sound when I enter the cage and before I pick them up from then on to help them know it’s me and what’s happening/cue them
* If they are receptive to your hand (sniffing, letting you touch them in the home cage) you can start petting them and interacting with them the same day. If not, just leave your hand in there as a benign object that doesn't cause startle/is aversive.
* Handling day 4: Next day repeat the slow hand introduction, interacting with the hand in the home cage. Skittish rats may just be allowed out onto the cart, more ‘sociable rats’ can be pet more and even start to be picked up onto the lap, picked up out of the cage. But they should be given plenty of freedom to return to the cage, leave your lap, or avoid your hand. Important that the rats learn that you and your hand are a positive or neutral stimulus and NOT an aversive one.
* Handling day 5: All rats should be picked up and be spending time on your lap (may be less time for certain rats, but they should still be getting used to the lap, give more skittish ones the option to escape onto the cart)
* Handling day 6+: Final stage of handling! Introduce your hand into the cage to let them know you are there, then pick up and weigh, and pour out into your lap and let them roam/practice injection pinches/practice head touching/give them pets!

I usually start picking up rats with the scoop method then transition into under the shoulders. NEVER pick up by the tail. You may HOLD the tail while you scoop when a rat is hard to corral, but do not pick them up solely by their tail.

Always clean everything with 70% EtOH between animals. Try to do this with some time to air out the ethanol smell before the next animal

Be very careful to look for startle responses like freezing (Whiskers stop moving when they’re startled)

Never interrupt grooming (it’s a coping mechanism for them)

**Introducing touch**

* Touch rats in ways they touch each other.
* Right below the shoulder blades is a good spot to touch.
* Really only use fingers because hands are too big.
* Faster rat like strokes of touching are better.

Be careful of sudden transitions - move slowly when handling them.

You can manipulate them by using your hand to get them comfortable with your hands and maneuvering them. You can make them turn right by using your hand etc use it as a platform they have to touch to get down from or up to some place.

There is a certain amount of pressure you can figure out to put on them to make them immobile without startling them so experiment with how much pressure that is.

Start touching them in ways you might use to pick them up.

Mess with the parts of the body that you want to touch and pick up from

Get them used to the sounds of gloves, any clicking, hand motions that loom, and changes of light in front of them.

Use short pick-ups with the immediate option of them being able to escape/get down.

Include chew block, enviro-pak, and cardboard tube in the cage

Prep

Order animal 1 week before (**7-9 weeks old**)

Handle

Need 2 fibers per animal made, plus extra

Calibrate fibers to laser

Book BAT rig

Have virus needles ready

Put screws in cetylcide

Virus and implant surgery (day 0)

5ul of virus over 3 injection sites

D/V: -4.8, 4.6 and 4.4mm

See [virus\_injection\_surgery\_KM2023.docx](https://docs.google.com/document/d/1leBkRjZqMWSSJCdhcRFKi8UpUYFw1hak/edit?usp=sharing&ouid=101010380107635377469&rtpof=true&sd=true)

Implant fibers in same surgery

1 week daily weighing

2 days post-operative care (melox, bacitracin)

Pre-Exposure Day 12, BAT habituation Day 15, Preference test Day 20, Conditioning Day + laser 21-26, test day 27,28

See: [Isaac Stuff](https://docs.google.com/presentation/d/1ejXYtA3JkyTGgH7K3ABn0VasRurC-oOAu1GH9TW5mQ0/edit?usp=sharing)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| -14  Order rats | -13 | -12 | -11 | -10 | -9 | -8 |
| -7  Rat handling 1 | -6  Rat handling 2 | -5  Rat handling 3 | -4 | -3  Rat handling 4 | -2 | -1 |
| 0  Virus  + implant | 1  post-ops | 2  post-ops | 3 | 4 | 5 | 6 |
| 7 | 8 | 9 | 10 | 11 | 12  pre-exposure | 13  pre-exposure |
| 14  pre-exposure | 15  BAT hab 1  pre-exposure | 16  BAT hab 2  pre-exposure  **Start water dep** | 17  BAT hab 3 | 18  BAT hab 4 | 19  BAT hab 5 | 20  Pre-preference test  60 trials |
| 21  Conditioning  48 trials  LASER | 22  Conditioning  LASER | 23  Conditioning  LASER | 22  Conditioning  LASER | 25  Conditioning  LASER | 26  Conditioning  LASER | 27  Condition Preference Test  60 trials |
| 28  Condition Preference Test  60 trials |  |  |  |  |  |  |

Odor paired with saccharin counterbalanced (mint v grass)

Unpaired (water+odor) and paired order (sacc+odor) start counterbalanced (AB v BA)

1 Animal - Enriched - Paired Odor = Carvone - Order = AB

1 Animal - Enriched - Paired Odor = Cis-3-hexen-1-ol - Order = BA

1 Animal - Enriched - Paired Odor = Carvone - Order = BA

1 Animal - Enriched - Paired Odor = Cis-3-hexen-1-ol - Order = AB

1 Animal - Unenriched - Paired Odor = Carvone - Order = AB

1 Animal - Unenriched - Paired Odor = Cis-3-hexen-1-ol - Order = BA

1 Animal - Unenriched - Paired Odor = Carvone - Order = BA

1 Animal - Unenriched - Paired Odor = Cis-3-hexen-1-ol - Order = AB

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Animal** | **Enriched (E) or Unenriched (U)** | **Enriched Odor** | **Paired Odor** | **AB or BA** |
| GW05 | E | Methyl valerate | Carvone | AB |
| GW06 | U | - | Carvone | AB |
|  |  |  |  |  |

Pre Exposure Begins - Day 12

Make fresh daily.

Use graduated cylinder to measure 100mL of DI/miliQ water. Add to amber 100mL bottle. In fume hood, select odorant. Use p10 to carefully pipette 10ul of odorant into 100mL bottle (pipette up and down to mix). Top 100mL bottle and shake. Label with animal and odor. Remove ad lib water bottle. Place lick spout with ball into amber bottle and into animal cage at same time daily (within the same hour timepoint). Measure how much water is consumed by animal.

Methyl valerate

Ethyl butyrate

Switch which odor is pre-exposed after 2-3 days (e.g. if started with MV, switch to EB)

Add infused water (0.01% concentration of each odor) into its home cage.

50ml of cis-3-hex in 100mL in conical tube with spout, remove ad lib water.

Unenriched also get conical tube and spout.

Remains until water dep starts day 16, end of BAT hab 2.

Habituation (hab) in BAT - Days 15-19

Weigh animal daily

Clean rig thoroughly with 70% EtOH, with 10min to air out in between animals

Fresh lick spouts used for each animal

MiliQ water fresh on hab1

**Hab 1**

Rig habituation,

no shutter or tastants. 30min

**Fan on**

**Hab 2**

Rig habituation,

no shutter or tastants. 30min

**Fan on**

Water restriction starts

**Hab 3**

Licking Habituation-

shutter open with 1 water bottle (stink\_hab3), 30 min

**Fan on**

9am 10-15ml water

**Hab 4**

2 bottles water - 30 trials, 15 each bottle (stink\_hab4)

**Fan on**

9am 10-15ml water

**Hab 5**

2 bottles water - 30 trials, 15 each bottle (stink\_hab5)

**Fan on**

9am 10-15ml water

Make Tastants

For Preference tests (pre and post):

Bottles:

1. 20mL MiliQ water
2. 20mL MiliQ water
3. 20mL 0.01% Carvone - **2μL Carvone and 20mL distilled water**
4. 20mL 0.01% Carvone -  **2μL Carvone and 20mL distilled water**
5. 20mL 0.01% cis-3-hexen-1-ol - **2μL cis-3-hexen-1-ol and 20mL distilled water**
6. 20mL 0.01% cis-3-hexen-1-ol - **2μL cis-3-hexen-1-ol and 20mL distilled water**

→ 48 presentations of 6 bottles (10 presentations each; hab4/5 = 2 bottles presented 15 times)

Number of presentations: 48

Licktime: 5s

IPI: 30s

Maxwaittime: 60s

Session time limit: 100min

Store in glass bottles

\*\* FOR CONDITIONING DAYS, 2 BOTTLE PROTOCOL DESCRIBED BELOW\*\*

Paired odor: in 20mL miliQ (DI water) (2ul paired odor) + 1.37g sucrose

In 50mL miliQ, 5ul paired odor + 3.423g sucrose

Unpaired odor: in 20mL miliQ (DI water) 2ul odor

In 50mL miliQ, 5ul odor

Con 1/3/5 (**paired odor**), bottles:

1. 0.2M sucrose + paired odor
2. 0.2M sucrose+ paired odor

Con 2/4/6 (**unpaired odor**), bottles:

1. 2μL unpaired odor in 20mL distilled water
2. 2μL unpaired odor in 20mL distilled water

→ 30 presentations of 2 bottles (15 presentations each)

Number of presentations: 30

Licktime: 5s

IPI: 30s

Maxwaittime: 60s

Session time limit: 100min

Pre-preference test - Day 20

6 bottles: 2 bottles water, 2 bottles Carvone, 2 bottles cis-3-hexen-1-ol - 48 trials, 10 each bottle (stink\_prepref\_test)

**Fan on**

9am 10-15ml water

Conditioning (con) - Days 21-26 (AB)

**Con 1**

2 bottles **paired odor** - 30 trials, 24 each bottle (stink\_con\_paired1)

**Fan on**

9am 10-15ml water

**Con 2**

2 bottles **unpaired odor** - 48 trials, 24 each bottle (stink\_con\_un1)

**Fan on**

9am 10-15ml water

**Con 3**

2 bottles **paired odor** - 48 trials, 24 each bottle (stink\_con\_paired2)

**Fan on**

9am 10-15ml water

**Con 4**

2 bottles **unpaired odor** -48 trials, 24 each bottle (stink\_con\_un2)

**Fan on**

9am 10-15ml water

**Con 5**

2 bottles **paired odor** - 48 trials, 24 each bottle (stink\_con\_paired3)

**Fan on**

9am 10-15ml water

**Con 6**

2 bottles **unpaired odor** - 48 trials, 24 each bottle (stink\_con\_un3)

**Fan on**

9am 10-15ml water

Post-preference tests - Day 27-28

6 bottles: 2 bottles water, 2 bottles Carvone, 2 bottles cis-3-hexen-1-ol - 48 trials, 10 each bottle (stink\_prepref\_test1)

**Fan on**

9am 10-15ml water

6 bottles: 2 bottles water, 2 bottles Carvone, 2 bottles cis-3-hexen-1-ol - 48 trials, 10 each bottle (stink\_prepref\_test2)

**Fan on**

9am 10-15ml water

Run Experiment

⚠️ See [LASER use SOP](https://docs.google.com/document/d/1wnVBU3T7piEQ9_zCmP_KmQEwbspqm-1_Xjjfc_p7vQs/edit?usp=sharing) for detailed laser use instructions

Prep

1. Make odors daily (paired or unpaired), Use miliQ (di-H2O)
2. Turn on laser, but do not turn key to “on” yet. Let it warm up for at least 15min
3. Fill bottles appropriately labeled with tastant at least ½ up (bottle 1- MiliQ, bottle 3-Carvone, etc.)
4. Make sure air bubble is out of the spout and liquid comes out of the spout when you dot it on your hand
5. **Weigh bottles (on side so air bubble stays out of spout) before experiment and note weight in notebook**
6. Double check bottle numbers and clear and place in appropriate door (1 is left most slot)
7. Turn the fan on by plugging the orange pin into any port by the red line (check it is on by placing your hand below the fan).

**A white mouse with black text

Description automatically generated**Computer setup

1. Use icon with ‘data collection’ to start
2. Select .pro file (or hab4 or test or test\_1, etc.)  from drop-down menu and press ‘show’ to check everything is correct
3. ‘Test Hardware’ and check licks are registering by touching metal (completing circuit, seeing licks are counted in the program), check it moves to the correct door#, door opens and closes
4. ‘Exit’
5. Enter animal ID
   1. Animal ID MUST BE 4 characters. E.g. if animal is BT5 input animal ID as ‘BT05’’

Computer setup for LASER days

1. Open VNC viewer for the BAT pi
2. In Desktop right click on “shutter\_lick\_laser\_codes” and select “open in terminal”
3. Terminal should open, enter the code as follows:
   1. Sudo ipython
   2. From Laser\_lick\_trigger  import \*
   3. laser\_trigger()
4. It will ask for the sequence of your bottle presentations. Open the \*.pro in notepad and copy the sequence into the easygui box, i.e. stink\_con\_paired1.pro sequence is: 2,1,2,1,2,2,1,1,2,2,1,2,2,2,1,1,1,2,1,1,2,1,2,1,2,2,1,2,1,1

A screenshot of a computer

Description automatically generated

1. Enter animal ID, but do not enter unless all davis rig program is set up

Animal in

1. Retrieve the animal, weigh the animal
2. For test days:
   1. Place the animal in your lap and conn ect the optic fibers to the laser lines, noting which laser line is on which side of the head.
3. Place animal in box and secure lid
4. Tape off part of the roof of box so not a t-shape, just a line
5. For test days:
   1. Adjust laser output to match the optic fiber with the lowest output (if fiber 2 needs 750 to put out 40mW, and fiber 1 needs only 600, then set it to 750). **Turn key to ON**
6. Press ‘Run’
7. Wait until animal licks to leave. Set a timer for 25 min (alternatively, watch pi on VNC viewer)
8. Return to check 30 presentations have occurred
9. Remove animal by simultaneously pulling out grate and animal
10. Save data
    1. MMDDAn##\_TEST#
    2. MMDDAn##\_HAB#
    3. Pull data from computer with a flashdrive

a.   Just need the .txt files (not .MS8)

1. Pull laser trials off of BAT pi
2. Turn laser key off, then laser off
3. Weigh bottles
4. If any bedding is in the box remove with dust blaster, careful not to send any bedding into gears
5. Clean every surface with 70% etoh, careful around shutter
6. Remove spouts from doors, remove from bottle and either replace with clean spouts for the next animal or clean spouts with 70% etoh then water
7. If running a second animal make sure to remove air bubble again