

Testing Effects of Chronic Chemogenetic Neuronal Stimulation on Energy Balance by Indirect Calorimetry

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[Abstract] The fundamental of neuroscience is to connect the firing of neurons to physiological and behavioral outcomes. Chemogenetics enables researchers to control the activity of a genetically defined population of neurons *in vivo* through the expression of designer receptor exclusively activated by designer drug (DREADD) in specific neurons and the administration of its synthetic ligand clozapine N-oxide (CNO) (Sternson and Roth, 2014). Using stimulatory Gq-coupled DREADD (hM3Dq) in mice, we showed that leptin receptor (LepRb)-expressing neurons in the preoptic area (POA) of the hypothalamus are warm-sensitive neurons that mediate warm-responsive metabolic and behavioral adaptations by reducing energy expenditure and food intake (Yu *et al.*, 2016). We also used DREADD technology to test effects of chronic stimulation of POA LepRb neurons on energy expenditure, food intake, and body weight with the TSE indirect calorimetry system. Here we describe the detailed protocol of how we used indirect calorimetry to study the outcome of chronic stimulation of POA LepRb neurons. This protocol can be adapted to study long-term metabolic and behavioral consequences of other neuronal modulations, with possible modifications to the type of DREADD, duration of CNO treatment, or method of CNO delivery.

Keywords: Chemogenetics, DREADD, Energy expenditure, Food intake, Indirect calorimetry, TSE

[Background] The POA is a central hub for body temperature homeostasis, which receives thermosensory information from the periphery and tunes the degree of sympathetic output to brown adipose tissue (BAT), cutaneous blood vessels, and heart to control the amount of heat generation and dissipation (Nakamura, 2011). We discovered that LepRb neurons in the POA are stimulated by warm ambient temperature and mediate warm-adaptive responses that include suppression of BAT thermogenesis and food intake (Yu *et al.*, 2016). This discovery was made mainly through chemogenetic stimulation of POA LepRb neurons by virally expressing Gq-coupled DREADD, hM3Dq, in POA LepRb neurons and injecting CNO in mice. A single IP injection of CNO can stimulate target neurons up to 10 h (Krashes *et al.*, 2011; Rezai-Zadeh *et al.*, 2014). This long-lasting CNO effect allows researchers to modulate target neuron activity chronically with two IP injections of CNO per day.

Optogenetics and chemogenetics have revolutionized the field of neuroscience by providing tools to selectively manipulate target neuron activity with its own unique advantages and disadvantages. For studying neurons that modulate energy balance, researchers often use an indirect calorimetry system to simultaneously measure energy expenditure and food intake for an extended period of time

(Rezai-Zadeh *et al.*, 2014; Correa *et al.*, 2015; Qualls-Creekmore *et al.*, 2017). Chemogenetics is best suited for this type of study thanks to slow clearance of CNO from the body and no requirement of optical devices as in optogenetics. In our study, we investigated consequences of chronic stimulation of POA LepRb neurons by DREADD with the PhenoMaster indirect calorimetry system. CNO was injected at 0.3 mg/kg twice per day for six days, and energy expenditure and food intake were continuously measured every 25 min. Body weight was measured once every morning to monitor how changed energy expenditure and food intake affected body weight. In this protocol, we describe a step-by-step procedure of using the TSE PhenoMaster indirect calorimetry system to measure energy expenditure and food intake during chronic stimulation of POA LepRb neurons by DREADD in mice.

Materials and Reagents

1. ½ CC Lo-dose U-100 insulin syringe 28 G ½ (BD, catalog number: 329461)
2. 1.5 ml microcentrifuge tube (SARSTEDT, catalog number: 72.690.301)
3. *LepRb-Cre* mice

Notes:

- a. *In-house bred and derived from original breeders kindly provided by Dr. Martin Myers, Jr., University of Michigan (Leshan et al., 2006); that were injected with AAV5-hSyn-DIO-mCherry (Vector Core, University of North Carolina at Chapel Hill) in the POA (control group, 3 month old, 3 males and 2 females, n = 5).*
 - b. *LepRb-Cre mice that were injected with AAV5-hSyn-DIO-hM3Dq-mCherry (Vector Core, University of North Carolina at Chapel Hill, kindly made available by Dr. Bryan Roth) in the POA (experimental group, 3 month old, 3 males and 2 females, n = 5).*
 - c. *All experiments were approved by the Institutional Animal Care and Use Committee at Pennington Biomedical Research Center.*
4. CNO (Sigma-Aldrich, catalog number: C0832)
 5. Saline/0.9% NaCl (B. Braun Medical, catalog number: L8001)
 6. CNO stock (see Recipes)
 7. CNO working solution (see Recipes)

Equipment

1. PhenoMaster (TSE systems)
2. Scale (Ohaus, Scout-Pro, model: SPE601)
3. Vortex Mixer (American Scientific Products, catalog number: S8223-1)

Software

1. LabMaster V5.0.8 (TSE systems)
2. Excel 2010 (Microsoft)
3. SPSS 22 (IBM)

Procedure

A. Setting up the PhenoMaster TSE system

1. Measure body weight of each mouse that will be used for the experiment.

Note: Age of mice was matched to eliminate the confounding effect of age on energy expenditure and food intake and to minimize the body weight difference between mice. At the beginning of the experiment, mouse body weight ranged between 20 g and 30 g. It is important to use mice with similar body composition to minimize errors during data analysis when body weight is used to normalize energy expenditure and food intake.

2. Open LabMaster and calibrate gas sensors for O₂ and CO₂, and weight scales for food and water according to manufacturer's manual. It is not necessary to turn on the system before calibration.
3. Turn on climate chambers and set the temperature at 23 °C (Figure 1).



Figure 1. The PhenoMaster TSE system. One climate chamber can harbor 6 boxes that can measure O₂, CO₂, food intake, water consumption.

4. Transfer mice to housing boxes (1 mouse/box; Figure 2) and securely seal lids using 2 latches per cage (one front and one back; Figure 2).



Figure 2. A mouse housing box for the PhenoMaster TSE system. Each box is equipped with tubing for gas in and out, weight sensors for water and food, and an infrared light beam frame for locomotor activity.

5. Fill water bottles with tap water and food hoppers with regular chow, and hang them at corresponding weight sensors. It is important to check each water bottle and food hopper for good water flow and accessibility of food pellets, respectively.
6. Under the 'Setup' tab in LabMaster, input animal IDs, body weight, and set up other parameters such as a measurement interval. In our experiment, the measurement interval was 25 min.
7. Under the 'Measurement' tab, select 'Start' to begin measurements.

B. Daily injections and body weight measurement

Notes:

- The room had a 12:12 light:dark cycle with the light on at 7 AM and off at 7 PM.
- Whenever opening a box to measure body weight, refill water or food, or perform injections, it is important not to open the lid while the system is measuring that specific box to prevent outside air from coming into the box. It is safest to open the lid of a box right after the system has just finished measuring that box. Select 'Status > Calo' in LabMaster to identify the box the system is actively measuring.
- Before opening the box, select 'Status > Trial Monitor Drink/Feed' in LabMaster to open the box monitoring screen (Figure 3). Right click on the green circle underneath a box number, and then select 'Pause Box' to temporarily pause measurements of that box (Figure 3). The circle turns yellow. Once the box lid is back on and sealed, right click the same circle and select 'Continue Box' to resume measurements (Figure 4).
- Once a week during the experiment, replace dirty mouse box bottoms with clean ones and refill water bottles with fresh water.
- When an accident happens, such as opening of a box while the system is reading the same box, data from that time point for that box should not be used for later analysis.

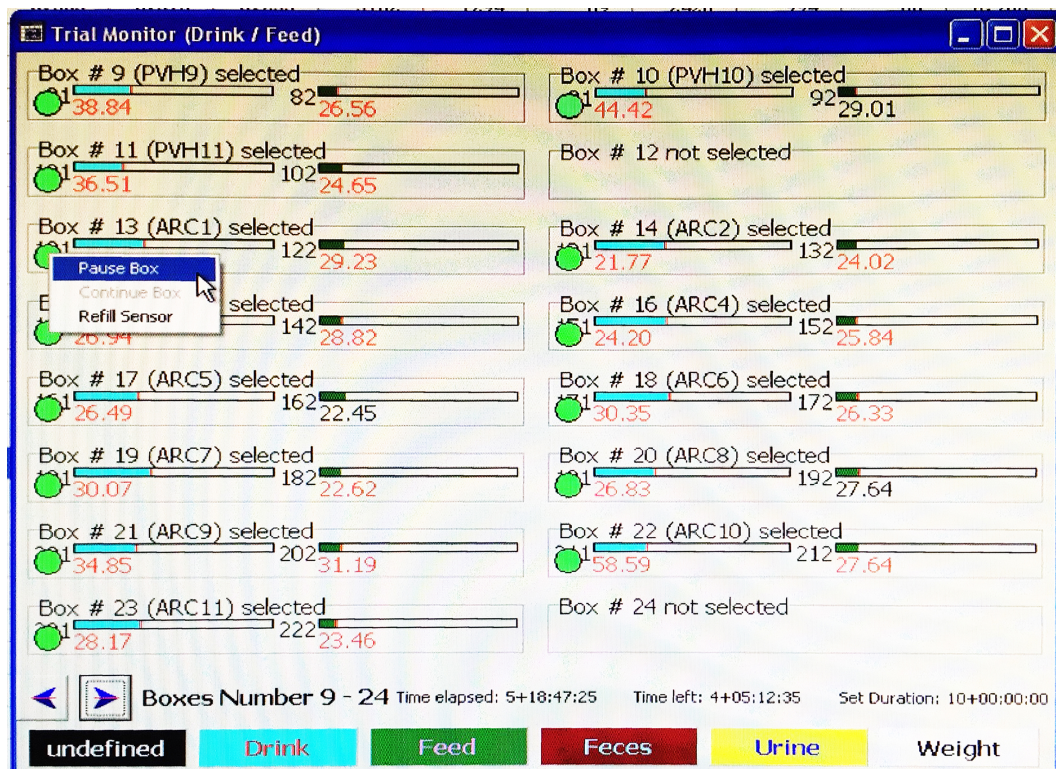


Figure 3. Example image of Trial Monitor Drink/Feed screen. The green circles under box numbers indicate boxes included for measurements. In this example, boxes #12 and #24 are not included in the experiment.

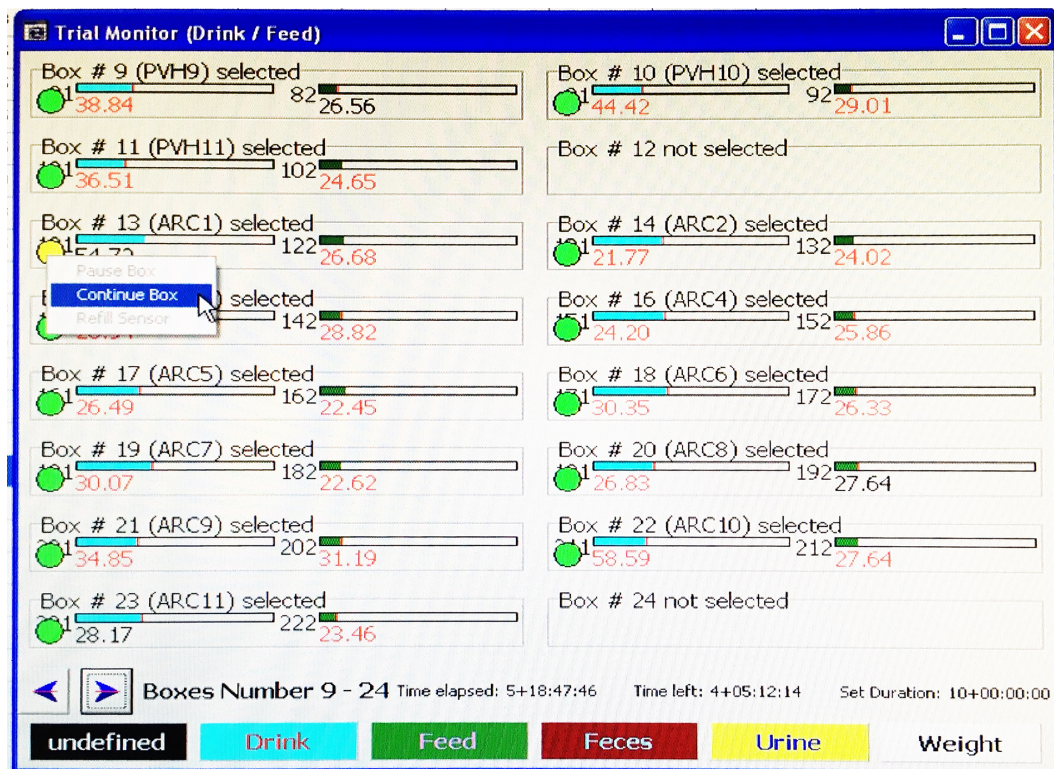


Figure 4. Example image showing how to continue measurements of a temporarily paused box

1. For overall experimental scheme, see Figure 5.

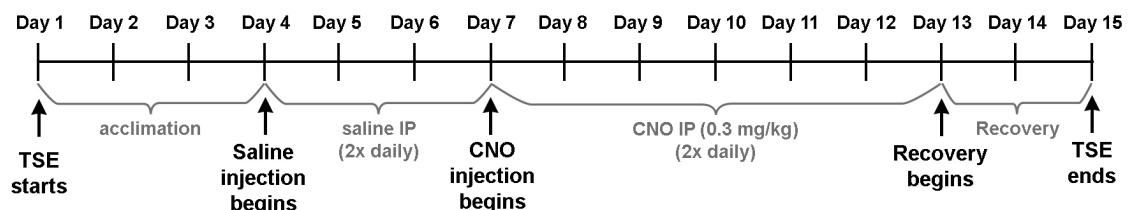


Figure 5. Overall experimental scheme. Mice were first acclimated to a new environment and then injected with 3 days of saline followed by 6 days of CNO. Body weight was measured every morning.

2. Day 1-3: Let mice acclimate to a new environment without any disturbance other than refilling water or food. During this period, if water leaks and floods the bedding, replace the faulty parts (e.g., sipper or entire bottle) and the box bottom.
3. Day 4-15: body weight measured daily at 9 AM for each mouse.
4. Day 4-6: saline IP injection twice daily at 9:30 AM and 6 PM for each mouse. Determine the amount of saline injection by the body weight of the day, such that it matches the amount of CNO working solution (0.06 mg/ml) that would have been needed for that body weight to achieve the dose of 0.3 mg/kg. For example, 20 g mouse receives 100 μ l of saline.

- Day 7-12: CNO IP injection (0.3 mg/kg) twice daily at 9:30 AM and 6 PM for each mouse. The amount of CNO injection is determined by the body weight of the day as described above.
- Day 13-15: mice recover from daily injections (no injection).

C. Finishing the experiment and retrieving data

- On day 14 after morning body weight measurement, stop measurements by selecting 'Measurement > Stop' in LabMaster.
- Take out mice and put them in regular housing cages with water and food.
- To export data, first go to 'View' to select parameters and box numbers to export data.
- Select 'Export > Table', then designate file name and location.

Data analysis

- Open the exported file in Microsoft Excel (Figure 6A).
- Copy energy expenditure (H(1) in kcal/h/kg) and food intake (Feed in g) data to a new tab or file.
- Make a table in Excel for daily body weight (Figure 6B).

A

Date	Time	Box-1 H(1)	Box-2 H(1)	Box-3 H(1)	Box-4 H(1)	Box-5 H(1)	Box-8 H(1)	Box-9 H(1)	Box-10 H(1)	Box-13 H(1)	Box-15 H(1)
Stop		[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]	[kcal/h/kg]
4/4/2014	15:01	18.647	15.857	17.133	21.037	19.529	18.514	23.556	17.769	19.716	22.151
	15:26	21.838	19.252	18.655	25.799	21.972	19.514	22.628	16.136	19.34	16.536
	15:51	21.801	18.696	15.306	27.076	19.957	24.893	19.15	15.384	14.374	15.109
	16:16	19.047	18.117	14.474	26.194	17.396	21.826	18.849	15.957	14.706	21.008
	16:41	15.475	18.065	13.83	19.572	16.102	22.021	20.844	14.36	13.249	21.548
	17:06	14.425	20.646	17.636	17.972	16.344	18.377	23.009	18.678	14.648	16.48
	17:31	14.735	21.372	13.822	16.809	14.286	25.278	20.122	20.583	14.379	15.973
	17:56	21.967	19.007	13.681	25.837	15.47	23.337	22.665	24.014	14.65	18.831
	18:21	17.11	21.269	14.682	23.974	20.726	22.286	17.598	16.936	19.643	16.595
	18:46	17.82	22.006	17.549	19.389	16.571	21.116	15.05	19.042	20.151	22.023
	19:11	22.505	21.347	14.252	24.708	21.867	24.299	22.739	21.444	21.194	21.671
	19:36	25.039	23.753	26.513	28.553	20.635	25.472	18.046	21.124	22.515	25.306
	20:01	26.709	20.619	23.675	26.205	22.887	21.394	21.203	18.341	22.774	23.094
	20:26	25.313	23.298	23.471	23.959	18.058	22.127	16.152	21.161	25.425	23.27
	20:51	26.806	23.103	21.173	19.99	19.049	23.156	22.478	22.903	21.813	16.323
	21:16	24.778	19.7	27.721	28.249	21.323	22.537	20.335	22.346	22.071	29.337
	21:41	24.266	23.334	24.378	22.28	21.205	20.149	24.626	20.718	24.272	24.841
	22:06	25.071	26.837	20.419	20.12	19.155	24.462	19.299	20.042	21.891	23.248
	22:31	25.856	23.281	28.731	25.675	20.461	18.236	20.176	27.58	21.419	22.817
	22:56	27.46	23.549	22.102	27.586	19.006	23.787	22.814	16.236	23.271	22.985
	23:21	27.411	20.465	22.652	18.705	21.361	22.77	21.085	19.722	21.887	24.63
	23:46	23.177	22.508	32.429	20.669	22.225	27.22	23.726	16.223	22.384	22.464
4/5/2014	0:11	22.539	22.203	27.312	26.764	21.206	25.632	23.359	17.94	20.859	17.537
	0:36	16.976	23.258	24.253	28.194	17.847	23.593	22.934	20.398	18.558	15.259
	1:01	19.76	23.217	27.137	28.347	24.219	25.461	21.42	17.443	18.994	16.922
	1:26	23.37	22.176	28.635	25.136	22.49	22.575	21.356	18.504	14.044	18.423

B

< BW change (g) >

Animal ID	Saline				CNO				Recovery												
	4/4/2014	4/7/2014	4/9/2014	4/10/2014	4/11/2014	4/12/2014	4/13/2014	4/14/2014	4/15/2014	4/16/2014	4/17/2014	4/18/2014									
Leptin-Cherry-Con mPOM	30.3	29.9	-0.3	30.2	0	29.9	-0.3	30	-0.2	30	-0.2	29.8	-0.4	30.2	0	30.1	-0.1	30.5	0.3	31	0.8
Leptin-Cherry-Con mPOM	29.6	29.5	0.1	29.8	0.4	29.7	0.3	29.4	0	28.9	-0.5	29.3	-0.1	29.2	-0.2	29.4	0	28.9	-0.5	29	-0.4
Leptin-Cherry-Con mPOM	22	22.3	-0.3	22.3	-0.1	22.5	0.1	22.4	0	22.4	0	22.2	-0.2	22.4	0	22.3	-0.1	22.5	0.1	22.4	0
Leptin-Cherry-Con mPOM	23.5	23.5	-0.6	23.4	-0.7	24.2	0.1	24.1	0	23.9	-0.2	23.8	-0.3	23.7	-0.4	23.9	-0.2	24.6	0.5	24.7	0.6
Leptin-Cherry-Con mPOM	25.1	24.5	0.4	24.3	0.2	24.3	0.2	24.1	0	23.4	-0.7	23.8	-0.3	23.7	-0.4	23.5	-0.6	23.6	-0.5	23.7	-0.4
Mean	26.1	25.9	-0.1	26.0	0.0	26.1	0.1	26.0	0.0	25.7	-0.3	25.8	-0.2	25.7	-0.3	26.0	0.0	26.0	0.0	26.1	0.1
SEM	1.7	1.6	0.2	1.7	0.2	1.5	0.1	1.6	0.0	1.5	0.1	1.6	0.0	1.6	0.1	1.6	0.2	1.5	0.2	1.6	0.2
Leptin-DREADD-STW mPOM	26.4	26.2	-0.2	26.7	0.3	26.2	-0.2	26.4	0	26.3	-0.1	26.2	-0.2	26.4	0	26	-0.4	26	-0.4	25.8	-0.6
Leptin-DREADD-STW mPOM	24.7	23.5	-1.5	24.5	-0.5	24.7	0.3	25	0	25	0	24.8	-0.2	24.5	-0.5	24.7	-0.3	24.8	-0.2	24.3	-0.7
Leptin-DREADD-STW mPOM	25.2	25.3	-0.3	25.6	0	26.1	0.5	25.9	0	26.4	-0.2	26	-0.6	25	-0.6	25.8	-1.7	25.5	-2.1	24.5	-1.1
Leptin-DREADD-STW mPOM	25.4	25	0.2	24.6	-0.2	24.8	0	24.8	0	24.6	-0.2	24.3	-0.3	24	-0.8	23.9	-0.9	23.8	-1	24.3	-0.5
Leptin-DREADD-STW mPOM	21	21.2	-0.5	22.1	0.4	21.7	0	21.7	0	21.4	-0.3	20.8	-0.6	20.6	-1.1	20.8	-0.9	20.6	-1.1	21	-0.7
Mean	24.5	24.2	-0.5	24.7	0.0	24.7	0.0	24.5	-0.2	24.3	-0.4	24.1	-0.6	23.9	-0.8	23.7	-1.0	23.8	-0.9	23.9	-0.8
SEM	0.9	0.9	0.3	0.8	0.2	0.8	0.1	0.8	0.0	0.8	0.1	0.9	0.1	1.0	0.2	0.9	0.2	0.9	0.3	0.9	0.2

Figure 6. Example tables for data analysis. A. A part of the energy expenditure data table that was exported by LabMaster. B. The body weight table that shows daily body weight of all mice, group means, and standard errors of the means during the experiment.

- Divide data based on the group and calculate means and standard errors for all time points in each group.
- For body weight analysis, calculate body weight change for each day in reference to the body weight on the first CNO injection day.
- For energy expenditure and food intake analysis, calculate daily means (kcal/g/day) separately for saline injection (3 days), CNO injection (6 days), and recovery (2 days) (Figure 7).
- Perform statistical analysis by repeated measures ANOVA (analysis of variance) in SPSS. Multiple pairwise comparisons were corrected by the Bonferroni method.

< FI/BW (kcal/g/day) - 24hr average >	Saline				CNO							Recovery		
Animal ID	4/7	4/8	4/9	Mean	4/10	4/11	4/12	4/13	4/14	4/15	Mean	4/16	4/17	Mean
LepRb mCherry-Con mPOA9	0.64	0.68	0.64	0.65	0.62	0.57	0.55	0.59	0.59	0.50	0.57	0.54	0.60	0.57
LepRb mCherry-Con mPOA10	0.47	0.47	0.45	0.46	0.42	0.49	0.47	0.41	0.43	0.42	0.44	0.34	0.35	0.34
LepRb mCherry-Con mPOA11	0.52	0.58	0.51	0.54	0.53	0.55	0.29	0.56	0.51	0.49	0.49	0.49	0.53	0.51
LepRb mCherry-Con mPOA12	0.57	0.67	0.66	0.63	0.58	0.58	0.56	0.69	0.75	0.68	0.64	0.66	0.62	0.64
LepRb mCherry-Con mPOA13	0.44	0.50	0.50	0.48	0.40	0.59	0.50	0.55	0.54	0.55	0.52	0.40	0.50	0.45
Mean	0.528	0.581	0.550	0.553	0.509	0.558	0.474	0.560	0.564	0.526	0.532	0.485	0.518	0.502
SEM	0.036	0.043	0.041	0.039	0.044	0.018	0.048	0.045	0.054	0.042	0.035	0.055	0.048	0.050
LepRb DREADD-STIM mPOA33	0.50	0.48	0.51	0.50	0.40	0.38	0.40	0.35	0.41	0.39	0.39	0.42	0.39	0.40
LepRb DREADD-STIM mPOA34	0.63	0.62	0.62	0.62	0.52	0.47	0.39	0.47	0.43	0.46	0.46	0.45	0.40	0.43
LepRb DREADD-STIM mPOA35	0.49	0.72	0.90	0.71	0.51	0.20	0.24	0.15	0.17	0.21	0.25	0.50	0.55	0.53
LepRb DREADD-STIM mPOA39	0.51	0.56	0.47	0.51	0.33	0.37	0.37	0.42	0.47	0.45	0.40	0.65	0.68	0.66
LepRb DREADD-STIM mPOA41	0.63	0.59	0.57	0.59	0.36	0.41	0.41	0.47	0.40	0.44	0.42	0.38	0.50	0.44
Mean	0.553	0.594	0.613	0.587	0.423	0.366	0.363	0.372	0.375	0.393	0.382	0.478	0.505	0.492
SEM	0.032	0.039	0.077	0.038	0.039	0.044	0.032	0.059	0.053	0.046	0.036	0.047	0.053	0.048

Figure 7. The table for daily food intake analysis. Daily food intake was averaged for each treatment condition, and group means were compared.

Notes

Homozygous *LepRb-Cre* mice were used to maximize Cre expression for more efficient viral gene expression.

Recipes

- CNO stock (5 mg/ml)
 - Add 1 ml saline to 5 mg of CNO powder
 - Vortex until the powder is fully dissolved
 - Transfer to a 1.5 ml microcentrifuge tube and keep the stock at 4 °C

Note: Stock CNO is stable at 4 °C for 6 months at least. However, CNO may precipitate over time. Adding a small amount of DMSO (3-10%) may help dissolving CNO and preventing precipitation.
- CNO working solution (0.06 mg/ml)
 - Take out and equilibrate the CNO stock solution to room temperature
 - Vortex to mix and dissolve any possible CNO pellets
 - Dilute the CNO stock 83.3-fold with saline to achieve a final concentration of 0.06 mg/ml.
For example, to make 1 ml working solution, add 83 µl CNO stock to 917 µl saline

- d. Make it fresh on the day of injection and use it as room temperature solution without warming it up to body temperature

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