Agouti-related peptide neural circuits mediate adaptive behaviors in the starved state

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In the face of starvation, animals will engage in high-risk behaviors that would normally be considered maladaptive. Starving rodents, for example, will forage in areas that are more susceptible to predators and will also modulate aggressive behavior within a territory of limited or depleted nutrients. The neural basis of these adaptive behaviors likely involves circuits that link innate feeding, aggression and fear. Hypothalamic agouti-related peptide (AgRP)-expressing neurons are critically important for driving feeding and project axons to brain regions implicated in aggression and fear. Using circuit-mapping techniques in mice, we define a disynaptic network originating from a subset of AgRP neurons that project to the medial nucleus of the amygdala and then to the principal bed nucleus of the stria terminalis, which suppresses territorial aggression and reduces contextual fear. We propose that AgRP neurons serve as a master switch capable of coordinating behavioral decisions relative to internal state and environmental cues.

Ecological studies reveal that prey species display cost–benefit decision making when foraging for food. The costs of foraging include food-seeking energy demands along with environmental threats such as predation and thermal challenges. Many prey species forage within a familiar territory, in zones that are protected from predators and have moderate temperatures1–3. However, when challenged with starvation, behavioral priorities adapt and prey species display higher-risk behavior to find food4–7.

Orexigenic AgRP neurons are active in a starved state8,9 and elicit signals that are paramount to the sensation of hunger10–12. Named for their expression of agouti-related peptide (AgRP), AgRP neurons are inhibitory projections neurons; they are GABAergic and express two inhibitory neuropeptides, neuropeptide Y (NPY) and AgRP8,13–15. Somewhat paradoxically, AgRP neurons appear to stimulate hunger by inhibiting downstream brain regions involved in satiety. AgRP neurons are derived from at least two progenitors16 and project (with minimal collaterals) to approximately 15 distinct brain regions14,17. Activation of distinct AgRP projections reveals a ‘parallel and redundant’ signaling network, but interestingly, some AgRP target regions do not evoke a feeding response17. We propose that the heterogeneous AgRP population functions to coordinate numerous behavioral and physiological adaptations that prioritize food seeking and energy conservation under conditions of starvation.

AgRP neurons may influence behavioral decisions by signaling to brain regions that are involved in sensory processing. For example, a subset of AgRP neurons project to the medial amygdala (MeA)14,18,19, a brain region implicated in innate social behaviors including aggression20. Chemosensory cues from conspecifics activate cells in the mouse MeA, as indicated by the expression of Fos21,22, and acute activation of GABAergic cells in the posterior dorsal MeA can induce attack behavior23. Under conditions of starvation, AgRP signaling to the MeA may alter an animal’s normal response to chemosensory cues, shifting behavior away from protecting an energy-depleted territory and toward exploratory, food-seeking behavior. To test this idea, we used a combination of viral and genetic tools to activate AgRP neurons and compared the behavior of these mice to those in the fasted state. We describe a specific starved-state neural circuit that influences innate and learned behavioral responses (Supplementary Fig. 1).

RESULTS
AgRP circuits promote risk taking and reduce territoriality

Starvation promotes higher-risk foraging behavior such that prey species become willing to search for food in exposed areas outside of their territorial safe zone4. To model this behavioral shift in a laboratory setting, we designed an experiment that challenges mice to search for food in a chamber that they are conditioned to associate with a mild foot shock (Fig. 1a). We observed that, under normal conditions, mice avoided the shock-associated area, spending only 24.5 ± 2.5% (± values represent s.e.m. throughout) of the trial in this chamber. Fasted animals, however, overcame the conditioned threat and spent more than 40% of the time in the shock-associated side. During habituation and training, food was present below the floor grid in the shock-associated chamber. On test day, the food either remained under the floor grid (food-blocked group) or was presented in the chamber and available for.

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consumption (food-access group). The food-access group spent 46.9 ± 4.6% of the trial in the shock chamber; similarly, the food-blocked group spent 43.2 ± 1.6% of the trial in the shock chamber (Fig. 1d; all statistical comparisons are detailed in the Supplementary Methods Checklist). We questioned whether the fasted state or the food cues biased the animals’ behavior. However, when the experiment was performed in the absence of food entirely, fasted mice behaved similarly to fed controls, spending only 25.7 ± 4.7% of the trial in the shock chamber (Supplementary Fig. 2a).

We reasoned that AgRP neurons may promote high-risk exploration in the starved state. To test this idea, we made AgRP neurons excitable by transducing Agprcre mice with a conditional virus containing the stimulatory DREADD (designer receptor exclusively activated by designer drugs) hM3Dq (designer receptor ligand, clozapine N-oxide (CNO)), induces Gqα-mediated signal transduction and can be used to activate AgRP neurons via intraparietal delivery11. We activated AgRP neurons in fed mice and asked whether this isolated circuit could recapitulate the foraging behavior of fasted mice in the food-challenge assay described above. Like fasted animals, AgRP neuron–stimulated mice spent more time in the shock-associated chamber relative to controls. Both food-available and food-blocked groups spent 40.0 ± 5.6% and 45.6 ± 5.6% of the trial in the shock chamber, respectively; while control animals displayed an aversion to the shock-associated side and spent only 22.7 ± 3.2% of the trial in this chamber (Fig. 1d). These data support the idea that, beyond promoting food intake, AgRP neurons can influence the behavioral response to environmental threats.

In a second test, we evaluated innate anxiety-like behavior by assessing the willingness of animals to enter an exposed platform on an elevated maze. In support of previous literature22, both fasted and AgRP neuron–stimulated animals spent significantly more time in the exposed platforms than controls (Fig. 1e). AgRP neuron activation has been demonstrated to promote locomotor activity11, and, consistent with this, stimulated mice moved an average total distance of 3.148 ± 148.3 cm on the maze, while controls moved an average of 2.024 ± 186.5 cm.

During starvation, the costs associated with foraging are not limited to environmental threats, but also include the threat of dwindling energy reserves. Consequently, organisms forage in a way that minimizes the energy costs associated with food seeking1–3,26. For example, territorial-defense behavior is not an efficient use of energy if a territory is depleted of resources27. Experimentally, we evaluated territorial behavior using the resident–intruder assay and defined territory as a defended area. Resident males were sexually experienced and territorialized to an isolated home cage. The intruder test evaluated aggressive territorial behaviors including holding, fighting (boxing, attacking, mounting), high-speed chasing and nudging when an intruder (younger, sexually naive, group-housed littermates that do not display aggression toward residents) was placed in the cage (Supplementary Fig. 2b). Compared to those in a fed state, fasted animals displayed less home-cage aggression toward an intruder (Fig. 1f). We observed that fasted residents spent significantly (P = 0.0001) more time investigating the snout of the intruder—perhaps smelling food odors on the intruder’s snout—and displayed escape behaviors including rearing and jumping (Supplementary Fig. 2d–f). We used a 48-h fast to maximally activate the feeding circuits; however, 24-h fasted residents also displayed decreased home-cage aggression (Supplementary Fig. 2c).

To test the role of AgRP neurons in fasting-related territorial behavior, we evaluated activated AgRP neurons in fed resident mice. Like fasted mice, AgRP neuron–stimulated mice displayed less home-cage aggression toward an intruder (Fig. 1f). If food was presented during the trial, AgRP neuron–stimulated residents spent the majority of the 10-min trial eating, consuming 0.34 ± 0.03 g (Supplementary Fig. 3). AgRP  $\rightarrow$ MeA signaling influences territorial behavior

Because MeA neurons are involved in innate social behavior, including territoriality, we reasoned that the inhibitory AgRP  $\rightarrow$ MeA
circuit may be responsible for starved-state decreases in territorial aggression. To test whether AgRP fibers can directly inhibit cells in the MeA, we transduced Agrpcre mice with a conditional channelrhodopsin-2 (ChR2)-expressing virus28 (Fig. 2a). We photostimulated AgRP fibers in the MeA and performed whole-cell recordings in slice preparations. MeA somata in close proximity to fluorescent AgRP fibers were recorded in patch-clamp mode; 4 of 11 cells from 2 mice displayed a light-evoked inhibitory postsynaptic current (IPSC) that was blocked by the GABAA receptor antagonist picrotoxin but not by glutamate receptor antagonists (Fig. 2b). We used retrograde tracing to quantify the subset of AgRPMeA-projecting neurons. Fluorescent RetroBeads injected into the MeA (Supplementary Fig. 4a) were retained in 7.1 ± 0.6% of NPY-expressing ARH cells (arrowheads). Scale bar, 100 µm; outlined region is magnified at right. (d) Schematic illustrating the projection of a subset of AgRP neurons (black filled) to the MeA. (e) Bilateral injection of AAV1-DIO-ChR2:YFP into the ARH with dual fiber-optic cannulas implanted above either the MeA or PVH. (f) Stimulation protocol for behavioral studies: 10 Hz with 5-ms pulses that continue for 5 s, followed by 2 s light-off recovery. (g) Paired two-tailed Student’s t-test analysis of home-cage aggressive behavior, comparing light on versus light off conditions; 10-min trial conducted during the first 2 h of the dark cycle (17:00–19:00). (h) Cumulative food intake measured during the light cycle (10:00–14:00) compared using a one-way ANOVA with Bonferroni’s multiple comparison tests. See Online Methods for detailed statistics. **P < 0.01, ***P < 0.001.

Manipulating cells downstream of AgRP in the MeA

AgRP neurons co-express NPY3,14; therefore, postsynaptic targets of AgRP neurons are likely to express NPY 1 or 5 receptors (Npy1R or Npy5R)33 and the melanocortin 4 receptor (MC4R). To evaluate the function of MeA cells that receive information from AgRP neurons, we generated an Npy1Rcre knock-in mouse line (Fig. 3a). We validated the correct targeting of this knock-in using multiple approaches. Before injection, neomycin-resistant embryonic stem cell colonies were screened for the proper insertion of cre in the targeted Npy1r allele by Southern blot analysis. We also evaluated transcripts expressed in Npy1rcre cells by crossing this line to a Cre-dependent RiboTag mouse that expresses an epitope-tagged ribosomal protein (RPL22:HA)34. The conditional expression of the hemagglutinin (HA)-tagged ribosomes in Cre-positive cells allowed us to isolate mRNA transcripts from these cells. Npy1rcre RiboTagged cells in the MeA (Fig. 3b) were enriched in both Npy1r and cre transcripts relative to transcripts expressed in all cells in the same region (Fig. 3c).

To further profile the Npy1RMeA cells, we mined the RiboTag-isolated transcriptome, probing for genes characteristic of excitatory, inhibitory and glial cells. We found that Npy1R-RiboTagged cells were enriched for both Mc4r and Gad2 transcripts. The glutamate transporter Slc17a6 (Vglut2) was not enriched and the glial cell marker Cnp was de-enriched (Fig. 3c). These data suggest that Npy1R cells in the MeA may be inhibitory neurons.

Npy1RMeA cells are anatomically distributed throughout the MeA, with a slight bias toward the anteroventral subdivision
*Figure 3* Npy1R neurons in the MeA can evoke aggression and inhibit feeding. (a) Npy1Rcre::GFP knock-in targeting construct (not to scale; see Online Methods for details). ORF, open reading frame; ex, exon. (b) RiboTag mice contain a Cre-dependent epitope-tagged polyribosome gene. MeA tissue was harvested (red circles) from Npy1rcre::RiboTag mice; breg, distance from bregma (mm). (c) Comparison of immunoprecipitated (IP) transcripts versus input sample, demonstrating enrichment of Npy1r and cre transcripts along with Gad2 and Mc4r. (d) Top, Npy1R expression in the anterior-dorsal (ad) and anterior-ventral (av) MeA (top); bottom, expression in posterior-dorsal (pd) and posterior-ventral (pv) MeA. Three animals were evaluated, demonstrating a similar expression pattern. Scale bar, 200 μm. (e) Left, bilateral injection of AAV1-DIO-hM3Dq:YFP into the MeA of Npy1rcre mice; right, histology demonstrating the injection site and Cre-dependent YFP fluorescence. Two animals were excluded from the study following histological analysis due to lack of reporter expression. Scale bar, 200 μm. (f,g) Stimulation of Npy1RMeA cells of resident animals evokes aggressive behavior (assessed during between 17:00–19:00; f) and decreased food consumption during the dark cycle (17:00–21:00; g); compared using unpaired two-tailed Student’s t-tests. (h) Bilateral injection of AAV1-DIO-GFP:TetTox into the MeA of Npy1rcre mice with histology to demonstrate the injection site. Scale bar, 200 μm. (i-k) Npy1RMeA neuron silencing resulted in decreased threat avoidance behavior (i), measured by time spent in the shock-associated side on test day (see Fig. 1a); compared using an unpaired two-tailed Student’s t-test. Silencing increased body weight, compared using a two-way repeated measures ANOVA (j); error bars, s.e.m. We did not observe a difference in home-cage aggression in Npy1RMeA silenced animals, compared using an unpaired two-tailed Student’s t-test (P = 0.296; k). *P < 0.05, **P < 0.01, ***P < 0.001. See Online Methods for detailed statistics.

(See Figs. 3d and Supplementary Fig. 5), a pattern that resembles that of Mc4r expression. To investigate whether Npy1RMeA cells are involved in feeding behavior or aggression, we bilaterally transduced the MeA of Npy1rcre mice with AAV1-DIO-hM3Dq:YFP (Fig. 3e). Because inhibitory AgRP → MeA fiber stimulation decreased territorial aggression, we predicted that activation of target Npy1R neurons would have the opposite effect. We found that CNO-induced Npy1RMeA neuron activation significantly increased territorial aggression (Fig. 3f,g). To determine whether these were secondary targets of AgRP neurons, we injected a Cre-dependent and trans-synaptic anterograde tracing virus, H129∆-fs-TK-TT into the pBNST. Likewise, we observed few, if any, fluorescent cell bodies in the ARH following injection of fluorescent RetroBeads into the pBNST (Supplementary Fig. 7a,b). To test the idea that the pBNST is a secondary target of AgRP neurons via the MeA, we co-injected RetroBeads into the pBNST and H129∆-fs-TK-TT into the ARH of Agrpcre mice (Supplementary Fig. 7c,d). We observed expression of both reporters in the MeA (Fig. 4d), consistent with the idea that the pBNST is a secondary target of the AgRP → MeA
Figure 4 The posterior BNST is a secondary target of AgRP and receives direct input from Npy1R<sup>MeA</sup> neurons. (a) Injection of AAV1-DIO-Synaptophysin:YFP into the MeA of Npy1<sup>Cre</sup>/+ mice. (b) Immunoreactive YFP fibers in the LS (lateral septal nucleus, rostroventral part), PO (preoptic area), aco (anterior commissure), RCH (retrochiasmatic area), LHA (lateral hypothalamic area), PAG (periaqueductal gray), VMH (ventromedial hypothalamus), MeA (medial amygdala), PB (parabrachial nucleus) and NTS (nucleus of the solitary tract). Findings were similar in three animals with properly targeted viral injections. Scale bar, 200 µm (see breg – 1.6, where breg is distance from bregma in mm). (c) AgRP neurons relay information to the pBNST and MeA. The trans-synaptic virus, H129∆-fs-TK-TT, was injected into the ARH of Agrp<sup>Cre</sup> mice (top panel). Immunoreactive DsRed cells were present in the MeA (middle panel) and pBNST (bottom panel). Scale bar, 200 µm. (d) The MeA relays signals from AgRP neurons to the pBNST. Diagram shows co-injection of green RetroBeads into the pBNST and H129∆-fs-TK-TT into the ARH of Agrp<sup>Cre</sup> mice. DsRed immunoreactive cell bodies and RetroBead-positive cells are present in the MeA. Scale bars, 100 µm; boxed region is magnified at right. Two animals were evaluated with proper targeting of both the ARH and pBNST demonstrating a similar expression profile in the MeA. (e) Model of the AgRP → MeA circuit.

To investigate the properties of pBNST-projecting MeA neurons, we injected RetroBeads into the pBNST and performed whole-cell recordings on bead-labeled cells in the MeA (Fig. 5). When subjected to current-step injections, the cells showed a prominent hyperpolarization-activated voltage sag (h-current) in 10 of 13 cells recorded. This h-current has been described in type 1 GABAAergic projections in the posterior MeA<sup>42</sup>. In addition, we discovered...
Figure 6 Npy1RMeA neurons that project to the pBNST evoke territoriality. (a) Unilateral injection of AAV-DIO-ChR2:YFP into the MeA of Npy1RMeA mice, with ipsilateral optic cannulas implanted above the pBNST or VMH. (b) Stimulation of Npy1RMeA fibers in pBNST increased aggression in a home-cage intruder assay (left pair; **P = 0.004), while stimulation of fibers in VMH did not (right pair; not significant, P = 0.074); assessed during the dark cycle (17:00–19:00) and compared using paired two-tailed Student’s t-tests.

that that a subset of these neurons (7 of 13 cells) expressed a T-type calcium current (Fig. 5c).

We determined that AgRP neurons could evoke GABA-mediated IPSCs in MeA neurons. Using the RetroBead labeling described here, we sought to determine whether ChR2-expressing AgRP neurons could evoke light-induced responses in pBNST-projecting MeA cells (Fig. 5a,b). Bead-positive MeA somata in proximity to YFP fibers demonstrated light-evoked IPSPs in 3 of 11 recorded cells (Fig. 5d). As in the recordings in Figure 2b, the light-evoked inhibitory response occurred with a short latency to the photostimulation, suggesting a direct connection. To support this idea, we identified a shifted (smaller and longer latency) light-evoked IPSP in the presence of the action-potential blocker TTX along with the potassium channel blocker 4-aminopyridine—a property indicative of monosynaptic connections in ChR2-assisted circuit mapping (Fig. 5d)

The light-responsive, bead-positive MeA cells also responded to NPY. Bath application of NPY in the presence of TTX resulted in an outward current when the cell was held at a membrane potential of ~60 mV in 4 out of 4 cells tested (Fig. 5e). To investigate the NPY-induced current, voltage ramps were performed in the presence and absence of NPY (Fig. 5f). Consistent with the idea that NPY-induced current is mediated by G-protein-coupled, inwardly rectifying potassium channel activation and reversal potential for potassium.

On the basis of the firing properties recorded in bead-positive cells (Fig. 5c) along with enrichment of Gad2 in Npy1R- RiboTagged cells (Fig. 3c), we predicted that these neurons were GABAergic. Following recording, we harvested the cytosol of the cells and performed single-cell RT-PCR to test for Slc32a1 (Vgat) expression. The majority of cells were Vgat-positive: out of 11 successfully harvested Actb-positive cells, 7 were positive for Vgat expression, while the remaining 4 did not amplify either Vgat or Vgat2 templates (Fig. 5g). These data are consistent with the idea that a subset of AgRP neurons synapse on a population of inhibitory, NPY-responsive cells in the MeA that project to the pBNST.

Npy1RMeA → pBNST signaling influences territorial behavior

The Npy1RMeA population projects to numerous efferent targets, some of which have been implicated in aggressive behavior, including the VMH and PAG. Because AgRP neurons make a disynaptic connection to the pBNST via the MeA, we hypothesized that this circuit may be involved in territorial aggression. We virally transduced Npy1RMeA mice unilaterally with ChR2:YFP virus and placed a fiber-optic cannula above either the ipsilateral VMH or pBNST (Fig. 6a). Optogenetic stimulation of Npy1RMeA fibers in the pBNST evoked significantly more territorial defensive behavior (Fig. 6b).

However, rather than overt attack behavior, the pBNST-fiber stimulation increased nudging activity: the resident mouse followed the intruder for the majority of the assay, constantly nudging the intruder into the wall of the cage (Supplementary Movie 3). A nudging display, as opposed to violent aggression, may be adequate for territorial defense from most competitors (Supplementary Fig. 7e). We activated Npy1RMeA somata using metabotropic hM3Dq DREADD receptors whereas we stimulated the Npy1RMeA fibers in the pBNST using ionotropic photostimulation; hence, we cannot exclude the possibility that this difference accounts for the behavioral difference.

Optogenetic stimulation of Npy1RMeA fibers in the VMH did not result in significant differences in territorial aggression (Fig. 6b). We also measured the effect of photostimulating Npy1RMeA axons in the pBNST or VMH on food intake, but did not observe a significant effect of stimulating either of these projections (Fig. 6c). Because Npy1R neurons project to numerous downstream targets, it is likely that the violent aggression observed following Npy1RMeA neuron stimulation (Fig. 3f and Supplementary Movies 1 and 2) and also feeding behavior (Fig. 3g) are orchestrated by projections to targets other than the pBNST or VMH. The modulatory effect of AgRP MeA fiber stimulation on territorial behavior likely involves Npy1R neurons that project to the pBNST, a circuit that can be modulated under physiological conditions of negative energy balance.

DISCUSSION

Risk assessment and territoriality require sensory processing of environmental cues. Rodents select a territorial domain for nesting and foraging with respect to the risk of predation and will defend the limited resources of this area from conspecific intruders. However, under conditions of starvation, mice forage in more exposed or threatening areas and are less willing to defend a territory that is depleted of resources, exemplifying a behavioral adaptation that is associated with an internal state change.

Hungry animals will aggressively defend limited food resources from competitors. However, if food is depleted, starving mice try to escape from their territorialized home cage (Supplementary Fig. 1a,c), display less aggression toward an intruding conspecific, are less anxious, and engage in risky exploration to seek food. This shift in behavior is accompanied by a coincident change in AgRP neuron activity. In the absence of food, AgRP neurons are activated by interoceptive cues of negative energy balance, but when food or food-related cues are present, AgRP neuron activity is rapidly silenced.

We demonstrate that a subset of AgRP neurons can evoke GABA-mediated inhibition of the MeA and argue that this circuit is responsible for modulating aggressive territorial behavior when food is limited. If food is discovered during foraging, this cue should rapidly relieve GABA-mediated AgRP inhibition of the MeA, providing a switch to adjust behavior for...
food acquisition. This behavioral switch is difficult to model in the confines of a small arena and isolated housing conditions. When food was presented to residents that were hungry (artificially induced by AgRP neuron stimulation) during an intruder trial, they chose to eat rather than interact with the intruder (Supplementary Fig. 3). Fluctuations in territorial aggression with respect to a limited or depleted food source could be evaluated with the development of techniques to study and track individual animals in a large, group-housed arena.

We proposed that the AgRP → MeA circuit is involved in territorial adaptations during starvation, but questioned whether hunger itself could influence territoriality. In this experiment we targeted the AgRP → PVH circuit, which has been demonstrated to evoke robust feeding behavior equivalent to that following a fast. Unlike activating AgRP → MeA fibers, stimulation of the AgRP → PVH circuit did not reduce territorial aggression (Fig. 2g). These data support the idea that hunger itself does not change territorial behavior. We define an MeA,opt←→pBNST circuit that is downstream of AgRP and can alter territorial behavior. There are, however, many other AgRP targets throughout the brain, and, as in feeding, they may play redundant roles in territorial adaptations.

The behavioral adaptations that occur during starvation facilitate food acquisition and minimize unnecessary energy expenditure, a complex state-change likely attributable to the broad projection profile of AgRP neurons throughout the brain. Not all anatomically distinct AgRP subsets contribute equally to food-intake behavior. Optogenetic stimulation of AgRP fibers in the PVH evokes maximal food consumption, while stimulation of AgRP fibers in the paraventricular thalamus and parabrachial nucleus have less, if any, influence on food consumption. Similarly, we find that AgRP fibers in the MeA can evoke food intake, but the magnitude of this effect is much less than that of equivalent fiber stimulation in the PVH. The long-term consequence of inhibiting cells in the MeA is a significant increase in body weight and food intake (Fig. 3j and ref. 31). One idea to resolve the differing degrees of food consumption observed by activating distinct AgRP neuron target regions is that their contributions are additive; however, because PVH stimulation is equivalent to stimulating AgRP cell bodies, that explanation is unlikely. Instead, brain regions where AgRP-axon stimulation promotes less food consumption may help coordinate nonfeeding behaviors with hunger. For example, AgRP-mediated inhibition of the MeA induces feeding and suppresses territoriality, providing a circuit that can function independently to coordinate two behaviors. It is also possible that, under some conditions, select populations of AgRP neurons become activated. The potential for AgRP neurons to orchestrate a complex behavioral response is broad. Future studies detailing the behavioral and physiological contribution of other AgRP targets will provide a complete profile of the starved-state behavioral response.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.L.P. designed the study under the guidance of R.D.P.; I.Q. gathered and analyzed electrophysiological data in the laboratories of M.J.K. and O.K.R.; M.E.S. gathered and analyzed electrophysiology data in the laboratory of L.S.Z.; E.S. performed the RiboTag pulldown and quantitative PCR in the laboratory of A.Q.; C.C.N. performed single-cell PCR on harvested cells after whole-cell recordings; R.D.P. generated the AgRPCre and Npy1r1+ lines of mice; F.D.B. assisted with behavior experiments and blind scoring; R.D.P., M.J.K., O.K.R. and L.S.Z. provided laboratory space and resources to conduct the experiments; S.L.P. and R.D.P. wrote the manuscipt with revisions and input from all contributing authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Adult male mice (2–6 months old) were bred onto a C57BL/6 background and housed on a 12-h light cycle (5:00–17:00). All experiments were approved by The Animal Care and Use Committee at the University of Washington and were in accordance with NIH guidelines. Apgrpcre/GFP and RiboTag (Rpl22tm1(Hsu)Jo) were backcrossed into C57BL/6J mice and were characterized. The cre-dependent reporter strain, Rosa26Sorcre-ERT2, was acquired from Jackson Laboratories (stock no. 007914).

Npy1rcre/GFP knock-in mice were generated by gene targeting in ES cells. A creGFP fusion gene was inserted just 5′ of the normal initiation codon for Npyy1r. The targeting construct had 3.5 kb 5′ and 3′ arms that were prepared by PCR (Npyy1r gene) and inserted into a targeting vector with StvNeo for positive selection and Hsv-TK and PGK-DTA for negative selection (Npyy1rcre targeting construct, Fig. 3a). After targeting, creGFP expression is under control of Npyy1r regulatory elements. The Sve-Neo selectable gene can be removed by action of FLP recombinase.

Stereotoxic injections and tissue preparation. Stereotoxic surgery and injection coordinates. Mice were anesthetized and positioned on a stereotaxic alignment device (David Kopf instruments). During the procedure, body temperature was maintained with a heating pad and a nose cone delivered isoflurane (1.5–2%). Either a Hamilton syringe (88000) or pulled glass capillaries was used to inject the target brain regions: ARH, bregma −1.25, lateral ± 0.25, ventral − 5.8; MeA (viral injections), bregma − 1.0, lateral ± 2.25, ventral − 5.55; pRNST, bregma + 0.4, lateral ± 0.9, ventral − 4.25. Cannulas were implanted 0.5 mm above the position of the injection except in the MeA, in which the cannula was implanted caudal to the injection at bregma − 1.5.

Fiber placement. MeA and PVH tracks were evaluated at bregma − 1.5 mm and − 0.8 mm, respectively. Terminal placement is shown in Supplementary Figure 4. It is likely that some of the MeA-targeted fibers terminated in the lateral ventricle, as we did not find terminals for every implant.

Viruses and neuronal tracers. AAV serotype 1 viruses (AAV1-Ef1α-DIO-hM3Dq:YFP, AAV1-Ef1α-DIO-hM3Dq-mCh, AAV1-CAG-DIO-GFP:TeTx, AAV1-Ef1α-DIO-Chr2:YFP, AAV1-Ef1α-DIO-Synaptophysin:YFP, AAV1-Ef1α-DIO-GFP) were generated at the University of Washington as described. We did not observe side effects in animals injected with any of the AAV1 viruses. AAV1 virus (500 nL) was injectected at a dose of 1.0 mg/kg CNO administration for Fos analysis.

Histology. Mice were anesthetized and transcardially perfused with saline, followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were postfixed (6 h), washed, cryoprotected in 1:0.1 MB with 30% sucrose (w/v), embedded in OCT and frozen at −80 °C. For immunohistochemistry, 30-μm floating sections were stained for YFP or tdTomato with the primary antibodies: rabbit anti-GFP (Life Technology A11212; diluted 1:2000) or rabbit anti-DsRed (Clontech 632496; diluted 1:1000). Antibodies were diluted in 0.1 MB with 0.1% Triton and 2% donkey serum and developed overnight at 4 °C.

CNO administration for Fos analysis. CNO was injected at a dose of 1.0 mg/kg body weight 2 h before euthanizing the animals. The tissue was stained with goat anti-Fos (Santa Cruz 48869, diluted 1:300).

Electrophysiology. Figure 2b. Whole-cell recordings were made using an Axopatch 700B amplifier (Molecular Devices) with filtering at 1 kHz using 4–6 MΩ electrodes. Coronal brain slices (250 μm) were prepared in an ice slush solution containing (in mM) 250 sucrose, 3 KCl, 2 MgSO4, 1.2 NaH2PO4, 10 t-glucose, 25 NaHCO3, 0.1 CaCl2. Slices recovered for 1 h at 34 °C in artificial cerebrospinal fluid (ACSF) continually bubbled with O2/CO2 and containing (in mM) 126 NaCl, 2.5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 11 t-glucose, 18 NaHCO3, 2.4 CaCl2. Patch electrodes were filled with an internal solution containing (in mM) 130 CsCl, 2 MgCl2, 0.5 EGTA, 10 HEPES, 0.25 Na-GTP and 2.5 Mg-ATP, pH 7.2–7.4, 280 mosm. ACSF at 32 °C was continually perfused over slices at a rate of ~2 ml/min during recording. Drugs were purchased from Abcam and were applied to the bath where indicated at the following concentrations: CNQX, 10 μM; APV, 100 μM; picrotoxin, 100 μM. For light-evoked responses the fiber-optic cable was lowered into the bath and 10–ms light pulses (10 mW) were delivered at a rate of 0.1 Hz while cells were held in voltage-clamp mode at −70 mV. Example traces are averages of 15 sweeps.

Behavior. To minimize the hierarchical dominance behavior observed in adult group-housed male mice, our behavior studies were performed on singly housed mice. The behavior results were scored blindly.

AgRP neuron–stimulated cohort: prescreening. Numerous studies using viral targeting as a means of activating AgRP neurons have found that the efficiency (number of cells transduced) correlates with the magnitude of the feeding response when activated. On this basis, we used a prescreening criterion to select animals that consumed at least 1 g of food 4 h after administration of CNO (1mg/kg).

Food-challenge assay. Rooms of two-chamber arena were distinguished by visual (vertical striped or wood grain wall paper), olfactory (nestlet below the floor containing three drops of either almond or orange extract) and tactile (wire mesh versus metal bar flooring) cues (Fig. 1a). Mice were exposed to this context on 3 consecutive days during the light cycle (5:00–17:00). On habituation day 1, mice were placed in the almond side of the cage and allowed to explore both chambers of the arena for a 30-min session. Mice were excluded from the test if they displayed ≥70% preference for either room. On training day 2, mice were again placed in the almond side; upon movement into the orange chamber, the door dividing the chambers was closed and a shock protocol (2 s, 0.3-mA shock, every 3 min over the course of 30 min) was initiated. Immediately following the last shock interval, mice were removed and placed in their home cage. Following a 2-h inter-trial interval, mice were returned to the almond chamber, the dividing door was shut and the mice did not receive shocks during this 30-min session. Food was present below the floor grid on the orange side on days 1 and 2. On testing day 3, mice were placed on the almond side with the chamber door open and their movement was tracked for a 30-min session. Depending on the experimental conditions, food was either available above the floor grid (food-available cohort) or was again placed below the floor grid (food-blocked cohort) in the orange/shock chamber. Food-available mice were excluded from the test if they moved the food pellet into the almond side during the test session. Video recordings on days 1 and 3 were evaluated manually in a blinded manner. Group 1, fed versus fasted: all animals were food deprived between day 1 and day 2 training. Food was returned to fed animals following training, while fasted animals continued to be deprived. Group 2, AgRP stimulated (hM3Dq + CNO) versus AgRP controls (YFP + CNO): on test day, food was removed from the home-cage hopper 1 h before testing and animals were injected with CNO (1 mg/kg body weight). Group 3, Npy1r silenced (TeTox) versus Npy1r controls (YFP): food was provided ad libitum throughout the trial. We did not repeat this test on individual subjects; each animal was evaluated only once.
Elevated plus maze. Movement within the arms of a plus maze was recorded and scored for time spent in the open arms. The trial was conducted during the light cycle (5:00–17:00). The open-arm score is the cumulative time the animal spent in either open arm during the 10-min session. The center of the maze was excluded from scoring. The animal’s movement was video recorded and scored using Ethovision XT (Noldus) video-tracking software. Mice were excluded from the study if they failed to stay on the maze for the duration of the trial. Group 1, fed versus fasted: fasted animals were food deprived for 48 h before testing. A separate cohort of age-matched mice given ad libitum access to food was used as controls. Group 2, AgRP-stimulated (hM3Dq + CNO) versus AgRP controls (YFP + CNO): on test day, food was removed from the home-cage hopper 1 h before testing and animals were injected with CNO (1 mg/kg). Group 3, Npy1R (YFP + CNO): on test day, food was removed from the home-cage hopper 1 h before testing and animals were injected with CNO (1 mg/kg). Sample size: 6; ChR2 off versus ChR2 stimPVH, n = 57.61, s.d. = 28.16) versus hM3Dq + CNO (M = 11.73, s.d. = 9.76): t(8) = 1.96, **P < 0.006.

Figure 1d. Sample size: ad libitum, n = 15; fasted food-available, n = 7; fasted food-blocked, n = 7; YFP + CNO, n = 13; hM3Dq + CNO food-available, n = 6; hM3Dq + CNO food-blocked, n = 7. One-way ANOVA: F(5,49) = 10.35, ***P < 0.0001. Bonferroni’s multiple comparison tests: ad libitum versus fasted food available, 95% CI (10.67 to 2.78) ***P, ad libitum versus fasted food-blocked, 95% CI (−9.58 to −1.69) ***P, YFP + CNO versus hM3Dq + CNO food available, 95% CI (−9.44 to −3.92) ***P, YFP + CNO versus hM3Dq + CNO food-blocked, 95% CI (−10.93 to −2.85) ***P.

Figure 1e. Sample size: ad libitum, n = 8; fasted, n = 7; YFP + CNO, n = 12; hM3Dq + CNO, n = 8. One-way ANOVA: F(3,11) = 5.88, ***P = 0.0003. Bonferroni’s multiple comparison tests: ad libitum versus fasted, 95% CI (−3.58 to −0.05) *P, YFP + CNO versus hM3Dq + CNO, 95% CI (−3.79 to −0.68) ***P = 0.01.

Figure 1f. Sample size: ad libitum versus fasted, n = 12; hM3Dq + saline versus hM3Dq + CNO, n = 10. Paired two-tailed Student’s t-test: ad libitum (M = 52.42, s.d. = 21.10) versus fasted (M = 33.58, s.d. = 13.60): t(11) = 2.87, *P = 0.015; hM3Dq + saline (M = 57.61, s.d. = 28.16) versus hM3Dq + CNO (M = 11.73, s.d. = 9.76): t(8) = 5.16, ***P < 0.0006.

Figure 2g. Sample size: ChR2 off versus ChR2 stimPVH, n = 6; ChR2 off versus ChR2 stimMeA, n = 6. Paired two-tailed Student’s t-test: ChR2 off (M = 58.62, s.d. = 17.97) versus ChR2 stimMeA (M = 27.93, s.d. = 11.27): t(7) = 4.67, ***P = 0.0023; ChR2 off (M = 58.73, s.d. = 12.54) versus ChR2 stimPVH (M = 76.37, s.d. = 22.85): t(8) = 2.02, not significant P = 0.0999.

Figure 2h. Sample size: ChR2 off n = 18; ChR2 stimMeA, n = 8; ChR2 stimPVH, n = 10. One-way ANOVA: F(2,33) = 58.68, ***P < 0.0001. Bonferroni’s multiple comparison tests: ChR2 off versus ChR2 stimMeA, 95% CI (−1.29 to −0.25) **P, ChR2 off versus ChR2 stimPVH, 95% CI (−2.56 to −1.59) ***P.

Figure 3a. Sample size: YFP + CNO, n = 8 versus hM3Dq + CNO, n = 10. Unpaired two-tailed Student’s t-test: YFP + CNO (M = 57.88, s.d. = 16.42) versus hM3Dq + CNO (M = 40.00, s.d. = 54.23); t(16) = 4.11, ***P = 0.0008.

Figure 3b. Sample size: YFP + CNO versus hM3Dq + CNO, n = 8. Sample size: ChR2 off versus ChR2 stimMeA, n = 6; ChR2 off versus ChR2 stimPVH, 95% CI (−2.36 to −1.59) ***P.

Figure 3c. Sample size: YFP + CNO, n = 8 versus hM3Dq + CNO, n = 10. Unpaired two-tailed Student’s t-test: YFP + CNO (M = 57.88, s.d. = 16.42) versus hM3Dq + CNO (M = 40.00, s.d. = 54.23); t(16) = 4.11, ***P = 0.0008.

Figure 3d. Sample size: YFP + CNO versus hM3Dq + CNO, n = 8. Sample size: ChR2 off versus ChR2 stimMeA, n = 6; ChR2 off versus ChR2 stimPVH, 95% CI (−2.36 to −1.59) ***P.

Figure 3e. Sample size: YFP + CNO, n = 8 versus hM3Dq + CNO, n = 10. Unpaired two-tailed Student’s t-test: YFP + CNO (M = 57.88, s.d. = 16.42) versus hM3Dq + CNO (M = 40.00, s.d. = 54.23); t(16) = 4.11, ***P = 0.0008.
s.d. = 20.45) versus ChR2 stim\textsuperscript{BNST} (M = 211.0, s.d. = 82.79): t(4) = 5.86, **P = 0.004; ChR2-off (M = 38.33, s.d. = 8.02) versus ChR2 stim\textsuperscript{VMH} not significant (M = 86.67, s.d. = 16.17): t(2) = 3.46, not significant P = 0.074.

**Figure 6c.** Sample size: ChR2-off versus ChR2 stim\textsuperscript{BNST}, n = 5; ChR2-off versus ChR2 stim\textsuperscript{VMH} n = 3. Paired two-tailed Student’s t-test: ChR2-off (M = 1.34, s.d. = 0.12) versus ChR2 stim\textsuperscript{BNST} (M = 1.47, s.d. = 0.04): t(2) = 1.60, not significant P = 0.251; ChR2-off (M = 1.00, s.d. = 0.58) versus ChR2 stim\textsuperscript{VMH} (M = 1.16, s.d. = 0.56): t(2) = 0.73, not significant P = 0.540.

**Supplementary Figure 2a.** Sample size: ad libitum (n = 6) versus 48-h fasted (n = 6). Unpaired two-tailed Student’s t-test: ad libitum (M = 7.65, s.d. = 3.09) versus 48-h fasted (M = 7.71, s.d. = 3.45): t(10) = 0.03, not significant P = 0.976.

**Supplementary Figure 2b.** Sample size: intruders (n = 23); residents, ad libitum (n = 12); residents, fasted (n = 12). One-way ANOVA: F(2,44) = 46.64, ***P < 0.0001. Bonferroni’s multiple comparison tests: intruder versus resident, ad libitum, 95% CI (−9.17 to −5.61) ***P; intruder versus resident, fasted, 95% CI (−4.85 to −1.29) ***P.

**Supplementary Figure 2c.** Sample size: ad libitum versus 24-h fasted (n = 6). Paired two-tailed Student’s t-test: ad libitum (M = 27.62, s.d. = 18.09): t(5) = 3.41, *P = 0.019.

**Supplementary Figure 2e.** Sample size: ad libitum versus 48-h fasted (n = 9). Paired two-tailed Student’s t-test: ad libitum (M = 57.82, s.d. = 13.53) versus fasted (M = 38.33, s.d. = 8.02): t(8) = 3.98, **P = 0.004; ChR2-off (M = 38.33, s.d. = 8.02) versus ChR2 stim\textsuperscript{VMH} not significant (M = 86.67, s.d. = 16.17): t(2) = 3.46, not significant P = 0.074.

**Supplementary Figure 2f.** Sample size: ad libitum versus 48-h fasted (n = 9). Paired two-tailed Student’s t-test: ad libitum (M = 7.29, s.d. = 4.10) versus fasted, (M = 3.75, s.d. = 22.17): t(8) = 3.63, **P = 0.007.

**Supplementary Figure 3a.** Sample size: hM3Dq + saline or hM3Dq + CNO (no food), n = 10; hM3Dq + CNO (with food), n = 7. One-way ANOVA: F(2,24) = 20.90, ***P < 0.0001. Bonferroni’s multiple comparison tests: saline versus CNO (no food), 95% CI (25.91 to 65.85) ***P; saline versus CNO (with food), 95% CI (28.89 to 72.90) *P.

**Supplementary Figure 6.** Sample size: YFP versus TetTox, n = 4. Unpaired two-tailed Student’s t-test: YFP (M = 2.11, s.d. = 0.84) versus TetTox (M = 1.72, s.d. = 0.84): t(6) = 0.75, P = 0.479.

A Supplementary Methods Checklist is available.

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