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Supporting Information for

Dynamic processing of hunger and thirst by common mesolimbic neural ensembles.

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Supporting Information Text

SI Methods.

Experimental model and mouse strains. Male adult mice (8-24 weeks old) were used for experiments. We obtained Drd1-Cre mice (Drd1-Cre120Mxu/Mmjax, stock no. 37156) and wild-type mice (C57BL/6J, stock no. 000664) from the Jackson Laboratory. Drd2-Cre mice were obtained from Dr. E. Azevedo at The Rockefeller University as previously described (25). All mice were maintained in temperature- and humidity-controlled facilities on a 12-h light-dark cycle (light on at 7:00 am) and had ad libitum access to food and water except when noted otherwise. All feeding experiments used standard rodent chow pellets. For fasting or dehydration experiments, mice were overnight fasted or dehydrated (16-24 hours of food or water deprivation). We conducted the behavioral and ad libitum food and water consumption experiments during the dark cycle with the red light as the background light source. For overnight fasting or water-dehydration studies, food or water were removed during light cycle and experiments were conducted during the light cycle. All studies were made using a uniform set of conditions with respect to the light dark cycle.

Viral vectors. The following AAV viruses were purchased from the vector core at the University of North Carolina: AAV5-EF1a-DIO-ChR2-YFP, AAV5-EF1a-DIO-Arch3.0-YFP, AAV5-EF1a-DIO-YFP, AAV5-EF1a-DIO-mCherry. The following AAV viruses were purchased from Addgene: AAV5-DIO-hM3Dq-mCherry and AAV5-DIO-hM4Di-mCherry, AAV1-hSyn-FLEX-GCaMP6s.

Stereotaxic surgery. Mice were used for behavioral experiments 2-3 weeks after surgeries. For in vivo two-photon imaging experiments, mice were used no earlier than four weeks after surgery to allow sufficient and stable GCaMP6s expression. Mice were anesthetized with 3% isoflurane in oxygen, placed in a stereotaxic apparatus (Kopf Instruments) and kept at 1.5% isoflurane during surgery. Viruses were bilaterally injected in the nucleus accumbens core (200-300 nl per side, 100 nl/min) using the following coordinates relative to the bregma: AP: +1.32; ML: ±1.1; DV: -4.25. For optogenetic experiments, optic fibers with 200-μm diameter core (Thorlabs CFML12U-20) were placed 0.3-0.5 mm above the virus injection site. For in vivo two-photon imaging surgeries, in order to obtain sufficient expression of GCaMP6s, 600 nl of virus were delivered (300 nl per coordinate) at AP: +1.22; ML: +1.2; DV: -4.25 and AP: +1.42; ML: +1.2; DV: -4.25 or at AP: +1.32; ML: +1; DV: -4.25 and AP: +1.32; ML: +1.3; DV: -4.25. A gradient-index (GRIN) lens with 1 mm diameter and 4.38 mm length (GRINTECH NEM-100-25-10-860-S) was implanted at the following coordinates: AP: +1.32; ML: +1.2; DV: -4.15, 0.1 mm above the injection site.

In vivo two-photon imaging paradigm. Two-photon (2P) calcium imaging was performed on a Scientifica SliceScope galvo-scanning 2P microscope with a Nikon 16×/0.8 water-dipping objective and a Coherent Chameleon Ultra II Ti:Sapphire laser source. The objective was focused onto the rear image plane of the implanted GRIN lens, so that the excitation laser beam was relayed into the sample by the GRIN lens, and conversely fluorescence was relayed out of the sample and recorded using photon-multiplier tubes in the SliceScope's non-descanned detection head. Microscope hardware and data acquisition was controlled using the ScanImage software (versions 5.5 and 5.6, Vidrio Technologies), which is based on Matlab (The Mathworks). The field-of-view of the microscope was sufficient to record the diameter of the GRIN lens image, 500 μm, at a frame rate of 4.82 Hz. Liquid food (Ensure) and water were provided via a spout connected to a touch detector (lickometer) during the imaging. Mice were adapted to liquid food as the only nutrition source for at least three days before the refeeding experiments.

Optogenetic modulations. For photostimulating ChR2, a 473-nm laser (OEM Lasers/OptoEngine) was used to generate laser pulses (5-7 mW at the tip of the fiber, 5 ms, 20 Hz) throughout the behavioral session, except when noted otherwise, controlled by a waveform generator (Keysight). For Arch3.0 photostimulation, a 532-nm laser (OEM Lasers/OptoEngine) generated constant light of 5-10 mW power at each fiber tip.

Caged object assay. The caged-object zone was manually defined in the Ethovision 9.0 (Noldus) behavioral tracking software, which automatically tracks and evaluates mouse behavior

parameters such as the frequency of visits in the caged-object zone, time spent inside the zone, as well as locomotor activity level (defined as total movement distance divided by total duration of movement). Photostimulation was delivered during each 20-minute session across different conditions: fasted, dehydrated, ad libitum access to food and water. Before behavioral tests, mice were habituated to the transparent jars (without caps) that were pre-filled with food or water gel as containers of available food or water gels for at least three days. On test day, a clean transparent jar containing fresh food chow or water gel was sealed with a cap pre-drilled with multiple 5-mm diameter holes. Caged food or caged water gel was fixed at the corner of a new cage. Mice were introduced and acclimated to the new cage for 5 minutes before experiments. Data were recorded by the software aforementioned.

Histology. Mice were transcardially perfused with PBS followed by 10% formalin. Brains were dissected and post-fixed in 10% formalin at 4°C overnight. Brains were sectioned into 50-μm or 100-μm coronal slices using a vibratome (Leica). For immunohistochemistry, brain sections were blocked (0.1% Triton X-100 in PBS, 3% bovine serum albumin, 2% normal goal serum) and then incubated with primary antibody (rabbit anti-c-fos, Cell Signaling, 1:500 for 100-μm sections, 1:1000 for 50-μm sections; chicken anti-mCherry, Abcam, 1:1000 for 50-μm sections) for 1-3 days at 4°C. Sections were then washed and incubated with secondary antibody (goat anti-rabbit IgG Alexa 488, Invitrogen, 1:500 for 100-μm sections, 1:1000 for 50-μm sections; goat anti-rabbit IgG Alexa 594, Invitrogen, 1:1000 for 50-μm sections; goat anti-chicken IgG Alexa 594, Invitrogen, 1:1000 for 50-μm sections) for 1 hour at room temperature, washed again, mounted with DAPI Fluoromount-G (Southern Biotech) and imaged with a confocal microscope (Inverted LSM 780 laser scanning confocal microscope, 20× objective; Zeiss). Fluorescent in situ hybridization (FISH) was carried out in frozen brain sections (15-20 μm) using the RNAscope fluorescent multiplex kit (Advanced Cell Diagnostics) following the manufacturer's protocols. Images underwent minimal processing (such as adjusting brightness and contrast) performed using ImageJ. The "Analyze particles" tool in ImageJ was used to quantify c-Fos-positive cells. The CellCounter plugin for ImageJ was used to quantify numbers and percentages of cell types from FISH sections.

Data Analysis. Multiple trials across days were all concatenated first, then processed by the Suite2p pipeline with non-rigid motion correction, to extract all neurons recorded across trials and days. Neurons extracted by the pipeline were subsequently curated manually in the Suite2p graphical user interface.

To preprocess the data from food and water gel sensory responses, total fluorescence of each individual neuron was normalized using the formula $z = (Fraw – \mu)/\sigma$, where Fraw is the raw fluorescence as extracted by the Suite2p pipeline, μ is the mean of Fraw during the baseline period (30 seconds prior to food and water gel presentation), and σ is the standard deviation of Fraw during the baseline period. For each condition, neural responses recorded across 3 trials were averaged to obtain neural response vectors.

The data from liquid food and water consumption experiments was preprocessed as follows in order to reduce variabilities for multiple-day comparisons: The raw fluorescence was z-scored according to the formula $z = (F– \mu)/\sigma$, where F is calculated from the raw fluorescence by applying a second order Butterworth filter with normalized cut-on frequency 0.266; μ is the mean baseline fluorescence (taken over the traces with activity below the median during the baseline period), σ is the standard deviation of fluorescence during the baseline period (taken over the traces with activity below the median during the baseline period). For this analysis, neurons with averaged responses larger than 3σ from 10 seconds after consumption start were considered to be activated.

Clustered neural traces during consumption were identified by k-means clustering. The neuronal states based on the k-means clustering label were projected to two-dimensional space using the t-SNE algorithm.

Statistics and reproducibility. P-values for pair-wise comparisons were obtained using the two-tailed Wilcoxon signed-rank test. P-values for comparisons across multiple groups were conducted using ANOVA (with repeated measures when possible) and corrected for multiple comparisons using Turkey's test or Sidak's test, respectively. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. Representative images were selected from 3 to 5 original biological replicates.

Legends for Figures S1 to S5.

Fig. S1 Selective c-Fos induction in NAc core versus shell after short-term refeeding and rehydration. a, Representative images showing c-Fos mRNA expression in NAc shell across different states. Scale bar, 100 μm. b, Representative images showing c-Fos mRNA expression in NAc core across different states, confirming the results obtained by anti-c-Fos antibody staining. Scale bar, 100 μm. c, c-Fos mRNA expression in NAc shell does not differ between fasted versus 0.5h-refed, or dehydrated versus 0.5h-rehydrated animals (n = 3 sections from 3 different mice per group, two-tailed Student's t-tests). d-g, Representative images of c-Fos (green), Drd1 (red), Drd2 (white) mRNA expression in NAc core. Scale bar, 100 μm. All error bars represent mean ± s.e.m. NS, not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All error bars represent mean ± s.e.m. NS, not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. S2 Co-localization of c-Fos+ and ChAT+ neurons after half an hour refeeding in NAc. a, Representative images showing DAPI, c-Fos, ChAT expressions in Nac. b, Percentage quantification of c-Fos+ and ChAT+ neurons versus the others (n = 11 versus 467 neurons pooled from 5 brain slices collected from 3 mice).

Fig. S3 Individual D1 and D2 neurons respond similarly to food versus water. a, Post-check of GCaMP6s expression in NAc core and GRIN lens implantation position. b, c, Example heatmaps of z-scored GCaMP6s fluorescence time traces of D1 and D2 individual neurons with water consumption in thirsty state (n = 120 neurons from one example D1-Cre transgenic mouse, n = 109 neurons from one example D2-Cre transgenic mouse, neurons are session-matched as shown in Fig. 3). All error bars represent mean ± s.e.m. NS, not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. S4 Complementary functions of D1 and D2 neurons regulate goal-seeking behaviors. a, b, c, d, Representative heatmaps showing the spatial distribution of time spent by mice in the cage. Black dashed circles are zones where caged food or caged water gel was located. e, Post-check of NAc core virus expression. Scale bar, 100 μm.

Fig. S5 Validation of chemogenetic perturbation of NAccD1 or NAccD2 on food and water consumption by PBS injections. a, I.p. injections of PBS in D1-Cre:hM3D(Gq) mice does not regulate food or water consumption in physiological ad libitum state compared to the control group received PBS (n = 7, 5 mice per group; two-way ANOVA, with Sidak's multiple comparisons). b, I.p. injections of PBS in D1-Cre:hM4D(Gi) or control mice does not affect food and water consumption in fasted animals compared to the control group received PBS (n = 8, 5 mice per group; two-way ANOVA, with Sidak's multiple comparisons) . c, I.p. injections of PBS in D1-Cre:hM4D(Gi) mice does not affect food and water consumption in water-deprived animals compared to the control group received PBS (n = 8, 5 mice per group; two-way ANOVA, with Sidak's multiple comparisons). d, I.p. injections of PBS in D2-Cre:hM3D(Gq) mice does not regulate food or water consumption in physiological ad libitum state compared to the control group received PBS (n = 10 mice per group; two-way ANOVA, with Sidak's multiple comparisons). e, I.p. injections of PBS in D2-Cre:hM4D(Gi) mice does not affect food and water consumption in fasted animals compared to the control group received PBS (n = 6, 5 mice per group; two-way ANOVA, with Sidak's multiple comparisons). f, I.p. injections of PBS in D2-Cre:hM4D(Gi) mice does not affect food and water consumption in water-deprived animals compared to the control group received PBS (n = 6, 5 mice per group; two-way ANOVA, with

Sidak’s multiple comparisons). g, h, Summary tables of optogenetic and chemogenetic modulation results. All error bars represent mean ± s.e.m. NS, not significant, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. S1

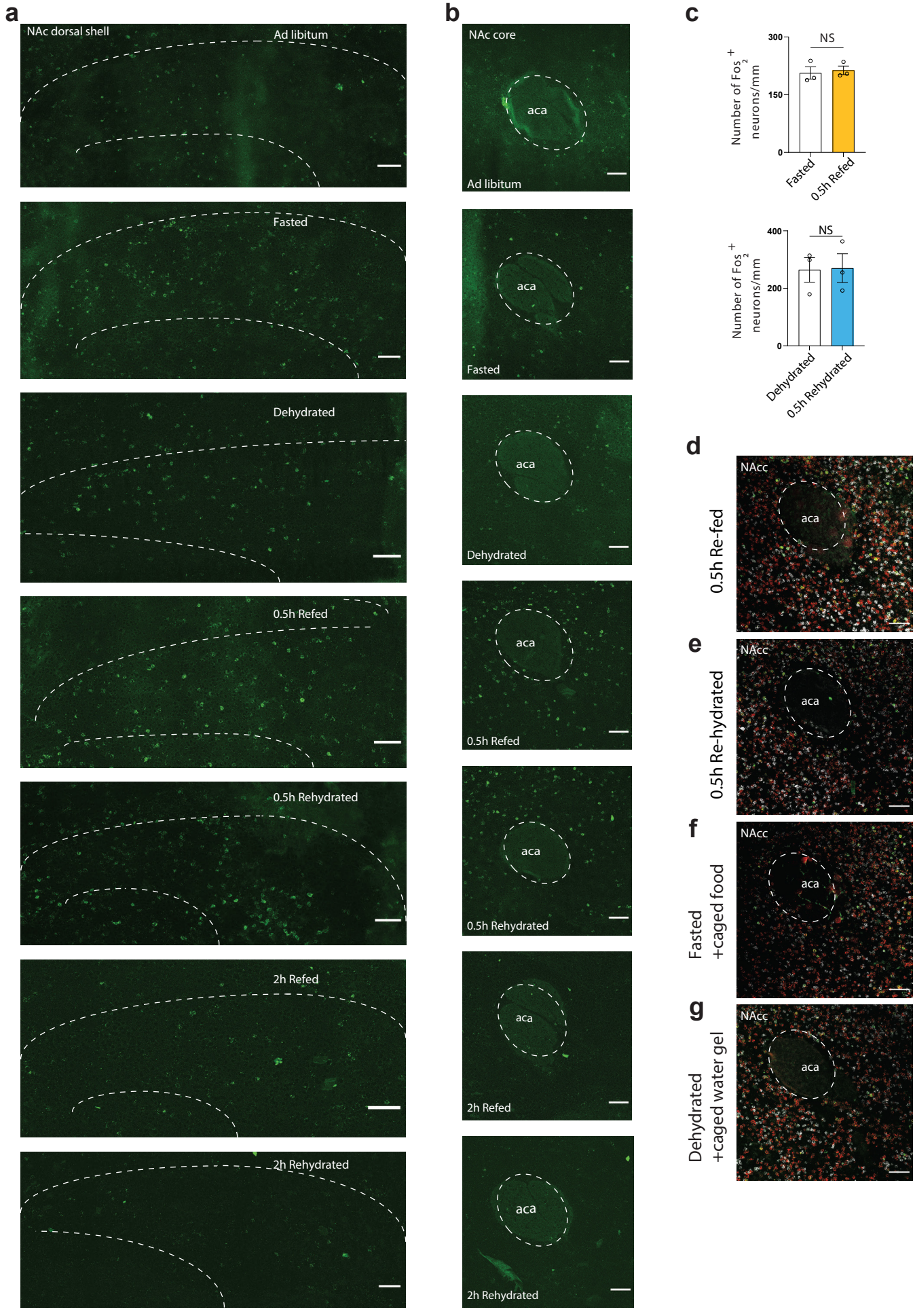

a
NAc dorsal shell
Ad libitum
Fasted
Dehydrated
0.5h Refed
0.5h Rehydrated
2h Refed
2h Rehydrated
b
NAc core
aca
Ad libitum
Fasted
Dehydrated
0.5h Refed
0.5h Rehydrated
2h Refed
2h Rehydrated
c
Number of Fos+ neurons/mm2
300
150
0
NS
Fasted
0.5h Refed
400
200
0
NS
Dehydrated
0.5h Rehydrated
d
0.5h Re-fed
NAcc
aca
e
0.5h Re-hydrated
NAcc
aca
f
Fasted +caged food
NAcc
aca
g
Dehydrated +caged water gel
NAcc
aca

Fig. S2

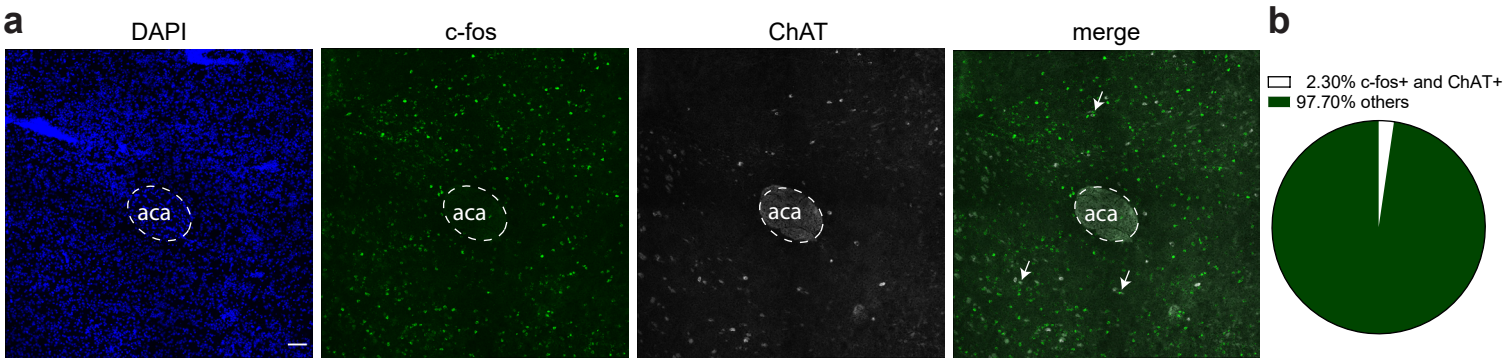

a
DAPI
c-fos
ChAT
merge
aca
aca
aca
aca
b
2.30% c-fos+ and ChAT+
97.70% others

Fig. S3

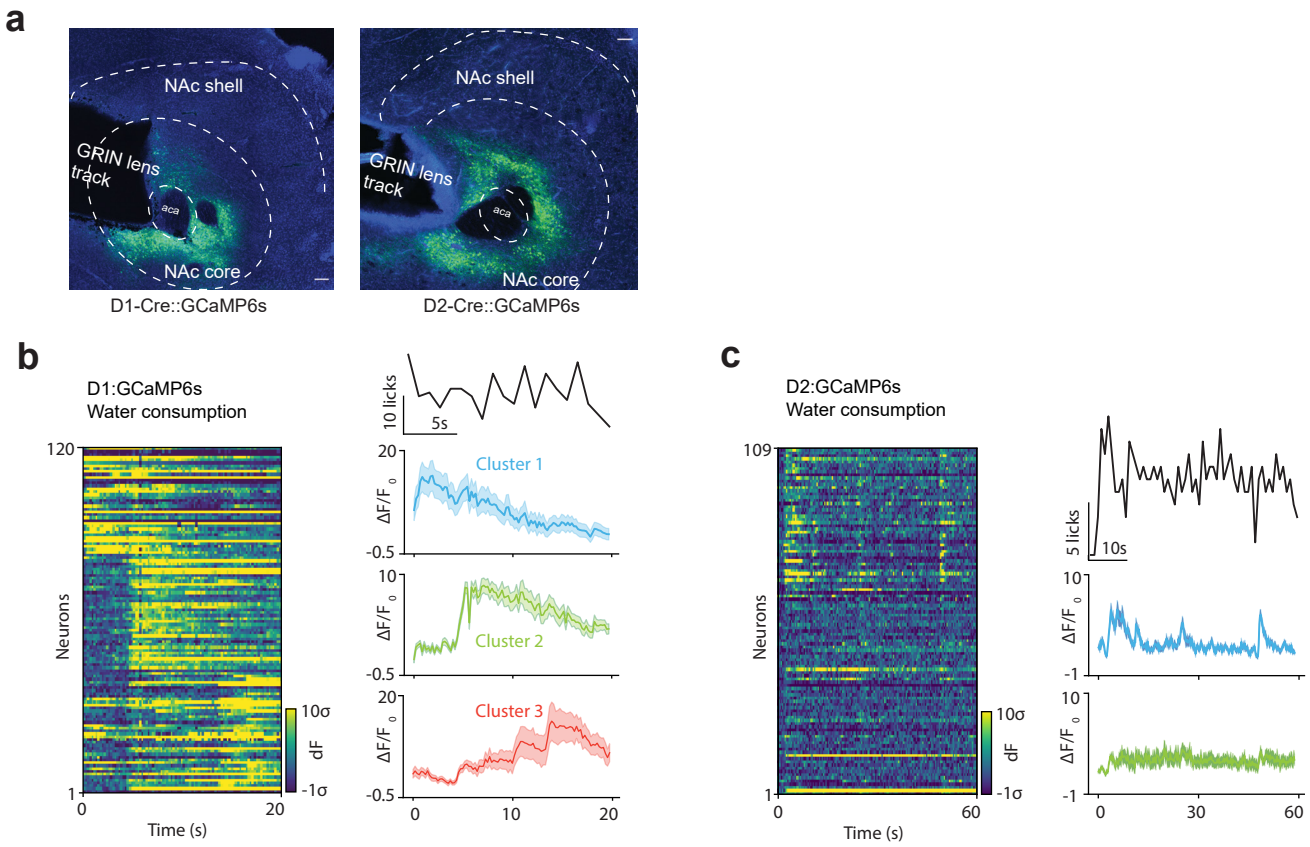

a
NAc shell
GRIN lens track
aca
NAc core
D1-Cre::GCaMP6s
NAc shell
GRIN lens track
aca
NAc core
D2-Cre::GCaMP6s
b
D1:GCaMP6s
Water consumption
120
Neurons
1
0
20
Time (s)
10σ
dF
-1σ
10 licks
5s
Cluster 1
Cluster 2
Cluster 3
ΔF/F0
20
-0.5
10
0
20
c
D2:GCaMP6s
Water consumption
109
Neurons
1
0
60
Time (s)
10σ
dF
-1σ
5 licks
10s
ΔF/F0
10
-1
0
30
60

Fig. S4

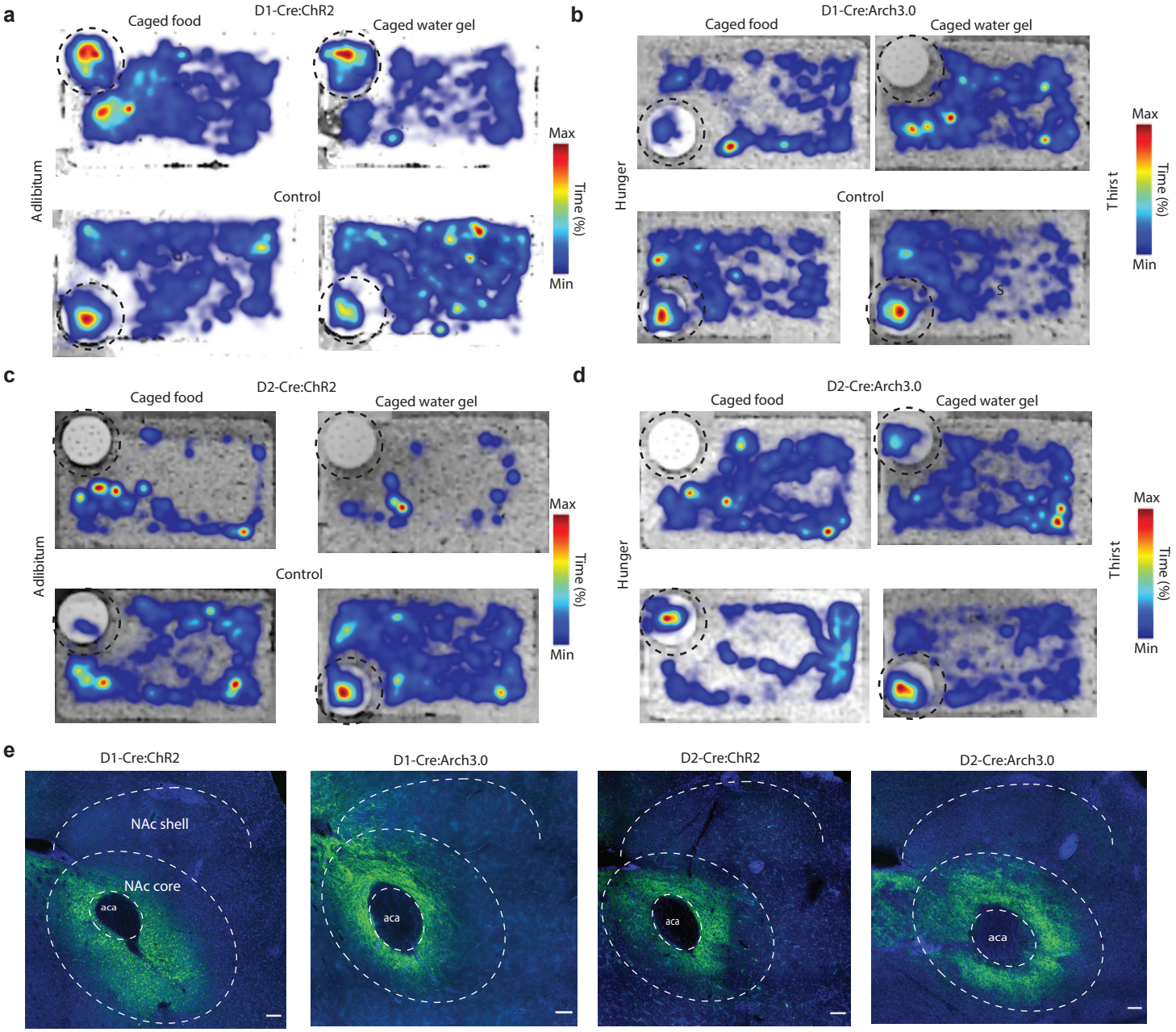
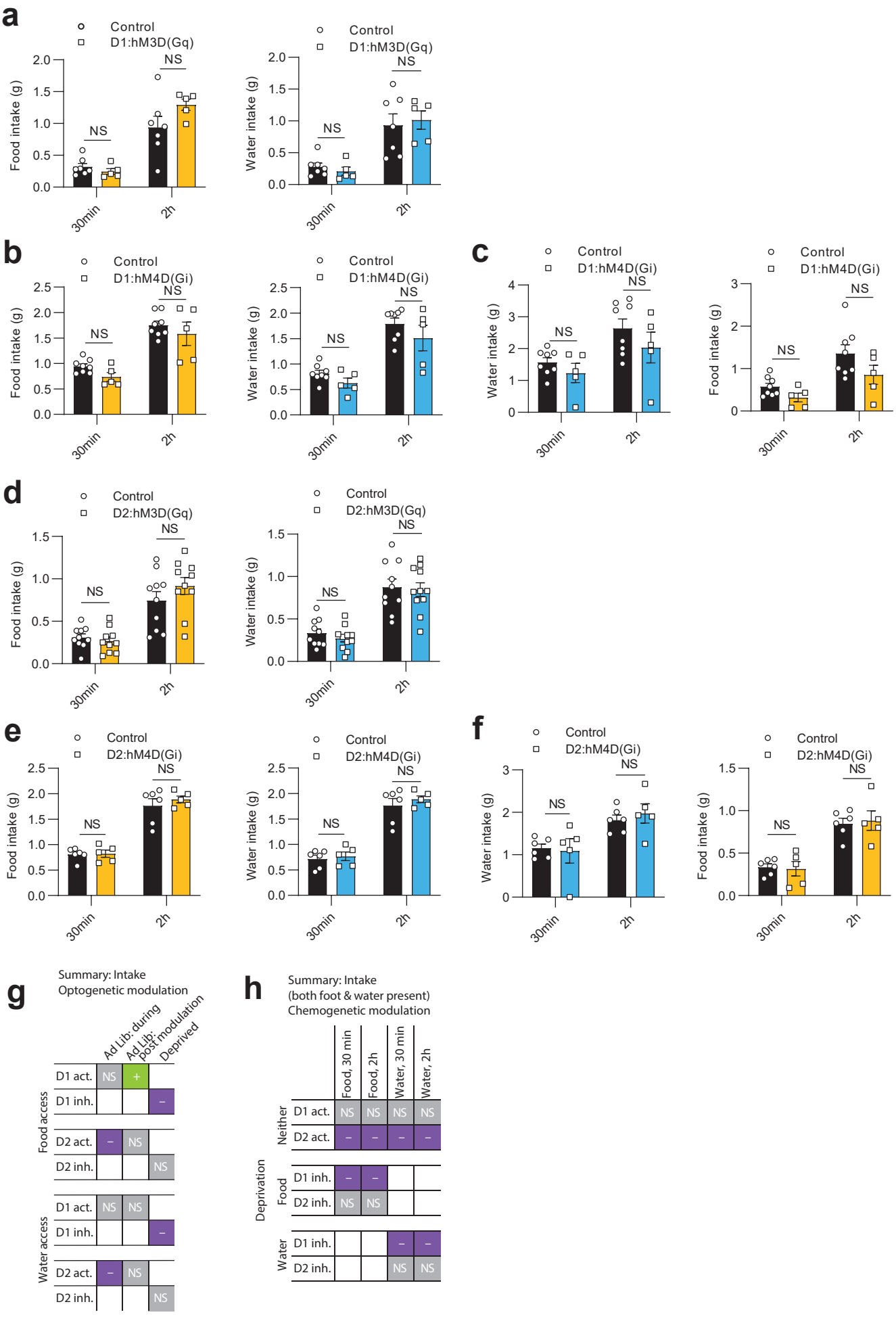

a
D1-Cre:ChR2
Caged food
Caged water gel
Adlibitum
Control
Max
Time (%)
Min
b
D1-Cre:Arch3.0
Caged food
Caged water gel
Hunger
Thirst
Control
Max
Time (%)
Min
c
D2-Cre:ChR2
Caged food
Caged water gel
Adlibitum
Control
Max
Time (%)
Min
d
D2-Cre:Arch3.0
Caged food
Caged water gel
Hunger
Thirst
Max
Time (%)
Min
e
D1-Cre:ChR2
NAc shell
NAc core
aca
D1-Cre:Arch3.0
aca
D2-Cre:ChR2
aca
D2-Cre:Arch3.0
aca

Fig. S5


a
Control
D1:hM3D(Gq)
Food intake (g)
Water intake (g)
NS
30min
2h
b
Control
D1:hM4D(Gi)
Food intake (g)
Water intake (g)
NS
30min
2h
c
Control
D1:hM4D(Gi)
Water intake (g)
Food intake (g)
NS
30min
2h
d
Control
D2:hM3D(Gq)
Food intake (g)
Water intake (g)
NS
30min
2h
e
Control
D2:hM4D(Gi)
Food intake (g)
Water intake (g)
NS
30min
2h
f
Control
D2:hM4D(Gi)
Water intake (g)
Food intake (g)
NS
30min
2h
g
Summary: Intake
Optogenetic modulation
Ad Lib. during
Ad Lib. post modulation
Deprived
Food access
D1 act.
D1 inh.
D2 act.
D2 inh.
Water access
D1 act.
D1 inh.
D2 act.
D2 inh.
NS
+
–
h
Summary: Intake
(both foot & water present)
Chemogenetic modulation
Food, 30 min
Food, 2h
Water, 30 min
Water, 2h
Deprivation
Neither
D1 act.
D2 act.
Food
D1 inh.
D2 inh.
Water
D1 inh.
D2 inh.
NS
–