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TITLE: Genetically Dissecting Basal Forebrain Circuits Underlying Eating Disorders

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14. ABSTRACT Abnormal eating habits that drive excessive food seeking- or avoidance, can manifest as health-threatening and socially impactful eating disorders. Comorbidity often exists between eating disorders and stress, an emotional state tightly associated with military personnel and their families. Studies investigating the neural contribution to eating habits and body weight control have focused largely on neuropeptidergic signaling in the hypothalamus, a key brain region involved in feeding behavior. We have previously reported a potent role for basal forebrain cholinergic neurons in regulating feeding behavior, and recently discovered that cell type-specific manipulations of their immediate downstream targets are capable of directly toggling a spectrum of eating-associated behaviors that range from increased food seeking and excessive food consumption to severe hypophagia, overt food avoidance, and starvation. We found that cholinergic (ACh+) neurons within the basal forebrain make synaptic connections onto neighboring glutamatergic (Glu+) projection neurons, which in turn directly govern feeding behavior. Our preliminary data identify prominent targets of basal forebrain Glu+ neurons within the LH, an area associated with feeding, reward processing, and addiction. This discovery provides intriguing insight into a previously unknown circuit that links the cholinergic basal forebrain, which is known for its role in attention, wakefulness, and sensory processing, to value based components of eating associated behaviors. Experiments designed in this award will elucidate the mechanisms by which basal forebrain circuits govern food seeking and/or food aversion.					
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INTRODUCTION

Abnormal eating habits that drive excessive food seeking- or avoidance, can manifest as health-threatening and socially impactful eating disorders. To date, studies investigating the neural contribution to eating habits and body weight control have focused largely on neuropeptidergic signaling in the hypothalamus, a key brain region involved in feeding behavior. Although signaling from extra-hypothalamic brain regions has also been implicated in regulating nutrient metabolism, appetite, and satiety, it is unclear which brain regions and pathways are involved. We previously found that cholinergic (ACh+) neurons within the basal forebrain make synaptic connections onto neighboring glutamatergic (Glu+) projection neurons, which in turn directly govern feeding behavior. Our preliminary data identify prominent targets of basal forebrain Glu+ neurons within the lateral hypothalamus, an area associated with feeding, reward processing, and addiction. Based on these findings, we aim to test whether basal forebrain glutamatergic projection neurons serve as a primary circuit node to govern eating-related behaviors. The scope of research includes 3 main aims: 1) to determine how basal forebrain ACh+ and Glu+ neurons are connected; and 2) to determine how Glu+ neurons in the basal forebrain influence feeding behavior; and 3) to identify downstream targets of basal forebrain Glu+ neurons that govern feeding behavior. The results will provide insights on how basal forebrain ACh+ and Glu+ neurons are connected; how Glu+ neurons in the basal forebrain influence feeding behavior and related behaviors; and downstream targets that mediate basal forebrain Glu+ neurons in regulating eating and related behaviors.

KEYWORDS

Basal forebrain, eating disorder, glutamate, acetylcholine, lateral hypothalamus, optogenetics, in vivo Ca²⁺ imaging, metabolism

ACCOMPLISHMENTS

Major goals

Major goal 1: identify subtypes of basal forebrain neurons responding to eating; SOW completion date and site: 1-12 months in Site 1 (Dr. Arenkiel). This goal has been accomplished 1-12 months.

Major goal 2: map local inputs onto basal forebrain Glu+ neurons; SOW completion data and site: 3-12 months in Site 2 (Dr. Tong). This goal has been delayed because of unavailability of SAD rabies virus during the pandemic. We have generated the required animal models and the rabies virus has just recently arrived, and we will resume the experiment immediately. This goal has been accomplished approximately 50% completion.

Major goal 3: Profile Ach receptor expression in basal forebrain Glu+ neurons; SOW completion data and site: 1-12 months in Site 1 (Dr. Arenkiel). This goal has been partially accomplished. Initial expression analysis has been conducted using FISH, but we are still confirming basal forebrain cell types of expression. This has been delayed due to the pandemic.

Major goal 4: decipher the nature of neurotransmitter between basal forebrain Ach+ neurons and Glu+ neurons; SOW completion date and site: 3-12 months in Site 1 (Dr. Arenkiel). This goal has been accomplished 3-12 months.

Major goal 5: determine how Glu+ neurons in the basal forebrain influence feeding behavior; SOW completion data and site: 6-20 months in Site 1 (Dr. Arenkiel). This goal has been accomplished approximately 50%.

Major goal 6: modulate basal forebrain Glu+ neuron activity and assess impact on feeding and physiology; SOW completion date and site: 6-24 months and Site 2 (Dr. Tong). This goal has been accomplished approximately 50%.

Major goal 7: Inhibition of basal forebrain Glu+ neurons and impact on feeding; SOW completion date and site: 12-28 months and both Sites 1 and 2 (Drs. Arenkiel and Tong). This goal has not been started yet.

Major goal 8: basal forebrain Glu+ neuron activity and assess feeding; SOW completion date and site: 12-28 months and Site 2 (Dr. Tong). This goal has not been started yet.

Major goal 9: basal forebrain Glu+ neurons mediating the action of Ach+ neurons in feeding regulation and body weight; SOW complete date and site: 12-36 months and Site 2 (Dr. Tong). This goal has not been started yet.

Major goal 10: Genetic rescue of obesity; SOW completion date and site: 6-36 months and Site 1 (Dr. Arenkiel). This goal has been accomplished approximately 25%.

Major goal 11: downstream targets of basal forebrain Glu+ neurons that govern feeding behavior; SOW completion date and site: 18-36 months and both Sites 1 and 2 (Drs. Arenkiel and Tong). This goal has not been started yet.

Major goal 12: basal forebrain Glu+ neuron to LH neuron projection in feeding motivation; SOW completion date and site: 12-30 months and Site 2 (Dr. Tong). This goal has not been started yet.

Major goal 13: determine whether LH Glu+ neurons are directly downstream of basal forebrain Glu+ neurons; SOW completion date and site: 6-18 months and Site 1 (Dr. Arenkiel). This goal has been accomplished approximately 25%.

Major goal 14: determine whether LH Glu+ neurons mediate the effect of basal forebrain Glu+ neurons in feeding regulation; SOW completion date and site: 6-36 months and Site 2 (Dr. Tong). This goal has been accomplished approximately 10%.

Accomplishment during the reporting period:

During the reporting period, we have made progress in major goals 1-6, 10, 13 and 14.

Major goal 1 (Dr. Arenkiel): the goal is to test differential responses in neuron activity to fasting vs fed states and to identify specific types of basal forebrain neurons responding to fasting and refeeding. We have used Vglut2-Cre and Chat-Cre reporter mice to identify glutamatergic and Ach+ neurons. These mice were subjected to fasting or fast-refeeding, and then c-Fos immunostaining was performed. The results as shown in Figure 1 demonstrate that a subset of both glutamatergic neurons (Figs. 1A-C) and cholinergic neurons (Fig. 1D and 1E) exhibited more c-Fos when mice were switched from fasting to refeeding, suggesting that these neurons are activated during refeeding, implying a role in feeding inhibition. The milestone for this goal has been achieved.

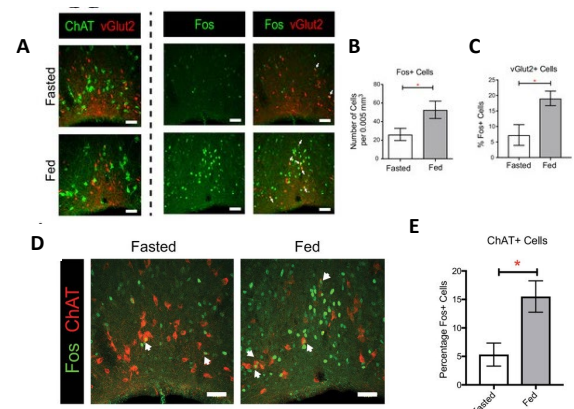


Figure 1

Major goal 2 (Dr. Tong): the goal is to determine whether Chat neurons in the basal forebrain send monosynaptic projections to Glu+ neurons. We plan to use monosynaptic pseudorabies viral approach to trace monosynaptic upstream neurons. Toward this, we have prepared Vglut2-Cre mice, in which AAV-Flex-TVA-mCherry and AAV-Flex-G viral vectors will be injections to the basal forebrain of these mice. One week later, SADdeltaG-GFP virus will be injected to the same location. Two weeks later, mice will be perfused to document the viral expression. However, due to the pandemic, the viral core for the pseudorabies virus

SADdeltaG-GFP had been shut down and without this virus, we have to delay this experiment. We have just received this virus and will start the experiment shortly.

Major goal 3 (Dr. Arenkiel): the goal is to analyze ACh receptors in basal forebrain Glu+ neurons. Toward this, RNA probes have been synthesized, tested for efficacy, and initial expression analysis has been completed. Due to the shutdown we are now just resuming further experimentation to validate the cell type-specific patterns of AChR expression.

Major goal 4 (Dr. Arenkiel): the goal is to determine the nature of neurotransmission between basal forebrain ACh+ neurons and Glu+ neurons. We have generated ChAT-ChR2::Vglut2-Cre mice and delivered AAV-Flex-mRuby2 virus to the basal forebrain of these mice (Fig. 2A, left). Brain sections containing the basal forebrain were used to record laser stimulation induced currents in mRuby2-labelled Vglut2 target neurons (Fig. 2A, right). We could detect reliable non-glutamate, non-GABA mediated currents, which were sensitive to nicotinic Acetylcholine receptor antagonist (Fig. 2B and 2C), suggesting a major for nicotinic AChRs in mediating the cholinergic action between Chat neurons and Glu+ neurons within the basal forebrain. The milestone in this goal has been achieved.

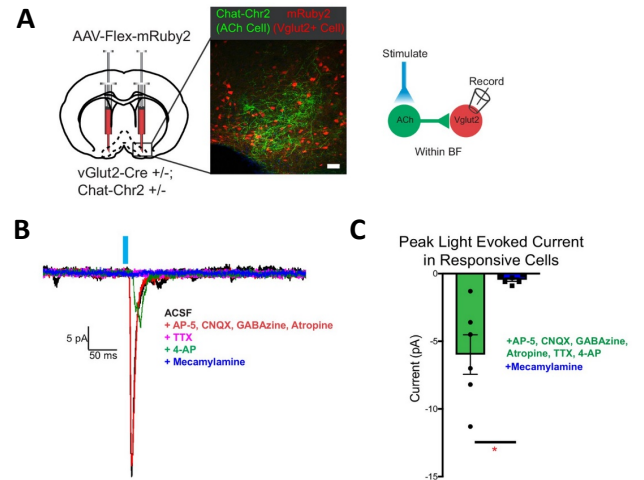


Figure 2

Major goal 5 (Dr. Arenkiel): the goal is to determine the *in vivo* responses of basal forebrain Glu+ neurons to fed, fasting, and food-related odors. We have prepared Vglut2-Cre mice, delivered AAV-Flex-GCamp6m virus, and successfully implanted GRIN lens to these animals. Initial experiments showed that we could reliably capture Ca²⁺ imaging from individual Vglut2 neurons in live animals. Ongoing studies focus on the response to these neurons to various feeding statuses and odors. It is estimated that the milestone in this goal will be met within the planned time frame.

Major goal 6 (Dr. Tong): the goal is to determine feeding and body weight in mice with basal forebrain Glu+ neurons expressing NaChBac. We have prepared Vglut2-Cre mice and delivered AAV-Flex-NaChBac virus or control GFP virus to the basal forebrain (Fig. 3A). We found that NaChBac injected mice exhibited rapid body weight reduction (Fig. 3B and 3C), due to dramatic reduction of feeding (Fig. 3C).

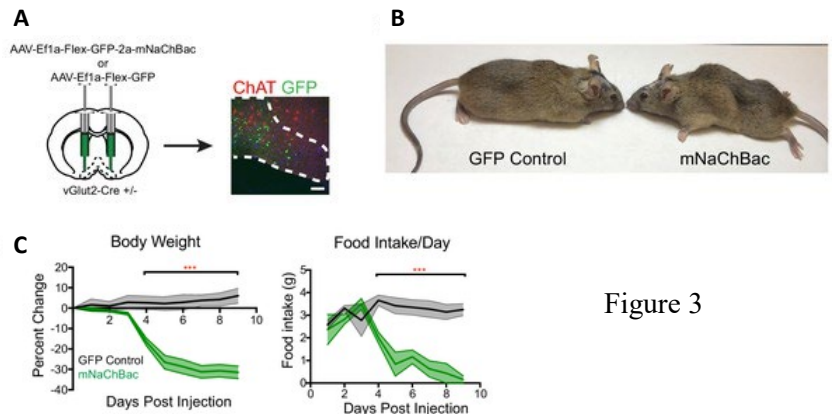


Figure 3

Ongoing experiments focus on whether the reduced body weight can be rescued by forced feeding, i.e. food gavage. It is estimated that the milestone of this goal can be accomplished within the planned time window.

Major goal 10 (Dr. Arenkiel): the goal is to determine whether hypophagia by basal forebrain Glu+ neuron activation can rescue the ob/ob obesity. Vglut2-Cre::ob/ob mice have been generated and will be used to deliver AAV-Flex-NaChBac virus to the basal forebrain region. It is estimated that the milestone in this goal will be met within the planned time window.

Major goal 13 (Dr. Arenkiel): the goal is to determine whether LH Glu+ neurons are direct downstream target of basal forebrain Glu+ neurons. AAV-Flex-ChR2 virus has been delivered to the basal forebrain and AAV-Flex-

mCherry virus has been delivered to the LH of Vglut2-Cre mice. Ongoing studies focus on in vitro brain slice recording to examine the laser induced currents in mCherry labelled LH neurons. It is estimated that the milestone in this goal will be met within the planned time window.

Major goal 14 (Dr. Tong): the goal is to determine whether LH Glu+ neurons mediate the effect of basal forebrain Glu+ neurons in feeding. Vglut2-Cre mice were prepared and AAV-Flex-ChR2 virus has been delivered to the basal forebrain and the AAV-Flex-hM4Di-mCherry virus has been delivered to the LH. Studies are ongoing to examine the effect on feeding.

Training and professional development

Dr. Zhiying Jiang, a postdoc and Ms. Jing Cai, a graduate student (Site 2, Dr. Tong) are currently involved in this project and are supported by this funding. Both of them are required to attend our departmental and center seminar series. The departmental seminar series invites well-known scientists across the US to present their new findings and the center seminar series will mainly presented by postdocs and students from each lab to share their research. Thus, both of them will have the opportunities to be exposed to research outside the campus and to present their own ongoing research. In addition, Ms. Cai will have planned activity and training classed within the graduate school, including required committee meetings in which Dr. Arenkiel is a committee member. We also have a regular lab meeting in which all lab members require to present their own research as well present a journal club on the papers from peers. Importantly, both of them have regular weekly meeting with me. Through these various platforms, they will get trainings in research. With the pandemic, almost all seminar series with the UT and TMC use virtual format, in which they regularly attend. Unfortunately, with the impact of the pandemic, it is not feasible for us to go to local/national meetings and we are trying to participate virtual meetings organized by various research foundations and other institutions, in order to get better exposure to peers.

Lab personnel in Arenkiel lab include Ms. Mayuri Patel and Ms. Peyshyuan Chin who are current graduate students focused on this project as part of their thesis work. Mayuri and Peyshyuan have received direct training in in vivo imaging, electrophysiological, and behavioral analysis. They are utilizing these skills towards the proposed studies. Both are making excellent progress towards the stated project goals, and have maintained holding committee meeting progress reports. Also, as partial contributors, Mr. Benjamin Belfort and Mr. Suyang Bao (two new students to the laboratory) are serving as support and collaborators to the individuals focusing 100% effort towards the project. Although practical research experimentation has been paused during the funding period due to the pandemic, the listed personnel were able to continue analysis of existing data, and reinstate new experimentation. Additionally, the Arenkiel Lab members have been actively involved with a program called the McNair Teaching Fellows program, where they interface with high school teachers and their classrooms in North Carolina to host lectures and virtual lab tours on their projects. This has been highly beneficial towards them gaining practical teaching experience and to share current scientific findings associated with their work.

Dissemination to community

Nothing to report

Plan for the next reporting period

We will execute the research plan outlined in SOW. There might be a slowdown in research progress because the required mouse colony reduction during the mandatory lockdown period of the pandemic. We will fully attempt to achieve the planned milestones, with some consideration of the delayed research plan.

IMPACT

Impact on development of principle disciplines

Nothing to report

Impact on other disciplines

Nothing to report

Impact on technology transfer

Nothing to report

Impact on society beyond science and technology

Nothing to report

CHANGES/PROBLEMS

It is not anticipated that there is a significant change in the approach or use of animals. However, due to the current pandemic, there might be a change in time to accomplish the planned and stated milestones. Unfortunately, during the initial lockdown, it was mandatory for us to significantly reduce mouse breeding/cages, which has caused a major delay in the availability of mouse study subjects. In addition, we are operating at a reduced personnel effort to achieve effective social distancing, which also delays the progress of research and associated research expenditure. One example is in goal 2, due to lockdown, we couldn't obtain the pseudo-typed rabies virus for the planned experiment, which will have to be delayed. Also, reducing the numbers of initially listed animals for behavioral analysis will require additional time to reestablish the needed sample size to complete the proposed experiments. The only significant foreseeable change to the project is related to needed additional time to complete the proposed work. This has directly resulted from the mandatory laboratory operations shutdown associated with the coronavirus pandemic.

PRODUCTS

During the course this funding period, we have published the following papers which indirectly benefited from this funding.

1. Paraventricular hypothalamus mediates diurnal rhythm of metabolism. Kim ER, Xu Y, Cassidy RM, Lu Y, Yang Y, Tian J, Li DP, Van Drunen R, Ribas-Latre A, Cai ZL, Xue M, Arenkiel BR, Eckel-Mahan K, Xu Y, Tong Q. *Nat Commun.* 2020 Jul 30;11(1):3794. doi: 10.1038/s41467-020-17578-7. PMID: 32732906; Acknowledgement of the federal support: Yes.
2. Profound and redundant functions of arcuate neurons in obesity development. Zhu C, Jiang Z, Xu Y, Cai ZL, Jiang Q, Xu Y, Xue M, Arenkiel BR, Wu Q, Shu G, Tong Q. *Nat Metab.* 2020 Jul 27. doi: 10.1038/s42255-020-0229-2. Online ahead of print. PMID: 32719538; Acknowledgement of the federal support: Yes.
3. A neural basis for brain leptin action on reducing type 1 diabetic hyperglycemia. Fan S, Xu Y, Lu Y, Jiang Z, Morrill J, Cai J, Wu Q, Xu Y, Xue M, Arenkiel BR, Huang C and Tong Q. Under review. Acknowledgement of the federal support: Yes.

Nothing to report on products in other categories listed in the report instruction.

PARTICIPANTS \$ OTHER COLLABORATING ORGANIZATIONS

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Contribution to project	Supervision
Funding Support	This Award and NIH grants

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Contribution to project	Supervision
Funding Support	This Award and NIH grants

Name	Zhiying Jiang (Site 2)
Project Role:	Postdoctoral researcher
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Nearest person month worked	11
Contribution to project	Viral injections, feeding, body weight and recording
Funding Support	This Award

Name	Jing Cai (Site 2)
Project Role:	Graduate student
ORCID ID	Not available
Nearest person month worked	6
Contribution to project	Viral injections, body weight, feeding
Funding Support	This Award

Name	Mayuri Patel
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Nearest person month worked	12
Contribution to project	Circuit labeling, viral injections, and behavioral analysis.
Funding Support	This Award

Name	Peyshyuan Chin
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Nearest person month worked	12
Contribution to project	Circuit labeling, viral injections, and behavioral analysis.
Funding Support	This Award

Name	Benjamin Belfort
Project Role:	Graduate Student
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Nearest person month worked	3
Contribution to project	Imaging, viral labeling, circuit analysis
Funding Support	This Award

Name	Suyang Bao
Project Role:	Graduate student

ORCID ID	Not available
Nearest person month worked	6
Contribution to project	Circuit labeling, cellular analysis, and behavioral analysis.
Funding Support	This Award

There is no change in PI or key personnel during the reporting period.

Nothing to report on other organization involved as partners.

SPECIAL REPORTING REQUIREMENTS

Nothing to report

APPENDICES:

3 reprints of publications. The Patel et al., manuscript listed last contains the essential preliminary data used in the preparation of the initial grant application and was officially published just prior to award activation. Thus, the formal acknowledgement of award is not included in published document.



PI: Benjamin Arenkiel, Baylor College of Medicine

Budget: \$1,199,500.00 **Topic Area:** Eating Disorders **Mechanism:** FY 2018 Peer Reviewed Medical Research Program Investigator-Initiated Research Award

Research Area: 1400

Award Status: 01 Sept 2019 – 31 Aug 2022

Study Goals:

The study is designed to test whether basal forebrain glutamatergic projection neurons serve as a primary circuit node to govern eating-related behaviors and the goal is to elucidate the mechanisms by which basal forebrain circuits govern food seeking and/or food aversion.

Specific Aims:

To accomplish the study goal, experiments are designed to 1) (Aim 1) determine how basal forebrain ACh+ and Glu+ neurons are connected in we will decipher how ACh+ neurons communicate with local Glu+ neurons is critical towards understanding the cholinergic basal forebrain circuit mechanisms that underlie eating behavior; 2) (Aim 2): determine how Glu+ neurons in the basal forebrain influence feeding behavior, in which we will test how food cues influence basal forebrain Glu+ neurons and how perturbations to these cells influence feeding behavior; and 3) (Aim 3): reveal downstream targets of basal forebrain Glu+ neurons that govern feeding behavior, in which we will test the function of LH neurons in mediating basal forebrain-linked feeding output.

Key Accomplishments & Outcomes:

Publications:

1. Paraventricular hypothalamus mediates diurnal rhythm of metabolism. Kim ER, Xu Y, Cassidy RM, Lu Y, Yang Y, Tian J, Li DP, Van Drunen R, Ribas-Latre A, Cai ZL, Xue M, Arenkiel BR, Eckel-Mahan K, Xu Y, Tong Q. Nat Commun. 2020 Jul 30;11(1):3794. doi: 10.1038/s41467-020-17578-7. PMID: 32732906
2. Profound and redundant functions of arcuate neurons in obesity development. Zhu C, Jiang Z, Xu Y, Cai ZL, Jiang Q, Xu Y, Xue M, Arenkiel BR, Wu Q, Shu G, Tong Q. Nat Metab. 2020 Jul 27. doi: 10.1038/s42255-020-0229-2. PMID: 32719538
- 3 .A neural basis for brain leptin action on reducing type 1 diabetic hyperglycemia. Fan S, Xu Y, Lu Y, Jiang Z, Morrill J, Cai J, Wu Q, Xu Y, Xue M, Arenkiel BR, Huang C and Tong Q. Under review.

Patents: Nothing to report

Funding Obtained: Nothing to report

Disrupted hypothalamic CRH neuron responsiveness contributes to diet-induced obesity

Canjun Zhu^{1,2}, Yuanzhong Xu², Zhiying Jiang², Jin Bin Tian^{2,3}, Ryan M Cassidy^{2,4}, Zhao-Lin Cai^{5,6}, Gang Shu¹ , Yong Xu⁷, Mingshan Xue^{5,6}, Benjamin R Arenkiel⁸, Qingyan Jiang^{1,*} & Qingchun Tong^{2,4,9,**} 

Abstract

The current obesity epidemic mainly results from high-fat high-caloric diet (HFD) feeding and may also be contributed by chronic stress; however, the neural basis underlying stress-related diet-induced obesity remains unknown. Corticotropin-releasing hormone (CRH) neurons in the paraventricular hypothalamus (PVH), a known body weight-regulating region, represent one key group of stress-responsive neurons. Here, we found that HFD feeding blunted PVH CRH neuron response to nutritional challenges as well as stress stimuli and dexamethasone, which normally produce rapid activation and inhibition on these neurons, respectively. We generated mouse models with the activity of these neurons clamped at high or low levels, both of which showed HFD-mimicking, blunted PVH CRH neuron responsiveness. Strikingly, both models developed rapid HFD-induced obesity, associated with HFD-mimicking, reduced diurnal rhythmicity in feeding and energy expenditure. Thus, blunted responsiveness of PVH CRH neurons, but not their absolute activity levels, underlies HFD-induced obesity and may also contribute to stress-induced obesity.

Keywords CRH; HFD; obesity; PVH; stress

Subject Categories Metabolism; Neuroscience

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EMBO Reports (2020) e49210

Introduction

The current obesity epidemic coincides with social and economic improvements. Susceptibility to obesity development is thus speculated to be caused by a combination of genetics and environmental

changes, including easy access to high-fat high-caloric diets (HFD), and stresses imposed by increasing socioeconomic pressure [1–4]. Specifically, HFD feeding is associated with disrupted diurnal rhythms in feeding and metabolism, and time-scheduled feeding alleviates the obesity and associated metabolic syndromes by HFD [5], suggesting an important contribution of disrupted diurnal rhythms to diet-induced obesity (DIO). Recent studies have also suggested a potential role for stress in worsening DIO [6–9]. Despite intense research, the cellular mechanism in the brain underlying HFD feeding and its potential interaction with stress in obesity development remains elusive.

Corticotropin-releasing hormone-expressing (CRH) neurons, widely distributed in the brain [10], are known to be the major subset of brain neurons that orchestrate behavioral and hormonal responses to stress [11]. Anatomically, hypothalamic CRH neurons serve as a link between metabolism regulation and stress responses. These neurons are known to elicit neuroendocrine responses through the autonomic nervous system and hypothalamus–pituitary–adrenal axis (HPA) [12,13], although emerging studies suggest a role for PVH CRH neurons in mediating stress behaviors [14,15]. In addition to stress responses, these neurons are shown to be important for diet preference [16], suggesting potential relevance to obesity development. Pathologically, uncontrolled glucocorticoid levels are known to be associated with hyperphagia, insulin resistance, obesity, and diabetes, as observed in human Cushing's diseases and leptin-deficient *ob/ob* mouse models [17,18]. However, other studies have also suggested that PVH CRH neurons play a minimal role in feeding or obesity development [15,19,20]. Thus, the relevance of CRH function to DIO is not yet clear.

High-fat high-caloric diet feeding has been shown to blunt diurnal rhythms [5,21,22]. Specifically, HFD reduces HPA axis activity and diurnal corticosterone rhythms in mice [23–25], and a disrupted corticosterone diurnal pattern is also present in obese *ob/ob* and

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db/db mouse models [26], pointing to a potential importance of PVH CRH neuron activity changes in response to HFD feeding and in obesity development. In this study, we therefore aim to test the hypothesis that HFD-induced defective responsiveness of PVH CRH neurons contributes to DIO. Our results show that HFD disrupts the responsiveness of PVH CRH neurons and our animal models with PVH CRH neurons chronically clamped at high or low levels both exhibit disrupted neuron responsiveness, susceptibility to DIO, and defective diurnal rhythms in metabolism, thus revealing blunted responsiveness of these neurons as a novel contributor to DIO.

Results and Discussion

Paraventricular hypothalamus neurons are known to be activated during nutritional challenges in switching from fasting to refeeding [27,28]. While PVH neurons exhibit little *c-Fos* expression with *ad libitum* fed conditions either on chow or HFD (Fig EV1A), a subset of PVH neurons in chow-fed control mice exhibited *c-Fos* expression when switching from fast to refed conditions (Fig 1A and B). In contrast, with 4-week HFD feeding, the number of *c-Fos*-expressing neurons was dramatically reduced (Fig 1A and B). We used CRH-Cre::Ai9 reporter mice to specifically examine *c-Fos* expression in CRH neurons and found that HFD feeding largely eliminated *c-Fos* expression in PVH CRH neurons (Fig 1A and C), suggesting that HFD feeding greatly reduced the response of PVH CRH neurons to a switch from fasting to refed, a nutritional challenge frequently experienced in mice. As expected, mice during the 4-week HFD feeding exhibited increased energy intake, reduced energy expenditure, and increased body weight. In addition, these mice also reduced their diurnal rhythmicity in both feeding (Fig 1D and E) and energy expenditure (Fig 1F and G), supporting earlier findings on significant contributions of diminished diurnal feeding and energy expenditure patterns to DIO [5].

To examine whether HFD disrupts PVH CRH neuron responses to acute stress stimuli, we recorded the activity of these neurons in freely behaving animals with the fiber photometry method [29,30]. For this, we delivered AAV-Flex-GCaMP6m to the PVH of CRH-Cre mice with implanted optic fibers targeting the PVH (Fig EV1B).

Consistent with the role of PVH CRH neurons in stress responses, applications of stressors, i.e., water spray, induced a robust increase in PVH CRH neuron activity, which lasted 2–3 s (Fig 1H and J). The mice were then switched to HFD and maintained on the same diet for 4 weeks before a new round of fiber photometry measurement with the same water spray stimulus. Those PVH CRH neurons of the same mice greatly diminished their responses to water spray (Fig 1I and J). To examine whether the diminished response is specific to stress-induced activation, we also applied dexamethasone (Dex), an analog of corticosterone (Cort) known to inhibit PVH CRH neurons. While saline administration failed to produce changes (Fig EV1C), Dex inhibited PVH CRH neuron activity within minutes (Fig 1K and M), which is likely mediated by its membrane receptors [31]. This effect was greatly diminished in PVH CRH neurons 4 weeks after HFD feeding (Fig 1L and M). These results demonstrate that HFD feeding reduces PVH CRH neuron responsiveness.

To test whether dampening PVH CRH responsiveness contributes to DIO, we aimed to generate animal models with disrupted responsiveness in PVH CRH neurons by clamping the neuron activity at high levels. The expression of *NachBac*, a sodium channel from bacteria, is known to increase baseline neuronal activity [32,33]. We therefore delivered AAV-EF1a-Flex-EGFP-P2A-m*NachBac* to the PVH of CRH-Cre mice. We recorded CRH neurons with *NachBac* expression in one side of the PVH, compared to those recorded in the other side with control neurons of the same animal 4 weeks after viral delivery (Fig 2A). Compared to controls, which showed spontaneous tonic firing around -50 mV, spontaneous burst firing and long-lasting plateau depolarization were recorded at more negative membrane potential (-65 mV) from *NachBac* neurons (Fig 2B). This more negative membrane potential may result from a compensatory response to avoid overexcitation. With a step-wise voltage clamp protocol to evoke Na^+ channel activation, compared to the -40 mV threshold found in control cells (Fig EV2A), *NachBac* channels were activated at -50 mV, with much slower inactivation than the endogenous Na^+ channels (Fig EV2B). In total, while 12 out of 15 recorded *NachBac* neurons (80%) showed spontaneous firing despite their more negative membrane potentials than that in controls cells, in which only six of 10 control cells (60%) exhibited spontaneous firing (Fig 2C). The higher percentage of spontaneous firing in *NachBac*-expressing neurons suggest they are more active

Figure 1. HFD blunted PVH CRH neuron responsiveness.

- A–C CRH-Cre::Ai9 reporter male mice fed on chow or 4 weeks on HFD were used to examine *c-Fos* expression in the PVH in 24 h fasting or refed after 24 h fasting. (A) Representative images showing CRH neurons (red in the first column), *c-Fos* expression (green in the second column), merged images of red and green (the third column), and the amplified pictures for the boxed areas in the merged images (the fourth column). The experimental conditions for each group (fasting versus refed, chow versus HFD) are labeled on the left. Scale bar = 200 μm . (B, C) Comparison of average neuron numbers from cell counting on total number of *c-Fos*-positive neurons in the PVH (B, $^{**}P = 0.0032$: chow fasting versus chow refed and $^{*}P = 0.023$, HFD fasting versus HFD refed) and total number of *c-Fos*-positive CRH neurons (C, $^{**}P < 0.0001$: chow fasting versus chow refed and $P = 0.7158$: HFD fasting versus HFD refed), $n = 3–4$ each, data presented as mean $\pm$ SEM, two-way ANOVA tests.
- D–G Effects of HFD feeding on diurnal rhythmicity in feeding (D and E) and energy expenditure (F and G). The measurement was collected with male mice fed chow or after 4-week HFD feeding, $n = 6$ each, data presented as mean $\pm$ SEM, $^{**}P = 0.0066$ in E and $^{**}P = 0.0007$ in G, unpaired Student's *t*-tests.
- H–M CRH-Cre mice received delivery of AAV-Flex-GCaMP6m viral vectors to bilateral PVH and optic fiber implantation targeting PVH neurons and were used for *in vivo* live recording. (H, I) Representative traces showing recordings of activity changes in CRH neuron in response to stressor (water spray) in chow-fed controls (H) and 4 weeks after HFD (I). (J) Comparison of average responses in the two conditions, $n = 4$ each, $^{**}P = 0.0006$, paired Student's *t*-test. (K, L) Representative traces showing recordings of activity changes in CRH neurons in response to dexamethasone (Dex) injections in chow-fed controls (K) and after 4 weeks on HFD (L). (M) Comparison of average responses in the two conditions, $n = 4$ each, data presented as mean $\pm$ SEM, $^{**}P = 0.0012$, paired Student's *t*-tests.

Data information: 3V: the third ventricle; downward arrows indicating onset of stimuli or Dex injections.

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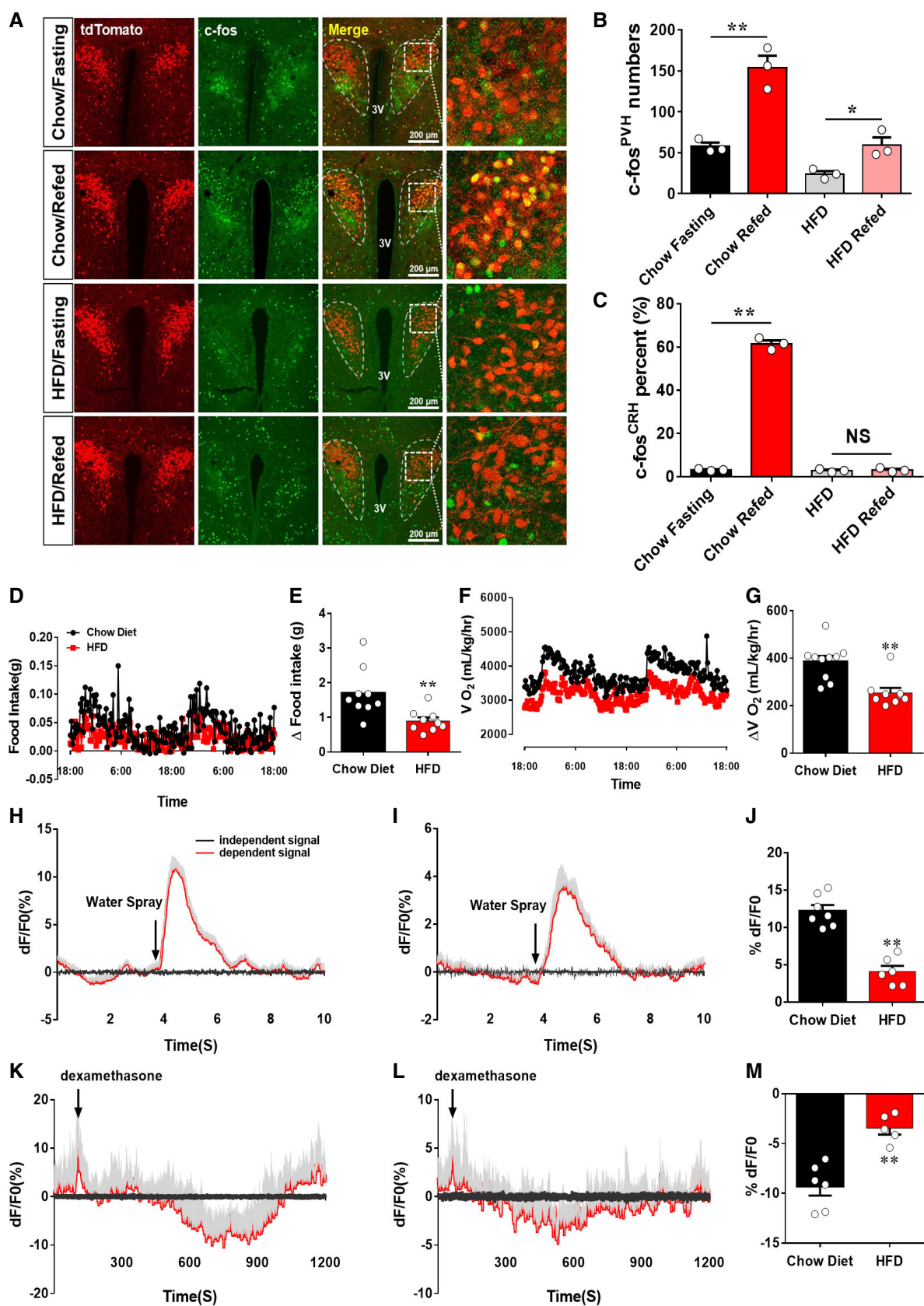


Figure 1.

than control neurons. To further confirm that NachBac-expressing neurons exhibit a high level of activity *in vivo*, we examined c-Fos expression in the PVH in response to a switch from fasting to refeeding. As expected, in control CRH-Cre mice with AAV-Flex-GFP injections to bilateral PVH, c-Fos was induced in the PVH (Fig 2D and E), especially in PVH GFP-labeled CRH neurons (Fig 2F). In CRH-Cre mice with AAV-EF1a-Flex-EGFP-P2A-mNachBac injection to bilateral PVH, a much greater number of PVH neurons exhibited c-Fos expression both at fasting and refed conditions (Fig 2D and E). Interestingly, in contrast to control CRH neurons, switching mice from fasting to refeeding failed to induce further c-Fos expression in NachBac-expressing neurons (Fig 2D and F), suggesting that NachBac expression effectively clamps CRH neuron activity at high levels, disrupting the ability of these neurons in responding to nutritional challenges.

In addition, we also examined the response of NachBac-expressed PVH CRH neurons to acute stressors and Dex. Toward this, we delivered AAV-Flex-GCaMP6m and AAV-EF1a-Flex-EGFP-P2A-mNachBac simultaneously to bilateral PVH of CRH-Cre mice for fiber photometry recording (Appendix Fig S1A). In contrast to rapid and strong activation on CRH neurons (Fig 1H), water spray failed to induce obvious changes in NachBac-expressing CRH neurons (Fig 2G and H). A similar observation was also made in response to Dex (Fig 2I and J). Notably, PVH CRH neurons with NachBac expression were greatly inhibited by exposure to isoflurane, an anesthetic with potent inhibitory action on neurons (Appendix Fig S1B), ruling out the possibility that failure in detecting CRH neuron response is an artifact of experimental defects. To examine the possibility that co-expression of additional fluorescent protein (e.g., GFP in the NachBac vector) might interfere with GCaMP6m measurements, we compared GCaMP6m measurements among three groups of CRH-Cre mice with delivery of AAV-Flex-GCaMP6m alone (Appendix Fig S1C), AAV-Flex-GCaMP6m plus AAV-Flex-GFP (Appendix Fig S1D), or AAV-Flex-GCaMP6m plus AAV-Flex-mCherry (Appendix Fig S1E). No difference was observed

among the three groups in the GCaMP6m signal in response to water spray (Appendix Fig S1F), suggesting that presence of other fluorescence (green or red) has no interference on measurements of GCaMP6m signals. Taking together, NachBac expression effectively increases the activity level and blunts the responsiveness of PVH CRH neurons.

Paraventricular hypothalamus CRH expression is known to exhibit a diurnal pattern, with a higher level at night periods [34], suggesting the activity of these neurons in a diurnal pattern. In addition to direct projections to other brain regions, PVH CRH neurons are known to regulate the HPA axis. To further test whether NachBac expression disrupts diurnal neuron activity pattern, we measured corticosterone (cort) levels across 24 h and found that NachBac mice exhibited much greater levels during night times, compared to controls (Fig EV2C). As cort is a remote indicator for CRH neuron activity and especially its level is controlled by complex feedback regulation through its own control at both CRH and pituitary levels, the diurnal pattern of cort may not necessarily reflect PVH CRH neuron activity pattern. Supporting this, mice with PVH-specific deletion of glucocorticoid receptor, known to inhibit CRH expression, show disrupted diurnal patterns in adrenocorticotrophic hormone (ACTH) and exhibit heightened diurnal changes in cort levels [35]. We therefore used CRH expression level as a proxy for CRH neuron activity. Notably, CRH immunostaining showed an increased CRH accumulation in the median eminence (ME), where CRH neuron fibers are located (Fig EV3D). Consistent with the fact that CRH is normally transported to terminals, CRH immunostaining failed to show clear immunoreactive structures in PVH CRH neurons during both morning and night periods in controls; however, NachBac mice showed abundant CRH-immunoreactive soma and fibers in the PVH at both day and night periods (Fig EV2E). These data, although on their own, cannot approve, nevertheless support, that NachBac expression increases PVH CRH neuron activity and disrupts diurnal patterns of activity.

Figure 2. Clamping PVH CRH neuron activity at high levels disrupted neuron responsiveness.

- A–C CRH-Cre male mice received stereotaxic delivery of AAV-EF1a-Flex-EGFP-P2A-mNachBac vectors at 7–8 weeks of age and recordings were made at least 4 weeks after viral delivery on neurons with NachBac (one side) or control neurons (contralateral side) of the same animal (A); typical action potentials (APs) in controls (B, top panel) and typical NachBac-mediated APs showing lower threshold and long duration (B, bottom, the inset highlighting the long duration) in NachBac mice; and the distribution of the membrane potential and firing cells in the two groups (C, ratios in the bracket denoting neuron number out of total number recorded with the indicated behavior).
- D–F CRH-Cre male mice were injected with either AAV-Flex-GFP or AAV-EF1a-Flex-EGFP-P2A-mNachBac to examine c-Fos expression in the PVH in 24 h fasting or refed after fasting. (D) Representative images showing CRH neurons expressing GFP or NachBac (GFP in the top two rows and NachBac in the bottom two rows of the first column), c-Fos expression (red in the second column), merged images of red and green (the third column), and the amplified pictures for the boxed areas in the merged images (the fourth column). The experimental conditions for each group (fasting versus refed) are labeled on the left. (E, F) Comparison of average neuron numbers from cell counting on total number of c-Fos-positive neurons in the PVH (E, $^{**}P < 0.0001$: fasting versus refed; $^{**}P = 0.0026$: fasting versus fasting/NachBac; $^{**}P < 0.0001$: fasting/NachBac versus refed/NachBac) and total number of c-Fos-positive CRH neurons (F, $^{**}P < 0.0001$: fasting versus refed; $^{**}P < 0.0001$: fasting versus fasting/NachBac; $P = 0.3423$: fasting/NachBac versus refed/NachBac), $n = 3$ –4 each, data presented as mean $\pm$ SEM, two-way ANOVA tests.
- G–J CRH-Cre male mice received stereotaxic delivery of a mixture of AAV-Flex-GCaMP6m and AAV-EF1a-Flex-EGFP-P2A-mNachBac vectors at 7–8 weeks of age with optic fiber implantation targeting PVH neurons. (G, H) Representative traces showing typical responses of PVH CRH neurons to water spray (arrows) in NachBac mice (G) and comparison of responsiveness to water spray between NachBac and control mice (H). (I, J) Representative traces showing typical responses of PVH CRH neurons to water spray (arrows) in NachBac mice (I) and comparison of responsiveness to water spray between NachBac and control mice (J). Gray traces showed Ca^{2+} -independent signal for system stability, and red ones showed Ca^{2+} -dependent signals for neuron activity, $N = 5$ –7 each, males, data presented as mean $\pm$ SEM, $^{**}P < 0.0001$ in both (H and J), unpaired Student's *t*-tests.

Data information: All mice were fed chow unless otherwise noted. 3V: the third ventricle; downward arrows indicating onset of stimuli or Dex injections. Scale bar = 200 μm .

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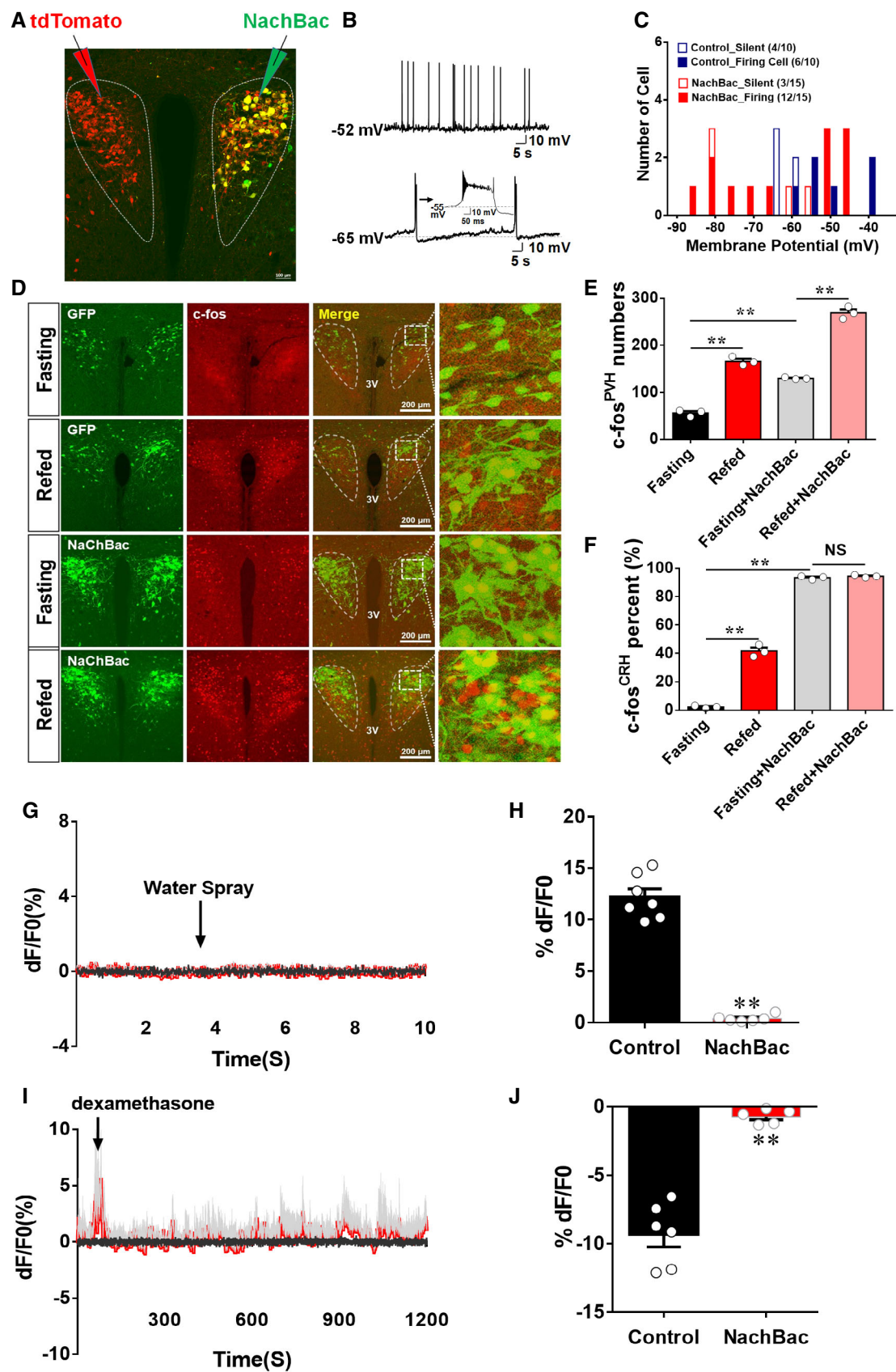


Figure 2.

We then used mice with NachBac vector injections to bilateral PVH of CRH-Cre mice to examine the effect on body weight. When fed chow, NachBac mice gained a slightly but significant more body weight than controls (Fig 3A). However, after exposure to HFD, these mice gained rapid body weight and were 10 g heavier than controls 4 weeks after HFD diet switch (Fig 3A). The observed body weight gain was associated with increased fat mass (Fig 3B), but no changes in lean mass (Fig 3C), suggesting obesity development. When measured at 7 weeks after viral delivery but before HFD switch, these mice exhibited lower energy expenditure (Fig 3D and E) and greater feeding levels (Fig 3G and H). Interestingly, when the difference between day and night periods was compared, NachBac mice showed much less difference in either energy expenditure (Fig 3F) or feeding (Fig 3I), suggesting reduced rhythmicity in both, which is reminiscent to the dampening effect on diurnal rhythmicity by HFD feeding [5]. When comparison between the two groups during light and dark periods was performed, we found that NachBac mice ate more food but spent less energy, especially during light periods (Fig EV3A–D), suggesting an increased feeding efficiency. A similar reduction in activity level in NachBac mice was also observed (Fig EV3E–I). Weekly glucose levels trended to increase on chow but were significantly elevated in HFD-fed conditions (Fig EV3J), likely due to a secondary effect of obesity development. In addition, NachBac mice exhibited an increased anxiety level in our behavioral tests (Appendix Fig S2). These data collectively demonstrate that clamping PVH CRH neuron activity at high levels diminishes the responsiveness of these neurons and leads to DIO.

The aforementioned results suggest that NachBac expression diminished PVH CRH responsiveness while increased the activity level of these neurons. To ascertain the role of diminished responsiveness in DIO and rule out a potential direct role from an increased neuron activity level, we aimed to generate a new mouse model with disrupted PVH CRH neuron responsiveness through clamping neuron activity at low levels. Towards this end, we used a mutated form of the Kir2.1 channel, which has previously used to reduce neuron activity [32]. We delivered either AAV-Flex-GFP vectors to one side as control or AAV-EF1a-DIO-Kir2.1-P2A-dTomato vectors to the other side of the PVH of CRH-Cre mice for recording experiments (Fig 4A). Compared to controls, Kir2.1 expression reduced resting membrane potential (Fig 4B and C) and significantly increased the size of minimum currents required to be injected to elicit action potential, i.e., rheobase (Fig EV4A–C), suggesting that expression of Kir2.1 effectively reduces the PVH neuron activity.

We next examined the effect of Kir2.1 expression on c-Fos induction in response to switch from fasting to refeeding. We delivered AAV-EF1a-DIO-Kir2.1-P2A-dTomato vectors to one side of the PVH of CRH-Cre mice (Fig 4D), which allows to effectively examine the effect of Kir2.1 on c-Fos expression by using the other side of PVH with no Kir2.1 expression within the same brain as control conditions. Compared to controls, Kir2.1 expression in PVH CRH neurons significantly reduced the number of c-Fos positive neurons in the PVH during fast-refeeding transitions (Fig 4D and E). When counting PVH CRH neurons with c-Fos expression, we found that while the control side with mCherry expression showed a great number of PVH CRH neurons with c-Fos expression, few of these neurons with Kir2.1 expression was found to express c-Fos during the fast-

refeeding switch (Fig 4D and F), suggesting that expression of Kir2.1 effectively clamped PVH CRH neuron activity at low levels and disrupted the ability of these neurons in responding to nutritional challenges during fast-refeeding transitions.

We further examined the impact of Kir2.1 expression on PVH CRH neuron response to acute stress and hormonal action. In CRH-Cre mice with co-injections of AAV-Flex-GCaMP6m and AAV-EF1a-DIO-Kir2.1-P2A-dTomato (Appendix Fig S3A), in contrast to controls, water spray (Fig 4G and H) or Dex administration (Fig 4I and J) failed to induce changes in the activity of CRH neurons. To verify that the failure in PVH CRH neuron response is not due to an experimental defect, we performed similar isoflurane inhalation as previously described and found that isoflurane was effective in reducing the activity of these neurons (Appendix Fig S3B). Thus, clamping PVH neuron activity at low levels with Kir2.1 expression is sufficient to blunt the responsiveness of these neurons, in a similar fashion to HFD feeding.

We also measure the HPX axis to confirm the effect of Kir2.1 inhibition. Compared to a nature diurnal pattern of cort, Kir2.1 mice exhibited a flat pattern across 24 h (Fig EV4D), suggesting chronic inhibition of PVH CRH neurons is sufficient to diminish diurnal cort pattern. We then used CRH-Cre::Ai9 mice (Fig EV4E and G) and CRH-Cre mice with one side injection of Kir2.1 vectors (Fig EV4F and H) to examine the effect of Kir2.1 inhibition on CRH expression. While control mice exhibited even CRH immunoreactivity between the two sides of ME, Kir2.1 mice showed much less CRH immunoreactivity in the side with more Kir2.1 expression (Fig EV4H), demonstrating inhibition of CRH expression by Kir2.1.

We then delivered the Kir2.1 vector to bilateral PVH of CRH1-Cre mice for body weight studies. While Kir2.1-expressing mice showed no difference in body weight on chow diet, they gained more body weight once switched to HFD (Fig 5A). The increased body weight is associated with increased fat mass (Fig 5B) but no changes in lean mass (Fig 5C), suggestive of obesity development. When measured between 7 and 8 weeks after viral delivery, energy expenditure was reduced (Fig 5D and E) and, importantly, the difference between night and day in energy expenditure was reduced in mice with Kir2.1 expression (Fig 5F). Food intake was increased (Fig 5G and H), and the difference between night and day in feeding was also reduced in mice with Kir2.1 expression (Fig 5I). When comparison during light and dark periods was performed, we found that Kir2.1 mice ate more food but spent less energy, especially during light periods (Fig EV5A–D), suggesting an increased feeding efficiency. A similar reduction in activity level in NachBac mice was also observed (Fig EV5E–I). These data demonstrate that Kir2.1 expression reduces PVH CRH neuron activity, disrupts the responsiveness of these neurons, and leads to DIO. Notably, despite no body weight difference when fed chow, these mice exhibited lower blood glucose levels, but rapidly increased their glucose levels to a similar level of controls after exposure to HFD, likely due to their development of obesity (Fig EV5J). Collectively, these results suggest that PVH CRH neuron activity clamped at either high or low levels both blunted responsiveness of these neurons and induced DIO. Thus, disrupted responsiveness of PVH CRH neurons, but not their absolute activity levels, predisposes to DIO.

In this study, we found that HFD feeding blunted the response of PVH CRH neurons to nutritional challenges, acute stressors, and hormonal action. To test whether the blunted responsiveness

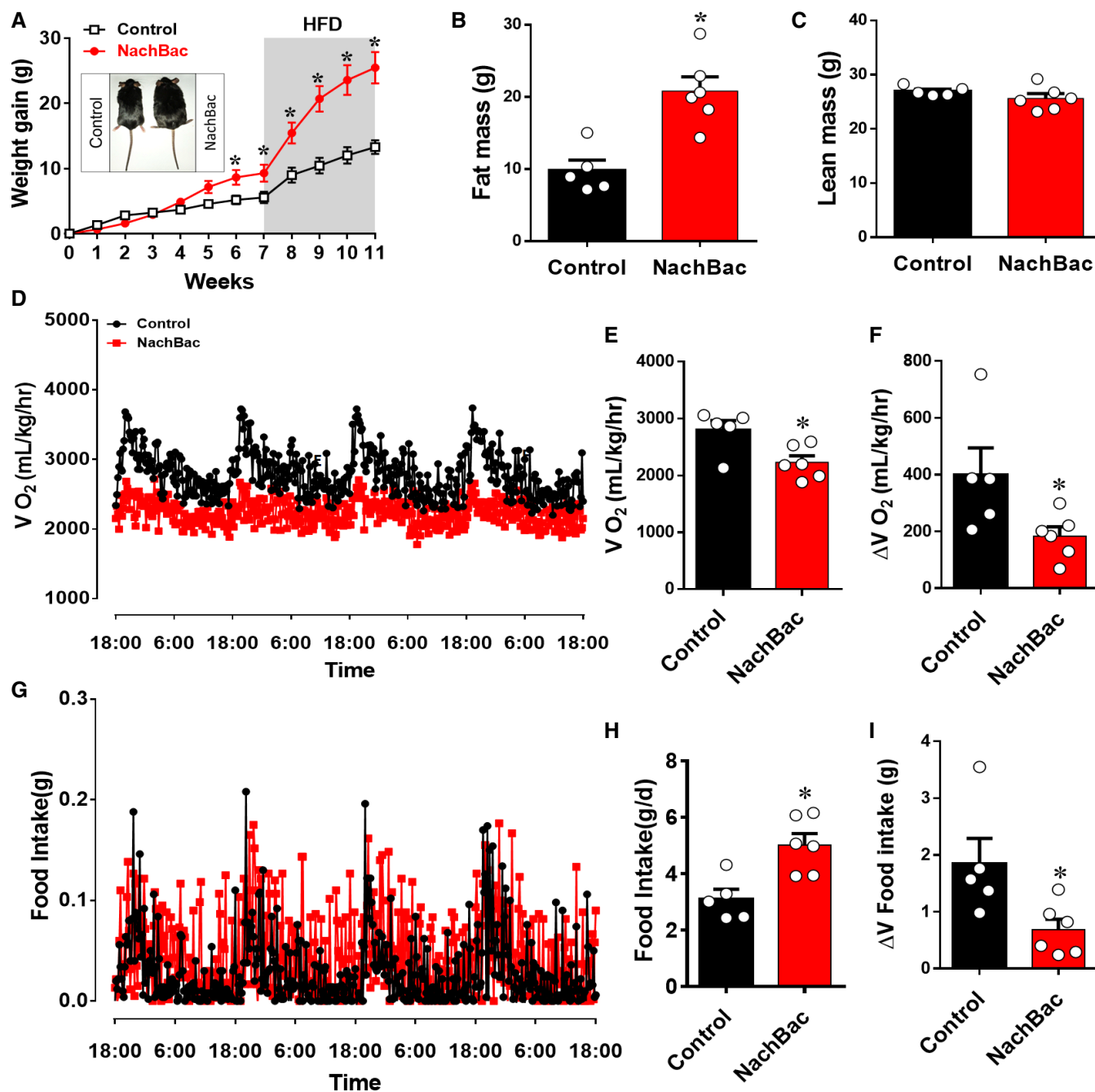


Figure 3. Clamping PVH CRH neuron activity at high levels causes rapid diet-induced obesity.

A–I CRH-Cre male mice received stereotaxic delivery of AAV-EF1a-Flex-EGFP-P2A-mNachBac or control GFP vectors at 7–8 weeks of age and were maintained on chow diet for 7 weeks before switching to HFD. Weekly body weight gain after viral delivery (A), and measurements of fat mass (B) and lean mass (C) at 11 weeks after viral delivery. (D–F) Measurements of energy expenditure during the 7th week after viral delivery. Comparison of real-time O_2 consumption traces during the 4-day measurement period (D), of average O_2 consumption between the two groups (E), and of the difference between day and night periods between the two groups (F). (G–I) Measurements of feeding during the 7th week after viral delivery. Comparison of real-time feeding traces during the 4-day measurement period (G), of average food intake between the two groups (H), and of the difference in food intake between day and night periods between the two groups (I). $n = 5–6$ each, * $P < 0.0001$ in A, * $P = 0.0019$ in B, $P = 0.2316$ in C, * $P = 0.0196$ in D, * $P = 0.0466$ in E, * $P = 0.0062$ in H, and * $P = 0.0298$ in I, unpaired Student's t -tests. All data presented as mean $\pm$ SEM.

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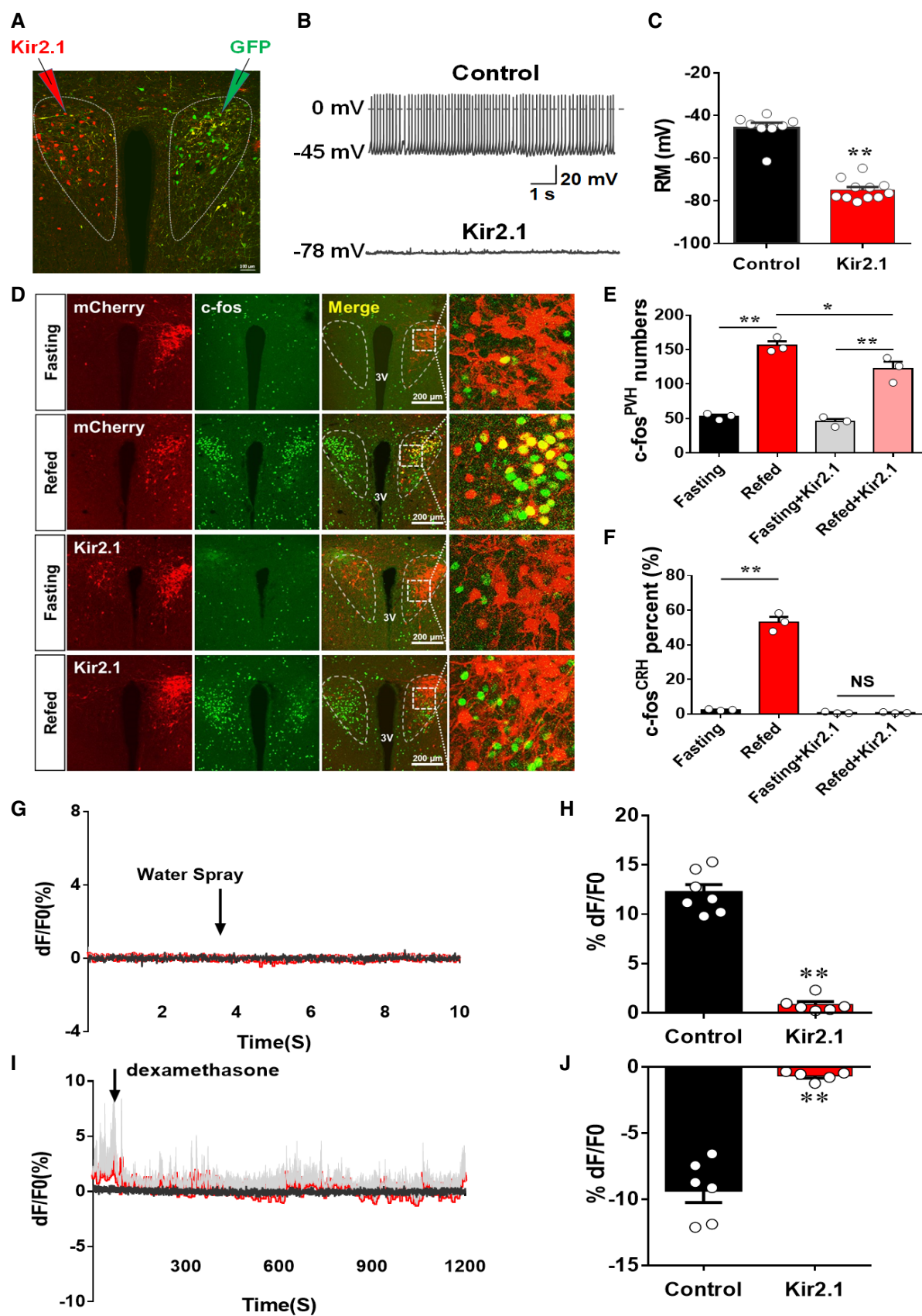


Figure 4.

Figure 4. Clamping PVH CRH neuron activity at low levels disrupted neuron responsiveness.

- A–C CRH-Cre male mice received stereotaxic delivery of AAV-EF1a-DIO-Kir2.1-P2A-dTomato vectors and AAV-Flex-GFP at 7–8 weeks of age, and recording was made at least 4 weeks after viral delivery on neurons with Kir2.1 (one side) or control GFP neurons (contralateral side) of the same animal (A); typical spontaneous neuron activity recorded in GFP controls (B, top panel) and Kir2.1 neurons (B, bottom); and comparison in resting membrane potentials between the two groups (C, $^{**}P < 0.0001$, unpaired Student's *t*-test).
- D–F CRH-Cre male mice were injected with either AAV-Flex-mCherry or AAV-EF1a-DIO-Kir2.1-P2A-dTomato to examine c-Fos expression in the PVH in 24 h fasting or refed after fasting. (D) Representative images showing CRH neurons expressing mCherry or Kir2.1 (mCherry in the top two rows and Kir2.1 in the bottom two rows of the second column), c-Fos expression (green in the first column), merged images of red and green (the third column), and the amplified pictures for the boxed areas in the merged images (the fourth column). The experimental conditions for each group (fasting versus refed) are labeled on the left. (E, F) Comparison of average neuron numbers from cell counting on total number of c-Fos-positive neurons in the PVH (E, $^{**}P = 0.0001$: fasting versus refed; $^{*}P = 0.0497$: refed versus refed/Kir2.1; $^{**}P = 0.0025$: fasting/Kir2.1 versus refed Kir2.1) and total number of c-Fos-positive CRH neurons (F, $^{**}P < 0.0001$: fasting versus refed; $P = 0.9586$: fasting/Kir2.1 versus refed/Kir2.1), $n = 3$ –4 each, two-way ANOVA tests. All data presented as mean $\pm$ SEM.
- G–J CRH-Cre male mice received stereotaxic delivery of a mixture of AAV-Flex-GCaMP6m and AAV-EF1a-DIO-Kir2.1-P2A-dTomato vectors at 7–8 weeks of age with optic fiber implantation targeting PVH neurons. (G–H) Representative traces showing typical responses of PVH CRH neurons to water spray (arrows) in Kir2.1 mice (G) and comparison of responsiveness to water spray between Kir2.1 and control mice (H). (I, J) Representative traces showing typical responses of PVH CRH neurons to water spray (arrows) in Kir2.1 mice (I) and comparison of responsiveness to water spray between Kir2.1 and control mice (J). Gray traces showed Ca^{2+} -independent signal for system stability, and red ones showed Ca^{2+} -dependent signals for neuron activity, $N = 5$ each, males, data presented as mean $\pm$ SEM, $^{**}P < 0.0001$ in both (H and J), unpaired Student's *t*-tests.

Data information: All mice were fed chow unless otherwise noted. 3V: the third ventricle; downward arrows indicating onset of stimuli or Dex injections. Scale bar = 200 μm .

Source data are available online for this figure.

mediates DIO, we created two complementary animal models with PVH CRH neuron activity clamped at either high or low levels, both confirmed to exhibit blunted responsiveness, mimicking the effect of HFD feeding. Interestingly, despite differences in body weight and glucose homeostasis on chow, both models developed rapid DIO. These results demonstrate that it is the responsiveness of PVH CRH neurons, but not their absolute activity level, dictates the susceptibility to DIO, thus providing a novel insight on the mechanism underlying DIO. This novel mechanism has important implications to prevalent investigations focusing on the role of neuron activity levels in the regulation of feeding and obesity development.

One potential mechanism for HFD to diminish neuron responsiveness may lie in alterations in synaptic neurotransmission from upstream neurons, given that HFD is known to cause rapid changes in synaptic currents on hypothalamic neurons [36–38]. One upstream site might be arcuate neurons, which are known to provide direct synaptic inputs to PVH neurons and respond to ambient signals including those induced by HFD feeding [39]. Disrupted neuron responsiveness may also lie in the changes within PVH CRH neurons induced by HFD diet as these neurons also exhibited reduced responses to Dex, a hormone that presumably acts directly on these neurons. The expression of either NachBac or Kir2.1 greatly blunted the responsiveness, suggesting that the action of NachBac and Kir2.1, which likely lock the neuron activity at respective elevated or reduced levels, may override the action induced by stressors and hormones. Notably, mice with NachBac expression exhibited a significant increase in body weight on chow, suggesting a sufficiency of chronic activation of PVH CRH neurons in causing a HFD-mimicking effect. No body weight difference observed in Kir2.1 mice on chow may be due to a beneficial effect of reducing PVH CRH neuron activity, as evidenced from improved glucose homeostasis in these mice, which was quickly reversed on HFD. The greater susceptibility of these mice to DIO demonstrates that appropriate responses of these neurons to HFD feeding are required to mount balanced body weight regulation on HFD feeding.

High-fat high-caloric diet-induced disruption in the responsiveness of PVH CRH neurons has important implications. As PVH CRH

neuron activity regulates the HPA axis, our findings are supported by previous studies showing that HFD blunts the HPA axis and alters diurnal rhythms of cort [23,24,40]. The reduced responsiveness of PVH CRH neurons may underlie the effect of chronic stress on exacerbating HFD-induced obesity. Chronic stress induces a sustained increase in the activity of PVH CRH neurons [41], which may mimic the effect induced by NachBac in clamping the neuron activity at high levels. Thus, disrupted neuron responsiveness may explain obesity development induced by chronic stressors (e.g., social stress) [42,43] and synergistic effects between chronic stress and HFD on obesity development [6–9]. Given the socioeconomic stress faced by humans is chronic in nature, and a positive correlation between cort levels and low socioeconomic status [44], our results support that chronic stress contributes significantly to obesity development, especially in the current HFD environment.

The PVH CRH neuron activity pattern is orchestrated by dynamic changes in synaptic inputs and hormonal action [13]. Thus, disruption in the response of these neurons to stressors and hormonal action may impact long-term activity patterns of these neurons. It is known that both CRH and cort levels exhibit a diurnal pattern in mice [34,45], indicating that PVH CRH neurons may exhibit a diurnal pattern of activity. Thus, although remains to be determined, HFD feeding, and NachBac or Kir2.1 expression may disrupt diurnal patterns of PVH CRH neurons. Given the reduced diurnal rhythmicity in feeding and energy expenditure as a common feature in all animal models presented in this study, it is likely that loss of diurnal metabolism in DIO may be contributed significantly by disrupted diurnal activity pattern of PVH CRH neurons owing to loss of responsiveness. As scheduled feeding is known to improve metabolism and diurnal rhythms [5], it would be interesting in future studies to examine whether HFD-induced disruption in responsiveness of PVH CRH neurons can be reversed by scheduled feeding. In summary, our current findings reveal a novel mechanism underlying DIO in which HFD feeding diminishes responsiveness of PVH CRH neurons, thereby affecting diurnal rhythms in feeding and metabolism and leading to obesity development, and that PVH CRH

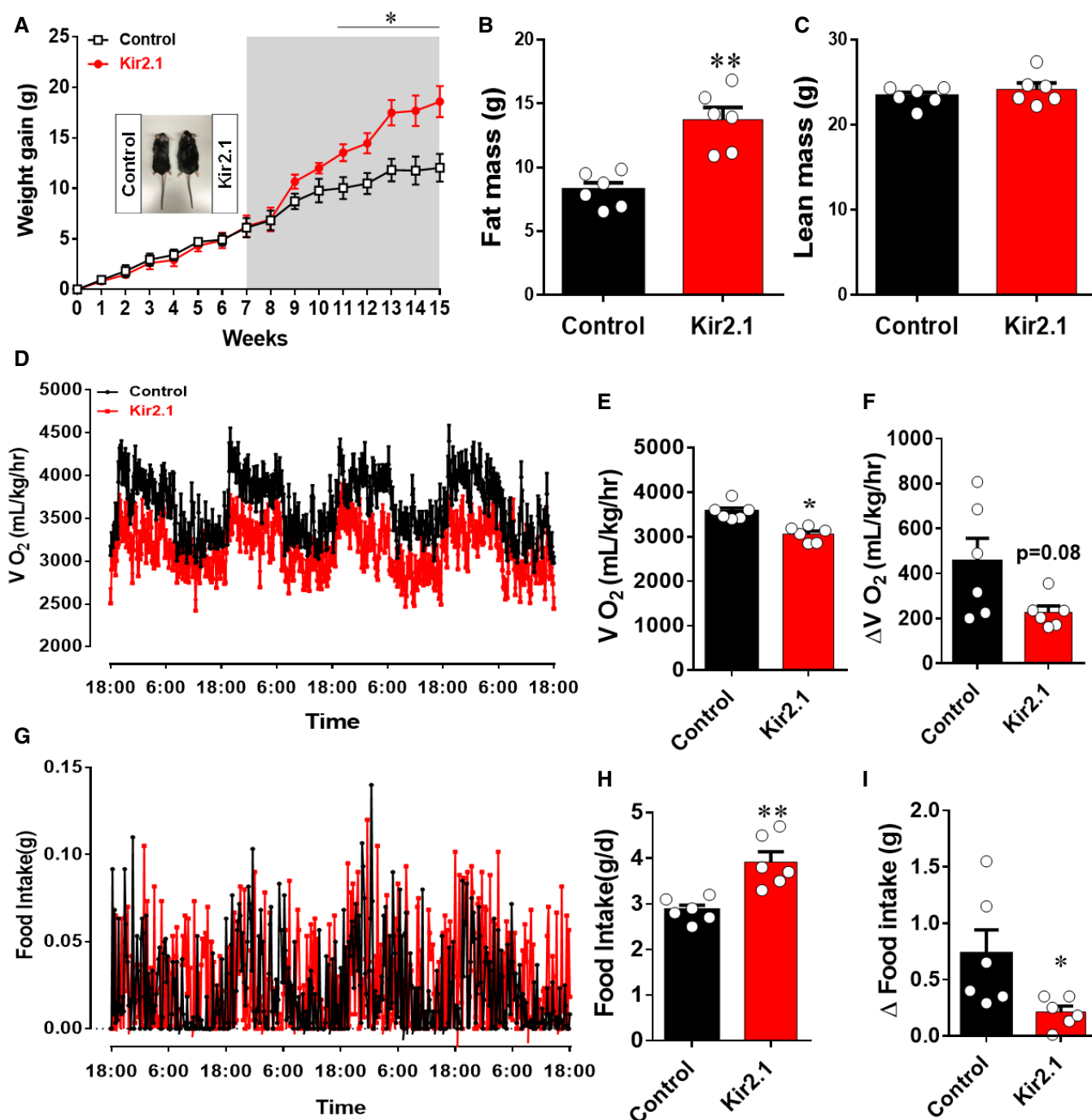


Figure 5. Clamping PVH CRH neuron activity at low levels causes diet-induced obesity.

A–I CRH-Cre male mice received stereotaxic delivery of AAV-EF1a-DIO-Kir2.1-P2A-dTomato or control AAV-Flex-mCherry vectors at 7–8 weeks of age and were maintained on chow diet for 7 weeks before switching to HFD. Weekly body weight gain after viral delivery (A, $*P < 0.0001$, unpaired Student's *t*-test), and measurements of fat mass (B, $**P = 0.0006$, unpaired Student's *t*-test) and lean mass (C, $P = 0.3728$, unpaired Student's *t*-test) at 15 weeks after viral delivery. (D–F) Measurements of energy expenditure during the 7th week after viral delivery. Comparison of real-time O_2 consumption traces during the 4-day measurement period (D), of average O_2 consumption between the two groups (E, $*P = 0.0007$, unpaired Student's *t*-test), and of the difference between day and night periods between the two groups (F, $P = 0.0791$, unpaired Student's *t*-test). (G–I) Measurements of feeding during the 7th week after viral delivery. Comparison of real-time feeding traces during the 4-day measurement period (G), of average food intake between the two groups (H, $**P = 0.0019$, unpaired Student's *t*-test), and of the difference in food intake between day and night periods between the two groups (I, $*P = 0.0363$, unpaired Student's *t*-test). $n = 6$ each. All data presented as mean $\pm$ SEM.

Source data are available online for this figure.

neurons may serve as a primary neural substrate in mediating converging effects on obesity development by HFD feeding and chronic stress.

Materials and Methods

Animal care

Mice were housed at 21–22°C with a 12-h light/12-h dark cycle with standard pellet chow and water provided *ad libitum* unless otherwise noted for fasting experiments. Animal care and procedures were approved by the University of Texas Health Science Center at Houston Institutional Animal Care and Use Committee. CRH-Cre mice were purchased from the Jax lab and described and confirmed to express Cre in PVH CRH neurons previously [10,46]. Additionally, in most breeding pairs, either male or female breeders (or both) contained the Ai9 reporter gene (Rosa-LSL-tdTomato) to allow RFP expression in the presence of cre recombination. The HFD used in this study were purchased from Research Diet (D12492). All study subjects were littermates and randomly distributed between study groups. Group size was estimated based on relevant literature reports and differences in average between groups from pilot studies. All mice that were used for stereotaxic injections were at least 8–10 weeks old.

Surgeries and viral constructs

Stereotaxic surgeries to deliver viral constructs and for optical fiber implantation were performed as previously described. Briefly, mice were anesthetized with a ketamine/xylazine cocktail (100 mg/kg and 10 mg/kg, respectively), and their heads were affixed to a stereotaxic apparatus. Viral vectors were delivered through a 0.5 μ l syringe (Neuros Model 7000.5 KH, point style 3; Hamilton, Reno, NV, USA) mounted on a motorized stereotaxic injector (Quintessential Stereotaxic Injector; Stoelting, Wood Dale, IL, USA) at a rate of 30 nl/min. Viral preparations were titrated at more than $\sim 1,012$ particles/ml. Viral delivery was targeted to the PVH (150 nl/side; anteroposterior [AP]: -0.9 mm; mediolateral [ML]: ± 0.1 mm; dorsoventral [DV]: -4.8 mm). AAV-Flex-GCaMP6m, AAV-EF1a-Flex-EGFP-P2A-mNachBac, or AAV-EF1a-DIO-Kir2.1-P2A-dTomato were delivered bilaterally into PVH of CRH-Cre mice. Two mutations (E224G and Y242F) were made in the Kir2.1 construct so that its activity will be less rectified by membrane potentials [32], thereby more effective in reducing neuron activity. AAV-Flex-GFP and AAV-Flex-mCherry injections were used as a control group. All viral vectors were either serotype 5 or DJ8, and provided through the Neuroconnectivity Core of Baylor College of Medicine with titer more than 1×10^{12} GC/ml. Behavioral testing was conducted following a minimum 4-week recovery period post-surgery.

Brain slice electrophysiological recordings

Mice were given an overdose of Avertin and then perfused intracardially with ice-cold cutting solution containing (in mM) 212 sucrose, 3 KCl, 1.25 NaH_2PO_4 , 26 NaHCO_3 , 7 MgCl_2 , and 10 glucose. Whole brain was excised quickly and immediately

immersed into ice-cold cutting solution. Coronal brain slices (250–300 μ m) containing the PVH from mice that had received stereotaxic injections of AAV-EF1a-Flex-EGFP-P2A-mNachBac, AAV-EF1a-DIO-Kir2.1-P2A-dTomato, or AAV-Flex-GFP to PVH at least 4 weeks prior to the recording were cut in oxygenized, ice-cold cutting solution. Slices were incubated for 60 min in artificial cerebrospinal fluid (aCSF) containing (in mM) 125 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 1.25 NaH_2PO_4 , 25 NaHCO_3 , and 11 D-glucose bubbling with 95% O_2 /5% CO_2 , at 32–34°C, for recovery. Individual slices were transferred to a recording chamber mounted on an upright microscope (Olympus BX51WI) and continuously superfused (2 ml/min) with aCSF warmed to 32–34°C by passing it through a feedback-controlled in-line heater (TC-324B; Warner Instruments). Cells were visualized through a 40 $\times$ water-immersion objective with differential interference contrast (DIC) optics and infrared illumination. Fluorescent-guided whole-cell patch clamp recordings were performed with a MultiClamp 700B amplifier (Axon Instruments). Patch pipettes were 3–5 M Ω when filled with an internal solution containing (in mM) 145 KClu, 10 HEPES, 0.2 EGTA, 1 MgCl_2 , 4 Mg-ATP, 0.3 Na_2 -GTP, and 10 Na_2 -Phosphocreatine (pH 7.3 adjusted with KOH; 295 mOsm). The whole-cell patch clamp recording was initiated by rupturing the plasma membrane of the cell voltage-clamped at -60 mV. Immediately after the formation of the whole-cell configuration, the clamp mode was switched to current clamp and the current injection was set to zero, membrane potential and spontaneous firing were recorded before a negative current pulse was applied to measure the input resistance, and a series of current steps were used to detect the action potential rheobase (the minimal injected current required for the generation of an action potential). Voltage clamp recordings were performed following current clamp recordings. The junction potential was not corrected throughout the study.

Body weight, food intake, and energy expenditure

Body weight studies

Weekly body weight was monitored on all genotypes mice fed standard mouse chow (Teklad F6 Rodent Diet 8664, 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat, Harlan Teklad, Madison, WI) from 4 to 20 weeks of age. Body composition (fat mass and lean mass) was measured at indicated times by using the Echo-MRI system (EchoMRI, Houston, TX). Littermate subjects were randomly assigned to study groups with a comparable initial body weight between groups. Animal IDs were blinded when body weight data were collected.

Food intake measurements

In young age mice, all study subjects were individually housed after weaning, and daily food intake was monitored for 1 week after 3 days acclimation. Body weights of these mice were also recorded at the beginning and end of the measurement period. In adult age mice (19–20 weeks old), all study subjects were pre-acclimated for at least 1 week by single housing, and then, daily food intake was monitored for 1 week. For both age groups, daily food intakes were calculated as the mean values of the 1-week measurement. All housing cages were changed daily during the measurement period.

Energy expenditure analysis

Energy expenditure was measured by oxygen consumption by indirect calorimetry. Individual housed mice maintained on chow diet at 4–5 weeks old were placed at room temperature (22–24°C) in chambers of a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Food and water were provided *ad libitum*. Mice were acclimatized in the chambers for at least 48 h prior to data collection.

Fast-refeeding studies

Mice received injections of viral vectors were used for fasting and fast-refeeding studies at least 4 weeks after viral delivery. For the HFD condition, mice were switched to HFD and maintained on HFD for at least another 4 weeks of period before fasting-refeeding studies. For the fast group, mice were fasted for 24 h and for the fast-refeeding group, mice were allowed to eat food *ad libitum* for 2 h after 24-h fasting. All mice were handled daily for a week to reduce stress levels before transcardial perfusion.

Behavioral assays

All behavioral assays were performed before body weight became different between groups fed on chow, and the animal ID was blinded when behavior tests were conducted.

Open field test

The apparatus consists of a white Plexiglas box and is brightly illuminated (120 lx). Each mouse was placed in the corner of the apparatus to initiate a 20-min test session. A camera (Noldus, Leesburg, VA, USA) mounted above the apparatus monitors the mice, and the track of its movements was recorded, which were analyzed by Etho-Vision XT software. Time spent in the center was recorded and subsequently analyzed, longer times spent exploring the arena in the center seen as less anxiety-like behaviors.

Elevated-plus maze

Elevated-plus maze that consists of two open and two closed arms was positioned 50 cm above the floor (Kinder Scientific, Poway, CA, USA). Mice were placed on the central facing an open arm, to initiate a 10-min session test. The time spent in the open or closed arms was recorded and analyzed by Kinder Scientific Motor Monitor software.

Light/dark room test

The light/dark room test apparatus consists of two same space parts, light room and the dark room (Kinder Scientific, Poway, CA, USA). Mice were placed in the center of apparatus with an opening to the both side, to initiate a 10-min session test. The time spent in the light room was recorded and analyzed by Kinder Scientific Motor Monitor software.

Brain tissue preparation, immunohistochemistry, imaging, and post hoc analysis

After behavioral experiments were completed, study subjects were anesthetized with a ketamine/xylazine cocktail (100 and 10 mg/kg, respectively) and subjected to transcardial perfusion. During

perfusion, animals were flushed with 20 ml of saline prior to fixation with 20 ml of 10% buffered formalin. Freshly fixed brains were then extracted and placed in 10% buffered formalin in 4°C overnight for post-fixation. The next day, brains were transferred to 30% sucrose solution and allowed to rock at room temperature for 24 h prior to sectioning. Brains were frozen and sectioned into 30 µm slices with a sliding microtome and mounted onto slides for *post hoc* visualization of injection sites and cannula placements. Primary antibodies used were rabbit mAb against c-Fos from cell signaling technology (#2250, 1:1,000 dilution) and rabbit polyclonal antibody against CRH from Peninsula Laboratories International Inc. (Cat. No: T-4037, 1:1,000 dilution). Mice with missed injections to the PVH were excluded from data analysis. Representative pictures of PVH injection sites were visualized with confocal microscopy (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany).

In vivo fiber photometry experiments

Corticotropin-releasing hormone-Cre mice with specific delivery of AAV-Flex-GCaMP6m [30] to the PVH and optic fiber implantation targeting PVH neurons were used for the *in vivo* fiber photometry Ca^{2+} imaging studies. The GCaMP6m virus was provided by the Baylor Neuroconnectivity Core. The experiments were conducted at least 4 weeks after the surgery. All fiber photometry was conducted using a Doric Lenses setup, with an LED Driver controlling two connectorized LEDs (405 and 465 nm) routed through a five port Fluorescence MiniCube (Order code: FMC5_AE(405)_AF(420-450)_E1(460-490)_F1(500-550)_S) to deliver excitation light for calcium-independent and calcium-dependent signals to the implanted optic fiber simultaneously. Emitted light was received through the MiniCube and split into two bands—420–450 nm (autofluorescence—calcium-independent signal) and 500–550 nm (GCaMP6 signal—calcium-dependent signal). Each band was collected by a Newport 2151 Visible Femtowatt Photoreceiver module (photometer) with an add-on fiber-optic adaptor. Output analog signal was converted to digital signal through the fiber photometry console and recorded using the “Analog-In” function on Doric Studios (V4.1.5.2). Mice were acclimated to the behavioral chamber for at least 15 min prior to the beginning of each testing session. After baseline recording for 10 s, water spray was started by spraying one time with water toward the head of mice with a sprayer and the recording will continue for about 2 min. The signal from the independent channel was used to control for potential fiber slippage or other mechanical issues.

Data were acquired through Doric Studios V4.1.5.2. and saved as comma-separated files (header was deleted) at a sampling rate of 1 kS/s. For water spray, the baseline was calculated from 10 s prior to stimulus onset, to 20 s after stimulus onset. For Dex injections, 1.5 min prior to IP injection was used to calculate baseline, and this period was averaged and compared to a 1.5-min time segment occurring 5 min after IP injection.

Statistics

GraphPad Prism 7.00 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses and construction of graphs except as described above. Single variable comparisons were made by paired or unpaired two-tailed Student's *t*-tests, or one-way

ANOVA followed by Tukey's multiple comparison *post hoc* tests. Error bars in graphs were represented as $\pm$ SEM.

Expanded View for this article is available online.

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Author contributions

CZ conducted the research with help from JBT and ZJ, YuX, and RMC; MX, BRA, GS, Z-LC, and YoX provided essential reagents; QT, QJ, and CZ conceived and designed the studies, and wrote the manuscript with significant inputs from GS, YoX, and BRA.

Conflict of interest

The authors declare that they have no conflict of interest.

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Profound and redundant functions of arcuate neurons in obesity development

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The current obesity epidemic faces a lack of mechanistic insights. It is known that the acute activity changes of a growing number of brain neurons rapidly alter feeding behaviour; however, how these changes translate to obesity development and the fundamental mechanism underlying brain neurons in controlling body weight remain elusive. Here, we show that chronic activation of hypothalamic arcuate GABAergic (GABA⁺), agouti-related protein (AgRP) neurons or arcuate non-AgRP GABA⁺ neurons leads to obesity, which is similar to the obese phenotype observed in *ob/ob* mice. Conversely, chronic inhibition of arcuate GABA⁺, but not AgRP, neurons reduces ageing-related weight gain and corrects *ob/ob* obesity. These results demonstrate that the modulation of Arc GABA⁺ neuron activity is a fundamental mechanism of body-weight regulation, and that arcuate GABA⁺ neurons are the major mediator of leptin action, with a profound and redundant role in obesity development.

Obesity has reached epidemic levels, and continues to cause an enormous burden to human health and society. Despite extensive research, and increasing civil and governmental efforts aiming to reverse obesity trends, worldwide obesity rates are still rising. The reasons underlying such susceptibility to obesity development and correlated difficulty in weight loss remain unclear. AgRP neurons, a subset of GABAergic (GABA⁺) neurons in the arcuate nucleus (Arc), potently regulate short-term feeding behaviour, as previously shown by acute manipulations of their activity^{1–4}. However, as AgRP-neuron activity is inhibited during natural feeding^{5–8}, how changes in short-term feeding relate to obesity development is unknown. For leptin action, it has been postulated that leptin's effects on body weight are mediated by a distributed neural network in the brain^{9–12}. Specifically, AgRP-neuron-specific deletion or restoration of leptin receptor (LepR) in the *db/db* background produces little effect on body weight^{13–15}, suggesting a relatively mild role for AgRP neurons in mediating leptin action. Deletion of LepR in GABAergic, but not glutamatergic, neurons produces obesity that is comparable to that of leptin-deficient *ob/ob* mice¹⁶, demonstrating a major role for GABAergic neurons. The contrasting outcome in body weight from LepR deletion in AgRP versus GABAergic neurons suggests there is an important role for non-AgRP GABAergic neurons in mediating leptin action. However, the location of those non-AgRP GABAergic neurons remains elusive. Surprisingly, a recent study on deletion of LepR in adult AgRP neurons suggested that AgRP neurons are the major mediator of leptin action on body-weight regulation¹⁷. Thus, despite the intense research focusing on AgRP neurons and leptin action, the relative importance of AgRP neurons and non-AgRP neurons in mediating leptin action on body-weight control remains controversial.

To more precisely elucidate the function of Arc neurons in obesity development, here we genetically targeted Arc neurons for condi-

tional expression of either the bacterial sodium channel (NachBac) or the inward rectifying potassium channel Kir2.1 to achieve chronic activation or inhibition of neuron activity, respectively^{18–20}. Surprisingly, we found that chronic activation of AgRP neurons, or even random unilateral or bilateral Arc GABA⁺ neurons in a variable number, caused a similar degree of hyperphagia and comparable obesity to what is observed with leptin deficiency. Strikingly, obesity via chronic activation of Arc GABA⁺ neurons can manifest independently of AgRP neurons, and involvement of AgRP neurons is not required for either leptin action on reducing obesity or obesity development in leptin deficiency. In addition, chronic inhibition of Arc GABA⁺ neurons, but not AgRP neurons, decreased normal ageing-related body-weight gain and rescued obesity in leptin deficiency. These results, taken together with the observations that obesity induced by chronic activation of Arc GABA⁺ neurons is refractory to leptin treatment, point to Arc GABA⁺ neurons as the major mediator of leptin action on body-weight regulation. Thus, our findings demonstrate profound and redundant roles for Arc GABA⁺ neurons, including previously unappreciated non-AgRP neurons, in obesity development. They also show the necessity of inhibiting the majority of, if not all, Arc GABA⁺ neurons to achieve obesity reversal, revealing that activation and inhibition of Arc GABA⁺ neurons are fundamental mechanisms underlying obesity development and reversal, respectively.

Results

Chronic activation of Arc GABAergic neurons leads to massive obesity. The Arc is positioned to rapidly sense nutritional indicators of feeding state and body weight, and contains mainly GABA⁺ neurons. Interestingly, although AgRP neurons have received intense attention for their roles in maintaining energy and body-weight homeostasis, they represent a relatively small subset of cells that

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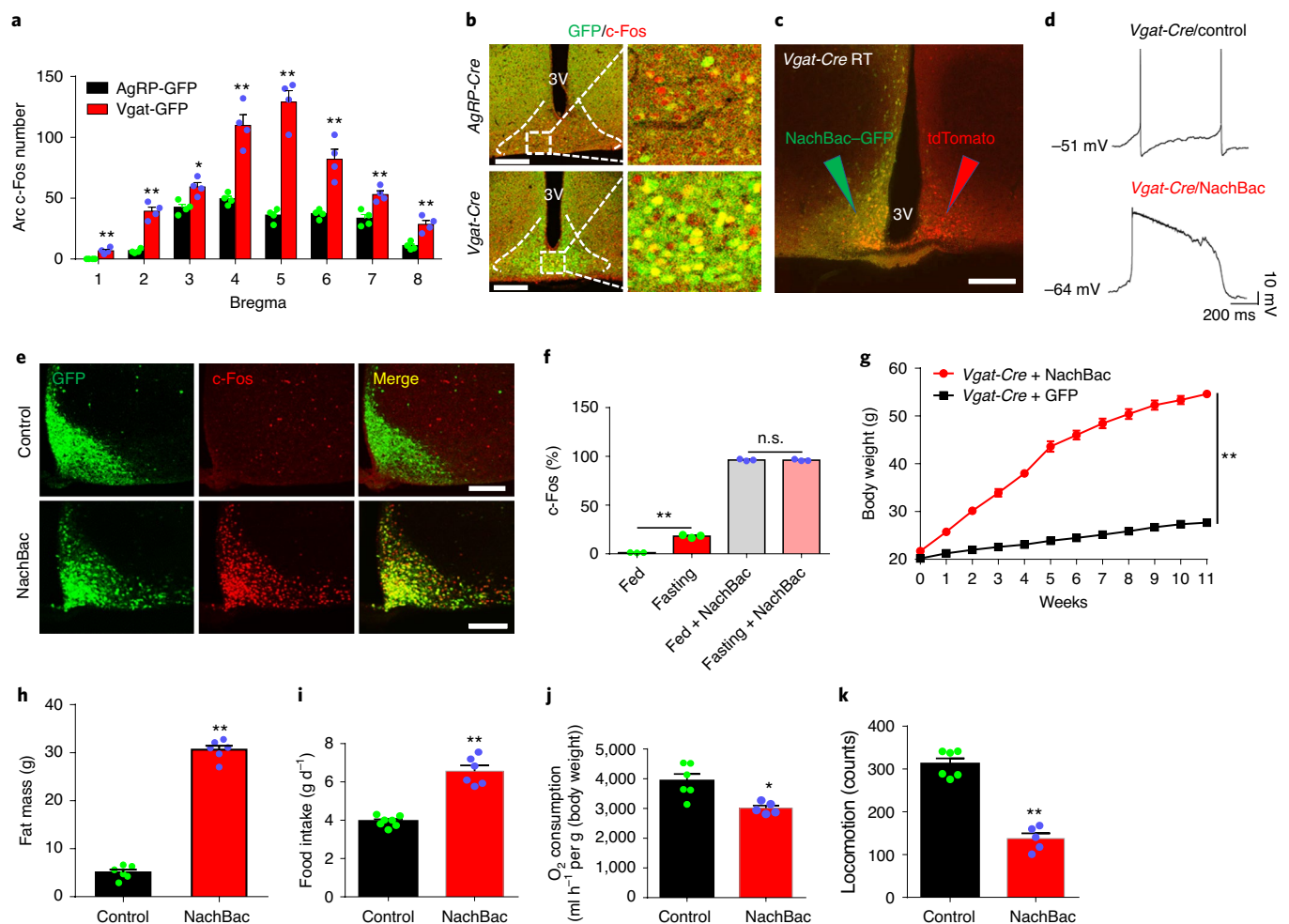


Fig. 1 | Chronic activation of Arc GABA⁺ neurons results in massive obesity. **a,b**, *Vgat-Cre* and *AgRP-Cre* GFP reporter mice were used to assess c-Fos induction in response to fasting. **a**, Quantitative comparison of c-Fos expression in GFP reporter neurons in brain sections at the indicated bregma levels. $n=4$ each. Comparisons were analysed with two-sided unpaired Student's *t*-tests. Bregma 1: -1.22 mm, $t=5$, d.f. = 6, $**P=0.0017$; bregma 2: -1.46 mm, $t=9.27$, d.f. = 6, $P<0.0001$; bregma 3: -1.7 mm, $t=3.85$, d.f. = 6, $**P=0.0084$; bregma 4: -1.82 mm, $t=3.96$, d.f. = 6, $*P=0.0245$; bregma 5: -2.06 mm, $t=6.6$, d.f. = 6, $**P=0.0006$; bregma 6: -2.3 mm, $t=5.3$, d.f. = 6, $**P=0.0018$; bregma 7: -2.46 mm, $t=4.46$, d.f. = 6, $**P=0.0043$; bregma 8: -2.7 mm, $t=4.96$, d.f. = 6, $**P=0.0026$. **b**, Representative pictures showing GFP and c-Fos colocalization at the level of bregma -1.46 mm in the two genotypes. **c-f**, NachBac expression resulted in neuron activation. **c**, A representative picture showing the expression of the NachBac virus injected into one side of the Arc of *Vgat-Cre::Ai9* reporter mice (the green arrow is pointing to the injected side with expression of both GFP and, and the red arrow is pointing to the non-injected side with red expression alone). **d**, Representative traces showing typical AP firing in neurons recorded from the control non-injected side (top) and NachBac-mediated AP in neurons recorded from the injected side (bottom). **e**, Representative pictures showing c-Fos immunostaining (red) in the Arc of ad-libitum-fed mice that received control GFP injections (top) or NachBac injections (bottom). **f**, Comparison of the percentage of c-Fos expression in Arc GABA⁺ neurons in response to fasting in both control and NachBac mice. $n=3$ mice per group. Comparisons were analysed with two-way ANOVA with Tukey's multiple-comparison test. $F(1,8)=7,566$, $**P<0.0001$ (Fed versus Fasting) and $P=0.7934$ (Fed + NachBac versus Fasting + NachBac). n.s., non-significant. **g**, Comparison of body weight between *Vgat-Cre* mice injected with a control GFP and *Vgat-Cre* mice injected with the NachBac virus into the bilateral Arc during the first 11 weeks after injection. Comparisons were analysed with two-way ANOVA. $F(1,204)=3,030$, $**P<0.0001$ (NachBac versus GFP). **h-k**, Comparison of fat mass between the 2 indicated mouse groups at week 11 after viral injection (**h**, $t=25.19$, d.f. = 10, $**P<0.0001$), and comparisons of food intake (**i**, $t=8.76$, d.f. = 11, $**P<0.0001$), O₂ consumption (**j**, $t=3.503$, d.f. = 9, $*P=0.0067$) and locomotion in beam breaks (**k**, $t=9.64$, d.f. = 9, $**P<0.0001$) during the first week following viral injection, when body-weight difference was minimal, $n=6$ mice per group. Comparisons were analysed with two-sided unpaired Student's *t*-tests. 3V, the third ventricle. Scale bars, 400 μm. All data are presented as mean ± s.e.m.

populate the Arc^{16,21}. To begin to investigate potential roles for non-AgRP Arc neurons, we first examined fasting-induced c-Fos expression, an indicator of neuronal activation, in both *AgRP-Cre* and vesicular GABA transporter (*Vgat*, also called *Slc36a1*)-*Cre* (that is, GABA⁺ neurons) green fluorescent protein (GFP) reporter mice. We noted that a substantial number of Arc GABA⁺ neurons that expressed c-Fos were *AgRP*-negative (*AgRP*⁻), as shown in serial rostral-to-caudal sections throughout the Arc (Fig. 1a,b and Extended Data Fig. 1a). To examine the physiological relevance of

the activation of GABA⁺ neurons to body-weight regulation, we aimed to activate these neurons through the targeted expression of NachBac^{18,19}, which elevates neuron activity without a need for ligand administration. To selectively target Arc GABA⁺ neurons, we delivered adeno-associated vectors (AAV-EF1a-Flex-EGFP-P 2A-mNachBac, referred to as AAV-Flex-NachBac) to the Arc of *Vgat-Cre* mice (Fig. 1c). To first validate functional modification of these neurons, we performed electrophysiological recordings and revealed the presence of modified action potentials (AP,

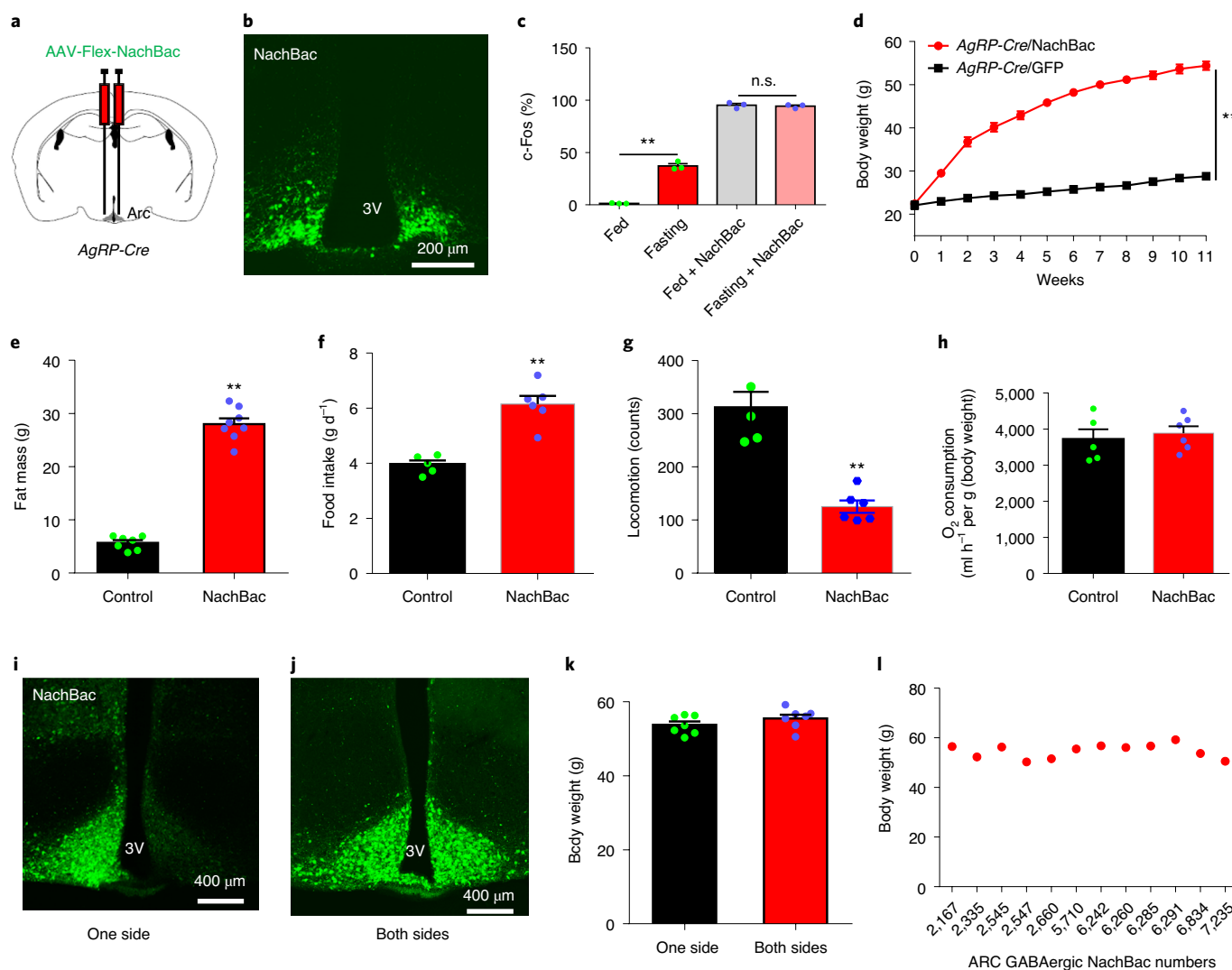


Fig. 2 | Chronic activation of AgRP neurons or random Arc GABA neurons results in massive obesity. **a**, Diagram showing NachBac viral injections to the bilateral Arc of AgRP-Cre mice. **b**, Representative expression pattern of AAV-Flex-NachBac in the Arc of AgRP-Cre mice. **c**, Comparison of the percentage of c-Fos expression in Arc AgRP neurons in response to fasting between control and NachBac mice; $n = 3$ mice per group. Comparisons were analysed with two-way ANOVA with Tukey's multiple-comparisons test. $F(3,8) = 770.2$, $^{**}P < 0.0001$ (Fed versus Fasting) and $P = 0.6422$ (Fed + NachBac versus Fasting + NachBac). **d**, Comparison of body weight between AgRP-Cre mice that received injections of control GFP or of NachBac vectors to the bilateral Arc, during the first 11 weeks after injection. The comparison was analysed with two-way ANOVA. $F(1,204) = 3,301$, $^{**}P < 0.0001$ (NachBac versus GFP). **e–h**, Comparisons of fat mass between the two mouse groups at week 11 after viral injection (**e**, $t = 18$, d.f. = 13, $^{**}P < 0.0001$), and comparisons of food intake (**f**, $t = 6.1$, d.f. = 9, $^{**}P = 0.0002$), locomotion (**g**, $t = 6.14$, d.f. = 9, $^{**}P < 0.0001$) and O₂ consumption (**h**, $t = 0.518$, d.f. = 9, $^{**}P = 0.6169$) during the first week following viral injection, when body-weight difference was minimal, $n = 6$ mice per group. Comparisons were analysed with two-sided unpaired Student's t -tests. **i–k**, Representative pictures showing NachBac expression following viral injections to one side (**i**) or both sides (**j**) of the Arc in Vgat-Cre mice, and comparison of body weight at week 11 after NachBac viral injection (**k**, $t = 1.266$, d.f. = 12, $P = 0.2296$). $n = 7$ mice (one side) or $n = 8$ mice (both sides). The comparison was analysed with two-sided unpaired Student's t -test. **l**, Plot of body weight of individual animals at week 11 after viral injection as a function of the number of Arc GABA⁺ neurons with NachBac viral expression. All data are presented as mean $\pm$ s.e.m.

Fig. 1d); reduced rheobase, AP firing threshold and input resistance; and an increased number of neurons with spontaneous firing (Extended Data Fig. 1b–j), all indicating increased neuron activity and excitability typically observed with NachBac expression^{18,19}. Interestingly, we routinely observed reduced resting membrane potential (Extended Data Fig. 1k), which may reflect a compensatory response to prevent overexcitation. However, we consistently observed that Arc neurons that expressed NachBac exhibited abundant c-Fos (Fig. 1e). GFP-injected control mice showed a dynamic increase in fasting-induced c-Fos expression in Arc GABA⁺ neurons. But mice in which NachBac was targeted exhibited no further increases, because nearly all GABA⁺ neurons expressed

c-Fos at baseline non-fasting conditions (Fig. 1f and Extended Data Fig. 2a), suggesting chronic activation of Arc GABA⁺ neurons by NachBac expression.

Anatomically, as the Arc is surrounded by brain structures with dense glutamatergic neurons, it is possible to achieve selective expression of AAV-Flex-NachBac vectors in the Arc with targeted delivery to the Arc of Vgat-Cre mice (Extended Data Fig. 2b). Notably, chronic activation of Arc GABA⁺ neurons caused rapid body-weight gain, with an average increase of nearly 30 g, within the 11 weeks following NachBac viral delivery (Fig. 1g). The observed weight gain was solely due to increased fat accumulation (Fig. 1h), with no change in lean mass (Extended Data Fig. 2c), suggesting

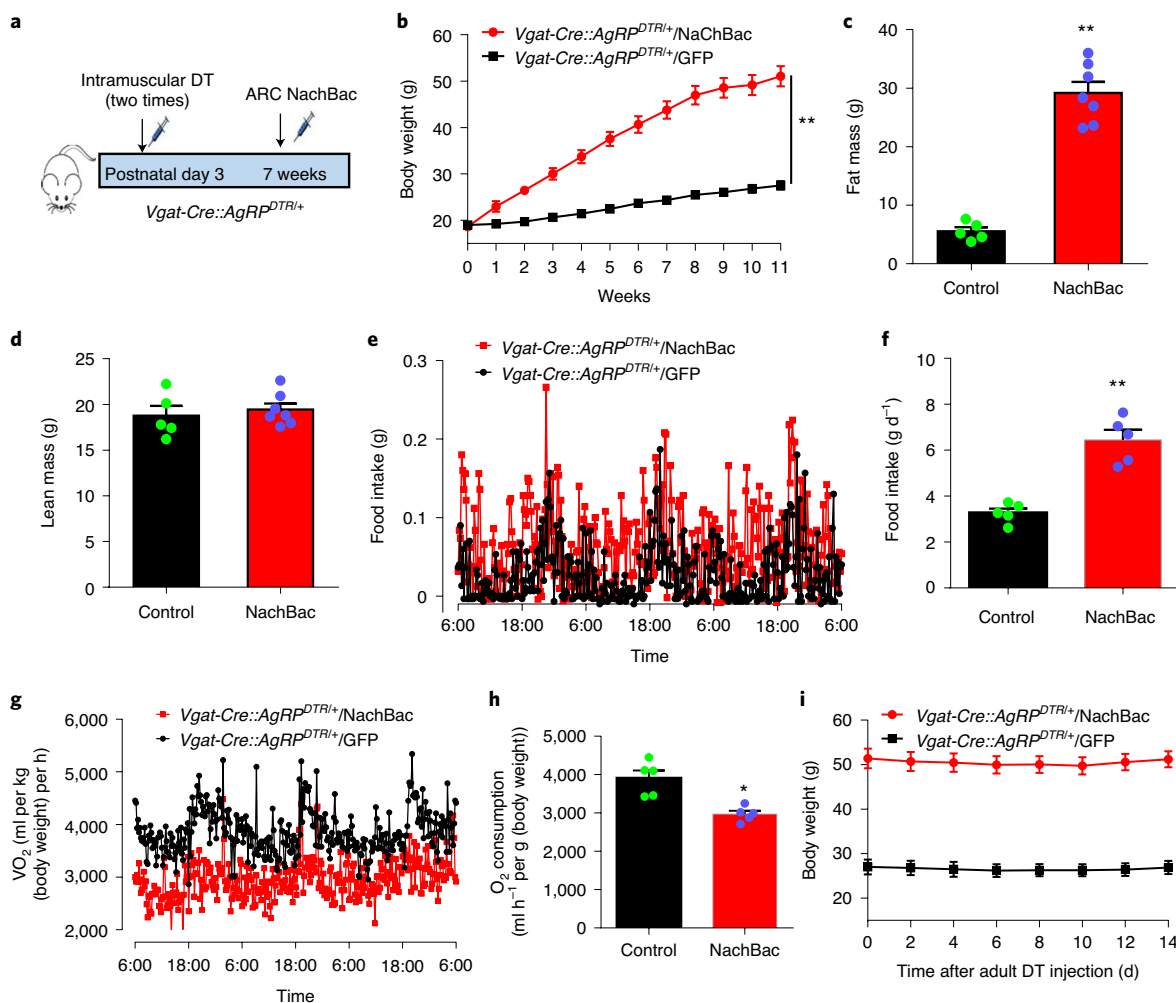


Fig. 3 | AgRP neurons are not required for obesity development by activation of Arc GABA⁺ neurons. **a**, Diagram showing injections of NachBac vectors to the bilateral Arc of *Vgat-Cre::AgRP^{DTR/+}* mice, with DTX (DT) treatment in neonates. **b–h**, Metabolic phenotypes of *Vgat-Cre::AgRP^{DTR/+}* mice that received AgRP lesion as neonates and NachBac injections to the Arc. Weekly body weight after viral injection (**b**, $F(1,156) = 3301$, $**P < 0.0001$, NachBac versus GFP), and fat (**c**, $t = 10.07$, d.f. = 10, $P < 0.0001$) and lean (**d**, $t = 0.5603$, d.f. = 10, $P = 0.5876$) mass at week 11 after viral injection. **e–h**, Food-intake pattern (**e**) and comparison of daily food intake (**f**, $t = 6.533$, d.f. = 8, $**P = 0.0002$), and O_2 -consumption pattern (**g**) and comparison of O_2 consumption (**h**, $t = 4.289$, d.f. = 8, $P = 0.0027$) measured in CLAMS during the first week after viral injection, when there was minimal body-weight difference between groups. $n = 5$ mice (control) or 7 mice (NachBac). Comparisons were analysed with two-sided unpaired Student's *t*-test in **c**, **d**, **f** and **h**, or with two-way ANOVA in **b**, **i**. Daily body weight of the *AgRP^{DTR/+}* controls and obese *Vgat-Cre::AgRP^{DTR/+}* mice presented in **b–h** after they received another dose of DT treatment at week 11 following NachBac or GFP viral delivery. All data are presented as mean $\pm$ s.e.m.

massive obesity development. Indeed, when these mice were placed in comprehensive lab-animal monitoring systems (CLAMS) to measure food intake, O_2 consumption and locomotion within the week following viral delivery and prior to any obvious differences in body weight (Extended Data Fig. 2d), GABA/NachBac mice exhibited hyperphagia (Fig. 1i and Extended Data Fig. 2e), reduced energy expenditure (Fig. 1j and Extended Data Fig. 2f) and reduced locomotion (Fig. 1k and Extended Data Fig. 2g), compared with those measures in controls. Consistent with severe obesity, GABA/NachBac mice also exhibited a dramatic increase in leptin levels (Extended Data Fig. 2h). In addition, chronic activation of Arc GABA⁺ neurons with NachBac expression in females also caused comparable obesity to that caused by leptin deficiency (Extended Data Figs. 2i–m). Thus, elevated activity of Arc GABA⁺ neurons leads to massive obesity and hyperleptinaemia.

Chronic activation of Arc AgRP neurons leads to massive obesity. Given the known importance of AgRP neurons in feeding^{2,3}, we used the same NachBac strategy to increase AgRP-neuron activity

(Fig. 2a,b). Similarly to what we observed in Arc GABA⁺ neurons, targeted NachBac expression in AgRP neurons significantly increased c-Fos expression, and no further increase was observed after fasting (Fig. 2c and Extended Data Fig. 3a). Chronic activation of AgRP neurons led to comparable obesity to that caused by activation of Arc GABA⁺ neurons (Fig. 2d) with a similar degree of fat-mass increase (Fig. 2e) and no obvious changes in lean mass (Extended Data Fig. 3b). When these mice were placed in CLAMS within 1 week of viral delivery, prior to any obvious differences in body weight (Extended Data Fig. 3c), AgRP/NachBac mice also exhibited hyperphagia (Fig. 2f and Extended Data Fig. 3d) and reduced locomotion (Fig. 2g and Extended Data Fig. 3e), but no change in energy expenditure (Fig. 2h and Extended Data Fig. 3f). Thus, activation of AgRP neurons causes a similar degree of obesity to that caused by activation of Arc GABA⁺ neurons. Interestingly, we noticed that mice with NachBac expression by GABA⁺ neurons that was exclusively in one side of the Arc exhibited a comparable level of obesity to those with such expression in both sides (Fig. 2i–k). Further analysis of mice that received various

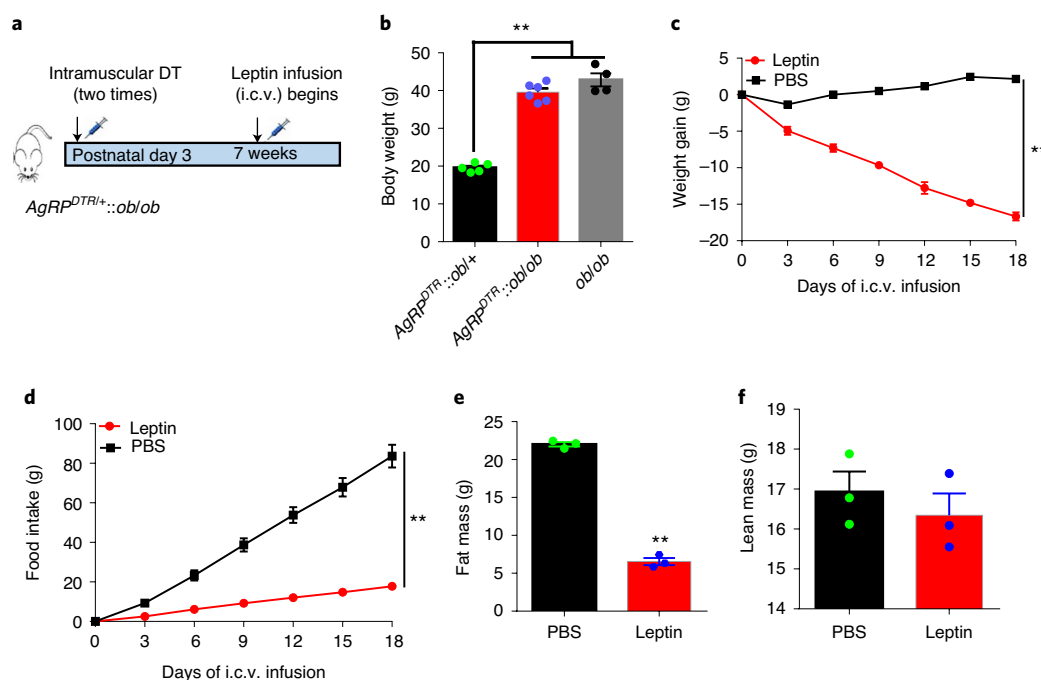


Fig. 4 | AgRP neurons are not required for *ob/ob* obesity or for leptin action on reducing body weight. **a–f**, The effects of leptin treatment on the body weight of *ob/ob* mice with neonatal AgRP-neuron lesion. **a**, Diagram showing DTX and leptin treatments in *AgRP^{DTR/+};;ob/ob* mice. **b**, Comparison of body weight between the indicated genotypes at the age of 7 weeks. Comparisons were analysed with one-way ANOVA. $F(2,12) = 131.9$, $**P < 0.0001$ (*AgRP^{DTR/+};;ob/+* versus *AgRP^{DTR/+};;ob/ob*); $**P < 0.0001$ (*AgRP^{DTR/+};;ob/+* versus *ob/ob*); $P = 0.1112$ (*AgRP^{DTR/+};;ob/ob* versus *ob/ob*). $n = 5$ (*AgRP^{DTR/+};;ob/+*), 6 (*AgRP^{DTR/+};;ob/ob*) or 4 (*ob/ob*) mice. **c,d**, Changes in daily body weight (**c**) and food intake (**d**) in *AgRP^{DTR/+};;ob/ob* mice that received either i.c.v. leptin ($n = 3$ mice) or control PBS ($n = 3$ mice). Comparisons were analysed with two-way ANOVA tests. For body weight, $F(1,21) = 1,510$, $**P < 0.0001$ (Leptin versus PBS); for food intake, $F(1,21) = 880.3$, $**P < 0.0001$ (Leptin versus PBS). **e,f**, Changes in fat mass (**e**, $t = 27.66$, d.f. = 4, $**P < 0.0001$, $n = 3$ mice per group) and lean mass (**f**, $t = 0.7783$, d.f. = 4, $P = 0.4798$, $n = 3$ mice per group) of *AgRP^{DTR/+};;ob/ob* mice at the end of day 18 of i.c.v. infusion. Comparisons were analysed with two-sided unpaired Student's *t*-tests. All data are presented as mean $\pm$ s.e.m.

viral-injection patterns in Arc GABA⁺ neurons showed that, despite a drastic difference in the number of Arc GABA⁺ neurons with NachBac expression, all mice exhibited comparable obesity (Fig. 2l). Thus, activation of AgRP neurons or randomly distributed GABA⁺ neurons located either unilaterally or bilaterally within the Arc is sufficient to drive massive obesity development.

AgRP neurons are not required for obesity development. To examine whether obesity induced by activation of Arc GABA⁺ neurons solely resulted from increased activity of AgRP neurons, we expressed NachBac in the Arc of *Vgat-Cre::AgRP^{DTR/+}* mice, in which AgRP neurons were lesioned in neonates, by using the previously established diphtheria toxin (DTX)-administration approach²² (Fig. 3a). In addition to validation of AgRP-neuron lesion (Extended Data Fig. 4a), to exclude the possibility that the toxin-induced pressure of killing AgRP neurons causes a selective suppression of AgRP expression, we used *NPY-GFP* mice and confirmed that DTX also eliminated NPY-GFP neurons (Extended Data Fig. 4b), confirming loss of AgRP neurons. Chronic activation of Arc non-AgRP GABA⁺ neurons also led to massive obesity (Fig. 3b), with a dramatic increase in fat mass (Fig. 3c) but no change in lean mass (Fig. 3d). When measured during the first week after viral delivery, when there were no obvious differences in body weight, these mice exhibited increased hyperphagic feeding (Fig. 3e,f) and reduced energy expenditure (Fig. 3g,h) and locomotion (Extended Data Fig. 4c–d). To rule out contributions from potential residual AgRP neurons as a result of unsuccessful ablation, we performed another injection of DTX at 12 weeks following viral delivery and found no changes in the obesity phenotype (Fig. 3i), confirming that AgRP neurons are not required for obesity development. Together,

these results reveal a previously unappreciated importance for Arc non-AgRP GABA⁺ neurons in body-weight regulation.

AgRP neurons are not required for leptin action on body-weight regulation. The Arc is known to mediate leptin action on body weight. While CRISPR-Cas9-mediated deletion of leptin receptor (LepR) in AgRP neurons causes comparable obesity to that due to leptin deficiency¹⁷, previous experiments with targeted deletion or restoration of LepR in AgRP neurons have revealed minor roles for AgRP neurons towards mediating leptin action on body weight^{13–15}. In fact, selective LepR deletion in GABA⁺ neurons causes comparable obesity to that due to leptin deficiency, but similar manipulations in AgRP neurons had only minor effects^{13,16}. Collectively, these data question the relative contributions of AgRP⁺ and AgRP[−] neurons within the Arc towards mediating leptin action on body-weight control. To more directly examine the role of non-AgRP neurons in obesity development of leptin-deficient *ob/ob* mice, we next generated *AgRP^{DTR/+};;ob/ob* mice with neonatal lesion of AgRP neurons (Fig. 4a). These mice developed morbid obesity that was comparable to that of *ob/ob* mice (Fig. 4b). In these mice, we used intracerebroventricular injection (i.c.v.) to infuse leptin, and confirmed its successful delivery and action via abundant phosphorylated signal transducer and activator of transcription 3 (p-STAT3) induction in the hypothalamus (Extended Data Fig. 4e), which is an established indicator of LepR-mediated signalling. Leptin potentially lowered body weight (Fig. 4c), reduced feeding (Fig. 4d) and decreased fat mass (Fig. 4e), with no change in lean mass (Fig. 4f). Thus, AgRP neurons are dispensable for both obesity development in *ob/ob* mice or leptin action on reducing obesity, suggesting an importance for AgRP[−] neurons in mediating leptin action on body-weight regulation.

Activation of Arc GABAergic neurons overrides leptin action on body-weight regulation. Given the concentrated LepR expression in the Arc and comparable obesity to leptin deficiency by chronic activation of Arc GABA⁺ neurons, we next examined whether obesity in NachBac mice can be reversed by leptin (Fig. 5a). Infusion of leptin via i.c.v. had no effect on NachBac-induced obesity (Fig. 5b), food intake (Fig. 5c), fat mass (Fig. 5d) or lean mass (Fig. 5e). However, as anticipated, leptin administration dramatically reduced body weight, feeding and fat mass in *ob/ob* mice (Fig. 5b–d). To further examine whether the resistance to leptin is mediated by blunted intracellular leptin signalling, we examined leptin-induced p-STAT3 expression. As expected, leptin-induced robust p-STAT3 expression in the Arc of *ob/ob* mice (Fig. 5f,g), and there was no difference in leptin-induced p-STAT3 expression between control and NachBac mice (Fig. 5f,g). To further correlate leptin signal with neuron activation, we performed intraperitoneal (i.p.) leptin treatment in mice with NachBac expression in Arc GABA⁺ neurons. Compared with that in controls, leptin dramatically increased the number of p-STAT3-positive neurons in the Arc of NachBac mice (Fig. 5h and Extended Data Fig. 5a,b). In contrast, we detected no difference in c-Fos-positive neurons between the groups, with the majority of Arc GABA⁺ neurons expressing c-Fos in both groups (Fig. 5i and Extended Data Figs. 5c,d). To rule out the possibility that the observed leptin resistance is caused by obesity in NachBac mice, we expressed NachBac in Arc GABA⁺ neurons in *ob/ob* mice (Extended Data Fig. 5e), which are known to exhibit increased leptin sensitivity²³, and we found that leptin was still incapable of reducing obesity (Extended Data Fig. 5f–k). We then directly examined leptin effects on electrical properties in Arc neurons, and found that the leptin's effect of reducing firing rates was blunted in neurons with NachBac expression, compared with that in controls (Fig. 5j and Extended Data Figs. 5l,m). These data suggest that NachBac expression causes a strong effect on neuron excitation that overrides the inhibitory action of leptin. Thus, mice with NachBac expression targeted in GABA⁺ neurons represent a unique obesity model with concurrent hyperleptinaemia and normal intracellular leptin signalling.

Chronic inhibition of Arc GABAergic, but not AgRP, neurons reduces body weight. We next examined whether baseline activity levels of Arc GABA⁺ neurons are required for body-weight regulation. Towards this aim, we expressed a mutated Kir2.1 channel by delivery of AAV-EF1a-DIO-Kir2.1-P2A-dTomato vectors (AAV-DIO-Kir2.1) in the Arc of *Vgat-Cre* mice (Fig. 6a), which effectively reduces neuron activity¹⁹. Whole-cell recordings from

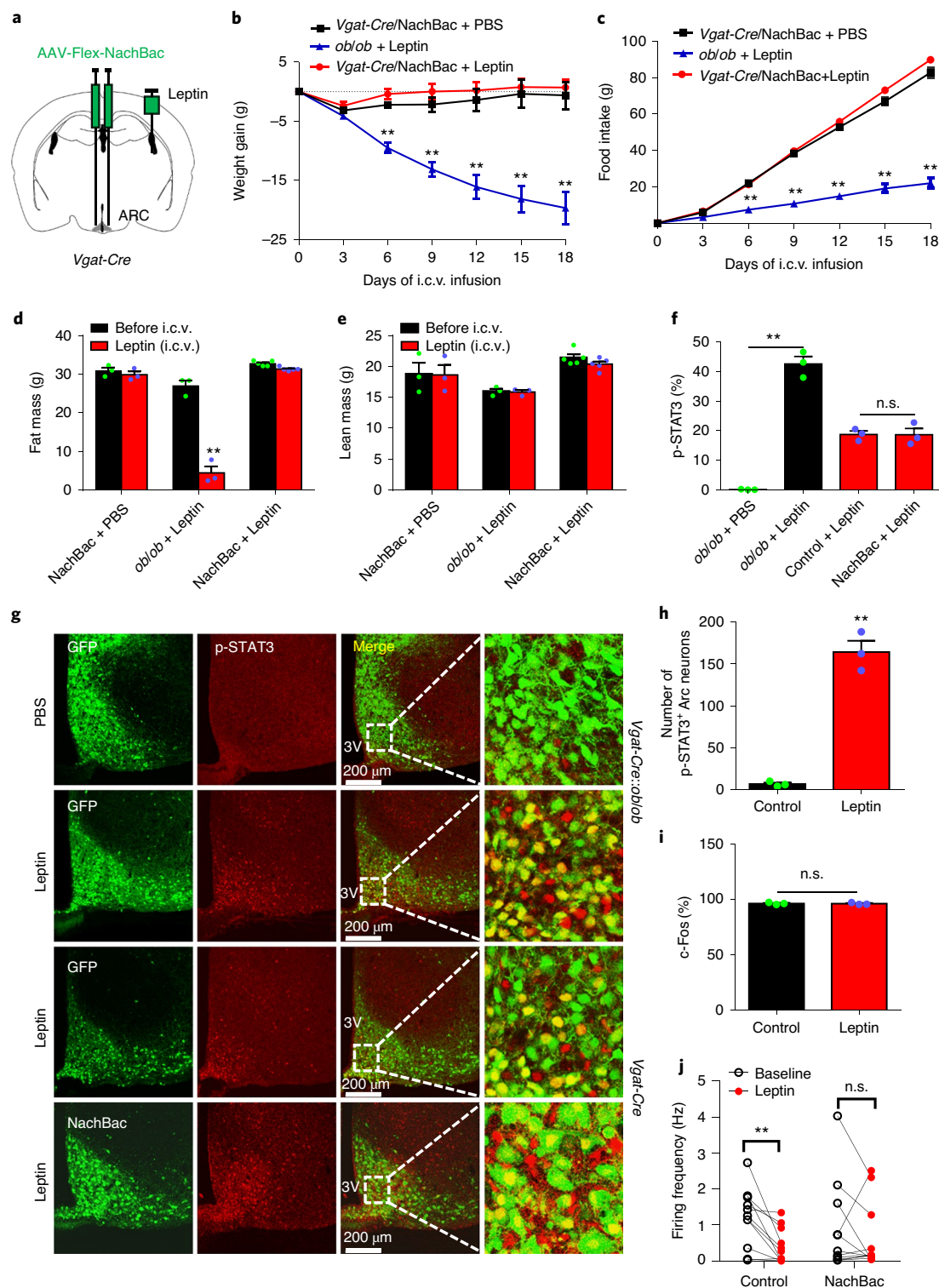
brain slices verified that neurons with Kir2.1 expression exhibited lower AP firing rates and resting membrane potential (Fig. 6b and Extended Data Fig. 6a,b), lower input resistance (Extended Data Fig. 6c) and elevated rheobase and AP firing threshold (Extended Data Figs. 6d–g), in accordance with reduced neuron activity. In response to fasting, Kir2.1 mice showed a much lower number c-Fos⁺ neurons in the Arc than did controls (Extended Data Fig. 7a,b), especially in GABA⁺ neurons (Fig. 6c). Together, these data indicate reduced levels of Arc GABA⁺ neuron activity in vivo. Interestingly, while controls showed a gradual increase in body weight, Kir2.1 mice had largely limited body-weight gain during ageing (Fig. 6d), which was associated with reduced fat mass (Fig. 6e) and slight reductions in lean mass (Fig. 6f). We further confirmed that the observed body-weight reduction was caused by a combination of reduced feeding (Fig. 6g and Extended Data Fig. 7c), elevated energy expenditure (Fig. 6h and Extended Data Fig. 7d) and increased locomotion (Fig. 6i and Extended Data Fig. 7e). To examine the potential contribution of AgRP neurons to body-weight regulation, we performed a similar manipulation in AgRP neurons using *AgRP-Cre* mice (Fig. 6j). *AgRP/Kir2.1* mice exhibited less c-Fos expression in response to fasting in Arc AgRP neurons than did controls (Fig. 6k and Extended Data Fig. 7f,g). Surprisingly, inhibition of AgRP neurons led to no changes in body weight (Fig. 6l), food intake (Extended Data Fig. 7h) or body composition (Extended Data Fig. 7i,j). Given the well-documented implication of AgRP neurons in feeding, we also used an independent approach using ablation of neurotransmission, mediated by tetanus-toxin light chain (TeLC), to confirm the role of AgRP neurons. We delivered AAV-DIO-TeLC-GFP viral vectors to the Arc of *AgRP-Cre* mice. The vector expression was confirmed in almost all AgRP neurons (Extended Data Fig. 8a), and these mice showed no difference in body weight, compared with control mice that received AAV-DIO-GFP vector injections (Extended Data Fig. 8b). Thus, normal baseline activity levels of Arc GABA⁺ neurons, but not of AgRP neurons, are required for ageing-related body-weight gain.

Chronic inhibition of Arc GABAergic, but not AgRP, neurons normalizes *ob/ob* obesity. To examine whether chronic inhibition of Arc GABA⁺ neurons can reverse obesity, we expressed Kir2.1 in the Arc of *Vgat-Cre::ob/ob* mice (Fig. 7a). Expression of Kir2.1 led to a dramatic reduction of fasting-induced c-Fos in GABA⁺ neurons in the Arc (Fig. 7b and Extended Data Fig. 9a,b), confirming chronic inhibition of these neurons in *ob/ob* mice. Strikingly, Kir2.1-mediated chronic inhibition of GABA⁺ neurons led to a near-complete reversal of obesity development in *ob/ob* mice (Fig. 7c),

Fig. 5 | Activation of GABA⁺ neurons in the Arc driven by NachBac overrides leptin action. **a**, Diagram showing i.c.v. treatment (for 3 weeks) with leptin or saline minipumps in *Vgat-Cre* or *Vgat-Cre::ob/ob* mice that were injected with NachBac vectors in the bilateral Arc. **b–e**, Body weight (**b**) and food intake (**c**) were measured once every 3 d in the indicated groups of mice, which received leptin or saline treatment, and body composition was examined at day 18 of leptin treatment for fat (**d**) and lean (**e**) mass. $n = 3$ (NachBac + PBS), 3 (*ob/ob* + Leptin) or 5 (NachBac + Leptin) mice. Comparisons were analysed by two-way ANOVA in **b** and **c**. **b**, $F(6,35) = 10.86$, $**P < 0.0001$, NachBac + PBS versus *ob/ob* + Leptin; $F(6,42) = 0.1529$, $P < 0.9874$, NachBac + PBS versus *ob/ob* + Leptin; $F(6,49) = 17.57$, $**P < 0.0001$, *ob/ob* + Leptin versus NachBac + Leptin. **c**, $F(6,35) = 112.1$, $**P < 0.0001$, NachBac + PBS versus *ob/ob* + Leptin; $F(6,42) = 6.368$, $P = 0.081$, NachBac + PBS versus *ob/ob* + Leptin; $F(6,69) = 290.2$, $**P < 0.0001$, *ob/ob* + Leptin versus NachBac + Leptin). Comparisons were analysed by two-sided unpaired Student's *t*-tests in **d** and **e**. **d**, $t = 10.40$, d.f. = 4, $**P = 0.0005$ in the *ob/ob* + Leptin group (before versus after leptin). **e**, $t = 1.491$, d.f. = 8, $P = 0.1778$ in the NachBac + Leptin group (before versus after leptin). **f**, Comparison of the percentage of p-STAT3 expression in Arc GABA⁺ neurons in control and NachBac mice that received saline or leptin infusion. $n = 3$ mice per group. Comparisons were analysed by one-way ANOVA. $F(3,8) = 99.83$, $**P < 0.0001$, *ob/ob* + PBS versus *ob/ob* + Leptin; $P = 0.9732$, Control + Leptin versus NachBac + Leptin. **g**, Representative pictures for data presented in **f**, showing GFP and p-STAT3 in *Vgat-Cre::ob/ob* mice that received saline or leptin infusion (top two rows), and showing NachBac and p-STAT3 in NachBac-injected *Vgat-Cre* mice that received leptin infusion (bottom two rows). **h–l**, In a new cohort of *Vgat-Cre* male mice with NachBac viral delivery to the Arc, we administered i.p. leptin (or saline), and the expression of p-STAT3 (**h**, $t = 11.71$, d.f. = 4, $**P = 0.0003$) and of c-Fos (**i**, $t = 0.28$, d.f. = 4, $P = 0.754$) was examined in the Arc and compared between leptin and control (PBS) groups. $n = 3$ mice per group. Comparisons were analysed by two-sided unpaired Student's *t*-tests. **j**, Comparison of leptin effects on firing frequency of control and NachBac-expressing AgRP neurons using patch-clamp recording on brain slices. $n = 11$ mice (control) or 12 mice (NachBac). Comparisons were analysed by two-sided unpaired Student's *t*-tests. $t = 3.63$, d.f. = 10, $**P = 0.0046$ in controls, and $t = 0.8136$, d.f. = 10, $P = 0.4348$ in the NachBac group. All data are presented as mean $\pm$ s.e.m.

accompanied by a dramatic reduction in fat mass (Fig. 7d) and a slight reduction in lean mass (Fig. 7e). The observed obesity reversal was due to a combination of reduced feeding (Fig. 7f and Extended Data Fig. 9c), elevated O_2 consumption (Fig. 7g and Extended Data Fig. 9d) and increased locomotor activity (Fig. 7h and Extended Data Fig. 9e). Similarly, we also bilaterally delivered Kir2.1 to the Arc of *AgRP-Cre::ob/ob* mice to evaluate potential effects on *ob/ob* obesity (Fig. 7i). As expected, Kir2.1 expression in AgRP neurons reduced fasting-induced c-Fos throughout the Arc (Extended Data Fig. 9f,g),

and largely eliminated c-Fos expression in AgRP neurons (Fig. 7j), indicative of chronic inhibition of AgRP neurons by Kir2.1 in *ob/ob* mice. Chronic inhibition of AgRP neurons had no effects on obesity development in *ob/ob* mice (Fig. 7k), food intake (Fig. 7l) or body composition (Extended Data Fig. 9h,i). To rule out the possibility that Arc AgRP neurons were partially inhibited due to insufficient viral delivery, we examined colocalization of Kir2.1-vector expression in *AgRP-Cre* reporter mice, and found that the vast majority of, if not all, AgRP neurons expressed Kir2.1 viral vectors (Extended



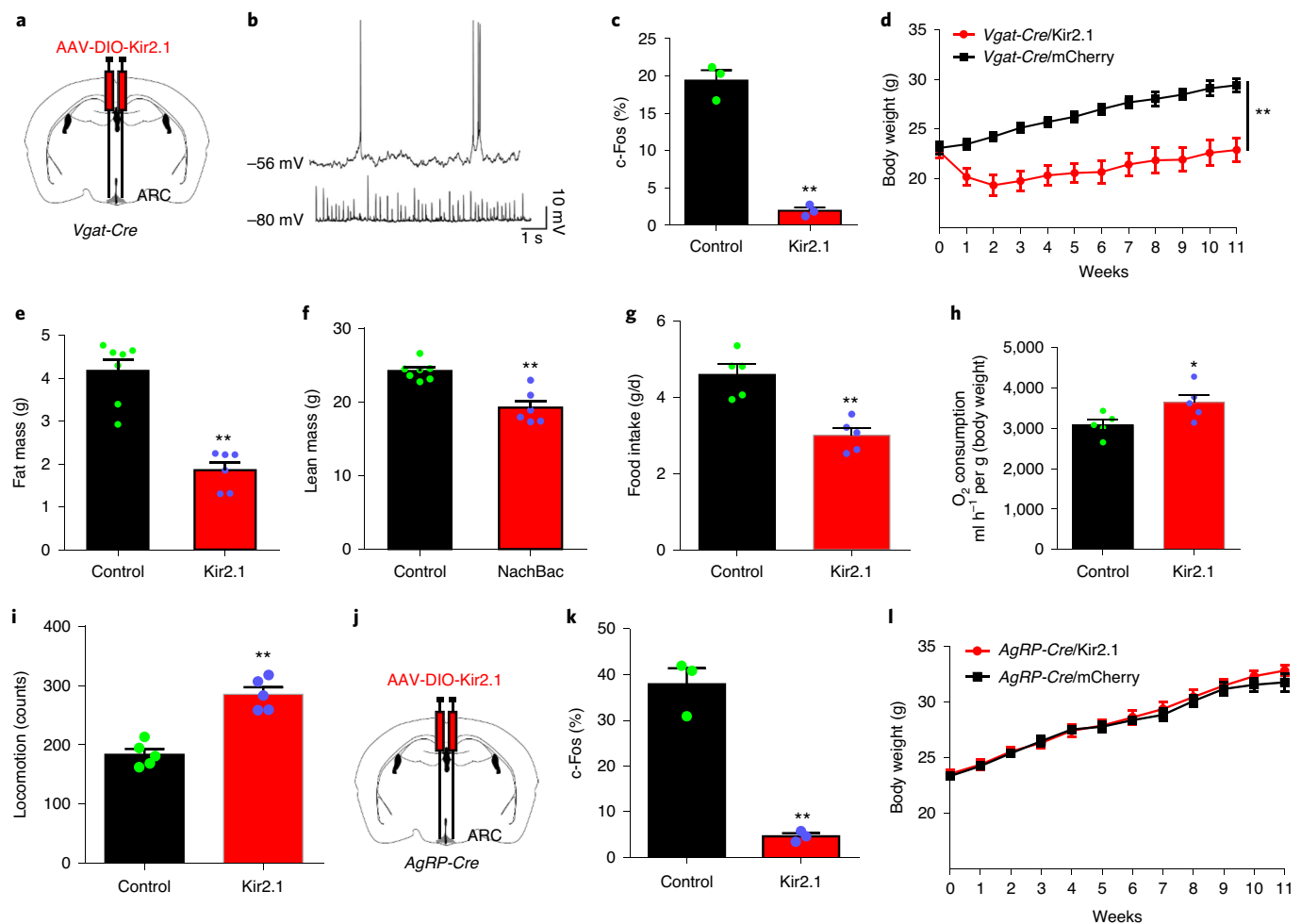


Fig. 6 | Arc GABA⁺, but not AgRP, neurons are necessary for ageing-related body-weight gain. **a**, Diagram showing the injection of Kir2.1 vectors into the bilateral Arc of 7- to 8-week-old *Vgat-Cre* mice. **b**, Representative traces showing spontaneous activity of control (top) and Kir2.1-expressing Arc GABA⁺ (bottom) neurons. **c**, Comparison of the percentage of Arc GABA⁺ neurons that express c-Fos in response to overnight fasting between control and Kir2.1 mice. **d–i**, Body weight was measured weekly (**d**, $F(1,120) = 226.8$, $**P < 0.0001$ Kir2.1 versus mCherry) and body composition in fat (**e**, $t = 6.816$, d.f. = 11, $**P < 0.0001$) and lean (**f**, $t = 5.015$, d.f. = 11, $**P = 0.0004$) mass was measured at week 11 after viral injection; food intake (**g**, $t = 4.923$, d.f. = 8, $**P = 0.0012$), O₂ consumption (**h**, $t = 2.428$, d.f. = 8, $*P = 0.0413$) and locomotion (**i**, $t = 6.649$, d.f. = 8, $**P = 0.0002$) were measured during the first week after viral injection, when the body-weight difference between groups was minimal. **j–l**, Body-weight effects of Kir2.1 expression in AgRP neurons. **j**, Diagram showing injections of Kir2.1 vectors to the bilateral Arc of 7- to 8-week-old *AgRP-Cre* mice. **k**, Comparison of the percentage of AgRP neurons expressing c-Fos in response to fasting between Kir2.1 and control mice. **l**, Comparison of weekly body weight between groups after viral injection. The comparison was analysed with two-way ANOVA. $F(1,120) = 1.926$, $P = 0.1645$ Kir2.1 versus mCherry. All data are presented as mean $\pm$ s.e.m.

Data Fig. 10a). Similarly, we also confirmed selective expression of Kir2.1 in all Arc sections harbouring GABA⁺ neurons (Extended Data Fig. 10b). Finally, we delivered Kir2.1 viral vectors to one side of the Arc and found that this failed to reduce ageing-related body-weight gain and *ob/ob* obesity (Extended Data Fig. 10c–f). Thus, chronic inhibition of most, if not all, Arc GABA⁺ neurons is required to reduce feeding and body weight under both ad libitum chow-fed or obese conditions.

Discussion

Here, we combined *Vgat-Cre* mice and targeted delivery of AAV vectors to achieve chronic changes to activity of Arc GABA⁺ neurons. Our targeting of expression to the Arc is highly specific, which in part benefited from the void of *Vgat* expression in the brain structures surrounding the Arc, preventing Cre-mediated expression

from potential viral-particle spillover beyond the Arc. This is evidenced by our data on viral-expression patterns. In some cases, we observed a small number of dorsal medial hypothalamic neurons with viral expression, which, however, caused no difference in the observed body-weight phenotype. Thus, although a role for dorsal medial hypothalamic neurons in body-weight regulation cannot be completely ruled out, the observed body-weight changes in this study are mainly contributed by Arc GABA⁺ neurons.

Our results reveal a profound effect of aberrant activation of Arc GABA⁺ neurons in driving rapid and massive obesity. As obesity with deficient leptin function is associated with increased Arc neuron activation²⁴, aberrant activation of these neurons may represent one major brain dysfunction that leads to hyperphagia and obesity. Despite normal leptin signalling, obesity through chronic activation of Arc GABA⁺ neurons is refractory to leptin treatment,

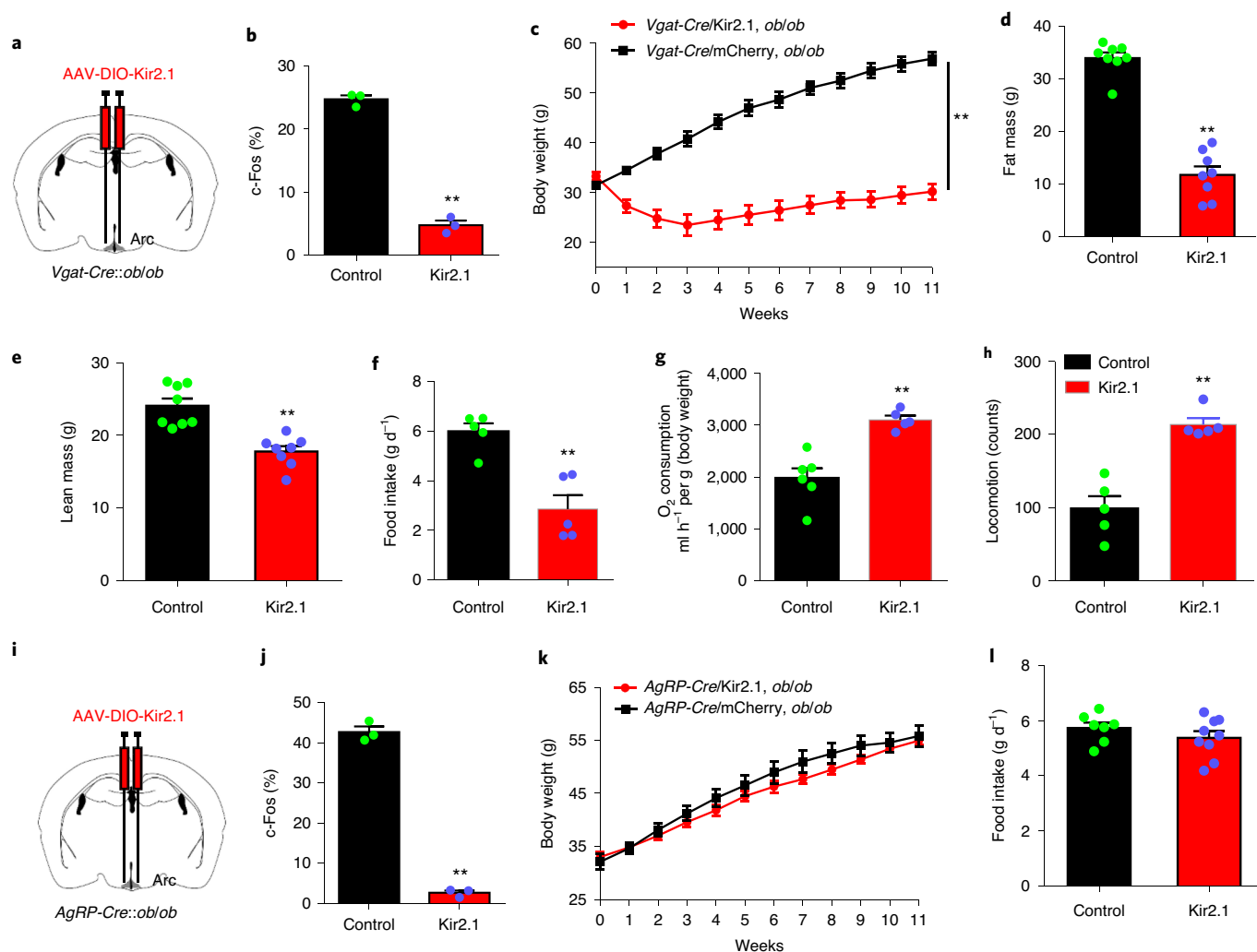


Fig. 7 | Chronic inhibition of Arc GABA⁺, but not AgRP, neurons is sufficient to normalize *ob/ob* obesity. a–i, Effects on body weight caused by Kir2.1 expression in Arc GABA⁺ neurons in obese *ob/ob* mice. **a**, Diagram showing injections of Kir2.1 vectors to the bilateral Arc of 7- to 8-week-old *Vgat-Cre::ob/ob* mice. **b**, Comparison of the percentage of Arc GABA⁺ neurons expressing c-Fos in response to overnight fasting between control and Kir2.1 mice, $n = 3$ mice per group. The comparison was analysed with two-sided unpaired Student's *t*-test. $t = 20.97$, d.f. = 4, $^{**}P < 0.0001$. **c–h**, Body weight was measured weekly (**c**, $n = 8$ mice per group, $F(11,168) = 16.28$, $^{**}P < 0.0001$, Kir2.1 versus mCherry), and body composition in fat (**d**, $n = 8$ mice per group, $t = 11.65$, d.f. = 14, $P < 0.0001$) and lean (**e**, $n = 8$ mice per group, $t = 5.09$, d.f. = 14, $^{**}P = 0.0002$) mass was measured at week 11 after viral injection; comparisons of food intake (**f**, $n = 5$ mice per group, $t = 4.785$, d.f. = 8, $^{**}P = 0.0014$), O₂ consumption (**g**, $n = 5$ mice per group, $t = 4.999$, d.f. = 9, $^{**}P = 0.0007$) and locomotion (**h**, $n = 5$ mice per group, $t = 5.949$, d.f. = 8, $^{**}P = 0.0003$) between groups, which were measured during the first week after viral injection, when the body-weight difference between groups was minimal. Comparisons were analysed with two-way ANOVA tests in **c** and two-sided unpaired Student's *t*-tests in **d–h**. **i–l**, Effects of Kir2.1 expression in AgRP neurons on body weight of obese *ob/ob* mice. **i**, Diagram showing injections of Kir2.1 vectors into the bilateral Arc of 7- to 8-week-old *AgRP-Cre::ob/ob* mice. **j**, Comparison of the percentage of AgRP neurons expressing c-Fos in response to fasting. $n = 3$ mice per group. The comparison was analysed with two-sided unpaired Student's *t*-test ($t = 26.02$, d.f. = 4, $^{**}P < 0.0001$). **k, l**, Comparison of weekly body weight (**k**, $n = 7$ Kir2.1 mice and $n = 9$ mCherry mice, $F(11,168) = 0.4810$, $P = 0.9131$, Kir2.1 versus mCherry) and food intake (**l**, $n = 7$ Kir2.1 mice and $n = 9$ mCherry mice, $t = 1.109$, d.f. = 14, $P = 0.2862$) between groups after viral injection. Comparisons were analysed with two-way ANOVA in **k** and two-sided unpaired Student's *t*-test in **l**. All data are presented as mean $\pm$ s.e.m.

representing a unique obesity model with hyperleptinaemia and intact leptin signalling. These findings echo emerging data suggesting that defective leptin signalling is not required for obesity development²⁵. However, inhibition of Arc GABA⁺ neurons, a leptin-mimicking effect, reduces ageing-associated body-weight gain. Complete rescues of *ob/ob* obesity via inhibition of Arc GABA⁺, but not AgRP, neurons and failure of leptin to reduce obesity in mice with activation of Arc GABA⁺ neurons collectively suggest that leptin action on body weight is largely mediated by GABA⁺ neurons within the Arc. Thus, the major function of leptin in body-weight regulation appears to modulate the activity of Arc GABA⁺ neurons, as opposed to the previous view that leptin

signals primarily through AgRP neurons. Supporting this, leptin action on Arc GABA⁺ neurons is inhibitory, and deletion of LepR from GABA⁺ neurons leads to a comparable level of obesity to that observed with leptin deficiency¹⁶. These findings are also in line with the observed mild effects on body weight mediated by LepR or LepR-expressing neurons in other non-Arc brain sites^{26–29}. Of note, our experiments were conducted in non-disturbed normal chow diet conditions, and it is conceivable that LepR neurons in non-Arc sites might be involved in stressful metabolic conditions, such as fasting and/or high-fat-diet feeding.

Notably, here we demonstrate that Arc GABA⁺ neurons promote feeding and obesity in a functionally redundant manner,

and aberrant activation of only a small number of these neurons is equally effective in driving a similar degree of obesity development to that of all Arc GABA⁺ neurons. One striking finding is that AgRP neurons are not required for either obesity development or the body-weight-reducing effects of leptin, revealing a previously unappreciated importance for Arc non-AgRP GABA⁺ neurons in body-weight regulation. In contrast to intense research on AgRP neurons, the function and underlying anatomy of non-AgRP GABA⁺ neurons remain poorly explored. Nevertheless, Arc neurons expressing tyrosine hydroxylase (TH) and nitric oxide synthetase 1 (NOS1) may represent non-AgRP neurons in promoting feeding and obesity^{11,30}. It is worth noting that activation of AgRP neurons caused only hyperphagia, whereas activating Arc GABA⁺ or non-AgRP GABA⁺ neurons caused both hyperphagia and reduced energy expenditure, suggesting a distinct role for AgRP GABA⁺ neurons in energy expenditure, in addition to feeding. Supporting this, previous studies on non-AgRP Arc neurons have revealed a role in energy expenditure³¹. Consistent with these redundant roles, inhibition of AgRP neurons alone was not sufficient, but rather inhibition of the whole population of GABA⁺ neurons within the Arc was required to reduce normal ageing-associated body-weight gain or *ob/ob* obesity, revealing a role for Arc non-AgRP GABA⁺ neurons in maintaining normal feeding or hyperphagia/obesity in *ob/ob* mice. Given the known efficient communication between Arc neurons and blood circulation for nutritional states, the redundant function of Arc GABA⁺ neurons in promoting feeding and body-weight gain provides an evolutionary advantage for survival.

Our results show that inhibition of AgRP neurons with no obvious effects on body weight contrasts with the impact of lesion of adult AgRP neurons, which leads to starvation and decreased survival²². The underlying reason for this remains unknown, but may involve residual activity of AgRP neurons with Kir2.1 inhibition, as shown by residual AP firing in our recording experiments, which is in contrast to complete loss of AgRP neurons by lesion. Such residual activity may more closely mimic natural physiologic inhibition of AgRP neurons, thus better reflecting AgRP-neuron function in normal physiology. Since AgRP-lesion-induced starvation can be alleviated and/or rescued non-specifically by increased fat reserves in *ob/ob* mice³², the starvation phenotype may also actually manifest as a secondary effect from acute perturbation of feeding by toxin. In addition, DTX-mediated AgRP-neuron lesion in *AgRP^{DTR/+}* mice caused a notable glial activation within the Arc³³. In contrast, little glial activation was identified with Kir2.1-mediated inhibition of AgRP neurons (Extended Data Fig. 10g). Although it remains to be examined, it is conceivable that local immune responses may potentially contribute to feeding, as demonstrated previously³⁴. Consistent with a sufficient, but not required, role for AgRP neurons in body-weight regulation, while peptide and GABA release from AgRP neurons promote potent feeding^{2,35}, deficiency of each of these factors alone or in combination produces no obvious body-weight phenotypes in mice on a chow diet^{36,37}. Despite the demonstrated role for loss of GABA in AgRP-lesion-induced starvation³⁸, adult ablation of GABA release from AgRP neurons causes no obvious body-weight phenotypes³⁹. In line with this, chronic inhibition, mediated by designer receptor exclusively activated by designer drugs, of AgRP neurons fails to reduce body weight, and re-expression of LepR in AgRP neurons also fails to rescue the *db/db* obesity^{14,40}.

In sum, our new approaches with chronic manipulation of brain neuron activity demonstrate Arc GABA⁺ neuron activation and inhibition as major mechanisms in promoting and inhibiting obesity development, respectively. Our results identify a profound and redundant role for Arc GABA⁺ neurons in promoting feeding and obesity. Reversal of aberrant activity of most, if not all, Arc neurons is required for obesity reversal in obese states. Our findings suggest that redundant functionality of Arc GABA⁺ neurons may underlie

the brain mechanism for easy susceptibility to obesity, but difficulty in weight loss. Thus, strategies to achieve efficient inhibition of Arc GABA⁺ neurons should represent effective therapeutic approaches against obesity with or without leptin resistance.

Methods

Animal care. Mice were housed at 21 °C–22 °C with a 12-h light–12-h dark cycle and with standard pellet chow and water provided ad libitum, unless otherwise noted for fasting experiments. Animal care and procedures were approved by the University of Texas Health Science Center at Houston Institutional Animal Care and Use Committee. *Vgat-Cre*, *ob/+*, *AgRP-Cre* and *NPY-GFP* mice were purchased from JAX and as previously described²². *AgRP^{DTR/+}* mice were provided by Q. Wu of Baylor College of Medicine, and were characterized previously²². Additionally, in most breeding pairs, either male or female breeders (ROSA)26Sortm9(CAG-tdTomato) (also called Ai9)⁴¹ or (ROSA)26Sortm1.1 (CAG-cas9*,-EGFP)⁴² were bred into the Cre lines to allow red fluorescent protein and GFP expression, respectively, in the presence of Cre recombination. All mice that were used for stereotaxic injections were at least 8–10 weeks old. AgRP lesion was performed by 2 doses (50 µg per kg (body weight)) of i.p. injections of DTX (Sigma) around days 3 and 5, similarly to that previously used for neonatal deletion²².

Surgeries and viral constructs. Stereotaxic surgeries to deliver viral constructs and for optical-fibre implantation were performed as previously described⁴³. Briefly, mice were anaesthetized with a ketamine/xylazine cocktail (100 mg per kg (body weight) and 10 mg per kg (body weight), respectively), and their heads were affixed to a stereotaxic apparatus. Viral vectors were delivered through a 0.5-µl syringe (Neuros Model 7000.5 KH, point style 3; Hamilton) mounted on a motorized stereotaxic injector (Quintessential Stereotaxic Injector; Stoelting) at a rate of 30 nl min⁻¹. Viral preparations were made through the Baylor NeuroConnectivity Core with either serotype 5 or DJ and titred at more than ~1 × 10¹² particles ml⁻¹. Viral delivery was targeted to the Arc through 4 local injections, with 2 each side (200 nl per side; anteroposterior (AP): –1.35 and –1.5 mm; mediolateral (ML): ±0.2 mm; dorsoventral (DV): –5.9 mm). AAV-EF1a-Flex-EGFP-P2A-mNachBac or AAV-EF1a-DIO-Kir2.1-P2A-dTomato was delivered bilaterally into the Arc. Two mutations (p.E224G and p.Y242F) were made in the Kir2.1 construct so that the channel would be more effective in reducing neuron activity. AAV-DIO-TelC-GFP, AAV-Flex-GFP and AAV-Flex-mCherry vectors were provided by B. Arenkiel at Baylor College of Medicine.

Brain-slice electrophysiological recordings. Mice were given an overdose of tribromoethanol then were perfused intracardially with ice-cold cutting solution containing (in mM): 212 sucrose, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 7 MgCl₂, 10 glucose. Whole brain was excised quickly and was immediately immersed into ice-cold cutting solution. Coronal brain slices (280 µm) containing arcuate nucleus were prepared from mice that had received stereotaxic injections of AAV-EF1a-Flex-EGFP-P2A-mNachBac, AAV-EF1a-DIO-Kir2.1-P2A-dTomato or AAV-Flex-GFP to Arc about 1 week prior to the recording were cut in oxygenized, ice-cold cutting solution. Slices were incubated for 30 min in artificial cerebrospinal fluid (aCSF) containing (in mM): 1,123 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 glucose, 1.3 MgCl₂, 2.5 CaCl₂, bubbling with 95% O₂/5% CO₂ at 32–34 °C for recovery. Individual slices were transferred to a recording chamber mounted on an upright microscope (Olympus BX51WI) and were continuously superfused (2 ml min⁻¹) with aCSF warmed to 32–34 °C by being passed through a feedback-controlled in-line heater (TC-324B; Warner Instruments). Cells were visualized through a ×40 water-immersion objective with differential interference contrast (DIC) optics and infrared illumination. Fluorescent-guided whole-cell patch-clamp recordings were performed with a MultiClamp 700B amplifier (Axon Instruments). Patch pipettes were 3–5 MΩ when filled with an internal solution containing (in mM): 142 K gluconate, 10 HEPES, 1 EGTA, 2.5 MgCl₂, 0.25 CaCl₂, 4 Mg ATP, 0.3 Na GTP, 10 Na₂ phosphocreatine (pH 7.3 adjusted with KOH, 300 mOsmol). To test leptin sensitivity, a phosphocreatine-free internal solution was used to prevent ATP-sensitive potassium blockade (in mM): 142 K gluconate, 10 HEPES, 2.5 EGTA, 2.5 MgCl₂, 0.25 CaCl₂, 2 Mg ATP, 0.3 Na GTP. The whole-cell patch-clamp recording was initiated by rupturing the plasma membrane of the cell that was voltage clamped at –60 mV. Immediately after the formation of the whole-cell configuration, the clamp mode was switched to current clamp and the current injection was set to zero, and membrane potential and spontaneous firing were recorded before a negative-current pulse was applied to measure the input resistance, and a series of current steps were used to detect the action potential rheobase (the minimal injected current required for the generation of an action potential). Voltage-clamp recordings were performed following current-clamp recordings. The junction potential was not corrected throughout the study.

Body weight, food intake and energy expenditure. *Body-weight studies.* Weekly body weight was monitored of all genotypes of mice fed 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) for 11 weeks after viral delivery. Body composition

(fat mass and lean mass) was measured at indicated times by using the Echo-MRI system (Echo MRI).

Food-intake measurements. For young mice, all study subjects were individually housed after weaning, and daily food intake was monitored for 10 d after viral delivery. Body weights of these mice were also recorded at the beginning and end of the measurement period. Daily food intakes were calculated as the mean values of the 1-week measurement. All housing cages were changed daily during the measurement period.

CLAMS analysis. Energy expenditure was measured by oxygen consumption by indirect calorimetry. Individually housed mice maintained on chow diet were used for the measurement 3–4 d after viral delivery when there would be no obvious difference in body weight between groups. Body weight was measured before and after CLAMS measurements were taken. Mice were placed at room temperature (22°C–24°C) in chambers of a CLAMS (Columbus Instruments) that had capacity of simultaneous measurement of food intake, O₂ consumption and locomotion (beam breaks). Food and water were provided ad libitum. Mice were acclimatized in the chambers for at least 48 h prior to data collection. Readings of O₂ consumption, locomotion and food intake were plotted and compared between groups.

Immunostaining and imaging. After behavioural experiments were completed, study subjects were anaesthetized with a ketamine/xylazine cocktail (100 mg per kg (body weight) and 10 mg per kg (body weight), respectively) and subjected to transcardial perfusion. During perfusion, animals were flushed with 20 ml of saline prior to fixation with 20 ml of 10% buffered formalin. Freshly fixed brains were then extracted and placed in 10% buffered formalin in 4°C overnight for postfixation. The next day, brains were transferred to 30% sucrose solution and allowed to rock at room temperature for 24 h prior to sectioning. Brains were frozen and sectioned into 30-µm slices with a sliding microtome and were mounted onto slides for post hoc visualization of injection sites and cannula placements. Brain sections were immunostained with antibodies against c-Fos (1:2,000, no. 2250S, Cell Signaling Technology), p-STAT3 (1:1,000, no. 9145S, Cell Signaling Technology) or GFAP (1:1,000, no. NB100–53809, Novusbio). Brain sections with reporter expression and/or immunostained fluoresceins were visualized with confocal microscopy (Leica TCS SP5; Leica Microsystems). For our analysis of c-Fos responses to fasting, mice were perfused with formalin when either fed ad libitum (fed) or overnight fasted (fasting). For c-Fos and p-STAT3 counting, matched sections at the same bregma levels from at least three different animals per group were used to count neurons with clear and strong signals of c-Fos immunostaining, and the counted numbers were averaged and compared between conditions and/or groups.

Leptin-treatment experiment. For i.c.v. leptin infusion, adult *ob/ob* mice and mice with NachBac expression with body weight > 40 g were implanted with minipumps of 3-week duration (DURECT Corporation), prefilled with leptin (A. Parlow, Harbor-UCLA, CA), which allows slow infusion of leptin (50 ng h⁻¹) for 3 weeks. Food consumption and body weight of mice were measured once every 3 d, and were analysed for body composition before the end of the experiment. After perfusion, brain sections were analysed for both NachBac-vector, p-STAT3 and c-Fos expression. For i.p. leptin injections, adult controls, *ob/ob* or NachBac mice fasted overnight and received a single bolus injection of 2 mg leptin per kg (body weight)⁴⁴, and mice were perfused 1 h later. Brain sections were analysed for NachBac vector, p-STAT3 and c-Fos expression.

Leptin measurement. Serum was obtained 11 weeks after NachBac delivery to the Arc of Vgat-Cre mice and was used for leptin measurements. Leptin was measured in the Mouse Metabolism and Phenotype Core at Baylor College of Medicine.

Statistical analyses. GraphPad Prism 7.00 (GraphPad Software) was used for all statistical analyses and construction of graphs for presentation of data comparison. Comparisons were made by paired or unpaired two-tailed Student's *t*-test, or one-way or two-way ANOVA followed by Tukey's multiple-comparison post hoc test. Error bars in graphs represent ± s.e.m.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

C.Z. conducted the research with help from Z.J. and Yuanzhong Xu; Z.-L.C., M.X., B.R.A., Q.W. and Yong Xu provided essential reagents; Q.J. and G.S. provided essential guidance and stipend support for C.Z.; Q.T. and C.Z. conceived and designed the experiments, and wrote the manuscript with significant inputs from M.X., Yong Xu and B.R.A.

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Sensory perception drives food avoidance through excitatory basal forebrain circuits

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Abstract Appetite is driven by nutritional state, environmental cues, mood, and reward pathways. Environmental cues strongly influence feeding behavior, as they can dramatically induce or diminish the drive to consume food despite homeostatic state. Here, we have uncovered an excitatory neuronal population in the basal forebrain that is activated by food-odor related stimuli, and potently drives hypophagia. Notably, we found that the basal forebrain directly integrates environmental sensory cues to govern feeding behavior, and that basal forebrain signaling, mediated through projections to the lateral hypothalamus, promotes selective avoidance of food and food-related stimuli. Together, these findings reveal a novel role for the excitatory basal forebrain in regulating appetite suppression through food avoidance mechanisms, highlighting a key function for this structure as a potent integrator of sensory information towards governing consummatory behaviors.

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Introduction

Sensory perception of food can induce salivation, increase appetite, and prepare the body for food intake through the release of gastric acid and insulin prior to consumption (Rogers and Hill, 1989). Similarly, the perception of rotting or spoiled food can elicit the opposite effect, including diminished appetite and overt food aversion (Li and Liberles, 2015). The integration of sensory information to either drive or diminish appetite involves a complex combination of both innate and learned behaviors (Li and Liberles, 2015; Rozin and Fallon, 1987). Food perception can thus be interpreted as a higher-cortical function, beyond the level of hypothalamic feeding centers, and necessarily requires communication between hypothalamic circuits and extra-hypothalamic regions.

Previous research has shown that hypothalamic feeding-associated neurons are rapidly modulated by sensory perception of food prior to consummatory behaviors (Betley et al., 2015; Chen et al., 2015). Additionally, nearby neurons within the lateral hypothalamus have also been shown to rapidly drive both appetitive and aversive feeding behaviors (Jennings et al., 2015; Jennings et al., 2013). How multi-sensory information is relayed to these hypothalamic nuclei to influence feeding or non-consummatory behaviors is not known.

In humans and rodents, olfaction plays a key sensory perceptive role in feeding. Anosmic humans exhibit abnormal food perception and changes in appetite (Bonfils *et al.*, 2005). Similarly, olfaction in mice has been shown to impact food intake and metabolism (Riera *et al.*, 2017). Additionally, olfactory perception of food itself has been shown to affect neuronal activity in hypothalamic feeding centers (Chen *et al.*, 2015). It remains unclear however, how olfactory perception of food is relayed to the hypothalamus to regulate feeding.

A recent non-hypothalamic structure that has been shown to influence feeding is the basal forebrain (Herman *et al.*, 2016; Zhu *et al.*, 2017). Traditionally identified as a cholinergic center, the basal forebrain has been shown to mediate aspects of wakefulness, attention, learning and plasticity, and sensory modulation (Golden *et al.*, 2016; Hangya *et al.*, 2015; Harrison *et al.*, 2016; Lin *et al.*, 2015; Pinto *et al.*, 2013; Ramanathan *et al.*, 2009; Xu *et al.*, 2015). Recent work, has also identified the cholinergic basal forebrain as a robust modulator of appetite (Herman *et al.*, 2016; Zhu *et al.*, 2017). However, these previous findings did not reveal what aspects of feeding activate basal forebrain neuronal subtypes, or the direct down-stream cellular connections that mediate feeding behaviors.

Here we identified an excitatory neuronal population within the basal forebrain (BF) marked by vGlut2+ expression (vGlut2^{BF} neurons) (Dumalska *et al.*, 2008; Hur and Zaborszky, 2005; Sotty *et al.*, 2003; Xu *et al.*, 2015), that potently influences feeding. Data have shown that vGlut2^{BF} neurons promote wakefulness, and exhibit diverse activation patterns in response to behavioral and training tasks (Harrison *et al.*, 2016; Xu *et al.*, 2015). In the current study, using a combination of Fos activity measures and in-vivo microendoscopy-imaging methods, we identified activation of vGlut2^{BF} neurons in response to feeding related events and food-odor stimuli. These data, alongside aforementioned work implicating the basal forebrain in appetite control, led us to further examine the role of vGlut2^{BF} neurons in feeding behavior.

Utilizing targeted genetic and viral manipulations of vGlut2^{BF} neurons, we discovered a novel and severe hypophagic phenotype that resulted in dramatic weight loss, starvation, and death, following genetically targeted vGlut2^{BF} neuron activation. Notably, the lethal starvation phenotype could be rescued by nutrient supplement and oral gavage, suggesting that vGlut2^{BF} circuits directly gate behavioral induced hypophagia mechanisms. Further in vivo microendoscopic imaging revealed that both odors associated with rotten food, and aversive odorant signaling through trace-amine-associated receptors in the main olfactory bulb (Dewan *et al.*, 2013; Osada *et al.*, 2018; Saraiva *et al.*, 2016) potently activated vGlut2^{BF} neurons. Cell type-specific synaptic tracing, and channelrhodopsin-assisted circuit mapping, revealed robust innervation of the lateral hypothalamic region by vGlut2^{BF} neurons. Furthermore, optogenetic activation of vGlut2^{BF} cell bodies or their hypothalamic projections was sufficient to induce hypophagia and led to food avoidance upon the olfactory perception of food. Together, these data reveal that vGlut2^{BF} neurons comprise an odor-responsive excitatory neuronal population that projects to the lateral hypothalamus and is capable of reducing feeding behavior by modulating avoidance to food and/or food-related odors.

Results

vGlut2^{BF} neurons respond to food odors

The basal forebrain (BF) comprises a heterogeneous population of neurons (Zaborszky *et al.*, 2015; Zaborszky *et al.*, 2012). In the adult mouse BF, vGlut2 +neurons define a distinct population of excitatory neurons that do not overlap with cholinergic neurons (Figure 1A). Re-feeding after an overnight fast activated BF neurons as detected by increased expression of the immediate early gene product Fos (Figure 1A–B) (Herman *et al.*, 2016). Similar to cholinergic BF neurons, we observed an increase in the percentage of Fos+ vGlut2^{BF} neurons in the fed state compared to periods of fasting (Figure 1A,C and Figure 1—figure supplement 1A–B). These data indicate that vGlut2^{BF} neurons are a distinct cellular population apart from cholinergic neurons, and are similarly activated by feeding.

To determine what aspect of feeding activates vGlut2^{BF} neurons, we performed in-vivo microendoscopy to image real-time activation patterns of vGlut2^{BF} neurons expressing the genetically encoded calcium indicator GCaMP6m. For this, vGlut2-Cre mice in which Cre recombinase was targeted to the Slc17a6 locus that encodes Vglut2(Slc17a6 Cre +/-) were stereotactically injected with

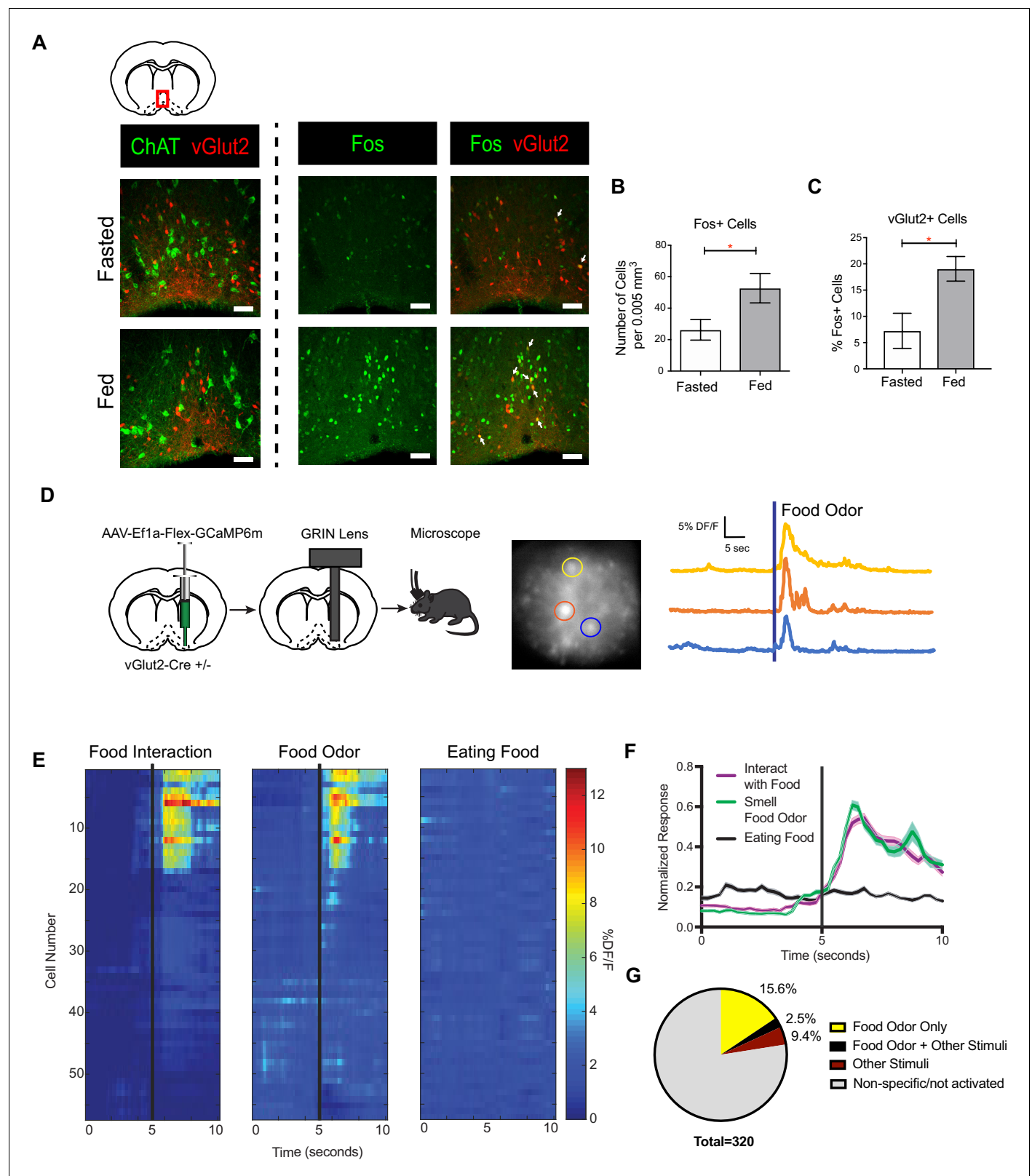


Figure 1. vGlut2^{BF} neurons are activated by feeding and food associated odors. (A) Coronal diagram showing slice location. Fos and ChAT immunohistochemistry in vGlut2-Cre^{+/−} animals injected with AAV-Flex-mRuby to label vGlut2⁺ neurons. Scale bar = 50 μ m. (B) Quantification of Fos positive cells in diagonal band of Broca (DBB) between fed and fasted animals. * $p < 0.05$, student's t-test, $n = 3$ animals per group. (C) Percentage of Fos positive DBB vGlut2⁺ cells in either fasted or fed state. * $p < 0.05$, student's t-test. $n = 3$ animals per group. scale bar = 50 μ m. (D) Schematic of viral

Figure 1 continued on next page

Figure 1 continued

injection and GRIN lens implantation, example raw image 350 μm x 350 μm , and individual neuronal traces showing changes in GCaMP6m calcium fluorescence in corresponding regions of interest when sniffing food odor. (E) Changes in calcium-mediated fluorescence of 56 neurons from one animal under multiple conditions for one trial. The same cell is in the same row across each condition. Black bars indicate stimulus onset. (F) Combined normalized calcium fluorescence activity from five mice, $n = 50$ neurons showing neuronal activation to food interaction and/or smelling food odor. ($p < 0.05$). (G) Percentage of total neurons recorded that showed activation to different stimuli. Other Stimuli include physical touch, grooming, and walking.

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The following figure supplement is available for figure 1:

Figure supplement 1. Cholinergic^{BF} neurons are activated by feeding, and vGlut2^{BF} neuron activation patterns.

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an adeno-associated virus (AAV) engineered to express Cre-dependent GCaMP6m (AAV-Flex-GCaMP6m) into the basal forebrain (Atasoy et al., 2008; Chen et al., 2013). We selectively targeted the horizontal limb of the Diagonal Band of Broca (DBB), since previous studies have shown that cell types associated with feeding are connected to the DBB (Sakurai et al., 2005; Wang et al., 2015). This was followed by implantation of a 500 μm diameter GRIN lens, which allowed recording of calcium dynamics from individual neurons (Figure 1D and Figure 1—figure supplement 1C). Following recovery, we used miniaturized fluorescence microscopy, combined with high resolution video tracking, to record vGlut2 +neuronal activity in the BF while mice freely moved throughout their environment.

We identified a population of activated vGlut2^{BF} neurons when fasted mice approached and interacted with food, prior to consummatory behavior (Figure 1E and Figure 1—figure supplement 1D). Notably, these same neurons were activated by food odor presentation alone but showed minimal responsivity while eating food (Figure 1E–F). Approximately 15.6% of DBB neurons were reactive to food odor alone, with 2.5% being responsive to not only food odors, but also other stimuli that included walking, grooming, or physical stimuli (Figure 1G and Figure 1—figure supplement 1D). 9.4% of neurons recorded responded to these other stimuli but not food, and the vast majority were unresponsive or showed non-specific activation (Figure 1G and Figure 1—figure supplement 1D). Together, these data identify a population of vGlut2^{BF} neurons that are selectively activated by food odors.

vGlut2^{BF} neurons drive hypophagic behavior

Given the responsivity of vGlut2^{BF} neurons to food odors, we next sought to selectively manipulate vGlut2^{BF} neuronal activity to determine their role in feeding behavior. Towards this, we engineered an AAV that expressed a mouse, codon-optimized version of the sodium bacterial channel, mNaChBac (AAV-Flex-eGFP-p2a-mNaChBac), which has previously been used as a genetic approach to upregulate neuronal activity by lowering firing thresholds to increase neuronal tone (Kelsch et al., 2009; Lin et al., 2010; Ren et al., 2001; Xue et al., 2014). We stereotactically injected this virus into the basal forebrains of adult vGlut2-Cre +/- mice, thereby allowing cell type-specific expression in vGlut2^{BF} neurons (Figure 2A). First, by performing whole cell recordings 7–10 days after viral injection, we measured that vGlut2^{BF} neurons that expressed mNaChBac showed increased spontaneous frequencies, action potential half-widths, and action potential voltage thresholds (Figure 2B–C and Figure 2—figure supplement 1B). Current-injection ramps showed increase area under the activation curve in mNaChBac versus control neurons, indicating neuronal activation was sustained and mNaChBac expressing neurons are capable of increasing the excitability of downstream targets (Figure 2—figure supplement 1C). We also performed extracellular single-unit recordings to measure mNaChBac expression effects in vivo. For this, vGlut2-Cre +/- animals were either injected with AAV containing Cre-dependent Channelrhodopsin-2 (ChR2) into the basal forebrain (AAV-Ef1a-Flex-hChR2::EYFP), or a 1:1 mixture of AAV containing Cre-dependent ChR2 and mNaChBac. Using photostimulation as confirmation of electrode placement and cell identification, we recorded spontaneous activity in the basal forebrain, and confirmed increased spontaneous frequency in animals containing mNaChBac (Figure 2—figure supplement 1D). Thus, cell type-specific targeting of vGlut2^{BF} neurons for expression of mNaChBac allowed for sustained and increased neuronal

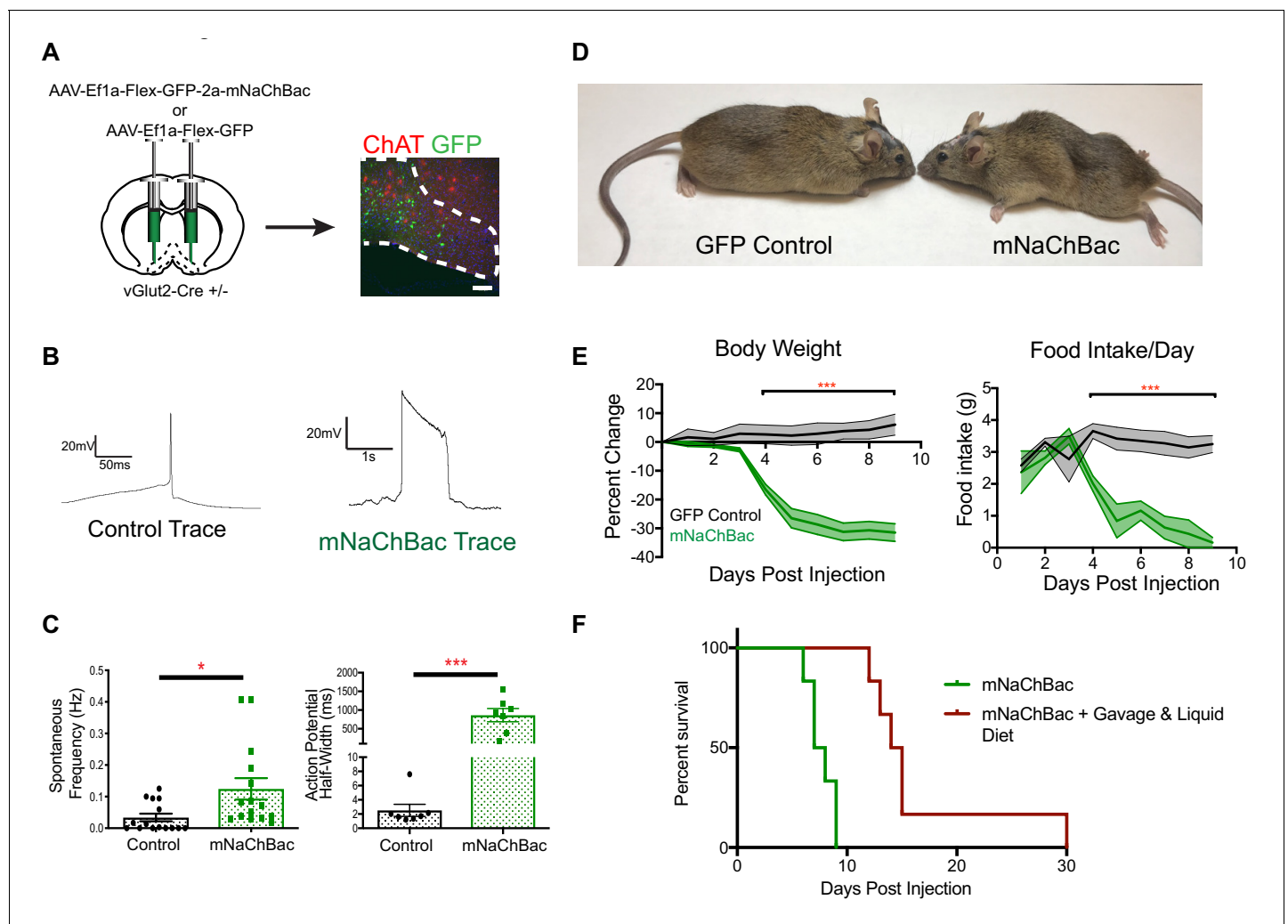


Figure 2. Over-activation of vGlut2^{BF} neurons using mNaChBac results in hypophagia, weight loss, and starvation. (A) Coronal brain section showing experimental procedure, viral labeling using eGFP-p2a-mNaChBac, and immunohistochemistry for ChAT in the basal forebrain. Scale bar = 100 μ M. (B) Example action potential traces from vGlut2^{BF} neurons 7 days post-injection of mNaChBac virus. (C) Spontaneous frequency and action potential half width between control vGlut2^{BF} and mNaChBac vGlut2^{BF} neurons 7 days post injection. * $p < 0.05$, *** $p < 0.001$. $n = 14$ neurons from six animals for frequency and $n = 7$ neurons from three animals for action potential half-width. (D) Representative vGlut2-Cre $\pm$ GFP (control) and mNaChBac injected animals 7 days post injection. (E) Percentage change in body weight from baseline (average 3 days prior to viral injection) and total daily food intake. *** $p < 0.001$, $n = 8$ animals 4 male/4 female per group. Data are mean $\pm$ SEM. (F) Survival curve showing percent animals surviving post injection. $n = 6$ animals per group. Log-Rank Mantel-Cox test $p < 0.001$. Gehan-Breslow-Wilcoxon test, $p < 0.01$. Median survival 7.5 days vs 14.5 days.

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The following figure supplements are available for figure 2:

Figure supplement 1. Electrophysiological properties of vGlut2^{BF} neurons expressing mNaChBac.

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Figure supplement 2. Endocrine hormones are not altered in vGlut2^{BF}-mNaChBac animals four days after viral injection.

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activation of this population, without the need for exogenous compound delivery (chemogenetics), or fiber-optic implantation for light (optogenetics).

Strikingly, approximately 4–5 days after viral injection, mice began to precipitously lose weight, while concurrently reducing their food intake (**Figure 2D–E**). Experimental animals rapidly lost 30% of their initial body weight as food intake ceased, eventually leading to starvation and death within 9–12 days after viral injection (**Figure 2D–F**). To determine whether the observed weight loss was initially due to either hypophagia, or metabolic dysfunction, we systematically measured a panel of

endocrine hormone levels 4 days following viral delivery, at a time point when animals just began to exhibit weight loss. We found no changes in pituitary hormones, or peripherally circulating thyroid hormones T3 and T4 (**Figure 2—figure supplement 2A–C**). Furthermore, animals did not show altered levels of glucose or insulin, indicating that hypo-insulinemia was not the cause of weight loss (**Figure 2—figure supplement 2D**). Additionally, leptin levels were also similar between experimental mice and controls, indicating that altered leptin signaling did not induce weight loss or decreased food intake (**Figure 2—figure supplement 2D**). In a separate experiment, we also measured ghrelin levels. For this, measurements were taken prior to viral injection under ad libitum and post 12 hr fasting conditions. We then injected half of the experimental mice with mNaChBac virus, while the other half served as GFP-injected controls. In control GFP animals, ghrelin levels were measured once again under ad libitum and 12 hr fast conditions, whereas experimental animals expressing mNaChBac were measured for ghrelin levels 4 days post injection. We detected elevated ghrelin levels in fasted compared to ad libitum fed animals in both pre-injection, and GFP injected animals, indicating viral injection did not alter ghrelin signaling (**Figure 2—figure supplement 2E**). Interestingly, mNaChBac injected animals exhibited elevated levels of ghrelin comparable to the fasted state, indicating ghrelin was indeed being released due to diminished food intake. However, elevated ghrelin signaling was unable to induce feeding, as mice continued to exhibit self-starvation (**Figure 2—figure supplement 2E**). Together, these data suggest that the observed rapid weight loss in mice with increased vGlut2^{BF} neuron activity was primarily due to diminished food intake, and not an altered metabolism. This was further supported by transitioning a group of mice to a liquid diet prior to viral injection, which allowed us to orally gavage a subset of experimental mice and test whether food supplementation could rescue starvation, and/or prolong life in the hypophagic mice. Indeed, we found that food supplementation via oral gavage 2x/day ameliorated the starvation phenotype (**Figure 2F**). Together, these data further substantiate that over-activity of vGlut2^{BF} neurons leads to hypophagia-induced weight loss.

To query the necessity of vGlut2^{BF} neurons in controlling food intake, we next implemented chemogenetic inhibition. Towards this, we stereotactically injected AAV engineered to express the inhibitory hM4Di DREADD receptor in a Cre-dependent manner (**Armbruster et al., 2007**) (AAV-Ef1a-Flex-hM4Di-mCherry) into the basal forebrains of adult vGlut2-Cre ± mice (**Figure 3A**). Three weeks post injection, we measured food intake of mice eating ad libitum, or following a 24 hr fast. Mice were then IP injected with either CNO or saline as a control, and again monitored for levels of food intake under normal feeding and fasted conditions. In both scenarios, mice treated with CNO displayed increased food intake compared to saline-treated controls (**Figure 3B–C**). Collectively, these data reveal a novel role for vGlut2^{BF} in regulating food intake and identified this neuronal population and their associated circuits as potent drivers of appetite suppression.

vGlut2^{BF} neuron induced hypophagia is resistant to leptin deficiency hyperphagia

We next sought to determine whether chronic activation of vGlut2^{BF} neurons could ameliorate excessive weight gain in previously described hyperphagic mouse models. For this, we implemented leptin-deficient mice (*Lep^{ob/ob}*) which show hyperphagia and become obese through increased food intake and subsequent altered metabolism (**Drel et al., 2006; Ingalls et al., 1950; Lindström, 2007**). First, we generated vGlut2-Cre^{+/−};*Lep^{ob/ob}* mice, then virally targeted vGlut2^{BF} neurons in obese animals (mean weight 51.56g ± 8.22 standard deviation) with the conditional mNaChBac AAV for cell type-specific activation of these neurons. Notably, we found that mice with overactive vGlut2^{BF} neurons via mNaChBac expression suppressed leptin deficiency-induced hyperphagia, and dramatically reduced their food intake with subsequent weight loss (**Figure 4A–B**). Together, these experiments reveal that food avoidance pathways driven by vGlut2^{BF} neurons can over-ride robust intrinsic signaling mechanisms that govern feeding.

vGlut2^{BF} neurons are activated by naturally aversive odorants

Given that food odors are often considered to be positive stimuli, it was intriguing that activation of food-responsive vGlut2^{BF} neurons resulted in severe hypophagia and starvation. To further investigate this, we performed in vivo calcium imaging as above; however, this time in awake head-fixed animals. We first injected vGlut2-Cre^{+/+} animals with AAV-Flex-GCaMP6m into the BF, and two

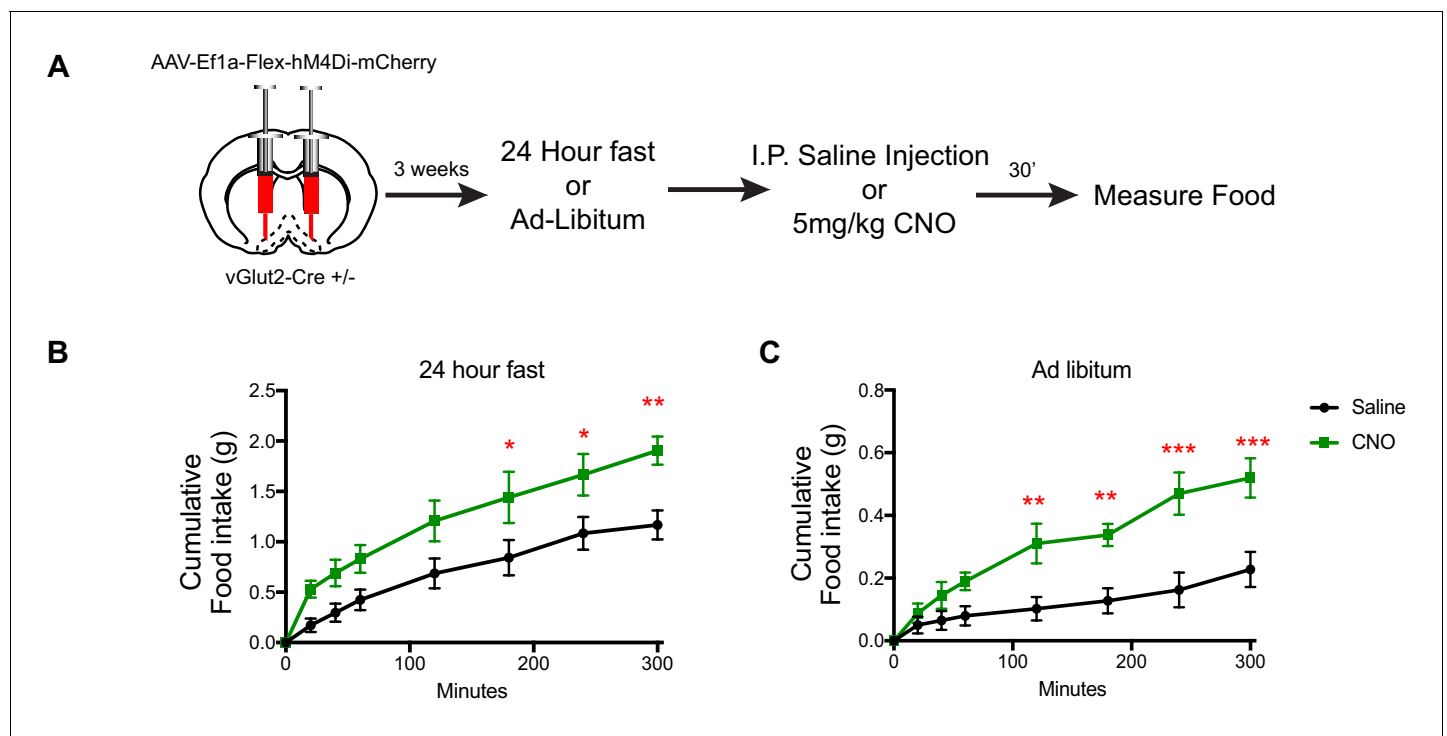


Figure 3. DREADD inhibition of vGlut2^{BF} neurons increases food-intake. (A) Schematic showing experimental paradigm. (B) Measurements of cumulative food intake in experimental and control mice following a 24 hr fast. (C) Measurements of cumulative food intake in experimental and control mice following ad-libitum over-night food access. Statistical measures include 2-way ANOVA multiple comparisons. Data are mean $\pm$ SEM. N = 5 animals per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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weeks later animals were placed on a head-fixed imaging apparatus that allowed for animals to move on a freely spinning wheel. Acutely, GRIN lenses were lowered over the BF, and cells were imaged using the same DORIC microendoscope as above. This setup allowed for precise odor delivery, and incorporation of multiple trials per animal. We presented mice with mineral oil as a control,

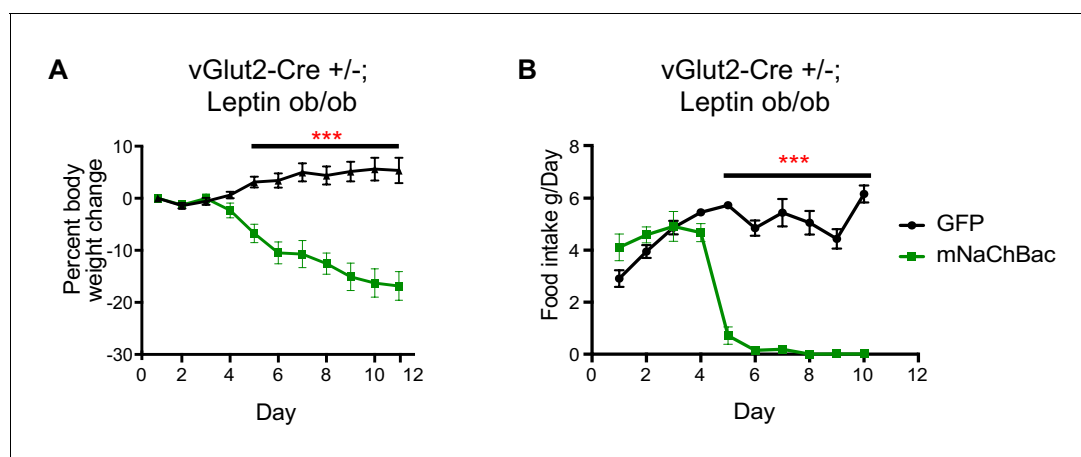


Figure 4. Chronic vGlut2^{BF} activation causes hypophagia in *Lep^{ob/ob}* mice. (A) Percent weight change from baseline in vGlut2-Cre^{+/−}; *Lep^{ob/ob}* mice injected with either AAV-Flex-GFP (control) or AAV-Flex-eGFP-p2a-mNaChBac into the basal forebrain. N = 5 animals per group (three males/2 female) (B) Food intake in the same animals from (A) N = 5 animals per group. *** $p < 0.001$, 2-way RM ANOVA with Sidak's post-hoc. Data are mean $\pm$ SEM.

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food odor (chow dissolved in mineral oil), and naturally aversive odorants, including butyric acid - which is normally associated with rotten/spoiled food, and 2-methylbutylamine - a known trace-amine associated receptor ligand (Dewan et al., 2013; Osada et al., 2018; Saraiva et al., 2016). We found that neurons were fairly unresponsive between odor presentations, and different neurons strongly responded to different groups of odors (Figure 5A). We also noted that food odors activated vGlut2^{BF} neurons similar to what we observed in freely behaving animals (Figure 1). Interestingly, these same neurons also responded strongly to the two aversive odorants (Figure 5A,B,C), and overall a greater percentage of vGlut2^{BF} neurons were responsive to the aversive odorants compared to food and/or control stimuli (Figure 5D). Together, these data indicate that vGlut2^{BF} neurons represent a heterogeneous population of neurons capable of responding to diverse odors with different valences, and that a greater number of the cells more robustly respond to aversive odorants.

vGlut2^{BF} neurons receive cholinergic input through nicotinic receptors

Cholinergic signaling from the basal forebrain has previously been implicated in appetite suppression (Herman et al., 2016). Activating cholinergic neurons in the basal forebrain causes cessation of feeding, while selectively activating their projections to the hypothalamus reduces feeding, but to a lesser extent (Herman et al., 2016). Further, nicotinic acetylcholine receptor activation in vivo is a known appetite suppressant (Mineur et al., 2011). Since vGlut2^{BF} neurons are a strong driver of appetite cessation, we hypothesized that they may represent an additional downstream target of cholinergic appetite suppression. To examine the synaptic connectivity from cholinergic neurons onto local glutamatergic projection neurons within the basal forebrain, we combined cell type-specific optogenetic activation of cholinergic neurons, with conditional viral labeling to visually target and record from vGlut2^{BF} neurons. Towards this, we generated *Chat-ChR2*^{+/−}; vGlut2-Cre ^{+/−} mice that expressed channelrhodopsin in cholinergic neurons, which allowed us to virally target vGlut2^{BF} neurons with a conditional virus engineered to express the red fluorescent protein mRuby2 (AAV-Flex-mRuby2) (Figure 6A). When performing whole-cell recordings from red labeled vGlut2^{BF} neurons while simultaneously photostimulating cholinergic inputs, we found that approximately 10% of patched vGlut2^{BF} neurons received fast, monosynaptic, nicotinic currents (six nicotinic responses from 52 recordings) (Figure 6B–C). In the presence of TTX and 4-AP, photo-evoked currents were blocked by the nAChR-specific antagonist mecamylamine, whereas the mAChR-specific antagonist atropine did not affect photo-evoked post-synaptic responses (Figure 6B). Together, these data suggest that a subset of cholinergic basal forebrain neurons are capable of acting via fast nicotinic signaling onto vGlut2^{BF} neurons, to potentially influence appetite suppression.

Basal forebrain excitatory neurons project onto vGlut2 +lateral hypothalamic neurons

To determine whether vGlut2^{BF} neurons functionally connect with known feeding centers in the brain to mediate appetite suppression, we performed targeted anterograde synaptic labeling using an AAV engineered to express Cre-dependent GFP-fused synaptophysin (AAV-Ef1a-Flex-Synaptophysin::eGFP), which localizes to presynaptic terminals. For this, we stereotactically injected AAV-Ef1a-Flex-Synaptophysin::eGFP into the basal forebrains of vGlut2 Cre ^{+/−} mice, followed by imaging vGlut2^{BF} neuron projection fields (Figure 7A). We observed dense projections to the lateral hypothalamus, with no GFP fluorescence in nearby structures such as the paraventricular nucleus or fornix (Figure 7B), as previously described (Do et al., 2016). Other prominent regions where we identified terminal fields included the piriform cortex, olfactory bulb, lateral habenula, and ventral tegmental area (Figure 7—figure supplement 1A–C). Relative intensities of these regions highlighted the lateral hypothalamus (LH) as a robustly innervated target of vGlut2^{BF} neurons (Figure 7—figure supplement 1B), and previous data have implicated the LH as a potent modulator of both feeding and non-consummatory behaviors (Jennings et al., 2015; Jennings et al., 2013; Stamatakis et al., 2016).

Based on these anatomical data, we sought to determine if functional connectivity between vGlut2^{BF} neurons and vGlut2^{LH} neurons could serve as a circuit to control appetite (Jennings et al., 2013; Stamatakis et al., 2016). To test whether vGlut2^{BF} neurons synapse onto vGlut2^{LH} neurons, we dual-injected vGlut2 Cre ^{+/−} mice with AAV containing Cre-dependent ChR2 into the basal

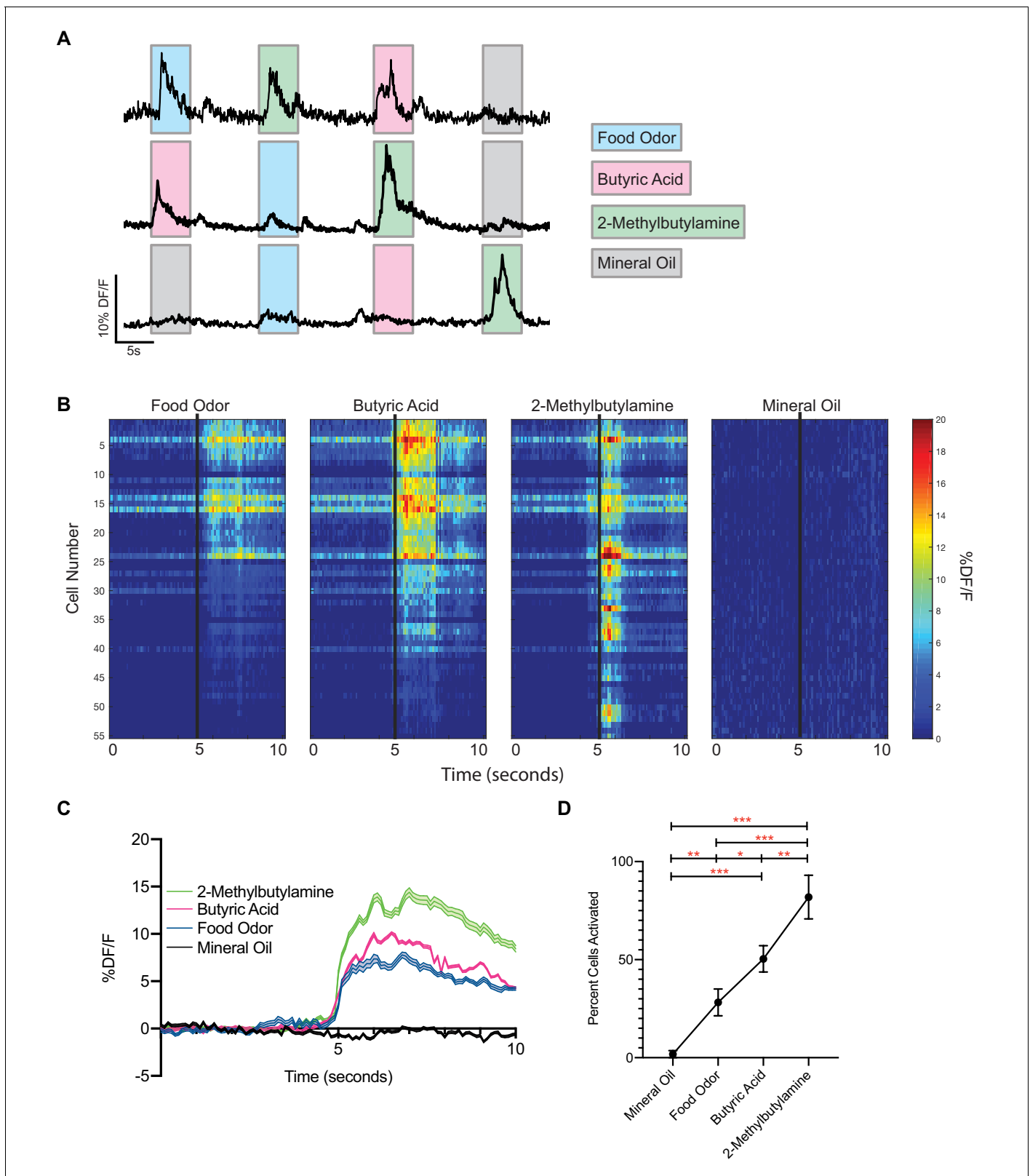


Figure 5. vGlut2^{BF} neurons are activated by aversive odorants. (A) Example calcium traces from vGlut2^{BF} neurons to different odors presented for 5 s each. (B) Representative heat map of all cells recorded from one animal showing % DF/F responsivity to different odors over a 10 s window. (C) Combined calcium fluorescence activity from $n = 5$ mice showing neuronal activation to different odors ($p < 0.05$ 2-way ANOVA with post-hoc Tukey's). Figure 5 continued on next page

Figure 5 continued

(64 mineral oil cells, 142 food odor cells, 168 butyric acid cells, 310 2-methylbutylamine cells). (D) Percent cells activated in each animal to different odors $n = 5$ mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, RM one-way ANOVA with Tukey's post-hoc analysis.

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forebrain (AAV-Ef1a-Flex-hChr2::EYFP), and a Cre-dependent red fluorescent reporter into the LH (AAV-Ef1a-Flex-mRuby2) (**Figure 7C**). Using slice electrophysiology, we made acute coronal slices that contained the lateral hypothalamus and patched onto red-labeled vGlut2^{LH} neurons. Through optogenetic mapping, and in the presence of TTX and 4-AP, we found that stimulation of vGlut2^{BF} nerve terminals in the LH resulted in monosynaptic glutamatergic neurotransmission onto vGlut2^{LH} neurons in approximately 88% of cells recorded (8 out of 9 cells recorded) (**Figure 7D–E**). These data directly link vGlut2^{BF} neurons to previously reported vGlut2^{LH} neurons as a possible upstream circuit node to control appetite suppression.

Optogenetic activation of vGlut2^{BF} neurons or their lateral hypothalamic projections decreased food intake

To further interrogate the functional connectivity between vGlut2^{BF} neurons and the lateral hypothalamus, and to determine if this circuit influences feeding behavior, we next stereotactically injected an AAV expressing Cre-dependent ChR2 AAV (AAV-Ef1a-Flex-hChR2) into the basal

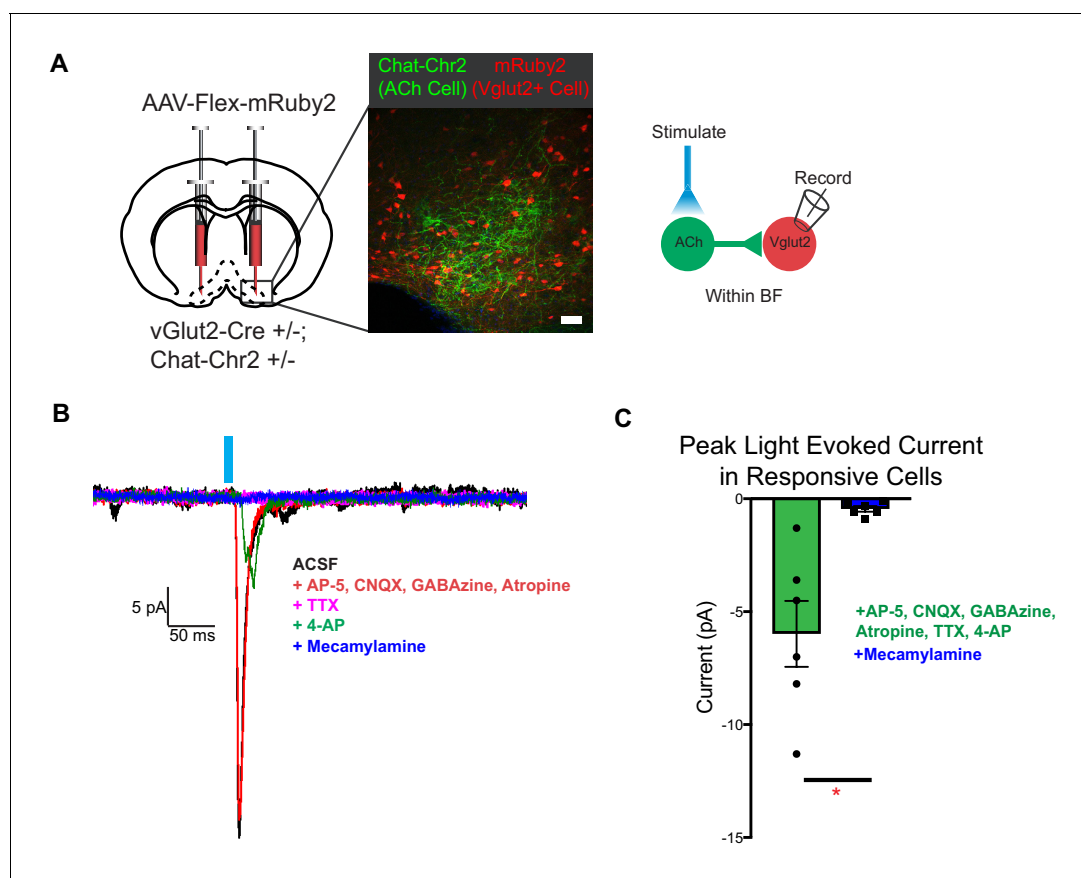


Figure 6. vGlut2^{BF} neurons receive fast mono-synaptic nicotinic current. (A) Schematic showing experimental paradigm with confocal picture of basal forebrain labeling cholinergic channelrhodopsin neurons (green) and vGlut2^{BF} neurons (red). Scale bar = 100 μ m. (B) Example trace from a vGlut2^{BF} neuron with cholinergic neuron photostimulation. (C) Average evoked monosynaptic nicotinic current onto vGlut2^{BF} neurons. $N = 6$ neurons out of 52 patched neurons from three animals. * $p < 0.05$, student's t-test.

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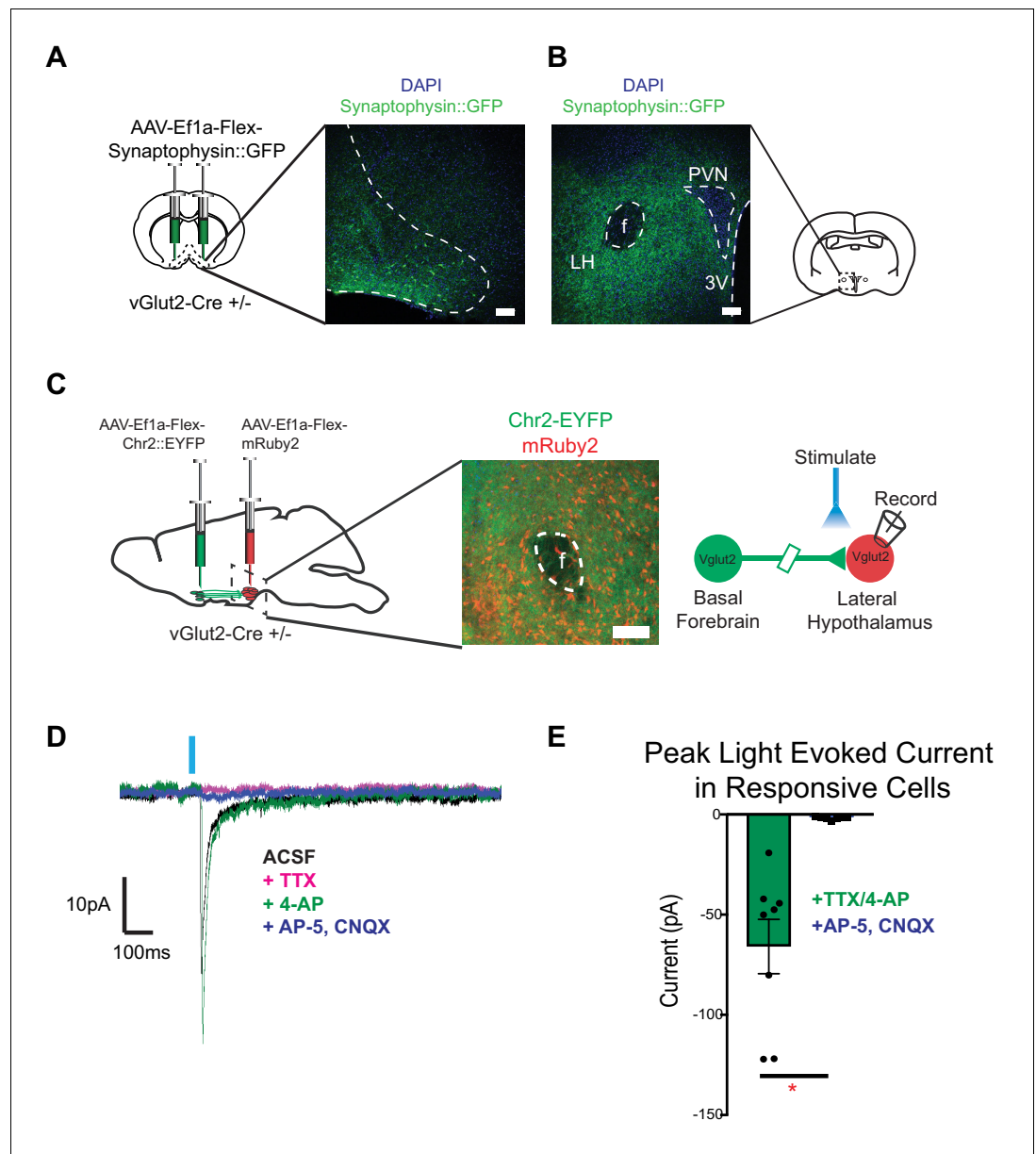


Figure 7. vGlut2^{BF} neurons project to lateral hypothalamus and make monosynaptic connections onto vGlut2^{LH} neurons. (A) Coronal diagram showing viral injection site, and immunofluorescence image showing labeled cell-bodies in the basal forebrain. Scale bar = 100 μ m (B) Coronal slice through hypothalamus showing synaptophysin::GFP nerve terminals. PVN = paraventricular nucleus, 3V = 3rd ventricle, f = fornix. LH = Lateral hypothalamic area. Scale bar = 100 μ m. (C) Schematic for dual injection electrophysiology experiment. 300 μ m thick acute slice showing green channelrhodopsin containing terminals from vGlut2^{BF} neurons and red vGlut2^{LH} cell bodies. Scale bar = 100 μ m. (D) Example traces from a vGlut2^{LH} neuron following photostimulation of vGlut2^{BF} neuron terminals. (E) Average evoked monosynaptic glutamatergic current onto vGlut2^{LH} neurons from vGlut2^{BF} neurons. N = 8 neurons out of 9 patched neurons from three animals. *p<0.05, student's t-test.

DOI: <https://doi.org/10.7554/eLife.44548.011>

The following figure supplement is available for figure 7:

Figure supplement 1. vGlut2^{BF} neurons project throughout the brain.

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forebrains of VGlut2 Cre $\pm$ mice (**Figure 8A**), followed by surgical implantation of bilateral fiber optics over both the basal forebrain and lateral hypothalamus, thereby allowing for differential stimulation of vGlut2^{BF} cell-bodies or their LH projections (**Figure 8A**). Whole-cell patch recording on vGlut2^{BF} cell-bodies demonstrated high-fidelity light-evoked action potentials at 5 Hz (**Figure 8B**) and represented a similar frequency utilized by other groups when photo-stimulating vGlut2 +neurons (*Jennings et al., 2013*). Following a 24 hr fast, implanted mice were presented with food, while concurrently photostimulated at 5 Hz either over the BF or LH. Compared to unstimulated control conditions, both BF and LH photo-stimulation resulted in approximately fifty percent reduction of food intake (**Figure 8C**). Increasing BF photo-stimulation frequencies led to a further reduction in food intake (**Figure 8D**), whereas photostimulation did not change the amount of food consumed by control GFP injected animals (**Figure 8C**). Together, results from these optogenetic

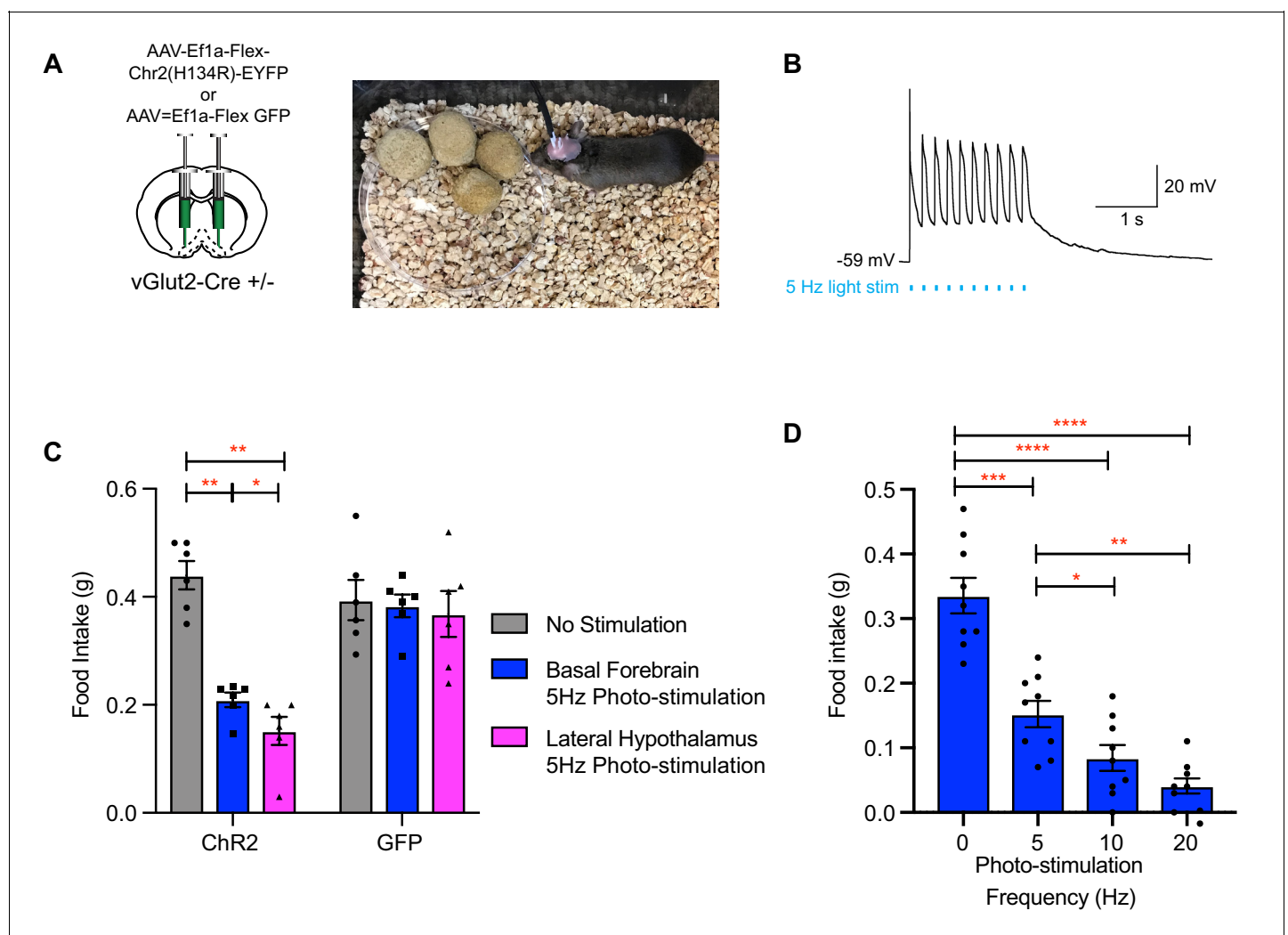


Figure 8. Photo-stimulation of vGlut2^{BF} neuron cell bodies and vGlut2^{BF} lateral hypothalamic projections decreases food-intake. (A) Schematic showing viral injection into the basal forebrain (left), and a picture of a mouse with bilateral fiberoptic implants over both the basal forebrain and lateral hypothalamus (right). (B) Whole cell recordings from a channelrhodopsin expressing vGlut2^{BF} neuron showing high fidelity activation with 5 Hz photo-stimulation. (C) Food intake of vGlut2^{BF} channelrhodopsin or GFP expressing mice during 30 min of photo-stimulation. * $p < 0.05$, ** $p < 0.01$. Two-way RM ANOVA, post-hoc Tukey's multiple comparison. $N = 6$ animals, three males/3 females. (D) Food intake of fasted vGlut2^{BF} channelrhodopsin expressing animals implanted only over the basal forebrain and subsequently photo-stimulated only over the basal forebrain at various photo-stimulation frequencies. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. RM One-way ANOVA, post-hoc Tukey's multiple comparison, $N = 9$ animals, five males/4 females.

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experiments corroborate findings from chronic activation mNaChBac, and further implicate the lateral hypothalamic projections of VGlut2^{BF} neurons in mediating appetite suppression.

Acute activation of vGlut2^{BF} neurons or their LH projections results in food avoidance

The temporal precision of optogenetics in previous experiments allowed us to monitor animal behavior during photostimulation. Interestingly, while performing these manipulations, we observed that experimental ChR2 animals tended to avoid food pellets during periods of photostimulation, while control GFP animals would spend most of their time eating during photostimulation, as would be expected following a 24 hr fasting period. This led us to question whether the dramatic hypophagic phenotype that we observed while manipulating vGlut2^{BF} neurons, may in fact be attributed to food avoidance. To directly investigate this, we placed food pellets in 3 corners of a square arena, while leaving one corner devoid of food. Whereas fasted control animals spent most of the monitored time near and/or eating food pellets, and very little time in the non-food corner, experimental animals with optogenetic stimulation of vGlut2^{BF} neurons showed a strong avoidance to areas of the arena that harbored food (**Figure 9A–B**). We photo-stimulated animals at 5 Hz since we observed a phenotype at this frequency, and it was comparable to the in vivo frequency we measured in mNaChBac animals using extra-cellular single unit recordings (**Figure 2—figure supplement 1D**). In fact, prolonged photo-stimulation of either the basal forebrain, or the terminal projection field within the lateral hypothalamus, led to a preference for the non-food corner (**Figure 9A–B**). Notably, fasted GFP injected animals showed no preference to the non-food corner and spent the majority of their time interacting with food (**Figure 9A–B**). Collectively, these data suggest that the induced hypophagia elicited by targeted activation of vGlut2^{BF} neurons or their projections to LH, may be attributed to a circuit-specific food avoidance.

Acute activation of vGlut2^{BF} neurons and their LH projections drives avoidance of food odors

Given that we identified that both food and aversive odors activated vGlut2^{BF} neurons, and that targeted manipulation of their activity elicited hypophagia and food avoidance, we next postulated that olfactory perception of food alone might also induce avoidance. To test this, we allowed mice expressing ChR2 in vGlut2^{BF} neurons with bilateral implants over basal forebrain cell bodies and lateral hypothalamic projections to form a passive association of food with the neutral odor R-limonene by spiking mouse chow for two weeks (**Figure 8A**). Following the odor association period, we then photostimulated either vGlut2^{BF} neuron cell bodies or their LH projections, while cotton swabs containing R-limonene were placed in 3 corners of the mouse arena (**Figure 10A–B**). Compared to non-stimulated conditions, optogenetically manipulated mice preferred the empty, non-odor corner. Importantly, swapping odorants to an unassociated odorant S-limonene, the enantiomer to R-limonene that mice can differentiate, did not lead to any detectable odor avoidance (**Figure 10A–B**). Together, these data indicate that photostimulation alone does not cause mice to prefer an empty corner (S-limonene experiment), and that photostimulation of vGlut2^{BF} neurons or their projections to the LH during odor perception of food (R-limonene) can lead to a potent food-odor avoidance behavior.

Discussion

Recent work has implicated discrete cell types in the basal forebrain as potent regulators of appetite and feeding. Cholinergic basal forebrain neurons have been shown to suppress appetite, while inhibitory VGAT-positive and somatostatin positive neurons promote general and high caloric food intake, respectively (**Herman et al., 2016; Zhu et al., 2017**). Here, we uncovered a previously unknown population of basal forebrain vGlut2 +excitatory neurons as extremely potent suppressors of appetite, some of which receive input from neighboring cholinergic neurons. We also found that a subset of these excitatory neurons responded to food-related odors, and an even larger portion of them responded to odors associated with aversive and/or spoiled food odorants. Therefore, it is plausible that by over-activating these neurons, we induced an overriding hypophagic state, where a typical rewarding food pellet became unappetizing. What remains unclear, is whether the valence assigned to consummatory odors versus aversive odors occurs at the level of the basal forebrain.

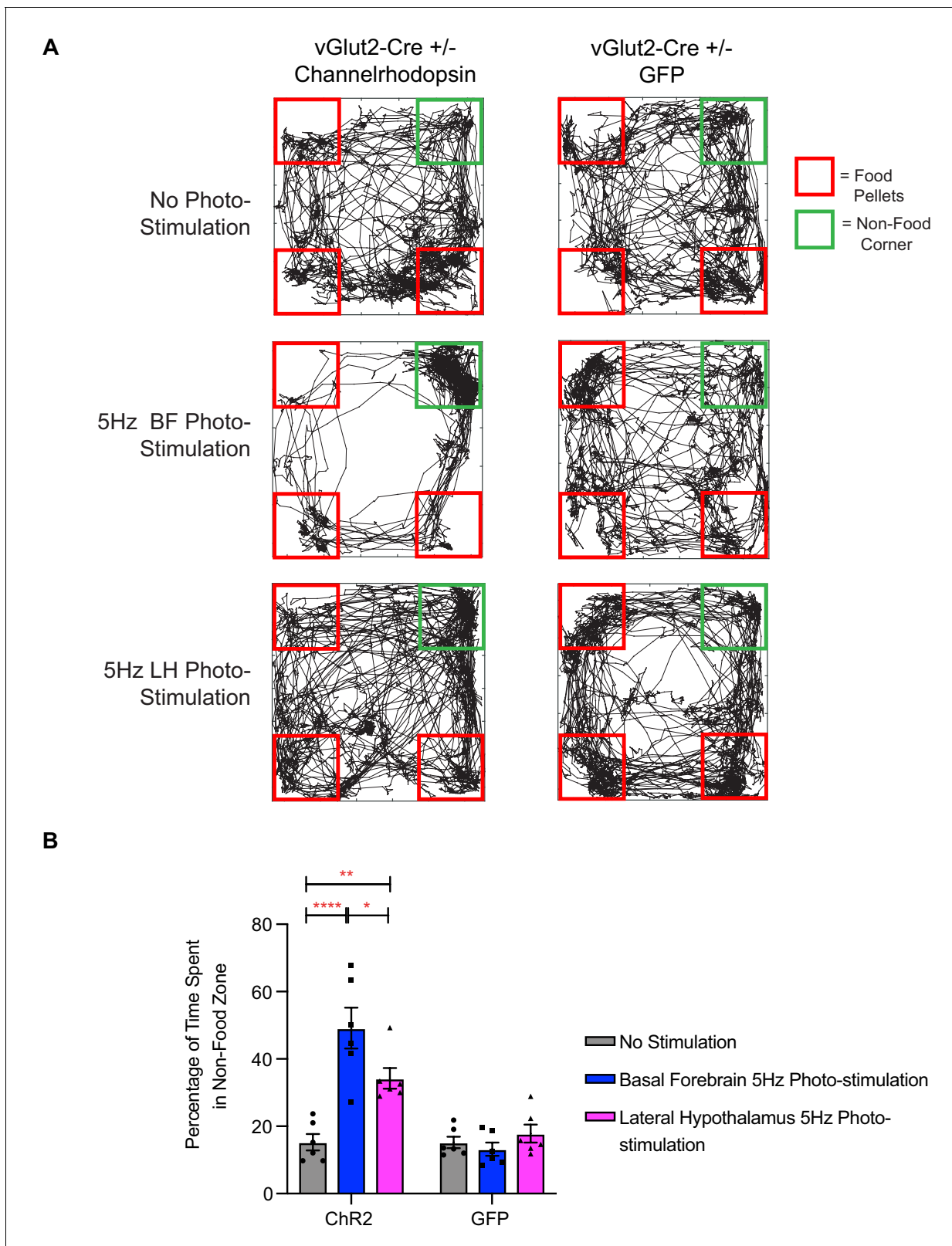


Figure 9. Photo-stimulation of vGlut2^{BF} neuron cell bodies and vGlut2^{BF} lateral hypothalamic projections induces food-avoidance behavior. (A) Example locomotor traces of the same channelrhodopsin and control GFP injected vGlut2-Cre animals under three different conditions for 30 min. (B) Quantification of percentage time spent by channelrhodopsin-expressing (left) or control mice (right) in non-food corner during 30 min of photo-stimulation. Figure 9 continued on next page

Figure 9 continued

stimulation. * $p < 0.05$. ** $p < 0.01$, **** $p < 0.0001$. Two-way RM ANOVA, post-hoc Tukey's multiple comparison test, $n = 6$ animals. three males/3 females. Data are mean $\pm$ SEM.

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Notably, given that activating vGlut2^{BF} neurons results in hypophagia, it is unlikely that valence assignment occurs downstream of the circuit. Further investigation into these sensory response features will be required to more fully understand vGlut2^{BF} neuron inputs from higher cortical regions, as well as diverse outputs to given stimulation paradigms. These data further add to the growing evidence of the importance of the basal forebrain as an integrator of multiple sensory stimuli. Additionally, non-cholinergic cells in the basal forebrain have been found to predict behavioral responses associated with attention, and their activity can be modulated by multiple sensory stimuli in a time scale of sub-seconds to seconds (Hangya et al., 2015; Harrison et al., 2016). Our imaging data support these claims as vGlut2^{BF} neurons were responsive to cues harboring both positive or negative valences, which would be important responses in an attentive state. Together, these findings reveal the robust interconnected dynamics of basal forebrain signaling in governing food intake, appetite, and downstream metabolic change.

Nicotine is a potent appetite suppressor (Stojakovic et al., 2017). Recent work has identified key roles for nicotinic signaling in suppressing appetite through POMC neuron activation within the arcuate nucleus of the hypothalamus (Mineur et al., 2011; Picciotto et al., 2012). Our electrophysiology results indicate that vGlut2^{BF} neurons functionally express acetylcholine nicotinic receptors, and thus

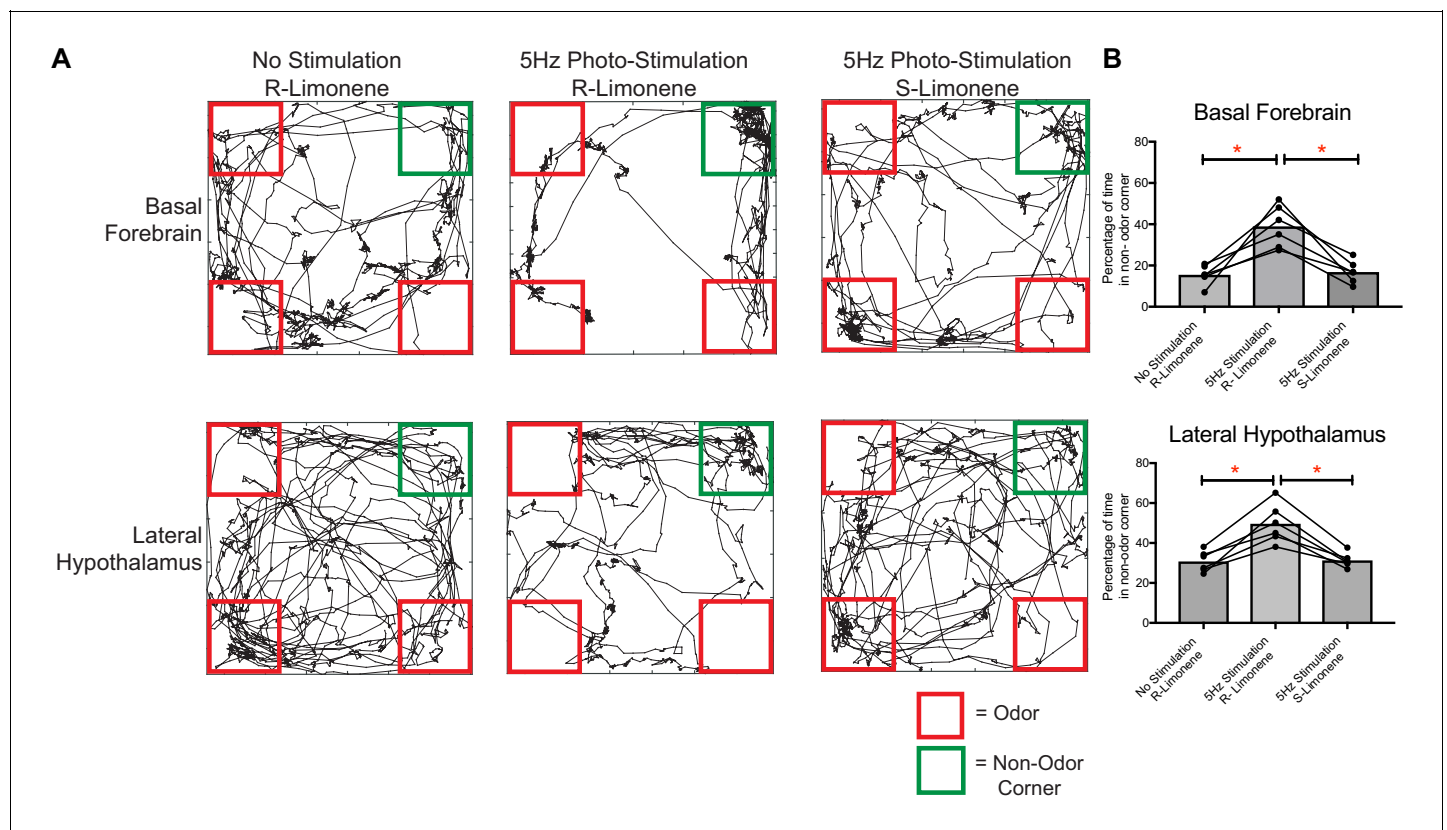


Figure 10. Food-associated odor induces avoidance behavior in vGlut2^{BF} neuron and vGlut2^{BF} LH projection photostimulated animals. (A) Locomotor trace of the same channelrhodopsin mouse photo-stimulated either over the basal forebrain or lateral hypothalamus under three different conditions. R-Limonene is passively associated with food, whereas S-Limonene represents the non-associated control. (B) Quantification of percentage of time spent in non-odor corner over 10 min. * $p < 0.05$, RM One-way ANOVA, post-hoc Tukey, $n = 6$ animals. 3 Males/3 females. Data are mean $\pm$ SEM.

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provide an additional target by which exogenous nicotine could suppress appetite through extra-hypothalamic neurons.

Chronic hyperactivation of vGlut2^{BF} neurons led to drastic hypophagia, and acute optogenetic activation of these neurons both reduced food intake and caused avoidance of food and food-associated odors. Such dramatic hypophagia has previously been reported in models of lateral hypothalamic lesions, permanent AgRP neuron ablation in the hypothalamic arcuate nucleus, and permanent loss of GABA signaling from AgRP neurons (Gropp et al., 2005; Luquet et al., 2005; Tong et al., 2008; Wu et al., 2009). To our knowledge, the current study is the first instance of a model with precisely targeted manipulations to the basal forebrain that leads to hypophagia and starvation. The dramatic phenotype of precipitous weight loss and complete appetite suppression within days of viral mNaChBac expression, indicates the high sensitivity of vGlut2^{BF} neuronal firing. While chronic activation persistent for tens of minutes to days most likely does not occur in nature, changes in appetite especially for neuropsychiatric conditions are often chronic in nature and do not manifest by simply avoiding a single food item once or skipping one meal. Given the sensitivity of this neuronal population and the correlated phenotype, it could be possible that small changes in the excitability of this neuronal population could have chronic phenotypes in altering feeding behaviors. Furthermore, the resistance of the phenotype in leptin deficient mice indicates the feeding pathways altered by our manipulation are either downstream or in parallel to leptin signaling, but capable of overriding leptin deficient induced hyperphagia. Importantly, the hypophagic phenotype seen here is similar to that observed with AgRP neuron ablation, suggesting that the activity of vGlut2^{BF} neurons is a fundamental regulator of feeding behavior (Gropp et al., 2005, p. 2005; Luquet et al., 2005). Further, moderately obese *Lep^{ob/ob}* animals survived AgRP neuron ablation (Wu et al., 2012), whereas viral mNaChBac manipulation of vGlut2^{BF} neurons in obese *Lep^{ob/ob}* animals did not rescue prolonged hypophagia and starvation. Accordingly, we and others have identified the lateral hypothalamus as a robust brain target of vGlut2^{BF} neurons involved in feeding regulation (Do et al., 2016).

Genetic subpopulations in the lateral hypothalamus (LH) have been extensively studied for their role in feeding related behavior. VGAT-positive inhibitory neurons in the LH have been shown to promote feeding (Jennings et al., 2015), while vGlut2 positive neurons in the LH elicit food aversion (Jennings et al., 2013). We revealed that vGlut2^{BF} neurons form direct synaptic connections onto vGlut2 positive neurons in the LH, and that their signaling onto LH neurons also diminished food intake and lead to food avoidance, thereby aligning with the previously proposed roles for the lateral hypothalamus. Notably, these findings highlight that a deeper understanding of the connectivity among and between the different cell types within the basal forebrain and lateral hypothalamus promises to reveal more precise mechanisms of how feeding and avoidance behaviors are fully relayed between these two circuit nodes.

An important consideration is the rewarding aspect of consuming food, particularly after a period of fasting. Previous work has shown that lateral hypothalamic projections to the lateral habenula modulate rewarding aspects of food intake, and that basal forebrain projections to the lateral habenula by somatostatin-expressing neurons can also regulate the rewarding aspects of feeding (Stamatakis et al., 2016; Zhu et al., 2017). The lateral habenula has been implicated in reward-related and aversive behaviors through indirect inhibition of the ventral tegmental area (VTA) dopaminergic neurons, which is another projection site of vGlut2^{BF} neurons. Therefore, given the interconnected nature of cell types between these structures, vGlut2^{BF} neurons could also play a pivotal role in encoding the rewarding aspects of food intake. In future experiments, it will be particularly interesting and important to determine the contribution of vGlut2^{BF} projections to other projection regions, including the lateral habenula and VTA, in controlling the valence of food and/or food-associated odors. Based on cell-body versus LH projection stimulation results, other nodes in this circuit are clearly important since lateral hypothalamic stimulation of vGlut2^{BF} terminals did not fully recapitulate the avoidance behavior seen when vGlut2^{BF} cell bodies were stimulated (Figures 8, 9 and 10). Although unlikely based on this evidence, we can not completely rule out that terminal excitation may backpropagate and activate cell-bodies in the optogenetic experimentation.

Recent investigations in the medial septum (MS) have also revealed neuronal populations that are capable of influencing feeding (Sweeney et al., 2017; Sweeney and Yang, 2016). Importantly, vGlut2 neurons in the MS reduced food intake through paraventricular hypothalamic projections, but to date, have not been shown to synapse onto lateral hypothalamic neurons (Sweeney et al.,

2017). In our study, we found that vGlut2^{BF} neurons make functional synapses onto LH neurons and mediate hypophagia in part through this connection. Additionally, anterograde tracing highlighted an absence of vGlut2^{BF} neuron innervation to the paraventricular hypothalamus. Excitatory neurons in the MS were also recently identified to influence auditory and somatosensory cue-based avoidance through lateral habenula projections (**Zhang et al., 2018**). That study also identified a unique locomotor phenotype, attributed to MS excitatory neuron projections to the preoptic region. Importantly, when we photostimulated excitatory-neuron cell bodies in the DBB, we did not observe any changes in locomotion, nor did we see increased cellular activity by calcium imaging when head-fixed animals moved on a walking ball. Additionally, our data indicate vGlut2^{BF} neurons cause an avoidance phenotype due to food olfactory sensory cues. Together, these studies suggest that vGlut2 neurons in the medial septum and the diagonal band of Broca represent unique basal forebrain populations with distinct projections that mediate appetite suppressing roles.

The robust hypophagia and starvation phenotype seen with the overactivation model described here draws parallels to hypophagic eating disorders. However, it is important to consider the structures investigated in this paper represent subcortical regions, and that neuropsychiatric eating disorders such as anorexia nervosa, binge eating disorders, and bulimia nervosa, are complex multifaceted conditions involving higher-order cortical regions (**Fuglset et al., 2016**). Additionally, the DBB of the basal forebrain is not considered a primary sensory cortex for olfactory information, and therefore the function of other presynaptic inputs onto vGlut2^{BF} neurons need to be further investigated to understand how more complex cognitive states can utilize sensory cues to suppress appetite. Pre-synaptic tracing onto vGlut2^{BF} neurons highlight the orbitofrontal and agranular insular cortex as potential targets which could integrate sensory perception and higher level cognition to suppress appetite through basal forebrain circuitry (**Do et al., 2016**).

Sensory perception of food can stimulate or decrease appetite by providing information on a food’s palatability, caloric value, and taste. Our results reveal a group of neurons capable of responding to the sensory perception of food, diminishing appetite, and eliciting avoidance to food and food-associated odors. Notably, these findings reveal a previously unknown circuit that links cholinergic signaling in the basal forebrain to feeding behavior, appetite suppression, and food avoidance. In the future, it will be important to uncover potential functions of this circuit in behaviors associated with nicotine-mediated appetite suppression, addiction, and/or learned food aversion associated with eating disorders.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (M.Muscularus)	Vglut2-Cre (Slc17a6-Cre)	The Jackson Laboratory	RRID:IMSR_JAX:028863	
Strain, strain background (M.Muscularus)	Chat-Chr2 (ChAT-ChR2-EYFP Line 6)	The Jackson Laboratory	RRID:IMSR_JAX:014546	
Strain, strain background (Adeno-Associated Virus Serotype 2/9)	AAV-Ef1a-Flex-hChR2(H134)-EYFP-WPRE-hGHpA (2/9); Flex-Chr2	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from RRID:Addgene_26973
Strain, strain background (Adeno-Associated Virus Serotype 2/9)	AAV-Ef1a-Flex-hM4Di-mCherry (2/9); Flex-hM4Di-mCherry	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from RRD:Addgene_44362

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Strain, strain background (Adeno-Associated Virus Serotype 2/DJ8)	AAV-Ef1a-Flex-GCaMP6m-WPRE-hGHpA (DJ8); Flex-GCaMP6m	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from RRID: Addgene_100839
Strain, strain background (Adeno-Associated Virus Serotype 2/DJ8)	AAV-Ef1a-Flex-eGFP-p2a-mNaChBac-WPRE-hGHpA (DJ8); Flex-mNaChBac; mNaChBac	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from plasmid gift from Dr. Mingshan Xue (mxue@bcm.edu)
Strain, strain background (Adeno-Associated Virus Serotype 2/DJ8)	AAV-Ef1a-Flex-Synaptophysin::eGFP-WPRE-hGHpA (DJ8); Flex-Synaptophysin	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from RRID: Addgene_73816
Strain, strain background (Adeno-Associated Virus Serotype 2/DJ8)	AAV-Ef1a-Flex-mRuby2 (DJ8); Flex-mRuby2	This Paper; Neuroconnectivity Core at the Jan and Dan Duncan Neurological Research Institute		Plasmid subcloned from Addgene:40260
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-hChR2(H134)-EYFP-WPRE-hGHpA	This paper	RRID: Addgene_26973	
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-hM4Di-mCherry	This paper	RRID: Addgene_44362	
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-GCaMP6m-WPRE-hGHpA	This paper	RRID: Addgene_100839	
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-eGFP-p2a-mNaChBac-WPRE-hGHpA	Plasmid subcloned from plasmid gift from Dr. Mingshan Xue (mxue@bcm.edu)		
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-Synaptophysin::eGFP-WPRE-hGHpA	This paper	RRID: Addgene_73816	
Recombinant DNA reagent (plasmid)	pAAV-Ef1a-Flex-mRuby2	This Paper	RRID: Addgene_40260	
Antibody	anti-ChAT (goat monoclonal)	Millipore	Millipore Cat# AB144P; RRID: AB_2079751	(1: 500 dilution)
Antibody	anti-cFos (rabbit polyclonal)	Abcam	Abcam Cat# ab190289; RRID: AB_2737414	(1:1000 dilution)
Chemical compound, drug	CNO (clozapine-n-oxide)	Tocris 4963		
Commercial assay or kit	Mouse/Rat T4 Elisa	Calbiotech, Inc	T4044T-100	
Commercial assay or kit	Active Ghrelin ELISA	Millipore	EZRGRA-90K	
Software, algorithm	Doric Neuroscience Studio	Doric Lenses		
Software, algorithm	MATLAB	Mathworks	RRID: SCR_001622	
Software, algorithm	Optimouse	PMID: 28506280		

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Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Software, algorithm	Prism 8	Graphpad	GraphPad Prism, RRID:SCR_002798	
Software, algorithm	Rodent Liquid diet	Test Diet	Test Diet:101 5LD1	
Other (Mouse Liquid Diet)	Synapse	Tucker-Davis Technologies	Tucker-Davis Technologies; RRID:SCR_006495	
Other (GRIN Lens)	Grin lens	Doric Lenses	Doric: SICL_E_500_80	

Animals

All mice in this study were treated in compliance with US Department of Health and Human Services and Baylor College of Medicine IACUC guidelines. Both male and female mice were used in analyses, littermates were randomly assigned to experimental groups, and further specified below. Mice were between 3–5 months of age for surgeries, and between 3–6 months for experiments. Standard mouse chow (Harlan, 2920X) was used for all experiments, and all animals were maintained on a 12 hr light-dark cycle. Depending on the experiment, mice were either group or singly housed (see below). vGlut2-Cre (*Slc17a6^{tm2(cre)Low}/J* Stock No. 016963) and Chat-Chr2 (*Chat-COP4^{H134R}/EYFP, Slc18a3 6Gfng/J* Stock No. 014546) mice were originally purchased and are available from Jackson Laboratories. Genotyping for Cre was done using the following primers: forward 5'-GCA TTACCGGTCGATGCAACGAGTGATGAG-3' and reverse 5'-GAGTGAACGAACCTGGTCGAAA TCAGTGCG-3'. Genotyping for Chat-Chr2 was done using the following primers: forward 5'-TCTG TTCCCAGGTCGGCAGC-3' and reverse 5'-GCAAGGTAGAGCATAGAGGG-3'.

Stereotaxic injections and viral constructs

For all stereotaxic injections and implantation surgeries, mice were anesthetized and maintained under anesthesia using vaporized isoflurane with O₂. All injections and implantations were performed using a stereotaxic apparatus synced to Angle Two software for coordinate guidelines. The basal forebrain was targeted through bilateral injections into the horizontal limb of the diagonal band of Broca (from bregma AP= +0.14 mm, DV = −5.8 mm, and ML= ±1.29 mm) or unilateral for calcium imaging experiments. The lateral hypothalamus was targeted using bilateral injections from bregma AP = −1.22 mm, DV = −5.12 mm, ML= ±0.97 mm. Viruses used in experiments include: AAV-Ef1a-Flex-GCaMP6m-WPRE-hGHpA Serotype DJ8, AAV-Ef1a-Flex-eGFP-p2a-mNaChBac-WPRE-hGHpA Serotype DJ8 (sub-cloned from plasmid gift from Dr. Mingshan Xue (mxue@bcm.edu), AAV-Ef1a-Flex-mRuby2 Serotype DJ8, AAV-Ef1a-Flex-Synaptophysin::eGFP-WPRE-hGHpA Serotype DJ8, AAV-Ef1a-Flex-hChr2(H134R)-EYFP-WPRE-hGHpA, serotype 2/9, AAV-Ef1a-Flex-hM4Di-mCherry Serotype 2/9. We injected 200–400 nL of virus for all experiments, except for synaptophysin tracing experiments where 50–100 nL of virus was injected.

Microscopy and immunohistochemistry

Animals were deeply anesthetized using isoflurane and were transcardially perfused with PBS followed by 4% PFA (Diluted from 16% Paraformaldehyde EM Grade No. 15710 Electron Microscopy Sciences). Brains were dissected and post-fixed in 4% PFA overnight at four degrees Celsius. Brains were cryoprotected in 20% sucrose/PBS solution for 1 day, followed by 30% sucrose/PBS solution for one more day, both at four degrees Celsius. Brains were then embedded and frozen in O.C.T. (Fisher HealthCare No. 4585) and stored at −80 degrees Celsius until being cut. Brains were cut using a cryostat (Leica CM1860) in coronal sections 40 μm. For ChAT and Fos immunohistochemistry, 40 μm free-floating sections were blocked at 1 hr at room temperature in 10% horse serum blocking solution made in PBS-T (1X PBS, 1% Triton-X 100, pH 7.35). Sections were incubated overnight at four degrees Celsius at 1:500 dilution of block solution containing goat anti-ChAT primary antibody (Millipore AB144P) and 1:1000 dilution of rabbit anti-Fos antibody (Abcam ab190289). Sections were washed 4 times for 10 min in plain PBS-T. Sections were then incubated in secondary antibodies

(1:500 donkey anti-goat Alexafluor-488, and donkey anti rabbit Alexafluor-647) each for 2 hr at room temperature. Sections were then washed 5 times at 10 min each in PBS-T. All sections were mounted using DAPI Fluoromount-G (southern Biotech, 0100–20). Sections for synaptophysin tracing were washed with PBS 4 times for 10 min and then mounted with DAPI Fluoromount-G. Detection of fluorescent expression was performed using a Leica TCS SPE confocal microscope, or Leica TCS SP8 STED microscope. For synaptophysin projection quantification all images were taken at 20X holding all imaging parameters constant for each animal. ROIs were drawn in imageJ over target fields and relative intensity was measured. These were normalized on a per animal basis to an ROI over the DBB.

Neuronal activation after feeding

Male vGlut2-Cre animals were injected with AAV-Flex-mRuby2. Three weeks after viral injection, animals were fasted for 24 hr. They were then placed in individually housed cages for 15 min. One group of animals were presented with standard pellet chow for 1 hr (fed), while a second group of mice lacked chow (fasted group). Mice were then immediately euthanized and processed using the microscopy and immunohistochemistry procedure above. Using identified positions from bregma, BF was identified by borders of cholinergic neuronal populations. Cells within this area were evaluated and counted in order to quantify positively labeled cells.

In vivo microendoscopy

vGlut2-Cre animals were injected with adeno-associated virus expressing a Cre-dependent fluorescent calcium reporter, GCaMP6M (AAV-Ef1a-Flex-GCaMP6m). For freely behaving experiments, following viral injection, two set screws were drilled into exposed skull tissue. Next, a DORIC GRIN lens (SICL_E_500_80) attached to a protrusion adjustment ring was lowered over the injection site, positioned approximately 80 μ m above the virally targeted location. GRIN lenses used had NAs of 0.5, focal distances of 80 μ m, 500 μ m diameters, and 8.39 mm lengths- adjustable between 5.3 mm to 8.39 mm dependent on the adjustment ring. Dental cement was used to fix the adjustment ring containing the GRIN lens onto the skull. Mice were then allowed to recover for three weeks. Prior to imaging, mice were fasted for 24 hr while having a dummy camera attached to their head. A DORIC microendoscope was next attached to the GRIN lens/protrusion adjustment ring. Mice were then imaged at an exposure rate of 50–250 ms per image, dependent on image quality (4–20 Hz). All results were down-sampled to 4 Hz for freely behaving experiments, and 10 Hz for head fixed animals, since there was better image quality for these experiments. LED power used for imaging was 1–3 mW. During imaging, mice were also video recorded using a Doric high-speed behavior camera at 60fps, which was time-locked with GCaMP imaging using Doric Neuroscience Studio software. For odorant delivery, mice were presented either monomolecular odorants or crushed food pellets dissolved in mineral oil on a cotton swab placed in the corner of a cage. Food interaction was defined as mice approaching and smelling food pellets, but not yet having eaten the food. Physical stimulation of mice was done by gently poking the mice with a cotton swab.

For head fixed experiments, 2 weeks after viral injection a metal head-plate was secured to the mouse skull using dental cement. 24–72 hr after head plate surgeries, mice were attached to a custom head-fix apparatus that includes a running-ball on which mice are able to walk on while their head is affixed to a side bar. The skull hole used for viral injection was re-drilled, and a DORIC GRIN lens (same as above) attached to the microscope was slowly lowered until the basal forebrain was in focus. Odorants were then delivered using a pressurized custom odor delivery robot while basal forebrain responses were imaged. Odorants were delivered for 10 s every 30 s, while being time locked with the beginning of microscope recording. Odors used include: 2-Methylbutylamine (241407 Sigma) dissolved in 5 mL mineral oil to a concentration of 4.5 μ M, 100 μ L Butyric Acid (103500 Sigma) dissolved in 5 mL mineral oil, food pellets were sonicated in mineral oil, and mineral oil alone. Gently touching mice with a cotton swab resulted in mice running on the wheel and was used to measure calcium activity from physical movement. Responses were averaged from two trials of randomized odor delivery.

Calcium image processing

Images, if necessary, were corrected for motion artifacts using DORIC neuroscience studio image analyzer. For freely moving animals, cells were identified by direct visualization due to accurate focusing. For head-fixed recordings, a combination of PCA cell identification and hand drawn ROIs were utilized to isolate cells in the DORIC neuroscience studio software. The software calculated Delta F/F for whole-field images and ROIs. Traces were extracted, and then further analyzed using existing plotting functions in MATLAB, including plot and images. For freely behaving animals, DF/F traces were normalized for individual animals from 0 to 1 using the animals' own maximum and minimum DF/F trace values. These normalized traces were then averaged across animals in order to compare activation. Cells were considered active if they achieved at least a two standard deviation increase in response for freely behaving animals, and three standard deviation increase in response for head-fixed animals.

Electrophysiology

For all slice experiments, animals were anesthetized with isoflurane and perfused with cold artificial cerebrospinal fluid (ACSF) solution pH 7.35 mOsm 305–315 containing: 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄-H₂O, 2 mM CaCl₂, 1 mM MgCl₂-6H₂O, 20 mM Glucose, 25 mM NaHCO₃. Brains were rapidly removed and transferred into sucrose-based cutting solution pH 7.35 containing: 87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH₂PO₄-H₂O, 0.5 mM CaCl₂, 7 mM MgCl₂-6H₂O, 13 mM Ascorbic Acid, 75 mM Sucrose, 10 mM Glucose, 25 mM NaHCO₃ and continuously bubbled with 5% CO₂/95% O₂. 300 µm thick coronal brain slices were prepared using a Leica VT1200 vibratome and placed in recover for 15 min at 37 degrees Celsius in 5% CO₂/95% O₂ bubbled ACSF solution. They were then gradually lowered to room temperature 25 degrees Celsius for and allowed to acclimate for at least 15 min before recording. For recording, slices were transferred into a recording chamber continuously perfused at 1–2 mL/min at 25 degrees Celsius. Neurons were identified by transmitted light DIC and fluorescent imaging (BX50WI, Olympus). Recordings were obtained using an Axon MultiClamp 700B amplifier digitized at 10 kHz (Axon Digidata 1440A). Recording electrodes (3–5 megaohms) were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm) with a micropipette puller (Sutter Instruments). For current clamp recordings, a potassium gluconate internal solution was used consisting of 120 mM K-gluconate, 5 mM KCl, 2 mM MgCl₂-6H₂O, 2 mM Mg-ATP, 10 mM Na₂ phosphocreatine, 0.4 mM Mg-GTP, 10 mM HEPES EGTA, 0.05 mM EGTA, pH 7.3 with KOH, 290 mOsm. For voltage clamp recordings, internal solution containing the following was used: 120 mM Cs-methanesulfonate, 2 mM MgCl₂-6H₂O, 0.05 mM CaCl₂, 6 mM CsCl, 20 mM HEPES, 0.2 mM EGTA, 10 mM phosphocreatine di(Na) salt, 4 mM ATP-Mg, 0.4 mM GTP-Na, pH 7.2 with CsOH, and 290–300mOsm with CsMeSO₄. *mNaChBac Recording*: Brain slices containing the basal forebrain were prepared from 12 to 16 week-old animals expressing eGFP-p2a-mNaChBac in one hemisphere, and mRuby2 in the contralateral hemisphere. Resting potential was recorded within seconds of breaking into a cell. Cells with a series resistance of less than 30 megaohms and <25% change for duration of the experiment were used for analysis. Analysis of current-clamp electrophysiological data was performed using pClamp10 (Molecular Devices). Single action potential parameters were measured at threshold, and action potential amplitudes were measured relative to action potential threshold.

Channelrhodopsin electrophysiology

Brain slices containing either the basal forebrain or lateral hypothalamus were prepared from 12 to 16 week-old animals. Cells were patched using voltage clamp internal solution. Cells were recorded for a minimum of 10 sweeps for each pharmacological condition. Each sweep consisted of 2 s of recording, with an excitation duration of 10 ms of blue light (470 nm) exposure with 30 s between each sweep. For evaluation of cholinergic connectivity onto VGlut2 +basal forebrain neurons, we used ACSF that had the following final pharmacological concentrations in the slice chamber: 20 µM AP-5 (Tocris), 10 µM CNQX (Tocris), 2 µM GABAzine (Tocris), 10 µM Atropine (Sigma), 1 µM TTX (Tocris), 0.5 µM 4-AP (Tocris), and/or 0.5 mM Mecamylamine (Tocris). For lateral hypothalamus recordings, ACSF with the following pharmacological concentrations in the slice chamber were used: 1 µM TTX (Tocris), 0.5 µM 4-AP (Tocris), 20 µM AP-5 (Tocris), 10 µM CNQX (Tocris).

In vivo extracellular recordings

12–16-week-old vGlut2-Cre $+/+$ mice were injected with either Flex-Chr2 or with both Flex-Chr2 and Flex-NaChBac. During the same surgery a metal head-plate was secured to the mouse skull using dental cement. 24–72 hr after head plate surgeries, mice were attached to a custom head-fix apparatus that includes a running-ball on which mice are able to walk on while their head is affixed to a side bar. The skull hole used for viral injection was re-drilled. A 32-channel NeuroNexus Optrode was stereotactically lowered into the brain to the depth of the basal forebrain. Cells were identified by applying 10 Hz light stimulation. Data for spontaneous frequency was recorded for 5 min, ending with a 10 Hz photostimulation using a 465 nm laser (DORIC). Data were recorded using a multichannel Tucker-Davis Technologies (TDT) system (Alachua, Florida), and single unit spikes were isolated using a 3.0 standard deviation cut-off from noise and a Bayesian clustering algorithm pre-built into the TDT Synapse software. Data were exported into MATLAB and further quantified to obtain spontaneous frequencies.

vGlut2 basal forebrain neuron activation with mNaChBac Assay

12–16 week-old equal number of male and female vGlut2-Cre $\pm$ mice were separated into individually housed cages for 1 week, and mice and chow were measured daily to establish average baseline weight and daily food intake. After 1 week, mice were injected with either Flex-eGFP-p2a-mNaChBac or Flex-GFP as described above. Mice and food were continuously measured daily. If during the daily mouse weighing, the mice lost more than 20% of baseline weight, the final weight was recorded, and the mouse was humanely sacrificed per IACUC guidelines. Additionally, if a mouse displayed anorexia for greater than 3 days, it was also sacrificed.

Liquid diet and oral gavage assay

12–16 week-old VGlut2-Cre $\pm$ mice were separated into individually housed cages and transitioned to a liquid diet 1 week prior to viral injection by placing both chow and liquid diet (Test Diet Rodent liquid diet 101 5LD1) in mouse water bottles. After viral injection, mouse food liquid diet intake, and body weight were measured twice daily. Based on manufacturer guidelines that mice should consume at least 20 g of liquid diet, any difference between actual intake and this minimum, more was administered as oral gavage twice daily to maintain mouse weight higher than IACUC sacrifice policy of 25% of baseline weight loss.

Hormone analysis

12–16 week-old VGlut2-Cre $\pm$ mice were injected with either Flex-eGFP-p2a-mNaChBac or Flex GFP virus. Blood was obtained using lancet puncture of the submandibular vein 4 days post viral injection. Blood was collected into 200 μ L lavender top EDTA tubes (microvette 200 K3E). Tubes were centrifuged at 2500 g at room temperature for 5 min. Supernatant plasma was immediately isolated, flash frozen on dry ice, and stored at -80 degrees. Mouse pituitary panels were measured by the Vanderbilt Hormone and Analytics Core using a Mouse Pituitary Luminex Panel. Mouse free T3 was measured by the Vanderbilt Hormone and Analytics Core using a free T3 radioimmunoassay kit. Mouse T4 was measured using a Mouse/rat T4 ELISA (Calbiotech, Inc). Mouse insulin, glucose, and leptin were measured by the Baylor College of Medicine Mouse Metabolic and Phenotyping Core. Ghrelin levels were measured using a Rat/Mouse Active Ghrelin ELISA kit (Millipore EZRGRA-90K).

Chemogenetic hM4Di assay

12–16 week-old, three male, two female VGlut2-Cre mice were injected bilaterally into basal forebrains with an hM4D expressing AAV. After two weeks of recovery, mice were separated and individually housed for 1 week and subjected to overnight fasting or ad-libitum feeding in their home cage. For control experiments mice were injected i.p. with sterile saline, for experimental conditions mice were injected i.p. with CNO (5 mg/kg mouse body weight). 30 min after injection, food was presented to animals and measured over the course of 5 hr. Trials were randomized, separated 1 week apart, and conducted on the same animals. Paired statistics were utilized to compare experimental and control conditions.

Optogenetic food intake assay

12–16-week-old mice were injected bilaterally into the basal forebrain with Flex-Chr2 or Flex GFP virus as described above. Two weeks after viral injection, mice were bilaterally implanted with 200 μm silica fiber optic implants made in-house (Thor Labs) with 230 μm attachment ferrules (Precision Fiber Products), and situated 1 mm above the viral injection sites in the basal forebrain and projection site in the lateral hypothalamus. Fiber optic implants were held in place by a cap made from adhesive cement (C and B Metabond Dental cement Parkell). On top of which a crosslinked flash acrylic (Yates-Motloid 44115 and 44119) cap was constructed. Mice were allowed another 2 weeks for recovery. Mice were acclimated to a behavior chamber for 30 min on day 1. On day two mice, were acclimated again while tethered to a dual fiber optic cord (Doric Lenses). Mice were subsequently fasted over-night. On day 3, mice were placed in behavior chambers while tethered to the dual fiber optic cord. One group was photostimulated at 5 Hz (10 ms pulses, ~4 mW) for 30 min over the basal forebrain, another group photostimulated over the lateral hypothalamus, and one group had no photostimulation. Food was placed on a petri dish in one corner of the cage. After 30 min food was measured. This three-day experiment was repeated twice more with 1 week between the start of each experiment, as the mice randomly switched photostimulation groups (Basal forebrain, lateral hypothalamus, or none).

Variable frequency optogenetic stimulation assay

12–16 week-old mice were bilaterally injected into the basal forebrain with Flex-Chr2 as described above. Two weeks after viral injection, mice were bilaterally implanted with 200 μm silica fiber optic implants made in-house (Thor Labs) with 230 μm attachment ferrules (Precision Fiber Products) and situated 1 mm above the viral injection sites in the basal forebrain. Fiber optic implants were held in place by a cap made from adhesive cement (C and B Metabond Dental cement Parkell). On top of which a crosslinked flash acrylic (Yates-Motloid 44115 and 44119) cap was constructed. Mice were allowed another 2 weeks for recovery. Mice were acclimated to a behavior chamber for 30 min on day 1. On day 2, mice were acclimated again while tethered to a dual fiber optic cord (Doric Lenses). Mice were subsequently fasted over-night. On day 3, mice were placed in behavior chambers while tethered to the dual fiber optic cord. Mice were photostimulated at variable frequencies 0–20 Hz (10 ms pulses, ~4 mW) for 10 min over the basal forebrain. This was repeated weekly with mice fasted overnight prior to the experiment. Photostimulation frequencies included 0, 5, 10, and 20 Hz. Pre-weighed food was placed on a petri dish in one corner of the cage. After 10 min, food weight was measured.

Optogenetic food pellet avoidance assay

Food pellets were placed in three random corners of a behavior chamber, leaving one corner devoid of food. Mice were recorded using a DORIC high speed behavior camera. The same mice from the food intake assay were used for these experiments in the same behavior chambers, so no re-acclimation was performed. On day 1, mice were separated into either BF stimulation, LH stimulation, or no photo-stimulation groups. They were fasted overnight prior to experiments. Mice were photostimulated at 5 Hz (10 ms pulses, ~4 mW) for the 30 min experimental timeframe. Experiments were repeated twice more, one week apart from each other as mice switched groups between stimulation locations. Unstimulated groups were still tethered to the fiber optic cord. Videos of mice were analyzed using open source software OptiMouse in MATLAB (Ben-Shaul, 2017).

Optogenetic food smell avoidance assay

The same mice from food intake and food pellet avoidance assays were used for these experiments in the same behavior chambers. Chow was spiked in mouse home cages with 1 mL of R-limonene dissolved in mineral oil for 2 weeks to form a passive association of R-limonene with food pellets. After 2 weeks, mice were fasted overnight. On the day of experiments, mice were placed in behavioral chambers and attached to fiber optic cords, either to the basal forebrain or lateral hypothalamus. For half of the animals, a q-tip with R-limonene dissolved in mineral oil was placed in an open 1.5 mL tube in three corners, leaving one corner empty. Mice spent 10 min in this situation. Next, 5 Hz photostimulation (10 ms pulses, ~4 mW) was presented for 10 min. Next, the q-tips/Eppendorf tubes with R-limonene were swapped with q-tips/Eppendorf tubes containing S-limonene dissolved

in mineral oil as controls. Mice were recorded in this situation for 10 min while being photostimulated. The other half of the animals went through the sequence in reverse (s-limonene with photostimulation, r-limonene with photostimulation, r-limonene with no photostimulation). This experimental paradigm was repeated one week later, as mice switched from having their basal forebrain to lateral hypothalamus photostimulated, or vice versa.

Quantification and statistics

Data were analyzed using GraphPad Prism seven for Mac OSX. For calcium imaging studies, a minimum of five animals with at least two animals of each sex were used. Active neurons were identified with fluorescent activity two standard deviations above baseline activity for freely behaving animals, and three standard deviations for head-fixed animals. Graphs with single time points were analyzed using repeated measures one way Anova tests, with Tukey's post hoc multiple comparison sets. Graphs with multiple time points, 2way ANOVA repeated measures were performed, with multiple comparisons using Tukey post hoc correction. For comparison of means between two groups, two tailed student's t-tests were used. No statistical methods were used to predetermine sample size. When relevant, randomization was carried out. Behavioral experiments were randomized. There was blinding during initial allocation of animals into groups, but not thereafter. All cell counts were blinded.

Contact for reagent and resource sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Benjamin R Arenkiel (arenkiel@bcm.edu).

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Author contributions

Jay M Patel, Conceptualization, Software, Formal analysis, Funding acquisition, Validation, Visualization, Methodology, Writing—original draft, Writing—review and editing; Jessica Swanson, Validation, Investigation, Writing—review and editing; Kevin Ung, Investigation, Writing—original draft, Writing—review and editing; Alexander Herman, Conceptualization, Methodology, Writing—original draft, Writing—review and editing; Elizabeth Hanson, Investigation, Writing—review and editing; Joshua Ortiz-Guzman, Investigation, Methodology; Jennifer Selever, Resources, Funding acquisition, Project administration; Qingchun Tong, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Methodology, Writing—original draft, Project administration, Writing—review and editing; Benjamin R Arenkiel, Conceptualization, Resources, Supervision, Funding acquisition, Investigation, Visualization, Methodology, Writing—original draft, Project administration, Writing—review and editing

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Ethics

Animal experimentation: All mice in this study were treated in compliance with US Department of Health and Human Services and Baylor College of Medicine and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocol (AN5966) at Baylor College of Medicine. All surgery was performed in accordance with the approved protocol, and every effort was made to minimize suffering.

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Supplementary files

- Transparent reporting form

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Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

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