doi:10.1038/nature22375

The role of InsCtx in the operant visual discrimination task

We found that InsCtx was necessary for performance of the operant visual discrimination task (**Fig. 1c**). However, it was not necessary for home-cage feeding on regular chow (**Fig. 1d**), nor for *ad libitum* feeding on Ensure in the same head-fixed context as the visual discrimination task but without visual cues (**Fig. 1e**). We interpret these results in the framework of InsCtx's role in associating external cues with their interoceptive outcomes (e.g., food cue and subsequent food ingestion). It is possible that certain aspects of gustatory/visceral processing might have been affected by InsCtx silencing (e.g., discrimination and preference of different tastants based on relative palatability[1]). This might then cause a reduction in the incentive salience of Ensure and of the associated food cue, resulting in suppressed cue-evoked licking in response to the food cue, specifically during the visual discrimination task (but not during *ad libitum* consumption). More generally, a lower incentive salience associated with the food cue may be due to changes in InsCtx encoding of the rewarding properties of Ensure (involving taste[2], as well as post-ingestive nutrient absorption[3]). Importantly, these interpretations do not affect our overall conclusion from the InsCtx silencing experiments, that InsCtx is important for the behavioral response to learned food-predicting cues, possibly due to lower incentive salience associated with these food cues.

Potential effects of orofacial movements on InsCtx cue responses

InsCtx neural activity can be modulated by orofacial movements[2,4]. Thus, we considered whether the robust InsCtx food cue response bias and hunger modulation we observed could potentially be artifacts of preparatory (pre-licking) orofacial movements that would occur only before the food cue and only during hunger. First, to directly address this issue, we used simultaneous videography of the orofacial region[5] together with InsCtx imaging, to directly assess the relationship between orofacial movements and InsCtx responses on single trials. Importantly, we found that only 3% of food cue-responsive neurons had cue responses that were positively correlated with orofacial movements (**Extended Data Fig. 4c-g**). Of note, orofacial videography is not as sensitive as electromyography. As such, it is possible that our videography measurements missed subtler orofacial movements.

Second, we analyzed the relationship between licking and cue responses at different pre-licking times. Specifically, in the analyses presented in **Figures 2** and **3**, we only used data up to 100 ms before the first lick in every trial, to avoid licking-related activity (**Methods**). We further analyzed this dataset using only data collected up to 200 ms or 300 ms before licking onset in each trial, thus further protecting against pre-lick preparatory orofacial movements. We found that food cue bias and hunger modulation were preserved even using these more restrictive analysis windows (**Extended Data Fig. 4a,b**). Together, these two approaches argue that food cue bias and hunger modulation in InsCtx cue responses are independent of licking and other orofacial movements. Furthermore, in the BLA$^{\rightarrow InsCtx}$ silencing experiments (**Fig. 5**), we found that InsCtx food cue responses were attenuated, while subsequent Ensure responses were unaffected. This provides a further dissociation between these two components of InsCtx neuronal responses.

Persistent AgRP activity during the visual discrimination task

Previous findings suggest that AgRP neuron activity increases with caloric deficit, and then drops upon the detection of food at the onset of feeding[6-8]. Indeed, using fiber photometry recordings from AgRP neurons[7], we also found a sustained drop in their activity upon consumption of relatively large amounts of food (0.2 gr

chow; **Extended Data Fig. 6b**). In contrast, InsCtx responses in hungry mice were constant across repeated food cue presentations and Ensure consumption (**Extended Data Fig. 3e**). This raises the following question: does AgRP neuron activity remain at a persistently high level during our visual discrimination task, or does task engagement cause an immediate and sustained drop in activity?

To answer this question, we used fiber photometry to record the activity of AgRP neurons during the visual discrimination task in hungry mice (food restriction) and following satiation on Ensure (same protocol used for InsCtx imaging in **Fig. 2**; **Extended Data Fig. 6c-f**). AgRP neuron activity decreased in response to the visual food cue, and decreased further upon consumption of a small drop of Ensure on each trial (~5 µL each, representing <0.2% of total Ensure consumption required for satiation). AgRP neurons responded to food cues but not to other visual cues, and these responses were abolished by the transition to satiety. Importantly, AgRP neuron activity returned to pre-cue baseline at 10-15 s after food cue onset, and AgRP neurons could respond to consecutive food cues. Furthermore, these transient AgRP neuron food cue responses did not systematically change in magnitude with increasing trial number within a session or with latency to onset of licking (**Extended Data Fig. 6c-f**). Therefore, these results demonstrate that AgRP neuron activity remains high in food-restricted mice during the visual discrimination task, and drops only transiently after every food cue and after consumption of a small amount of Ensure (see summary schematic in **Extended Data Fig. 6g**).

The difference between previously published work[6-8] and the above results is likely due to the very small amount of food the mice receive after each successful food cue trial in our task (~5 µL drop). For example, during our Ensure satiation protocol, mice will consume >400 of these drops to become sated, while during a 30 min imaging session, mice are allowed to consume only ~50-60 of these drops (see dashed lines vs. bars in **Fig. 1e**). These results, together with our recent finding that ongoing AgRP activity is necessary to maintain ongoing food consumption[9], validate the use of continuous AgRP neuron activation (e.g., using chemogenetics) in the context of our visual discrimination task.

Mapping potential pathways between AgRP neurons and InsCtx

We performed a series of circuit-mapping experiments in search of a pathway linking AgRP neurons to InsCtx (**Fig. 4**). Our projection-specific monosynaptic rabies tracing of inputs onto $BLA^{\rightarrow InsCtx}$ neurons labeled several sites, and we examined their colocalization with AgRP axons (**Fig. 4; Extended Data Fig. 8b-d**). Rabies sparsely labeled neurons in the BNST, but they did colocalize with AgRP axons and did not receive synaptic input from them (**Extended Data Fig. 8d,f**). While we also observed very sparse labeling in the ventrolateral periaqueductal gray, its involvement is unlikely, as AgRP projections to this area do not evoke feeding[10].

We performed several CRACM experiments testing several neuronal populations for GABAergic input from AgRP neurons. Of note, AgRP neurons can potentially release GABA, NPY and AgRP[11,12]. However, the CRACM protocol we used involved only brief light pulses (5 ms) with long inter-pulse intervals (1-8 s), and was designed to assess fast (GABAergic) transmission. As such, we cannot exclude the possibility of NPY or AgRP transmission at the sites we examined. Future experiments could develop a CRACM protocol for measuring NPY/AgRP transmission from AgRP neurons, thus allowing these issues to be addressed. Nevertheless, due to the delayed behavioral effects of AgRP release[12], slow AgRP signals would not explain the fast effects of AgRP neuron stimulation on our behavior *in vivo*. These effects were already apparent in the first trials of a behavioral session in sated mice, which began ~10 min following CNO injection.

Using this CRACM protocol, we also found that AgRP neurons preferentially target $PVT^{\rightarrow BLA}$ neurons within the PVT. For example, only ~7% (1/14) of $PVT^{\rightarrow AStr}$ neurons (AStr: amygdalo-striatal

transition area, a region adjacent to BLA) received AgRP input, demonstrating the synaptic input specificity of these distinct populations. Interestingly, ~45% (13/29) of PVT$^{\rightarrow CeA}$ neurons received AgRP input. As PVT$^{\rightarrow CeA}$ neurons are involved in fear memory[13,14], it is likely that, independently of feeding, AgRP neurons may also modulate affective processing to promote foraging and hunting through PVT$^{\rightarrow CeA}$ neurons[15-17]. Indeed, PVT has been recently shown to play a role in selecting between competing behaviors, such as food seeking and defensive behaviors[18].

Importantly, there is redundancy in these circuits (**Fig. 5h**), especially when considering additional intermediate nodes between AgRP neurons and InsCtx (e.g., AgRP neuron projections to other targets). Furthermore, it is possible that AgRP neuron activation also causes peripheral changes (e.g., enhanced gut motility) that affect insular cortex via viscerosensory pathways. However, our selective manipulations of components of the AgRP→PVT→BLA→InsCtx pathway (i.e., PVT$^{\rightarrow BLA}$ and BLA$^{\rightarrow InsCtx}$) suggest that they are important for InsCtx responses to predictive cues, while not excluding the contribution of additional pathways. As such, this pathway provides a useful entry point for further dissection of the mechanisms by which InsCtx develops response biases towards motivationally-relevant learned cues.

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Supplementary Methods

All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Animals were housed with standard mouse chow (Teklad F6 Rodent Diet 8664; 4.05 kcal g^{-1}, 3.3 kcal g^{-1} metabolizable energy, 12.5% kcal from fat; Harlan Teklad) and water provided ad libitum, unless specified otherwise. We used male mice only. Sample sizes were chosen to reliably measure experimental parameters while keeping with standards in the relevant fields[1-3], and remaining in compliance with ethical guidelines to minimize the number of animals used. Experiments did not involve experimenter-blinding, but randomization was used with respect to trial order and data collection. Animal subjects were not randomly allocated to experimental groups as all comparisons were performed within subject.

Behavioral training

After at least 1 week of post-surgical recovery, animals (10-16 weeks old) were food restricted to 85% of their free-feeding body weight. Animals were head-fixed on a styrofoam trackball for habituation prior to any behavioral training (10 minutes to 1 hour over the course of 2-3 days). If the mice displayed any signs of stress, they were immediately removed and additional head-fixation sessions were added until there were no visible signs of stress[4]. On the final head-fixation session, food-restricted animals were given Ensure (a high calorie liquid meal replacement) by hand via a syringe to acclimate them to the taste of Ensure. To train the animals to associate licking a lickspout with delivery of Ensure, we initially triggered delivery of Ensure (5 µL, 0.0075 calories) to occur with every lick (with a 2.5 s inter-trial minimum interval between Ensure deliveries). We tracked licking behavior via an infrared beam positioned directly in front of the animal's mouth. All behavioral training was performed using MonkeyLogic[5].

Once food-restricted and head-fixed mice reliably licked to obtain an Ensure reward, we introduced the food cue ('go' trials). We initially trained animals by presenting the food cue followed by unconditional delivery of the Ensure reward (Pavlovian reward; 5 µL, 0.0075 kcal). Once animals were regularly licking in response to the Pavlovian food cue but prior to reward delivery, we transitioned them to delivery of an operant reward, conditional on the animal licking during the response window (in the 2 s post stimulus offset, see below for more details). After animals demonstrated stable licking behavior to the operant food cue (licking in response to >80% of trials involving food cue presentation), we simultaneously introduced 'no-go' trials involving presentation of an operant quinine-predicting cue or of a neutral cue, for which licking during the response window resulted in the delivery of 5 µL of 1 mM quinine or nothing, respectively (**Fig. 1**). Initially, we biased the total number of trials towards the food cue (food cue : quinine cue : neutral cue, 2:1:1), but over several days we slowly increased the fraction of quinine cue and neutral cue trials so that all visual cues were presented in equal proportions. Animals typically learned to perform the visual discrimination task in ~2 weeks. We began all imaging and behavior sessions with 2-5 Pavlovian food cue trials, which served as "behavioral reminders." Pavlovian food cue trials also occurred sporadically during imaging (5-10% of trials). These trials were helpful in maintaining engagement, particularly during late stages of training. None of these Pavlovian food cue presentations were included in the data analysis.

The Go/NoGo task required food-restricted mice to discriminate between square-wave drifting gratings differing in orientation. The LCD screen (Dell) used to deliver visual stimuli was positioned 20 cm from the mouse's eye. All visual stimuli were presented as movies designed in Matlab (2 Hz and 0.04

cycles/degree, full-field square wave drifting gratings, 80% contrast; food cue: 0°, quinine cue: 270°, neutral cue: 135° [6] (see **Fig. 1)**. All drifting gratings were presented for 2 s, after which the mouse had a 2 s window to respond with a lick, detected using an IR photodetector. Licking during the visual cue was not punished, but also did not trigger delivery of the Ensure/quinine. Only the first lick (if any) occurring during the response window triggered delivery of Ensure/quinine. The lickspout was designed with two lick tubes (one for quinine and one for Ensure), epoxied together such that the tongue contacted both tubes on each lick, which served as an effective deterrent for lick responses following quinine cues. Well-trained mice had a high rate of correct food cue licking responses (criterion: >80% of trials, usually ~90-95%), and rarely licked following aversive cue presentations (usually ~10-20%, criterion: <50%), and thus rarely experienced the taste of quinine.

Switching cue-outcome associations in well-trained mice

We first trained mice with the same cue-outcome associations and training protocol described above (e.g., rightward drifting grating was the food cue, 2:1:1 incidence of food cue : quinine cue : neutral cue trials early in training, followed by a gradual shift to a 1:1:1 incidence across sessions), and imaged InsCtx once mice were well-trained. Then, we switched cue-outcome associations such that the visual grating associated with the neutral outcome became associated with the food outcome, and vice versa (the aversive cue remained unchanged). To address the potential effects of biased overexposure to one visual stimulus during early training, we kept the number of presentations of the 3 stimuli strictly equal throughout re-training on the new cue-outcome associations. Initially, all food cue trials were Pavlovian (aversive and neutral cues remained operant throughout training). Then, when mice started exhibiting anticipatory licking to the new food cue (within ~1-2 days), we gradually increased the ratio of operant-to-Pavlovian food cue trials, while keeping the total number of food cue trials strictly equal to that of the other two cues. Mice were deemed successfully re-trained (after ~2 weeks of re-training) once all food cue trials were operant, and once mice exhibited good behavioral performance (>80% correct food cue responses, <40% incorrect aversive and neutral cue responses). At this point, we re-imaged the same InsCtx fields-of-view in these mice.

Surgical procedures

Cannula implantation

AgRP-ires-Cre mice (10-16 weeks old) were anesthetized using isoflurane in 100% O_2 (induction, 3%; maintenance, 1%–1.5%) and placed into a stereotaxic apparatus (Kopf) above a heating pad (CWE). Ophthalmic ointment (Vetropolycin) was applied to the eyes. Using aseptic technique, the surface of the skull was exposed, and small holes were drilled above InsCtx (Bregma: AP: 0.4 mm, ML: ~4.0 mm). Stainless-steel guide cannulae (26-gauge; Plastics One) were implanted bilaterally through the holes to a depth of 2.5 mm ventral to Bregma. Cannulae were secured in place with C&B Metabond (Parkell) to form a permanent seal. A custom-made headpost was then also glued to the skull using C&B Metabond (Parkell). Meloxicam (0.5 mg per kg, s.c.) and a prophylactic antibiotic (cefazolin; 500 mg/kg, s.c.) were administered and the mouse was allowed to recover. Dummy cannulae were inserted into the guide cannulae (0.2 mm extension). Behavioral training started one week post-surgery.

Stereotactic injections

Stereotaxic injections were performed as previously described[7]. Mice were anesthetized with isoflurane in 100% O_2 (induction, 3%–5%; maintenance, 1%–2%), and placed into a stereotaxic apparatus (Kopf model

963 or Stoelting). After exposing the skull via a small incision, a small hole was drilled for injection. A pulled-glass pipette with 20–40 μm tip diameter was inserted into the brain, and virus was injected using an air pressure system (Picospritzer). A micromanipulator (Grass Technologies, model S48 stimulator) was used to deliver the injection at 25 nl/min and the pipette was withdrawn 5 min after injection. For postoperative care, mice were injected intraperitoneally with meloxicam (0.5 mg per kg). Mice were 8-14 weeks old at the time of injection, except from CRACM experiments, for which mice were 5-10 weeks old.

We used the following volumes of virus and injection coordinates: InsCtx (100–200 nl, Bregma: AP: 0.0, 0.4 mm, DV: −4.1, −4.3 mm, ML: ~4.0 mm), ARC (200 nl, Bregma: AP: −1.45 mm, DV: −5.85 mm, ML: ±0.25 mm), PVT (25–50 nl, Bregma: AP: −1.0, −1.3 mm, DV: −3.0, −3.0 mm, ML: 0.0, 0.0 mm), BLA (100 nl, Bregma: AP: −1.6 mm, DV: −4.5, −4.76 mm, ML: ±3.3 mm), CeA (50 nl, Bregma: AP: –0.75 mm, DV: −5.1 mm, ML: ±2.3 mm), NAc (100 nl, Bregma: AP: 1.4 mm, DV: −4.7 mm, ML: ±0.85 mm).

We used the following viruses: AAV1-hSyn-GCaMP6f (University of Pennsylvania Vector Core), AAV1-hSyn-GCaMP6s (University of Pennsylvania vector core), AAV8-DIO-hM3Dq-mCherry (University of North Carolina Vector Core), AAV8-DIO-hM4Di-mCherry (University of North Carolina Vector Core), AAV8-DIO-ChR2(H134R)-mCherry (University of North Carolina Vector Core), AAV5-hSyn-ChR2(H134R)-EYFP (University of North Carolina Vector Core), AAV8-FLEX-TVA-mCherry, (University of North Carolina Vector Core), AAV8-FLEX-RG-mCherry, (University of North Carolina Vector Core), SADΔG–EGFP (EnvA) rabies (Salk Gene Transfer Targeting and Therapeutics Core), AAV6-CAG-cre-GFP; (Boston Children's Hospital Vector Core), H129ΔTK-TT (Center for Neuroanatomy with Neurotropic Viruses, strain H356).

Optic fiber implantation for fiber photometry

First, mice were stereotactically injected with AAV1-hSyn-GCaMP6s into the ARC, as described above. An optic fiber with metal ferrule (400-μm diameter core; BFH37-400 Multimode; NA 0.37; Thor Labs) was then implanted unilaterally over the ARC (AP: −1.45 mm, DV: −5.8 mm, ML: 0.3 mm from Bregma). The fiber was fixed to the skull using C&B Metabond (Parkell). A custom-made headpost was then glued to the skull using C&B Metabond (Parkell). Mice were allowed at least 2 weeks for recovery before behavioral training started.

Microprism assembly and surgery

Glass microprism assemblies were fabricated using standard 2 mm prisms (#MCPH-1.0; Tower Optical) coated with aluminum along their hypotenuse[8]. Prisms were attached to a coverglass (#1 thickness), both along the hypotenuse (to prevent scratching of the reflective surface) and at the side of the prism that faces InsCtx, using Norland Optical Adhesive 71 cured using ultraviolet light.

Approximately 1-2 weeks following AAV-GCaMP6f injection into InsCtx, AgRP-ires-Cre (all experiments except inhibition of $BLA^{\rightarrow InsCtx}$ neurons) or C57BL/6 (for experiments involving inhibition of $BLA^{\rightarrow InsCtx}$ neurons) mice (10-16 weeks old) were given 0.03 ml of dexamethasone sodium phosphate (4 mg/ml, intramuscularly) ~3 hr prior to surgery in order to reduce brain edema. Mice were anesthetized using isoflurane in 100% O_2 (induction, 3%; maintenance, 1%–1.5%) and placed into a stereotaxic apparatus (Kopf) above a heating pad (CWE). Ophthalmic ointment (Vetropolycin) was applied to the eyes. Using aseptic technique, a custom-made headpost was secured using cyanoacrylate glue, dental acrylic and C&B Metabond (Parkell). A 2.2x2.2 mm^2 craniotomy was then performed over the left InsCtx and S2 (bottom edge of the craniotomy was just above the squamosal plate), centered around the AP location of the

previously performed AAV-GCaMP6f injections. A 2x2 mm^2 microprism was then stereotactically lowered into the craniotomy until contact with the InsCtx was made, and was then lowered further with concomitant movement medially (~100-200 μm) until contacting the top edge of the craniotomy, while verifying that the microprism's bottom edge was inserted below the squamosal plate. Once the prism was in place, the window edges were affixed to the skull using Vetbond (3M), followed by C&B Metabond (Parkell) to form a permanent seal. A 1:3 dental cement mix of black powder paint (Black) and white dental acrylic (Dentsply) was then applied for light shielding. Meloxicam (0.5 mg per kg, s.c.) and a prophylactic antibiotic (cefazolin; 500 mg/kg, s.c.) were administered and the mouse was allowed to recover. We imaged and included in analyses all animals with implanted microprisms and adequate microprism clarity for imaging (~70% of implanted animals).

Pharmacological silencing

Pharmacological silencing experiments started once mice were fully trained (see above). We removed the dummy cannulae and inserted stainless steel cannulae (internal: 33-gauge; Plastics One). For InsCtx silencing, the internal cannulae extended 1.6 mm beyond the external cannulae (i.e., total depth below Bregma: 4.1 mm), while for S2 silencing, the internal cannulae extended 0.5 mm beyond the external cannulae (i.e., total depth below Bregma: 3 mm). As previously described[9], we injected mice with 0.3 μL of muscimol/baclofen solution (0.05 μg/μL and 0.02 μg/μL, respectively) or saline at a rate of 0.1 μL/min. One minute after infusion, the injection cannulae were replaced with the dummy cannulae, and behavioral testing started 15 min later. We verified cannula location for every animal and included all animals with cannulae in InsCtx in subsequent analyses.

For testing performance on the *visual discrimination task*, food-restricted mice (~85% of free-feeding weight) performed two runs per day. The first run was always a saline infusion run to test initial performance on that day. The second run was either a drug infusion run, or another saline run (to control for time elapsed and Ensure consumed). Each run started with 10 Pavlovian food cue trials, then 10 operant food cue trials, and then 150 trials consisting of the 3 operant visual cues at equal proportions, presented in random order. Pavlovian food cue trials also occurred sporadically (5% of trials), and were helpful in maintaining engagement. None of these Pavlovian food cue presentations were included in the behavioral or neural data analyses.

For testing *locomotion in the home-cage*, food-restricted mice were head-fixed and infused with either saline of drug (on separate days) into InsCtx via the cannulae, as described above, except mice were then returned to their home-cage after the internal cannulae were replaced with the dummy cannulae. Fifteen min post infusion, a 30 min video recording began. Video recordings were performed using cameras (Point Grey, Flea3 FL3-U3-13Y3M) located directly above the home cage. Video data were recorded at a frame rate of 30 Hz, and recorded videos were sub-sampled to 15 Hz for subsequent analyses performed using custom software written in Matlab. For each camera frame analyzed, mice were segmented from the background in order to generate masks of the animal's body (using software adapted from the mousetrack.m function available at http://brodywiki.princeton.edu//wiki/index.php/MouseMove). From each mask, we obtained the coordinates for the center-of-mass and used these to compute mouse position and locomotion.

For testing *feeding on chow in the home-cage*, food-restricted mice were head-fixed and infused with either saline of drug (on separate days) into InsCtx via the cannulae, as described above, except mice were returned to their home-cage after the internal cannulae were replaced with the dummy cannulae. Fifteen min

post infusion, a large food pellet (regular chow, ~4 g) was inserted in to home-cage. The food pellet was weighed every 30 min.

For testing *feeding on Ensure while head-fixed on the trackball*, food-restricted mice were head-fixed and infused with either saline or drug (on separate days) into InsCtx via the cannulae. The same LCD screen (Dell) used to deliver visual stimuli was present, but displayed uniform gray background illumination. Fifteen min post infusion, we began a 30 min run in which licking triggered delivery of Ensure (5 µL; 2.5 s inter-trial minimum interval between Ensure deliveries). Mice usually consumed 2-3 mL of Ensure during this period.

Two-photon imaging across hunger, satiety, activation of AgRP neurons, inhibition of BLA$^{\rightarrow InsCtx}$ neurons, and in naïve mice

Two-photon imaging was performed using a resonant-scanning two-photon microscope with tiltable scanhead (Neurolabware; 31 frames/second; 1154x512 pixels). All imaging was performed with a 20x 0.45 NA air objective (Olympus) with a 540 x 360 µm^2 field of view. All imaged fields of view (FOV) were at a depth of 90-150 µm below the pial surface, using a Mai Tai DeepSee laser (Newport Corp.) with laser power at 920-960 nm of 35-80 mW at the front aperture of the objective (power at the sample was likely substantially less due to partial transmission via the microprism). Imaging depth was adjusted in between runs (every 30 min) to account for slow drift in the z plane (< 7 µm). We additionally created mean 3D movies of the neural response to each visual cue to confirm there were no large peri-stimulus changes in z-position.

To assay how changes in hunger state affect behavioral and neural activity, we imaged in two blocks of trials within a session, one during food restriction and a subsequent block immediately following re-feeding. At the start of each imaging session, food-restricted mice (~85% of free-feeding weight) performed the visual cue discrimination task. After ~180 trials (30-min imaging run), we provided the mouse with *ad libitum* access to Ensure for 45-75 minutes using the same protocol for acclimating mice to the lick-spout (see above). During this period of time, mice consumed ~3-5 mL of Ensure and then voluntarily stopped licking for rewards[6]. We then ran an additional ~180 trials (30 min imaging run) while mice were sated (operationally defined as the absence of voluntary licking). Using this satiation protocol, we found that mice may occasionally resume feeding within 30-60 minutes (see examples in **Extended Data Fig. 7a,b**). In such cases, the imaging run was aborted, and mice were allowed to consume more Ensure *ad libitum*. When mice voluntarily stopped licking, we restarted the imaging run and considered it to be a 'sated' run if mice did not lick in >70% of trials (see example in **Extended Data Fig. 7a,b**). This re-satiation was necessary only in a subset of mice and only in ~30% of post-satiation runs.

For experiments involving *chemogenetic activation of AgRP neurons*, following imaging during satiety, mice were returned to their home-cage with *ad libitum* access to regular chow. The next morning, mice were typically at 100-110% of their normal body weight. Mice were then returned to the imaging apparatus and allowed to consume Ensure *ad libitum*, as described above. Mice usually exhibited sporadic consumption of Ensure (<300 µl of Ensure, <10% of that consumed by food-restricted mice) and voluntarily stopped consumption within 1-5 min. Next, we imaged the same InsCtx field of view from the previous imaging day during ~180 trials (30-min imaging run) while the mouse was in this sated state. We then injected CNO (1-3 mg/kg, i.p.). Ten minutes after CNO injection, we initiated an additional imaging run of

~180 trials (30 min imaging run). For every mouse used for these experiments, we used postmortem histology and immunohistochemistry (see below) to verify hM3Dq-mCherry expression in the ARC.

The effects of CNO injections were not due to pain caused by the injection. First, we verified that saline injections did not restore behavioral responses or neuronal responses (**Extended Data Fig. 7d**). Second, all mice were habituated with several saline injections before performing the actual CNO injections. Third, it is highly unlikely that a painful stimulus, such as an intraperitoneal injection, would cause food cue-biased responses in InsCtx. It remains possible that this would cause a general long-lasting increase in arousal. However, as our pupil-tracking data demonstrate, this should result in a *non-specific* increase in responses to all 3 cues (**Fig. 2**). Therefore, because CNO injections restored food cue-biased responses (**Fig. 3**), and the food cue bias tracked motivational salience (**Extended Data Fig. 3g-m**), it is highly unlikely this can be explained by the actual needle injection, rather than by activation of AgRP neurons.

For experiments involving *chemogenetic inhibition of* $BLA^{\rightarrow InsCtx}$ *neurons*, we first performed bilateral injections of AAV6-Cre into InsCtx and AAV8-DIO-hM4Di-mCherry into the BLA of C57BL/6 mice. One to two weeks later, we injected AAV1-hSyn-GCaMP6f into the InsCtx. Between one and two weeks later, we performed the InsCtx microprism surgery. We started imaging mice ~8-12 weeks after the initial AAV6-Cre injections. We used a two-day protocol in which Day 1 included two consecutive behavioral blocks (180 trials each), both following a saline injection (Saline-1.1, Saline 1.2). Day 2 included two behavioral blocks, the first one following a saline injection and the second one following CNO injection (Saline-2.1, CNO-2.2; 10 mg/kg). We waited 10 min after each injection before starting the imaging session. For subsequent analyses, we only used mice that had either BLA hits ipsilateral to the InsCtx microprism, or bilateral BLA hits, based on postmortem histology. None of the mice had contralateral hits only.

For experiments involving *naïve mice* (prior to learning the behavioral task), we first habituated mice to head-restraint, as described above. Then, we food-restricted mice to ~85% of their normal body weight and placed them in the head-fixation setup under the two-photon microscope but in the absence of a lick-spout. Mice underwent one 30 min habituation session in which they were presented with the same visual cue sequence described above (~180 cues at random order, equal number of presentations of each cue), and InsCtx was then imaged during a second identical 30 min session.

Pupil and orofacial videography during two-photon imaging

We acquired data using a GigE Vision camera (Dalsa) with a 60 mm lens (Nikon MicroNikkor) from a pre-selected region of interest around the eye ipsilateral to the LCD monitor used to present visual cues (contralateral to the InsCtx microprism), or around the orofacial region. Acquisition of each frame (frame rate of 15.5 Hz) was triggered on every other frame of two-photon acquisition (acquired at 31 Hz) using Scanbox software (Neurolabware). The pupil was illuminated by spread within the brain of the IR light used for two-photon excitation during imaging. See below for details of data analysis.

Postmortem identification of imaging field location

Mice were terminally anesthetized with an overdose of chloral hydrate (Sigma Aldrich), and were then left in their homecage for several hours to reduce subsequent blood loss during decapitation. Mice were then decapitated, and heads were postfixed in 10% neutral buffered formalin (Fisher Scientific) overnight. The

brains were then carefully removed and washed. We then performed whole-mount imaging of the entire brain using light and fluorescence microscopy for visualization of surface vasculature and GCaMP6f fluorescence (**Extended Data Fig. 2c**). Microprism location was evident by a minor indentation of the tissue. We then aligned the postmortem surface vasculature to *in vivo* epifluorescence images (imaged through the microprism) for coarse localization of the imaging field. For a more precise localization of the imaging field of view, we further aligned the vasculature to vascular landmarks from *in vivo* two-photon imaging (**Extended Data Fig. 2c**). Finally, we used this information to localize imaging fields of view, relative to the middle cerebral artery and rhinal vein. We broadly classified imaging fields as being located either in granular or dysgranular InsCtx, based on proximity to the rhinal vein and based on subsequent examination of coronal sections (**Extended Data Fig. 2c,d; Fig. 2**).

Brain tissue preparation and immunohistochemistry

Mice were terminally anesthetized with chloral hydrate (Sigma Aldrich) and transcardially perfused with phosphate-buffered saline (PBS) followed by 10% neutral buffered formalin (Fisher Scientific). Brains were extracted, cryoprotected in 20% sucrose, and sectioned coronally on a freezing sliding microtome (Leica Biosystems) at 30-40 μm, and collected in 3-4 equal series.

Brain sections were washed in 0.1 M phosphate-buffered saline pH 7.4, blocked in 3% normal donkey serum/0.25% Triton X-100 in PBS for 1 hour at room temperature and then incubated overnight at room temperature in blocking solution containing primary antiserum. The next morning, sections were extensively washed in PBS and then incubated in Alexa fluorophore-conjugated secondary antibody (Molecular Probes, 1:1000) for 2 h at room temperature. After several washes in PBS, sections were mounted onto gelatin-coated slides and fluorescent images were captured with an Olympus VS120 slide scanner microscope. We used the following primary antibodies: rabbit anti-dsRed, Clonetech (#632496) 1:1000; chicken anti-GFP, Life Technologies (#A10262) 1:1000; goat anti-AgRP, Neuromics (#GT15023) 1:1000. rabbit anti-cfos, Life Technologies (#sc-52) 1:1000.

All antibodies used were previously verified[2]. For all experiments that involved stereotactic injections (e.g., anatomical tracing, *ex vivo* CRACM, chemogenetic behavioral and imaging studies), we verified infection in the desired brain region with minimal spillover outside it, and excluded animals with imprecise injections.

Anterograde HSV

AgRP-ires-cre mice were injected with H129ΔTK-TT into the ARC and perfused 2-3 days later. At 2 days post-injection, we only observed labeled neurons in known AgRP targets, colocalized with AgRP axons (visualized using immunohistochemistry, see above). At 3 days post-injection, we observed additional labeled sites that were not co-localized with AgRP axons[10,11].

Rabies collateral mapping

Similar to previously described procedures[12], three weeks after unilateral injection of AAV8-EFIα-DIO-TVA-mCherry into the PVT (in vglut2-ires-Cre mice) or BLA (in Emx1-ires-Cre mice), SADΔG–EGFP

(EnvA) rabies was unilaterally injected into the BLA or NAc (PVT collateral mapping), or into InsCtx (BLA collateral mapping; for coordinates see above). Animals were allowed 6 days for retrograde transport of rabies virus and EGFP transgene expression in long-range axons before perfusion, tissue collection and imaging (Olympus VS120 slide scanner microscope). The GFP signal was amplified using immunohistochemistry to visualize weakly labeled axons.

Projection-specific monosynaptic rabies tracing

Vglut2-ires-Cre mice or Emx1-ires-Cre mice were injected with a mixture (1:1) of AAV8-EFIα-DIO-TVA-mCherry and AAV8-EFIα-DIO-RG into the BLA. Three weeks later, SADΔG–EGFP (EnvA) rabies was injected unilaterally into InsCtx. Animals were allowed 6 days for retrograde transport of rabies virus and EGFP expression before perfusion and tissue collection. Sites of afferent input to $BLA^{\rightarrow InsCtx}$ neurons were assessed by the presence of EnvA-EGFP positive cell bodies in coronal sections imaged using an Olympus VS120 slide scanner microscope. The GFP signal was amplified using immunohistochemistry to visualize weakly labeled neurons. It should be noted that using this technique, rabies may spread within the BLA between excitatory Cre-expressing neurons that are synaptically connected to each other. Therefore, this will add one more synapse with the same sign (i.e., excitatory) in between the labeled upstream site and $BLA^{\rightarrow InsCtx}$ neurons. However, this is not the case for $PVT \rightarrow BLA^{\rightarrow InsCtx}$ as we have verified this connection using CRACM (**Extended Data Figure 8e**).

Slice electrophysiology and channelrhodopsin-2-assisted circuit mapping (CRACM)

Two weeks after a ChR2-expressing AAV was injected, and 1 week before electrophysiological recordings, we injected fluorescently-conjugated CTB (AF488, AF555 or AF594; Molecular Probes) into the relevant target region.

For brain slice preparation, mice 7–10 weeks old were anesthetized with isoflurane before decapitation and removal of the entire brain. Brains were immediately submerged in ice-cold, carbogen-saturated (95% O_2, 5% CO_2) sucrose-based cutting solution consisting of (in mM): 72 sucrose, 83 NaCl, 2.5 KCl, 1 NaH2PO4, 26 NaHCO3, 22 glucose, 5 MgCl2, 1 CaCl2 (measured osmolarity 310 – 320 mOsm/l) or a NMDG-based cutting solution consisting of (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 MgSO4, 0.5 CaCl2 (pH 7.3 adjusted with HCl, oxygenated with 95% O2/5% CO2, measured osmolarity 310 – 320 mOsm). Then, 300-µm-thick coronal sections were cut with a Leica VT1000S vibratome and incubated in oxygenated cutting solution at 34 °C for 10 min. Next, slices were transferred to oxygenated aCSF consisting of (in mM): 126 NaCl, 21.4 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 10 glucose and recovered for 30 min at 34 °C. Slices were stored in oxygenated aCSF at room temperature (20–24°C) for at least 60 min prior to recording. A single slice was placed in the recording chamber where it was continuously superfused at a rate of 3–4 ml per min with oxygenated aCSF. Neurons were visualized with an upright microscope equipped with infrared-differential interference contrast and fluorescence optics. Borosilicate glass microelectrodes (5–7 MΩ) were filled with internal solution.

For CRACM of inputs from AgRP neurons, light-evoked GABAergic IPSCs were recorded in whole-cell voltage-clamp mode, with membrane potential clamped at V_h = −70 mV using a CsCl-based internal

solution consisting of (in mM): 140 CsCl, 1 BAPTA, 10 HEPES, 5 $MgCl_2$, 2 Mg-ATP, and 0.3 Na_2-GTP (pH 7.35 adjusted with NaOH; 295 mOsm · kg^{-1}).

For CRACM of monosynaptic and polysynaptic glutamatergic and GABAergic inputs onto BLA neurons, following PVT axon stimulation, recordings were obtained using a Cs^+-based low Cl^- internal solution consisting of (in mM): 135 $CsMeSO_3$, 10 HEPES, 1 EGTA, 4 $MgCl_2$, 4 Na_2-ATP, 0.4 Na_2-GTP, 10 Na_2-phosphocreatine (pH 7.3 adjusted with CsOH; 295 mOsm · kg^{-1}; E_{Cl} = −70 mV). Light-evoked glutamatergic EPSCs or light-evoked GABAergic IPSCs were recorded in whole-cell voltage-clamp mode, with membrane potential clamped at V_h = −70 mV or 0 mV, respectively.

All recordings were made using a Multiclamp 700B amplifier, and data were filtered at 2 kHz and digitized at 10 kHz. To photostimulate channelrhodopsin2-positive fibers, a laser or LED light source (473 nm; Opto Engine; Thorlabs) was used. The blue light was focused onto the back aperture of the microscope objective, producing wide-field illumination of the recorded cell of 10–15 mW/mm^2. The light power at the specimen was measured using an optical power meter (PM100D, Thorlabs). The light output was controlled by a programmable pulse stimulator, Master-8 (A.M.P.I.) and pClamp 10.2 software (Axon Instruments). The photostimulation-evoked EPSC/IPSC detection protocol consisted of four blue light laser pulses administered 1 s apart during the first 4 s of an 8-s sweep, repeated for a total of 30 sweeps. We attempted to maximize our ability to detect light-evoked currents by biasing our recordings to cell bodies within the densest axon fields. Unless recorded from the same neurons, photostimulation-evoked IPSCs and EPSCs were recorded in presence of CNQX (20 μM) or picrotoxin (100 μM), respectively, to isolate glutamatergic or GABAergic postsynaptic currents. In some experiments TTX (1 μM) and 4-AP (100 μM) was added to the bath solution in order to identify monosynaptic connectivity. All CRACM results presented are from 3-6 mice per group.

Food intake studies following chemogenetic inhibition of $PVT^{\rightarrow BLA}$ neurons

All animals (C57BL/6) were singly housed for at least 2.5 weeks following surgery, and handled for 5-7 consecutive days before the assay to reduce stress. Studies were conducted in a home-cage environment with *ad libitum* access to food. We assessed food intake in these mice after they received injections of saline on Day 1 and 10 mg/kg CNO on Day 2. A complete experiment involved repetition of these measurements once a week for 3 weeks. The food intake data from all days were then averaged and combined for analysis. Mice with mis-targeted injections or expression extending outside the area of interest were excluded from analysis after post hoc examination of GFP and mCherry expression in BLA and PVT, respectively. Experiments were conducted during the light-cycle, between 13:00 and 16:00, and intake was monitored for three hours. Food intake studies began at 8-10 weeks after bilateral injection of retrogradely-trafficked AAV6-CAG-Cre-GFP into the BLA and AAV8-DIO-hM4Di-mCherry into the PVT.

Behavioral studies during the visual discrimination task following chemogenetic excitation of $PVT^{\rightarrow BLA}$ neurons

All animals (C57BL/6) started behavioral training (using the same protocol described above) at least 3 weeks following surgery. Behavioral testing began at 8-10 weeks after bilateral injection of AAV6-CAG-Cre-GFP into the BLA and AAV8-DIO-hM3Dq-mCherry into the PVT. We used a two-day protocol in which Day 1 included two consecutive behavioral blocks (180 trials each) with saline injections (Saline-1.1, Saline 1.2).

Day 2 included two behavioral blocks, the first one following a saline injection and the second one following CNO injection (Saline-2.1, CNO-2.2; 10 mg/kg). Mice with mis-targeted injections or expression extending outside the area of interest were excluded from analysis after post hoc examination of GFP and mCherry expression in BLA and PVT, respectively.

Fiber photometry in the home-cage and during the visual discrimination task

In vivo fiber photometry was conducted as previously described[15,16]. Fiber optic cables (1 m long) were attached to the implanted optic fibers (400 μm core diameter; metal ferrule; Doric Lenses) with zirconia sleeves (Doric Lenses). Blue light (465 nm; Plexon LED and driver) was focused on the opposite end of the fiber optic cable such that a light intensity of 0.2–0.3 mW entered the brain; light intensity was kept constant across sessions for each mouse. We collected light through a GFP minicube (Doric Lenses) and a photoreceiver (Newport 2151), connected with 0.48 NA, 400 μm core diameter patchcord (Doric Lenses). The signal was then digitized at 1 kHz with a National Instruments data acquisition card and collected using a custom Matlab script.

For home-cage feeding, *ad lib* fed mice were fasted for 24 hr and then put in their home-cage while connected to the optical fiber to collect photometry data. We collected several minutes of baseline data and then dropped a 0.2 gr pellet into the home-cage every 7-10 min. Mice were allowed to freely consume all dropped pellets.

For fiber photometry during the visual discrimination task, we only selected mice with robust responses to pellet drops in the home-cage (see e.g., **Extended Data Fig. 6b**). These mice were then food-restricted and trained to perform the behavioral task, as described above. Once mice were well-trained, we performed fiber photometry recordings across hunger (food-restriction) and satiety (after on-the-ball satiation on Ensure), using the exact same procedure described above for two-photon imaging of InsCtx.

Statistics

Statistical tests were performed using standard Matlab (MathWorks) functions. Differences across mice (e.g., behavior) were tested using the t-test due to relatively small sample sizes. Differences in neural activity across large populations of InsCtx neurons were tested using non-parametric tests (Kruskal-Wallis and Mann-Whitney tests) due to the non-normal distribution of the data. We did not assume equal/unequal variance in parametric t-tests as all t-tests were paired.

Data analysis

All data analyses were performed using custom scripts in Matlab (MathWorks) or ImageJ (NIH).

Image registration and timecourse extraction

First, each acquired image was spatially downsampled by 2X. To correct for motion along the imaged plane (x-y motion), each frame was registered to an average field-of-view using efficient subpixel registration methods[17]. Within each imaging session, each run (2-3 runs/session) was registered to the first run of the day. Image stacks were de-noised using principal components analyses (PCA) of every pixel across time, and by

user identification and removal of noise principal components (low eigenvalues; based on[18]). Cell masks and calcium activity timecourses ('F(t)') were extracted using custom implementation of common methods[18]. To avoid use of cell masks with overlapping pixels, we only included the top 75% of pixel weights for a given mask[19] and excluded any remaining pixels identified in multiple cell masks. We manually verified that all cell masks had typical cell body morphology and size.

Fluorescence timecourses were extracted by averaging the pixels within each region-of-interest ('ROI') mask. Fluorescence timecourses for neuropil within a 25 μm annulus surrounding each ROI (but excluding adjacent ROIs and a protected ring surrounding each ROI) were also extracted ($F_{neuropil}(t)$: median value from the neuropil ring on each frame). Fluorescence timecourses were calculated as $F_{neuropil\_corrected}(t) = F_{ROI}(t) - F_{neuropil}(t)$. The change in fluorescence was calculated by subtracting a running estimate of baseline fluorescence ($F_0(t)$) from $F_{neuropil\_corrected}(t)$, then dividing by $F_0(t)$: $\Delta F/F(t) = (F_{neuropil\_corrected}(t) - F_0(t))/ F_0(t)$. $F_0(t)$ was estimated as the 10th percentile of a 32 s sliding window[3]. All example cue-evoked timecourses were re-zeroed in the 1 s prior to visual stimulus onset for visualization purposes only.

Alignment of cell masks across runs and across days

We chose one set of cell masks for each day. All analyses for the alignment of cell masks across days were semi-automated with the aid of a custom Matlab GUI. To align masks across two days, we first aligned the mean image from each day using one of three methods (depending on the degree of across-day image warping): a rigid body translation, an affine image translation (which allowed for across-day image rotation), or a Delauney triangulation image transformation (for more complex image warping). The alignment transformation used to register the mean image from each day was then applied to each individual mask. We estimated a 2D correlation coefficient to obtain an initial estimate of candidate masks from the same cell across multiple days. We then manually confirmed all suggested candidate masks across days using a custom Matlab GUI. Note that the image registration and warping techniques were applied only to masks for alignment suggestion purposes, and were never applied to cell masks for fluorescence timecourse estimation. Identical procedures applied to primary visual cortex data resulted in high stability of functional properties across days[6].

Single-neuron response analyses

We sought to categorize cells as responsive to visual cues and/or licking and/or Ensure delivery. To determine if cells were responsive to *visual cues*, we independently tested the cue-evoked response of each cell to each cue for each day the cell was identified. For each cell, we compared activity in the 1 s pre-stimulus to activity in a 200-ms sliding window, until 100 ms before licking onset, to minimize contamination of estimates of early, visual cue-evoked responses from activity linked to licking behavior. Given the variability of licking onset across trials, we only analyzed timepoints that preceded lick onset by >100 ms in at least 10 trials. The comparison of this post-stimulus activity with pre-stimulus baseline was performed using the Wilcoxon Signed-Rank test, followed by an FDR correction for multiple comparisons ($p<0.05$). All data were analyzed using timepoints up to 100 ms before licking onset. However, we also separately repeated this analysis using data up to 200 ms or 300 ms before licking onset, and observed similar results (**Extended Data Fig. 4a,b**).

As previously shown, InsCtx neurons can exhibit responses that are temporally locked to licking in the absence of any prior sensory cues, and can begin either just before or just after lick-bout onset. The same

is true for gustatory responses to liquid tastants, such as those to Ensure (see e.g., refs. [20-22]). Licking or Ensure responses can occur either just before (e.g., anticipatory) or just after licking/Ensure onset (i.e., somatosensory/gustatory). As such, to determine if cells were responsive to *licking* onset, we first aligned each trial to licking onset and tested whether activity changed significantly either just before (e.g., anticipatory) or just after licking onset. To assess responses that preceded licking, we performed the same procedure described above, but now comparing 1 s pre-visual cue to the pre-licking period (post-visual cue onset). Additionally, to assess responses that occurred just after licking onset, we compared the 1 s period prior to licking onset to the period from licking onset until 100 ms before Ensure delivery. We followed a similar procedure for responses to *Ensure delivery*. We first aligned each trial to Ensure delivery. Then, to assess responses that preceded Ensure delivery, we performed the same procedure described above, but now comparing data in the 1 s prior to licking onset with data to a pre-Ensure delivery period. Additionally, to assess responses that occurred just after Ensure delivery, we compared the 1 s pre-Ensure period to the 4 s period following Ensure delivery. After these initial analyses, cells were categorized as responsive to licking if they were responsive to licking in either the pre-licking or post-licking periods (or both), and categorized as responsive to Ensure if they responded in either the pre-Ensure or post-Ensure periods (or both).

We ultimately sought to categorize cells as responsive to visual cues and/or licking and/or Ensure delivery. As described above, cells were initially *independently* classified as responsive to visual cues, licking and/or Ensure. However, cells may have responses that start at one epoch (e.g., excited by licking) and continue well into the following epoch (excited by Ensure). In such cases, we would consider the cell only responsive to the initial epoch, unless the response changed in sign in the second epoch (e.g., excited by licking and then suppressed by Ensure). Thus, if a cell was significantly responsive in more than one epoch (visual cue/licking/Ensure), in order for it to be categorized as licking or Ensure responsive, its response to either epoch had to be of opposite sign (excited/suppressed) as compared to the previous epoch, for cells in which the previous epoch also evoked a significant response.

The above analyses and categorization pertain mostly to food cue trials and subsequent licking/Ensure delivery. However, incorrectly licking in response to an aversive cue would lead to quinine delivery. InsCtx responses to quinine have been extensively investigated[23], and we thus sought to find responses to quinine delivery in our data. To this end, we performed the analyses described above for Ensure delivery but for quinine responses. However, we could not detect any significant quinine responses in the main datasets (**Figs. 2,3**). This is likely due to the fact that our well-trained mice rarely licked in response to the quinine cue, resulting in a very low number of trials with delivery of quinine (**Extended Data Fig. 1c**).

We also assessed response magnitude of the average cue-evoked response. For each neuron, we used the maximal absolute value of the average cue response (during the time of presentation of the 2-s cue) as its response magnitude. To assess trial-to-trial variability per neuron, we used the Fano factor (variance/mean). To examine the evolution of responses during a recording session across the InsCtx population, we first normalized each neuron's single-trial responses to the average response across all trials. Then we averaged these normalized single-trial responses across the entire population of neurons responsive to a given cue.

Pupil diameter and its effects of cue-evoked responses

To measure pupil diameter across hunger and satiety, we used only movies from both states acquired within the same imaging session. We first concatenated the movies from the 'Hungry' and 'Sated' sessions, and performed all pupil analyses on this concatenated movie. We warped and rotated the movie to achieve a circular pupil shape, and then used the Matlab function 'imfindcircles.m' to detect the pupil circumference in

every frame separately, from which we extracted pupil diameter. Movies were then warped and rotated back to their original size and orientation, and pupil diameter was also scaled accordingly. For every concatenated movie, we manually verified the precision of the detection of pupil circumference.

For analyses of the relationship between InsCtx activity and pupil diameter, we first upsampled the pupil diameter timecourse (from 15.5 Hz to 31 Hz) using linear interpolation. We then calculated the average pre-cue pupil diameter using the average of 1 s before the presentation of each cue during hunger and satiety. We then matched pre-cue pupil diameter using the following procedure. For every cue presentation (trial) during hunger, we searched for a matching trial during satiety. We first searched for all 'satiety trials' that were within ±10% of the 'hunger trial'. Of these, we then selected the 'satiety trial' that had the value nearest to the 'hunger trial'. Each trial from both states could only be analyzed once. Using this procedure, we could match ~50% of trials from both states (because pupil diameter was typically slightly smaller, on average, during satiety, usually the 'satiety trials' with higher pupil diameter values were matched to a given 'hunger trial'; see e.g., **Fig. 2g**). We then analyzed InsCtx data either from all trials, or only from trials matched for pre-cue pupil diameter (such that the distribution of pupil diameters across all trials used was similar for states of hunger and satiety within each session).

Orofacial movements and their effects on cue responses

We analyzed orofacial movements as previously described[24]. Briefly, for each image, we chose a region of interest (ROI) around the orofacial region and an adjacent "background" ROI. We then subtracted the average intensity of the pixel values in this background ROI from the pixel values in the orofacial ROI to correct for overall luminance changes. We then calculated the absolute difference in pixel intensity across consecutive frames. These differences were then averaged across the entire orofacial ROI, providing a single scalar estimate of orofacial movement per frame (Δpixel intensity/frame). We upsampled the timecourse of this estimate (Δpixel; from 15.5 Hz to 31 Hz) using linear interpolation, to match the acquisition rate of two-photon imaging data.

For analyses of the relationship between InsCtx activity and orofacial movements, we used the same procedure to analyze licking-independent orofacial movements and neuronal cue responses. In every trial, we only used data (both orofacial and neuronal) up to 100 ms before the first lick (as described above). To examine whether trial-to-trial variability in neuronal responses may be attributed to trial-to-trial variability in orofacial responses, we calculated the Pearson correlation coefficient between the absolute neuronal response and orofacial response per trial (average of 0-1.5 sec after cue onset), testing for a positive correlation coefficient.

Grouping of neurons with similar responses to the food cue, and/or to subsequent licking/Ensure

We grouped neurons with similar responses to the food cue, and/or to subsequent licking/Ensure, for visualization purposes only (**Extended Data Fig. 2b**). For cells that were significantly responsive to the food cue, we normalized food cue, licking and Ensure responses (average of all trials for each neuron) by normalizing each epoch as follows: we estimated $(xi-\bar{x})/S$, where xi is the ΔF/F at timepoint i, is the average ΔF/F of the 1 s pre-food cue period, and S is the standard deviation of ΔF/F in the 1 s pre-food cue period. To enable clustering based on distinct food cue and licking responses, we only included cells for which we had more than 10 trials without licking in the first 1 s following food cue onset (244/274 neurons). We then performed principal components analysis (PCA) on the average food cue and licking responses. Because licking responses were usually larger than food cue responses, we attempted to mitigate the associated bias in sensitivity of the PCA to licking vs. cue responses by scaling the food cue responses by the ratio between

licking and food cue responses per neuron. We then used complete agglomerative hierarchical clustering based on the first 2 PCs using a cosine similarity metric[25].

Evaluation of spatial clustering of neurons with similar functional properties

To test for spatial clustering of response properties at a relatively fine scale (tens of microns), we calculated pair-wise distances between all neurons. We then examined the distribution of distances between neurons that were either similar or different in their response type (i.e., food cue responsive *vs.* licking/Ensure responsive).

Comparisons across natural and artificial hunger states

We first aligned data from the two days of the experiment and only used neurons that were active on both days and could be reliably identified on both days. To facilitate comparisons of responses across states within an imaging day, and across states between days, we normalized the responses of each neuron within day across states, using a single transformation that was applied to all cue responses from a given neuron within the same-day session. We accomplished this by z-scoring each neuron's responses to the 3 visual cues across the two states within each day (i.e., z-scores from one distribution consisting of all cue presentations of all cue types during 'hungry-1' and 'sated-1', or during 'sated-2' and 'sated-2+AgRP'). Z-scoring was performed by , where is the ΔF/F at time-point *i*, is the average ΔF/F of all visual cue responses from that day (all timepoints from 1 s before cue onset up to 100 ms before the first lick per trial or 2 s post-cue onset in case of no licking in that trial, across all trials, all visual cues, and all states), and *S* is the standard deviation of ΔF/F from all visual cue presentations from that day (all timepoints from -1 s to ~2 s relative to cue onset (up to 100 ms before first lick), across all trials, all visual cues, and all states). Responses were re-zeroed such that pre-cue period mean was zero.

The 'hunger modulation index' (HMI) was calculated for each neuron as $(R_{hungry} - R_{sated})/ (R_{hungry} + R_{sated})$, where R is a neuron's average response during the entire visual cue (using timepoints up to 100 ms before the first lick in each trial, and for which such lick-free data existed for ≥10 trials). We assessed similarity across days by using a three-step approach. First, we calculated a 'state modulation index' (SMI) that was identical to the HMI, but that was used to compare any two states, either within or across days (e.g., 'hungry' vs. 'hungry next day', 'hungry-1' vs. 'Sated-2+AgRP'). Second, to compare across-state similarity to inherent variability of responses over time in individual neurons, we also compared the similarity within-state. We did this by assessing each neuron's reliability (or 'self-similarity') by randomly splitting up trials within a session and state into two halves and calculating the SMI between the two halves, and then repeating this analysis 100 times, to obtain a distribution of self-similarity. Third, we compared the actual SMI across states/days to the neuron's 'self-similarity' and classified it as similar if (i) both SMIs were between the 10^{th} and 90^{th} percentiles of the 'self-similarity' distribution and (ii) both SMIs had the same sign (excitation/suppression).

Comparisons across saline and CNO injections during inhibition of $BLA^{\rightarrow InsCtx}$ neurons

We first aligned data from the two days of the experiment and only used neurons that were active on both days and could be reliably identified on both days. For subsequent analyses, we included all neurons that were cue- and/or Ensure-responsive either on Saline-1.1 or on Saline-2.1. To facilitate comparisons across experimental conditions, we used the same within day z-scoring procedure described in the previous section. (i.e., z-scores from one distribution consisting of all timepoints in all trials across all cue types during 'Saline-1.1' and 'Saline-1.2', or during 'Saline-2.1' and 'CNO-2.2'). Z-scores were calculated as , where is

the ΔF/F at time-point *i*, is the average ΔF/F of the all visual cue data from that day (all trials, all visual cues, all states), and *S* is the standard deviation of ΔF/F from all visual cue presentations from that day (all trials, all visual cues, all states). Responses were re-zeroed such that pre-cue period mean was zero.

The 'modulation index' was calculated for each neuron per day as $(R_{session2} - R_{session1})/ (R_{session2} + R_{session1})$, where R is a neuron's average response during the entire visual cue (for timepoints up to 100 ms before the first lick in each trial, and for all neurons for which such lick-free data existed for ≥10 trials). For the calculation of the modulation index, we included neurons with the same sign of response across both conditions. However, a small subset of neurons appeared to putatively switch the sign of their responses across conditions (from excited to suppressed or vice versa, i.e., the second session had an average response of the opposite sign as compare to the first one). As such, for these neurons, if the (opposite sign) response in the second session was not statistically significant, we zeroed it, resulting in a modulation index of -1. We did not include in our analyses the small subset of neurons that did have a significant switch in the sign of their cue responses or their Ensure responses across two consecutive sessions.

Population decoding

The goal of our population analysis was to train a linear classifier to decode, from the pattern of responses of all simultaneously recorded InsCtx neurons on a single visual cue trial, *which visual cue* was presented. Our approach was to obtain average responses (across trials) to each cue across the population, and use these as the 'templates' (i.e., 3 templates in total, one for each visual cue type). Then we compared the single-trial population responses to each individual cue presentation with the 3 templates. As described below, the cue with the average response template that was most similar to the single-trial response pattern was chosen as the decoder's prediction as to which visual cue was presented.

For each mouse and each session, we used all simultaneously imaged neurons. We used within-day, across-state z-scored timecourses of responses to each cue, and re-zeroed the response to each cue using the 1-s pre-cue period. For each trial, we then obtained a population 'template vector' for each cue by calculating the average cue-evoked response of each neuron in the window from 0-2 s following cue onset (resulting in a vector with *n* points for *n* neurons) and normalized it to a vector of unit magnitude in order to explicitly classify based on the *pattern* (as opposed to magnitude) of neural responses across trials. For test trials, we used multiple durations of response, integrated from cue onset (in steps of 200 ms), for the entire population. For each trial, we obtained a 'trial vector' by taking the mean response pattern from cue onset across various durations post-cue onset. Each 'single trial vector' (response vector with *n* points for *n* neurons) was normalized to a vector of unit magnitude in order to examine the pattern of neural responses. For each trial, we then calculated the cosine similarity between the 'trial vector' and the 'template vectors' of each cue, at the different response durations. The decoder's prediction of which cue was being presented during that trial was the cue whose 'template vector' was most similar to the 'trial vector' (i.e., highest cosine similarity).

We quantified decoder accuracy as the fraction of food cue trials in which we correctly predicted the presentation of the food cue (chance: 33%). We assessed maximal decoder accuracy by creating a 'template vector' from a randomly-selected subset of 75% of trials for each cue, and testing the decoder on the remaining 25% of trials. This was repeated 1000 times, and the average of all these repetitions was used as a measure of maximal decoding accuracy (e.g., decoder trained on 'Hungry-1' and tested on 'Hungry-1'). For imaging experiments involving AgRP neuron activation, we examined decoder accuracy when training it on all trials during 'Hungry-1' and testing it on the other 3 states ('Sated-1', 'Sated-2', 'Sated-2+AgRP'). For two consecutive days imaged during hunger, we examined decoder accuracy when training it on all trials

during a hunger state and testing it on trials from the following day ('Hungry next day'). We compared average decoding accuracy (across 4 mice) to chance using a 1-tailed t-test ($p<0.05$).

Fiber photometry data analysis

For data analysis, fluorescence traces were down-sampled from 1 kHz to 100 Hz and smoothed using a 1-s running average. We calculated the fractional change in fluorescence using $\Delta F/F = (F - F_0)/F_0$. In home-cage pellet drop experiments, F_0 was the average of 30 s before the first pellet drop. In the context of the visual discrimination task, F_0 was the average of 1 s before each cue. To examine the evolution of responses during a recording session across mice, we first normalized the signal from each mouse to its average response across all trials. Then we averaged these normalized responses per trial across the three mice.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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