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

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AgRP 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 AgRP neurons are critically important for driving feeding and project axons to brain regions implicated in aggression and fear. Using circuit-mapping techniques, 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 principle bed nucleus of the stria terminalis, which plays a role in suppressing territorial aggression and reducing contextual fear. We propose that AgRP neurons serve as a master switch capable of coordinating behavioral decisions relative to internal state and environmental cues.
Seminal ecological studies reveal that prey species display cost-benefit decision making when foraging for food. The costs of foraging include the energy demands associated with food seeking along with environmental threats such as predators and thermal challenges. To maximize fitness, many prey species forage within a familiar territory, in zones that are relatively protected from predators and have moderate temperatures. However, when challenged with starvation, the behavioral priorities associated with foraging adapt and prey species display higher-risk behavior to find food.

Territorial species protect their home domain from conspecifics using aggression, ranging from threat displays to outright attacks; in this manuscript we define territory as a defended area. There are data to support that food is the ultimate resource governing territorial defensive behaviors. In the absence of food, group-housed Drosophila display relatively little aggression; however, when presented with food, flies will fight to obtain nutrients. This behavior change, in the presence or absence of food, exemplifies a complex decision associated with the cost of exerting energy to challenge a competitor relative to the benefit of a food reward.

Orexigenic AgRP neurons are active in a starved state and elicit signals that are paramount to the sensation of hunger. Coined for the expression of agouti-related peptide (AgRP), AgRP neurons are inhibitory projections neurons; they are GABAergic and express two inhibitory neuropeptides, neuropeptide Y (NPY) and AgRP. Somewhat paradoxically, AgRP neurons appear to stimulate hunger by inhibiting downstream brain regions involved in satiety. Immediate early gene expression and in vivo imaging studies demonstrate that most AgRP neurons are active in the fasted state, yet this population is both anatomically and functionally heterogeneous. AgRP neurons are derived from at least two progenitors and project (with minimal collaterals) to approximately 15 unique downstream brain regions. Activation of distinct AgRP projections revealed a “parallel and redundant” network of satiety centers downstream of AgRP, but interestingly, some AgRP target regions do not evoke a feeding response. 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)\(^{15,20,21}\), a brain region implicated in innate social behaviors including aggression\(^{22}\). Chemosensory cues of conspecific mice activate cells in the MeA, as indicated by the expression of Fos\(^{23,24}\), and acute activation of GABAergic cells in the posterior dorsal MeA can induce attack behavior\(^{25}\). 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 neuronal activation promotes risk taking and reduces territorialism.**

In the wild/natural state, starvation influences foraging behavior such that prey species, in particular rodents, are willing to forage in: 1) areas that are more exposed to predators, 2) areas that pose a thermal challenge, and/or 3) novel areas outside of the habitual foraging zone\(^4\). To model this behavioral adaptation in a laboratory setting, we designed an experiment that requires mice to forage in an arena that they are conditioned to avoid. We used Pavlovian conditioning to instate a context-dependent fear for one side of a two-chamber arena. The chambers of the arena contain distinct floor texture, visual cues, and odors. During conditioning, mice learned to associate one chamber with a paired foot shock (Fig. 1a). We observed that, under normal conditions, mice will develop an aversion to
the shock-associated side, spending only 24.5 ± 2.5% of the 30-min trial in this chamber post-
conditioning. 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 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, black outlined bars; Supplemental
STATISTCS provides details of all tests performed). We questioned whether the fasted state or the food
cues biased this experimental paradigm. To validate that the conditioned avoidance could be acquired in
fasted animals we repeated the assay in the absence of food. We found that similar to fed controls,
fasted animals avoided the shock-associated chamber, spending only 25.7 ± 4.7 % of the trial in the
shock chamber (Supplementary Fig. 2a).

We hypothesized that high-risk foraging is facilitated by a neural circuit that alters the behavioral
state during starvation. Given that AgRP neurons are active in the fasted state, we reasoned that they
may promote high-risk exploration. We made AgRP neurons excitable by transducing AgrpCre mice with
the stimulatory DREADD (designer receptor exclusively activated by designer drugs), hM3Dq, via viral
delivery of AAV1-DIO-hM3Dq:mCherry into the arcuate hypothalamic nucleus (ARH); control mice
received AAV1-DIO:mCherry virus (Fig. 1b, c). The designer receptor ligand, clozapine-n-oxide (CNO),
induces Gαq-mediated signal transduction, thereby activating the AgRP neurons. Using this model, we
exposed AgRP neuron-stimulated mice to the food-challenge test described above. Similar to fasted
animals, we found that 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, red bars). These data
support 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 literature, both fasted and AgRP neuron-stimulated animals spent significantly more time in the exposed platforms compared to controls (Fig. 1e). AgRP neuron-stimulated mice are reported to exhibit enhanced locomotor activity and, consistent with this, we found that during the maze trial, stimulated mice moved an average total distance of $3148 \pm 148.3$ cm, while controls moved an average of $2024 \pm 186.5$ cm. Considering this potential confound, we analyzed the percent of distance traveled in the exposed arms. Consistent with our previous results, stimulated mice traveled more in the open arms compared to controls (stimulated, 48.2 ± 3.1%; controls, 30.7 ± 4.5%).

In the state of starvation, the costs associated with foraging are not limited to environmental threats, but also include the threat of dwindling energy reserves. As a reflection of this, organisms forage in a way that minimizes the energy costs associated with food seeking. For example, territorial-defense behavior is not an efficient use of energy if a territory is depleted of resources.

Experimentally, we evaluated territorial behavior using the resident-intruder assay. Resident males were sexually experienced and territorialized to an isolated home cage; they were evaluated for aggressive territorial behaviors including: holding, fighting (boxing/attacking/mounting), high-speed chasing, and nudging. The intruders were sexually naïve, group-housed littermates that were younger, and smaller than the resident animals (Supplementary Fig. 2b). Compared to the fed state, fasted animals displayed less home-cage aggression toward an intruder (Fig. 1f, black bars). In this manuscript we used a 48 hr fast to maximally activate the feeding circuits; however, we found that 24-hr fasted residents also displayed decreased home-cage aggression (Supplementary Fig. 2c). We also observed that fasted
residents spent significantly more time investigating the snout of the intruder—perhaps smelling food odorants on the intruder’s snout—along with displaying escape behaviors including rearing and jumping (Supplementary Fig. 2b-d). To control for changes in aggression upon repeated exposure, the order of the baseline-state test was randomized and unique intruders were used in every trial. We did not observe intruder-initiated aggression toward resident animals.

To test the role of AgRP neurons in fasting-related territorial behavior, we evaluated hM3Dq-transduced *Agrp*\textsuperscript{Cre} animals (injected with either saline or CNO) in the resident-intruder assay. Similar to fasting, AgRP neuron-stimulated mice displayed less home-cage aggression toward an intruder (Fig. 1f, red bars). If food was presented during the trial, AgRP neuron-stimulated residents spent the majority of the trial engaged with the food, eating 0.34 ± 0.03 g of food during the 10-min trial (Supplementary Fig. 3).

**AgRP neuron projections to the MeA affect feeding and territorialism**

Neurons in the MeA are involved in innate social behavior, including territorialism. We reasoned that the inhibitory AgRP\textsuperscript{MeA} circuit may be responsible for starved-state decreases in territorial aggression. AgRP-immunoreactive fibers have been observed in the MeA, but the function of the MeA-projecting AgRP population (AgRP\textsuperscript{MeA}) has yet to be defined. To test whether AgRP fibers in the MeA make direct inhibitory connections to cells in this region, we transduced *Agrp*\textsuperscript{Cre}-expressing mice with a conditional channelrhodopsin-2 (ChR2)-expressing virus, AAV1-DIO-ChR2:YFP (Fig. 2a). We photostimulated AgRP fibers in the MeA and performed whole-cell recordings in slice preparations. MeA soma in close proximity to fluorescent AgRP fibers were patched; 4 of 11 cells from 2 mice displayed a light-evoked inhibitory postsynaptic current (IPSC) that was blocked by the GABA\textsubscript{A} receptor antagonist picrotoxin (PTX) but not by glutamate receptor antagonists (Fig. 2b). We used retrograde tracing to quantify the subset of AgRP\textsuperscript{MeA}-projecting neurons. Fluorescent RetroBeads injected into the MeA (Supplementary
Fig. 4a) were retained in 7.1 ± 0.6 % of AgRP-expressing cells (167 ± 15 of 2356.3 ± 146 total hemisphere, n = 3; Fig. 2c, d). These data establish that a subset of AgRP neurons make direct inhibitory connections onto MeA neurons.

In lieu of a genetic marker to define the subpopulation of AgRP<sup>MeA</sup> neurons, we used optogenetic fiber stimulation to probe this anatomically distinct AgRP circuit (Fig. 2e, f; Supplementary Fig. 4b). Similar to fasting, AgRP<sup>MeA</sup> fiber-stimulated animals displayed less home-cage aggression relative to a non-stimulated baseline state (Fig. 2g). To gauge the specificity of the AgRP<sup>MeA</sup> circuit on territorial behavior, we stimulated AgRP neurons that project to the PVH. Prior studies demonstrated that the AgRP<sup>PVH</sup> circuit can induce maximal food intake equivalent to that observed following a fast. If fasting-induced territorial behavior is a consequence of being hungry, then this circuit should also modulate home-cage aggression. We did not observe a significant change in home-cage aggression upon AgRP<sup>PVH</sup> stimulation (Fig. 2e, f; Supplementary Fig. 4c). Together, these data indicate that distinctly projecting AgRP neurons can mediate unique behaviors.

Along with social behavior, there is evidence suggesting that the MeA is also involved in feeding behavior and body-weight regulation. We found that AgRP<sup>MeA</sup> fiber-stimulated mice ate significantly more than non-stimulated, fiber-attached controls (0.87 ± 0.12 g vs 0.10 ± 0.02 g; Fig. 2h). To gauge the relative magnitude of this effect, we measured light-evoked food intake from AgRP<sup>PVH</sup> fibers. Under comparable conditions, the hyperphagia induced by AgRP<sup>PVH</sup> fiber stimulation was 2.5-fold higher than AgRP<sup>MeA</sup> fiber stimulation (Fig. 2h). These data add to growing evidence that there are parallel and redundant AgRP circuits involved in feeding behavior.

**Manipulating cells downstream of AgRP in the MeA**

AgRP neurons co-express NPY and AgRP and, therefore, post-synaptic targets of AgRP neurons likely express receptors for these neuropeptides including NPY 1 or 5 receptors (Npy1R or Npy5R) and the...
melanocortin 4 receptor (MC4R). To evaluate the function of MeA cells that receive information from AgRP neurons, we generated an $Npy1r^{Cre}$ knock-in mouse line (Fig. 3a).

We validated the correct targeting of this knock-in using multiple approaches. Prior to injection, neomycin-resistant ES 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 $Npy1r^{Cre}$ cells by crossing this line to a Cre-dependent RiboTag mouse that expresses an epitope-tagged ribosomal protein (RPL22:HA)35. The conditional expression of the tagged ribosomes in Cre-positive cells provided a means to isolate mRNA transcripts from these cells. $Npy1r^{Cre}$-RiboTagged cells in the MeA (Fig. 3b) were enriched in both $Npy1r$ and Cre transcripts relative to transcripts expressed in all cells within the same region (Fig. 3c). These experiments confirmed the positional insertion of Cre in the $Npy1r$ allele by Southern blot and demonstrated that Cre is expressed in Npy1R cells in the MeA ($Npy1R_{MeA}$) using RiboTag technology.

To further profile the $Npy1R_{MeA}$ 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 indicate that Npy1R cells in the MeA may be inhibitory neurons, an idea explored in more detail below.

$Npy1R_{MeA}$ cells are anatomically distributed throughout the MeA, with a slightly biased distribution in the anteroventral subdivision (Fig. 3d and Supplementary Fig. 5), a pattern that resembles Mc4r expression20,21. To investigate whether $Npy1R_{MeA}$ cells are involved in feeding behavior or aggression, we bilaterally transduced the MeA of $Npy1r^{Cre}$ 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. In line with this idea, we found that CNO-induced $Npy1R_{MeA}$ neuron activation significantly increased home-cage aggressive behavior (Fig. 3f and
Supplementary Movie 1). Consistent with previous work using pharmacogenetics to manipulate social behavior, we observed a scale of aggressive phenotypes upon CNO-induced activation. Four of nine stimulated animals displayed overt attack behavior, while the rest engaged in other aggressive behaviors including, nudging, aggressive grooming, and holding. To determine the degree of aggression evoked, we evaluated the overtly aggressive males in the presence of an anesthetized intruder; all of these mice attacked the anesthetized conspecific within 59.0 ± 5.9 s (Supplementary Movie 2), a behavior never observed in non-stimulated mice. Along with changes in aggression, activation of the Npy1RMeA neurons significantly decreased food consumption within the first 4 h of the dark cycle (Fig. 3g).

To determine the necessity of the Npy1RMeA population for satiety, territorial aggression, and high-risk exploration, we used a viral approach to chronically inhibit Npy1rCre-expressing cells in the MeA. Tetanus toxin light chain prevents synaptic transmission and AAV1-DIO-GFP:TetTox (Fig. 3h) has been used to silence Cre-expressing neurons. In the food-challenge assay, TetTox-silenced Npy1RMeA mice spent more time in the shock-associated chamber post-conditioning (Fig. 3i). They also gained significantly more weight after viral transduction relative to controls (Fig. 3j), but there was no statistical difference between TetTox-silenced mice relative to controls in territorial aggression (Fig. 3k). Given that acute inhibition from AgRPMeA fibers was sufficient to suppress home-cage aggression, this finding was surprising but may be the result of compensatory plasticity following chronic inhibition. We also evaluated anxiety using the elevated plus maze. There was no difference in open-arm exploration between TetTox-silenced mice relative to YFP controls (17.2 ± 4.2% for TetTox versus 21.2 ± 3.1% for YFP controls, P = 0.5; Supplementary Fig. 6).

A disynaptic network from ARHAgRP to the MeANpy1R and on to the pBNST
To identify candidate secondary targets of the AgRP → MeA circuit, we mapped the projection field of Npy1RMeA cells using a virus expressing a Cre-dependent, synapse-specific reporter (AAV1-DIO-synaptophysin:YFP, Fig. 4a). We observed dense reporter expression in the posterior principle region of the bed nucleus of the stria terminalis (pBNST), along with several other brain regions including the lateral hypothalamic area, periaqueductal grey, parabrachial nucleus, ventromedial hypothalamus and anterior olfactory bulb (Fig. 4b). To determine whether these were secondary targets of AgRP neurons, we injected a Cre-dependent and trans-synaptic anterograde tracing virus, H129Δ-fs-TK-TT, into the ARH of AgrpCre animals (Fig. 4c). TdTomato fluorescence was observed in many sites throughout the brain, notably the MeA and the pBNST (Fig. 4c).

The pBNST is an established target of the MeA39,40 and while AgRP fibers have been found in the anterior BNST15,17, they have not been observed in the pBNST. Consistent with this observation, 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 H129Δ-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 circuit (Fig. 4e). Due to the nature of H129 infection, the cells were not healthy enough to quantify the overlap of these reporters in the MeA. Instead, we use an alternative approach to further characterize the properties of cells in the MeA that receive input from AgRP neurons and project to the pBNST.

Numerous cell types in the posterior MeA have been defined based on electrophysiological and morphological properties41-43. 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, we observed a prominent hyperpolarization-
activated voltage sag (h-current, denoted by the arrow, in 10 of 13 cells recorded), which has been described in type 1 GABAergic projections neurons in the posterior MeA \(^{43}\). In addition, we discovered that that a subset of these neurons expressed a T-type calcium current (denoted by the arrow head, in 7 of 13 cells; Fig. 5c).

We previously determined that AgRP neurons can 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 soma in proximity to YFP fibers demonstrated light-evoked IPSPs in 3 of 11 recorded cells (Fig. 5d). Similar to the recordings in Fig 2b, the light-evoked inhibitory response occurred with a short latency to the photostimulation, suggesting a direct connection. To further 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 a potassium channel blocker, 4-aminopyridine (4-AP)—a property indicative of monosynaptic connections in ChR2-assisted circuit mapping (Fig. 5d, red trace) \(^{44}\).

We used pharmacology in the slice preparation to demonstrate that light-responsive, bead-positive cells in the MeA also respond to NPY. Bath application of NPY in the presence of TTX resulted in an outward current when 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 \(^{45,46}\), we found that the reversal potential for the outward current was at -85 mV, close to the Nernst equilibrium potential for potassium.

Based on 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\(^{43}\). Following recording, we harvested the cytosol of the cells \(^{47}\) and performed single-cell PCR to test for \(Slc32a1\)
(Vgat) expression. The majority of cells were Vgat-positive—out of 11 successfully harvested Actb-positive cells, 7 tested positive for Vgat expression, while the remaining 4 did not amplify either Vgat or Vglut templates (Fig. 5g). Together, our 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.

The behavioral influence of the ARH<sup>AgRP</sup> → MeA<sup>Npy1R</sup> → pBNST circuit

The Npy1R<sup>MeA</sup> population projects to numerous efferent targets, some of which have been implicated in aggressive behavior, including the VMH and PAG<sup>24,48</sup>. Because AgRP neurons make a disynaptic connection to the pBNST via the MeA, we hypothesized that this circuit may be involved in aggressive/territorial behaviors. To investigate the role of pBNST-projecting Npy1R<sup>MeA</sup> neurons, we virally transduced Npy1<sup>Cre</sup> mice unilaterally with AAV1-DIO-ChR2:YFP virus and placed fiber optic a cannula above either the ipsilateral VMH or pBNST (Fig. 6a). Optogenetic stimulation of Npy1R<sup>MeA</sup> 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). As opposed to violent aggression, a nudging threat display may be adequate for territorial defense from most competitors (Supplementary Fig. 7e). It should also be noted that the methods of stimulation in these two instances were different. We activated Npy1R<sup>MeA</sup> soma using metabotropic hM3Dq DREADD receptors while we stimulated the Npy1R<sup>MeA</sup> fibers in the pBNST using ionotropic photostimulation; hence, we cannot exclude the possibility that this difference accounts for the behavioral difference.

Optogenetic stimulation of Npy1R<sup>MeA</sup> fibers in the VMH did not result in significant differences in territorial aggression (Fig. 6b). We also measured the effect of photostimulating Npy1R<sup>MeA</sup> efferents 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 overt/violent aggression observed following Npy1R^{MeA} neuron stimulation (Fig. 3f and
Supplementary Movie 1,2), 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 territorialism 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
take more risks to forage in 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 49. We explored the influence of AgRP neurons in these starvation-related decisions. Similar to
fasted animals, acute activation of AgRP neurons was sufficient to induce food seeking in a threatening
environment and decrease territorial defense behavior. AgRP neurons send axonal projections to
downstream brain regions involved in sensory processing; hence, we propose that in the state of
starvation, these neurons tune behavioral and physiological processes to conserve energy and prioritize
food acquisition.

Both territorial aggression and the activation state of AgRP neurons depend on the availability of
food. 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 hungry residents (artificially induced by AgRP neuron-stimulation) during an intruder trial, they choose to eat rather than interact with the intruder (Supplementary Fig. 3). Fluctuations in territorial aggression levels 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 propose that the AgRP$^{MeA}$ circuit is involved in territorial adaptations during starvation, but questioned whether hunger itself could influence territorialism. 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 a pBNST-projecting MeA$^{Npy1R}$ circuit that is downstream of AgRP and can alter territorial behavior. There are, however, many other AgRP targets throughout the brain, and similar to 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 attributed to the broad projection profile 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

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evoke lesser, if any, influence on food consumption \(^{17,30}\). Similarly, we find that AgRP fibers in the MeA can evoke food intake, but the magnitude of this effect is much less compared to equivalent fiber stimulation in the PVH. The long-term consequence of inhibiting cells in the MeA is a significant increase in body weight (our data and ref \(^{32}\)). 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 non-feeding behaviors with hunger. For example, AgRP-mediated inhibition of the MeA induces feeding and suppresses territorialism, 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 associated references are available in the online version of this paper.

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AUTHOR CONTRIBUTIONS

SLP designed the study under the guidance of RDP; CZ performed and analyzed electrophysiological data in the laboratories of MJK and OKR; MES performed and analyzed electrophysiology data in the laboratory of LSZ; ES performed the RiboTag pulldown and qPCR in the laboratory of AQ; CCN performed single-cell PCR on harvested cells post whole-cell recordings; RDP generated the AgRP\textsuperscript{Cre} and Npy1r\textsuperscript{Cre} lines of mice; FDB assisted with behavior experiments and blind scoring; RDP, MJK, OKR and LSZ provided laboratory space and resources to conduct the experiments; SLP and RDP wrote the manuscript with revisions and input from all contributing authors.
COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

REFERENCES


FIGURE LEGENDS

Figure 1  AgRP stimulation recapitulates fasting-related foraging and reduced territorialism. (a) Schematic diagram of the Pavlovian food-challenge assay used to assess risk taking. (b) Viral construct: AAV1-DIO-hM3Dq:mCh, and diagram of the stereotaxic injection site in Agrp\textsuperscript{Cre} mice. (c) Viral expression of hM3Dq:mCh in AgRP neurons in conjunction with CNO-induced FOS immunoreactivity; scale bar, 100 \mu m. Animals included in the study (red dots) consumed \textgreater 1.0 g of food 4 hr post CNO (1 mg/kg IP). (d) Post-conditioning (TD) quantification of time spent in the shock-associated chamber (ad lib, n = 15; fasted food available, n = 7; fasted food blocked, n = 7; mCH + CNO, n = 13; hM3Dq + CNO food available, n = 6; hM3Dq + CNO food blocked, n = 7). (e) Time spent in the open arms of a plus maze (ad lib, n = 8; fasted, n = 7; mCH + CNO, n = 12; hM3Dq + CNO, n = 8). (f) Paired analysis of home-cage aggressive behavior (ad lib/fasted paired n = 12; hM3Dq + saline/CNO paired, n = 10). (d-f) Bar outlines indicate mouse genotype: red indicates Agrp\textsuperscript{Cre}, while black indicates C57BL/6. The condition or treatments of the animals are indicated below the x-axis. (d, e) Conducted during the light cycle (5:00 – 17:00), (f) Conducted in the dark (17:00 – 19:00).

Figure 2  MeA-projecting AgRP neurons evoke hunger and inhibit territorial aggression. (a) ChR2:YFP-expressing AgRP fibers observed in the MeA. Scale bar, 200 \mu m. (b) Whole-cell, patch clamp recordings (voltage-clamp, -70 mV) of MeA cells in proximity to AgRP::ChR2-expressing fibers. Light-evoked responses were measured with 10-ms light pulse (472 nm, 10 mW power at the tip) in the presence of CNQX and APV (black trace), followed by picrotoxin (red trace). (c) Green fluorescent RetroBeads injected into MeA were retained in 7.1 ± 0.6 % of Npy-expressing ARH cells; indicated by arrows. Scale bar, 100 \mu m. (d) Schmatic demonstrating that a subset of AgRP neurons (black filled) project 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 paradigm for behavioral studies: 10 Hz, 5-ms pulse, 5 s on, 2 s off. (g) Paired analysis of home-cage aggressive behavior, comparing light on versus light off conditions (MeA fibers, n = 8; PVH fibers, n = 9); conducted during the first 2 hr of the dark cycle (17:00 – 19:00). (h) Cumulative food intake (ChR2 off n = 17; ChR2 stimMeA, n = 8; ChR2 stimPVH, n = 9), measured during the light cycle (10:00 – 14:00).

Figure 3  Npy1R neurons in the MeA can evoke aggression and inhibit feeding. (a) Npy1rCre:GFP knock-in targeting construct (diagram not to scale; see methods for complete details). (b) RiboTag mice contain a Cre-dependent epitope-taged polyribosome gene. MeA tissue was harvested (red circles) from Npy1rCre; RiboTag mice. (c) Comparison of transcripts expressed in precipitated/RiboTagged cells versus those expressed in non-Cre-expressing MeA cells, demonstrated enrichment of Npy1r and Cre transcripts along with Gad2 and Mc4r. (d) Npy1R expression in the anterior-dorsal (AD) and anterior-ventral (AV) MeA; lower, posterior-dorsal (PD) and posterior-ventral (PV) MeA. Scale bar, 200 \mu m. (e) Bilateral injection of AAV1-DIO-hM3Dq:YFP into the MeA of Npy1r\textsuperscript{Cre} mice; histology demonstrating the injection site and Cre-dependent YFP fluorescence. Scale bar, 200 \mu m. (f) Stimulation of Npy1RMeA cells of resident animals evokes aggressive behavior (assessed during between 17:00 – 19:00) and (g) decreased food consumption (recorded from 17:00 – 21:00; hM3Dq + CNO, n = 9; YFP + CNO, n = 8) (h) Bilateral injection of AAV1-DIO-GFP:TetTox into the MeA of Npy1r\textsuperscript{Cre} mice with histology to demonstrate the injection site. (i - k) Npy1RMeA neuron silencing did not change home-cage aggression (f), but increased body weight (g). TetTox-silenced mice also presented decreased threat avoidance behavior (h). Conditioned avoidance was measured by time spent in the shock-associated side, on test day (detailed in Fig. 1a; TetTox, n = 6; YFP, n = 6).
Figure 4  The posterior BNST is a secondary target of AgRP and receives direct input from $Npy1R^{MeA}$ neurons. (a) Injection of AAV1-DIO-Synaptophysin:YFP into the MeA of $Npy1r^{Cre}$ mice. (b) Immunoreactive YFP fibers in the: AOBmi (accessory olfactory bulb, mitral layer), LSr (lateral septal nucleus, rostroventral part), PO (preoptic area), pBNST (bed nucleus of the stria terminalis, posterior division, principle nucleus), RCH (retrochiasmatic area), LHA (lateral hypothalamic area), VMH (ventromedial hypothalamus), MeA (medial amygdala), PAG (periaqueductal gray), PB (parabrachial nucleus), NTS (nucleus of the solitary tract). All images were scaled equally; scale bar, 200 µm (see breg -1.6). (c) AgRP neurons relay information to the pBNST and MeA. The trans-synaptic virus, H129Δ-fs-TK-TT, was injected into the ARH of $Agrp^{Cre}$ 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 H129Δ-fs-TK-TT into the ARH of $Agrp^{Cre}$ mice. DsRed immune-reactive cell bodies and retrobead-positive cells are present in the MeA. Scale bar, 100 µm. (e) Model of the AgRP$^{MeA}$ circuit.

Figure 5  Identification of Vgat-expressing NPY-responsive cells in the MeA that received direct input from AgRP neurons and project to the pBNST. (a) Sagittal diagram of injection and recording sites. (b) Coronal sections confirming targeted injections of RetroBeads into the pBNST (left) and DIO-ChR2:YFP into the ARH (center). Recordings were performed on bead-labeled cells in the MeA (right); zoom image (right) represents a red cell in proximity to YFP fibers. (c) Voltage response to current step injections. The majority of bead-positive neurons exhibited a prominent hyperpolarization-activated voltage sag (h-current; $n = 10$; arrow) and a T type calcium current ($n = 7$; arrow head). (d) Light-responsive, bead-positive cells in the MeA ($n = 3$); blue light-evoked fast IPSP (black trace) was abolished in the presence of TTX (blue trace), and rescued with the addition of the K+ channel blocker 4-AP (red trace). (e) Bead-positive neurons that displayed an outward current in response to bath application of NPY (1 µM; red line) ($14.5 \pm 4.5$ pA; $n = 4$) when held at -60 mV (note, gaps in the trace indicate the voltage ramp interval in f). (f) IV relationship during a voltage ramp performed on bead-positive, light-responsive cells, before (black) and after (red) NPY application. (g) PCR detection of $Slc32a1$ (Vgat) cDNA in cell lysates harvested from recorded cells. Reverse-transcribed cDNA from the hypothalamus was used as a positive control, while hypothalamic RNA was tested as the negative control.

Figure 6  $Npy1R^{MeA}$ neurons that project to the pBNST evoke territorialism. (a) Unilateral injection of AAV-DIO-ChR2:YFP into the MeA of $Npy1r^{Cre}$ mice, with ipsilateral optic cannulas implanted above the pBNST or the VMH. (b) Stimulation of $NpyR^{MeA}$ fibers over pBNST increased aggression in a home-cage intruder assay (left panel), while stimulation of fibers over VMH does not (right panel); VMH, $n = 3$, pBNST, $n = 5$, assessed during the dark cycle (17:00 – 19:00). (c) Neither VMH nor pBNST-projecting $Npy1R^{MeA}$ cells decreased feeding (recorded from 17:00 – 21:00).
Figure 1 (Padilla et al.)

a. Food Challenge - Contextual Fear

b. AAV1-DIO-hM3Dq:mCh in AgRP<sup>Cre</sup> neurons

b. hM3Dq:mCh in AgRP<sup>Cre</sup> neurons

- CNO-induced FOS

- CNO-induced Food Intake

- Agrp<sup>Cre/+</sup>

- CNO induced

- Food Intake (g)

- 0.0

- 1.0

- 2.0

d. Food Challenge
   - 30-min trial

- Time in shock chamber (min)

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- Ad Lib

- 48-hr Fast

- YFP

- CNO

- hM3Dq

- CNO

- Food available

- Food blocked

- Food available

- Food blocked

e. Elevated Plus Maze
   - 10-min trial

- Time in open arm (min)

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- Ad Lib

- 48-hr Fast

- mCh

- CNO

- hM3Dq

- CNO

- CNO

f. Resident-intruder Assay
   - 10-min trial

- Territorial aggression (s)

- ***

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- Ad Lib

- 48-hr Fast

- hM3Dq

- Sal

- CNO
**Figure 2 (Padilla et al.)**

(a) AAV1-DIO-ChR2:YFP

(b) Light-evoked IPSC: AgRP → MeA

(c) Beads from MeA

(d) Schematic of brain regions

(e) AAV1-DIO-ChR2:YFP

(f) Temporal sequence of light stimulation

(g) Resident-intruder Assay

(h) Food Intake

- **Resident-intruder Assay**
  - 10-min trial
  - Off, MeA<sub>dmn</sub>, Off, PVH<sub>dmn</sub>
  - Territorial aggression (s)

- **Food Intake**
  - 4-hr trial
  - Off, MeA<sub>dmn</sub>, PVH<sub>dmn</sub>
  - Food intake (g)
Figure 4 (Padilla et al.)

(a) AAV1-DIO-Syn::eYFP

(b) Images showing different brain regions labeled with AAV1-DIO-Syn::eYFP. The images are labeled with breg values indicating their location within the brain.

(c) Schematic diagram of H129Δ-fs-TK-TT

(d) H129Δ-TK-TT retrobeads

(e) Schematic diagram showing the distribution of AgRP fibers in the pBNST and MeA.
Figure 6 (Padilla et al.)

a) AAV1-DIO-ChR2:eYFP

b) Resident-intruder Assay
10-min trial

Rr2
ChR2
ChR2
ChR2
ChR2

Territorial aggression (s)

Off
stim
Off
stim

ChR2
ChR2
ChR2
ChR2

Off
stim
Off
stim

C) Food Intake
4-hr trial

Food intake (g)