

AWARD NUMBER: W81XWH-19-1-0429

TITLE: Genetically Dissecting Basal Forebrain Circuits Underlying Eating Disorders

PRINCIPAL INVESTIGATOR: Qingchun Tong

CONTRACTING ORGANIZATION: The University of Texas Health Science Center, Houston, TX

REPORT DATE: October 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE October 2022		2. REPORT TYPE Annual		3. DATES COVERED 01Sep2021-31Aug2022	
4. TITLE AND SUBTITLE Genetically Dissecting Basal Forebrain Circuits Underlying Eating Disorders				5a. CONTRACT NUMBER W81XWH-19-1-0429	
				5b. GRANT NUMBER W81XWH-19-1-0429	
				5c. PROGRAM ELEMENT NUMBER N/A	
6. AUTHOR(S) Tong, Qingchun, Ph.D.				5d. PROJECT NUMBER 0011327909	
				5e. TASK NUMBER N/A	
				5f. WORK UNIT NUMBER N/A	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) UNIVERSITY OF TEXAS HEALTH SCIENCE CENTER OFFICE OF SPONSORED PROJECTS 7000 FANNIN ST HOUSTON, TX 77030-5400				8. PERFORMING ORGANIZATION REPORT NUMBER N/A	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S) CDMRP	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S) N/A	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES None.					
14. ABSTRACT Abnormal eating habits that drive excessive food seeking- or avoidance can manifest as health-threatening and socially impactful eating disorders. The scope of research includes 3 main aims: 1) to determine how basal forebrain ACh+ and Glu+ neurons are connected; 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. Specific results from this reporting period have identified that specific activation of DBB Glu+ neurons severely reduced feeding, and importantly, specific activation of DBB Glu+ fibers in the lateral hypothalamus (LH) also induced similar reduction in feeding, suggesting LH as one major downstream target mediating the feeding effect. However, specific inhibition of DBB Glu+ fibers in LH failed to increase feeding, suggesting a sufficient but not necessary role for LH neurons in mediating the feeding effect. The remaining planned experiments including the examination of local DBB circuits between cholinergic and glutamatergic neurons, the genetic interaction between DBB Glu+ neurons and the ob gene and the effect of DBB Glu+ neurons in motivation feeding will be accomplished in the next year. Overall, the results of the supported work 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 identify the downstream targets that mediate basal forebrain Glu+ neurons in regulating eating and related behaviors.					
15. SUBJECT TERMS Basal forebrain, eating disorder, glutamate, acetylcholine, lateral hypothalamus, optogenetics, in vivo Ca2+ imaging, metabolism					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 45	19a. NAME OF RESPONSIBLE PERSON USAMRDC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	1
2. Keywords	1
3. Accomplishments	1-5
4. Impact	6
5. Changes/Problems	6
6. Products	6-7
7. Participants & Other Collaborating Organizations	7-8
8. Special Reporting Requirements	8
9. Appendices	8

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. The scope of this research includes 3 main aims: 1) to determine how basal forebrain ACh+ and Glu+ neurons are connected; 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. Specific results from this reporting period have identified that selective activation of DBB Glu+ neurons severely reduced feeding, and importantly, targeted activation of DBB Glu+ fibers in the lateral hypothalamus (LH) also reduced feeding, suggesting that the LH as a major downstream target that mediates DBB Glu+ neuron feeding effects. However, specific inhibition of DBB Glu+ fibers in LH failed to increase feeding, suggesting a sufficient but not necessary role for LH neurons in feeding behavior. The remaining planned experiments include the examination of local DBB circuits between cholinergic and glutamatergic neurons, the genetic interaction between DBB Glu+ neurons and the ob gene, and the effect of DBB Glu+ neurons in motivational feeding will be accomplished in the next year. Overall, the results of the supported work will provide insights on how basal forebrain ACh+ and Glu+ neurons are connected, how Glu+ neurons in the basal forebrain influence feeding and related behaviors, and identify the 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. However, we found some issues with the use of the SAD rabies virus in local circuit tracing. We are trying alternative ways to overcome the issues. This goal will be accomplished in the next 6 months.

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 mostly 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, but is currently nearly completed. We have also added the detailed profiling of ACh receptor subtypes in DBB Glu+ neuron targets.

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 and included in the last report.

Major goal 5: Image basal forebrain Glu+ neurons *in vivo* responses to eating; SOW completion data and site: 6-20 months in Site 1 (Dr. Arenkiel). This goal has been accomplished and included in the last report.

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 6-24 months and included in the last report.

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 been accomplished 12-36 months and included in this report.

Major goal 8: Modulate basal forebrain Glu+ neuron activity and assess feeding; SOW completion date and site: 12-28 months and Site 2 (Dr. Tong). This goal has been accomplished and included in this report.

Major goal 9: Evaluate roles for 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 been delayed due to issues encountered in animal models. We anticipate completion in the coming months.

Major goal 10: Genetic rescue of obesity; SOW completion date and site: 6-36 months and Site 1 (Dr. Arenkiel). This goal has been delayed due to breeding issues. Mice are currently available, and actively being investigated. We anticipate completion in the coming months.

Major goal 11: Evaluate 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 been accomplished 18-36 months and reported in this report.

Major goal 12: Decipher 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 been accomplished 50%, and we have established behavioral protocol for nose poke for food. We anticipate completion of analysis in the coming months.

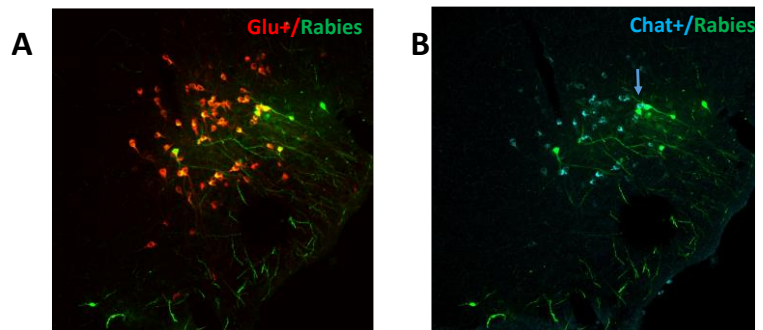
Major goal 13: Determine whether LH Glu+ neurons are directly downstream of basal forebrain Glu+ neurons; SOW completion date and site: 6-18 months and Site 1 (Dr. Arenkiel). This goal has been accomplished (6-18 months).

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 6-36 months and included in this report.

Accomplishment during the reporting period:

In the previous reports, we have accomplished major goals 1, 4, 5, and 6. During the reporting period, we have accomplished major goals 7, 8, 11, 13 and 14, and also made significant progress in major goals 2, 3, 9, 10 and 12.

Major goal 2 (Dr. Tong): Here the goal has been to determine whether ChAT neurons in the basal forebrain send monosynaptic projections to Glu+ neurons. We plan to use a monosynaptic pseudotyped rabies viral tracing approach to trace monosynaptic upstream neurons. Toward this, we have prepared *Vglut2-Cre* models, in which AAV-Flex-TVA-mCherry and AAV-Flex-G viral vectors will be injected into the basal forebrains 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 pseudotyped rabies virus SADdeltaG-GFP had been shut down and the experiments had to be delayed from year 1 to year 2. We have accomplished this experiment in year 2 (Fig. 1, arrow identifies ChAT+ neurons positive for rabies viral expression). However, we only found a limited number of neurons that are presynaptically traced. The low efficiency of this pseudorabies monosynaptic viral tracing has been reported previously. We just learned a new method based on the tracing ability of heavy chain of tetanus toxin for much more effective tracing from neuromodulatory cell types, and we will use this method to confirm the pseudotyped rabies virus tracing. We have already obtained the new heavy chain viral vectors and the experiments are under way and we project we will accomplish these experiments in 36-48 months.

Major goal 3 (Dr. Arenkiel): Here the goal has been 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 resuming further experimentation to validate the cell type-specific patterns of AChR expression. We have obtained single cell FISH results, and new approaches towards single cell sequencing of basal forebrain material are currently underway.

Major goal 5 (Dr. Arenkiel): Here 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 lenses into these animals. We have observed that GCamp6m signaling in the forebrain region responded to various external signals and found that, interestingly, these neurons responded to different food-related odors, but not to the mineral oil controls. These responses were rapid and reversible, suggesting a direct relevance of these neurons to feeding. This goal has been accomplished and reported in the last report.

Major goal 6 (Dr. Tong): Here the goal has been to determine feeding and body weight impacts in mice with basal forebrain Glu+ neurons expressing NaChBac. In the last report, we have prepared *Vglut2-Cre* mice and delivered AAV-Flex-NaChBac virus or control GFP virus to the basal forebrain. We found that NaChBac injected mice exhibited rapid body weight reduction, due to dramatic reduction of feeding.

Other experiments in this major goal are to test whether reduced food intake and associated reduced body weight can be rescued by forced feeding with food gavage. We found that by liquid food gavage, the survival rate of the animal with NaChBac expression was significantly improved, but not fully rescued. This goal has been accomplished and discussed in the last report.

Major goal 7 (Drs. Arenkiel and Tong): Here we sought to determine how loss-of-function manipulations in basal forebrain Glu+ neurons affects feeding (Dr. Arenkiel) and energy expenditure (Dr. Tong) by eliminating neurotransmitter release via expression of tetanus toxin light chain (TeNT). AAV-Flex-TeNT-p2A-GFP virus has been delivered to the forebrains of *Vglut2-Cre* mice, and we monitored feeding and body weight. However, we did not observe obvious differences in feeding, body weight, or O₂ consumption, and/or locomotion, which has been summarized in the last report. Verification of injection and expression, and confirmation of TeNT achieving loss of Glu release has been completed.

Major goal 8 (Dr. Tong): Here we sought to determine effects on feeding via manipulation of forebrain Glu+ neurons activated with ChR2 or inhibited with eNpHR3.0. Towards this, we delivered AAV-Flex-ChR2-GFP virus to the forebrains of *Vglut2-Cre* mice, and at the same time implanted optic fibers. We found that delivery of blue light significantly reduced fasting-induced refeeding (Fig. 2), suggesting an importance of forebrain Glu+ neurons in feeding control. However, when we delivered AAV-Flex-eNpHR3.0 virus to the same neurons with light stimulation, we failed to observe significant effects on fasting refeeding. All the injections have been verified.

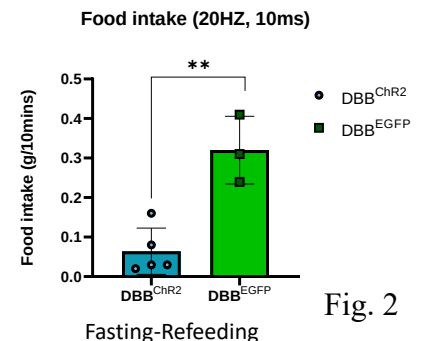


Fig. 2

Major goal 9 (Dr. Tong): Here the goal has been to determine whether basal forebrain Glu+ neurons mediate the action of ACh+ neurons in feeding and body weight. Towards this, we have crossed *ChAT-Cre* mice with *Vglut2-Flp* mice to obtain *ChAT-Cre::Vglut2-Flp* models, in which we delivered AAV-Flex-ChR2-GFP and AAV-fDIO-hM4Di-mCherry, or control AAV-fDIO-mCherry viruses to the forebrain. However, we found an issue with *Vglut2-Flp* mice in that this line does not seem to express Flp in *Vglut2* neurons in the DBB area. We are identifying issues and will try alternative ways to overcome this issue.

Major goal 10 (Dr. Arenkiel): Here the goal has been 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 the AAV-Flex-NaChBac-GFP or control AAV-Flex-GFP virus has been delivered to the basal forebrain region of some of these mice. Experiments are planned to measure weekly body weight and feeding of these mice. The breeding of ob/ob mice has been slow and we have not yet accumulated enough numbers of mice to complete this experiment. We project that we will accomplish this experiment in the next 6-12 months.

Major goal 11 (Drs. Arenkiel and Tong): Here the goal has been to determine whether the LH functions as a key downstream target to mediate hypophagia (Dr. Arenkiel) or aversion (Dr. Tong) induced by forebrain Glu+ neuron activation. Stereotaxic surgery has been performed to deliver AAV-Flex-ChR2 virus to the forebrains of *Vglut2-Cre* mice and at the same time, optic fibers implantation at the LH. Our results showed that stimulation of fibers in LH elicited aversion associated with food odor (Fig.3A), which represents a major part of the total aversion induced by the stimulation of DBB Glut+ neurons (Fig. 3B), suggesting a major role of LH neurons in mediating DBB neuron function in aversion associated with food odor.

Major goal 12 (Dr. Tong): the goal here has been to determine whether inhibition of basal forebrain Glu+ neuron projections to the LH promotes motivation to eat. Bilateral delivery of AAV-Flex-eNpHR3.0 virus has been made to the basal forebrain regions of *Vglut2-Cre* mice and optic fibers have also been implanted targeting the LH. These mice are scheduled to receive training in a nose poke chamber and then we will test whether light delivery will impact correct nose pokes and latency to nose poke. We have established the animal nose poke protocol in wild type animals (Figs. 4A and 4B) and we will perform the experiments in the experimental group in the coming months.

Major goal 13 (Dr. Arenkiel): Here the goal has been to determine whether LH Glu+ neurons are direct downstream

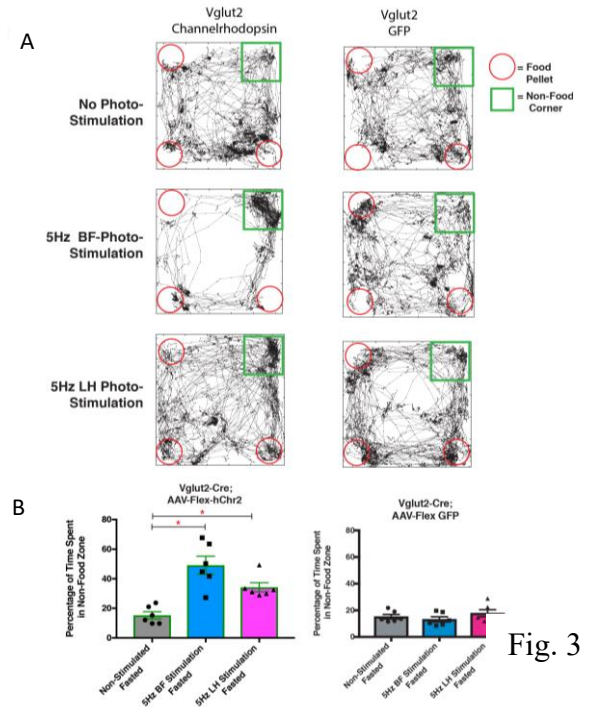


Fig. 3

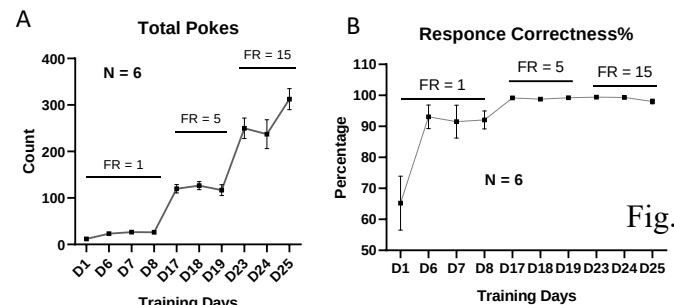


Fig. 4

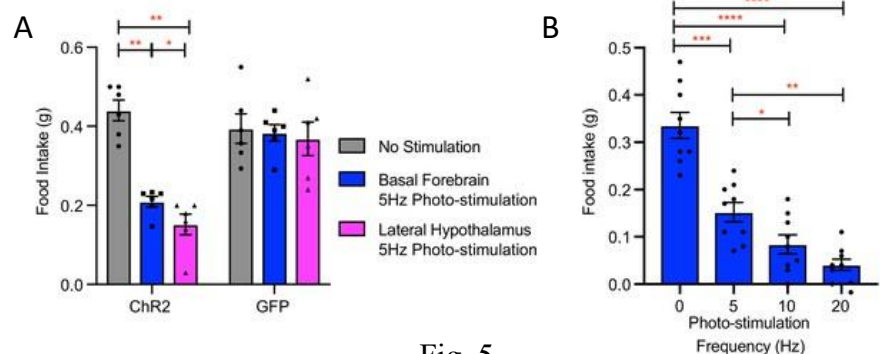


Fig. 5.

targets of basal forebrain Glu+ neurons. Towards this, AAV-Flex-ChR2-GFP virus has been delivered to the basal forebrain, while AAV-Flex-GFP virus has been delivered to the LH of *Vglut2-Cre* mice. We have finished the feeding experiments and as shown in Fig. 5, where local photostimulation in the LH potently inhibited feeding, the extent of which is similar to that observed with direct activation of basal forebrain Glu+ neurons (Fig. 5A). Notably, the effect on feeding inhibition was dependent on laser frequency (Fig. 5B). The studies are now wrapping up, with verification of accurate viral delivery and induction of c-Fos in the LH neurons after photostimulation have been completed.

Major goal 14 (Dr. Tong): Here the goal has been 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, while at the same time AAV-Flex-hM4Di-mCherry was delivered to the LH. Initial experiments on verification of viral delivery have been confirmed. Our results show that inhibition of LH neurons failed to reduce food inhibition effects by DDB *Vglut2* neuron activation, and we reasoned that the results observed might be due to activation of collaterals in other downstream neurons is sufficient to reduce feeding. We are currently testing this idea by examining whether DBB projections to the lateral habenula reduce feeding.

Training and professional development

Dr. Zhiying Jiang (postdoc) and Ms. Jing Cai (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 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 are required to present their own research, as well present a journal club on the papers from peers. Importantly, both trainees have regular weekly meetings with me. Through these various platforms, both trainees have ample exposure to extended mentoring and research exposure. With the pandemic, almost all seminar series with the UT and TMC have implemented a virtual format, in which they regularly attend. Unfortunately, with the impact of the pandemic, it is not easy 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. Of note, Ms. Cai and Dr. Tong have just attended this year's SfN meeting in November 2022 at San Diego

Lab personnel in Arenkiel lab include Ms. Mayuri Patel and Ms. Peyshyuan Chin. Both 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 recordings, and behavioral analysis, and have also been learning imaging and *in vivo* electrophysiological recording methods. They are utilizing these skills towards the proposed studies. All are making excellent progress towards the stated project goals, and have maintained timely committee meeting progress reports. As another training opportunity, both Ms. Patel and Ms. Chin have attended this year's SFN meeting. 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. Also due to school/daycare shut down with online course at home, the time spent on experiments on lab members with young kids has also been reduced. Despite facing these adversaries, we have managed to make the planned progress. The approved additional time will allow us to complete all proposed work.

PRODUCTS

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

1. Anatomy and Function of Ventral Tegmental Area Glutamate Neurons. Cai J, Tong Q. Front Neural Circuits. 2022 May 20;16:867053. doi: 10.3389/fncir.2022.867053. eCollection 2022. PMID: 35669454. Acknowledgement of the federal support: Yes.
2. An estrogen-sensitive hypothalamus-midbrain neural circuit controls thermogenesis and physical activity. Ye H, Feng B, Wang C, Saito K, Yang Y, Ibrahimi L, Schaul S, Patel N, Saenz L, Luo P, Lai P, Torres V, Kota M, Dixit D, Cai X, Qu N, Hyseni I, Yu K, Jiang Y, Tong Q, Sun Z, Arenkiel BR, He Y, Xu P, Xu Y. Sci Adv. 2022 Jan 21;8(3): eabk0185. doi: 10.1126/sciadv.abk0185. Epub 2022 Jan 19.

Program Director/Principal Investigator (Last, First, Middle): Arenkiel, Benjamin R. & Tong, Qingchun

PMID: 35044814. Acknowledgement of the federal support: Yes.

- Hypothalamic CRH neurons: A crossroad between stress and metabolism. Jiang Z. and Tong Q. Current Opinion in Endocrine and Metabolic Research 2022, 26:100384. Acknowledgement of the federal support: Yes.

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

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name	Benjamin Arenkiel
Project Role:	PI
ORCID ID	0000-0001-9047-2420
Nearest person month worked	12
Contribution to project	Supervision
Funding Support	This Award and NIH grants

Name	Qingchun Tong
Project Role:	PI
ORCID ID	0000-0002-4561-2540
Nearest person month worked	4
Contribution to project	Supervision
Funding Support	This Award and NIH grants

Name	Zhiying Jiang (Site 2)
Project Role:	Postdoctoral researcher
ORCID ID	0000-0002-3838-2791
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
Project Role:	Graduate student
ORCID ID	Not available
Nearest person month worked	12
Contribution to project	Circuit labeling, viral injections, and behavioral analysis.
Funding Support	This Award

Name	Peyshyuan Chin
Project Role:	Graduate student
ORCID ID	Not available
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
ORCID ID	Not available
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. Additional manuscripts are under review and will be reported in the next report.



Anatomy and Function of Ventral Tegmental Area Glutamate Neurons

Jing Cai^{1,2} and Qingchun Tong^{1,2*}

¹ Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, UTHealth McGovern Medical School, Houston, TX, United States, ² Neuroscience Program, The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences, Houston, TX, United States

The ventral tegmental area (VTA) is well known for regulating reward consumption, learning, memory, and addiction behaviors through mediating dopamine (DA) release in downstream regions. Other than DA neurons, the VTA is known to be heterogeneous and contains other types of neurons, including glutamate neurons. In contrast to the well-studied and established functions of DA neurons, the role of VTA glutamate neurons is understudied, presumably due to their relatively small quantity and a lack of effective means to study them. Yet, emerging studies have begun to reveal the importance of glutamate release from VTA neurons in regulating diverse behavioral repertoire through a complex intra-VTA and long-range neuronal network. In this review, we summarize the features of VTA glutamate neurons from three perspectives, namely, cellular properties, neural connections, and behavioral functions. Delineation of VTA glutamatergic pathways and their interactions with VTA DA neurons in regulating behaviors may reveal previously unappreciated functions of the VTA in other physiological processes.

OPEN ACCESS

Edited by:

Elizabeth Hanson Moss,
Baylor College of Medicine,
United States

Reviewed by:

Giulia Chiacchierini,
Italian Institute of Technology (IIT), Italy
Robert James Wickham,
Elizabethtown College, United States

*Correspondence:

Qingchun Tong
qingchun.tong@uth.tmc.edu

Received: 31 January 2022

Accepted: 30 March 2022

Published: 20 May 2022

Citation:

Cai J and Tong Q (2022) Anatomy and Function of Ventral Tegmental Area Glutamate Neurons.
Front. Neural Circuits 16:867053.
doi: 10.3389/fncir.2022.867053

Keywords: VTA, VGLUT2, dopamine, neural circuits, reward, addiction

INTRODUCTION

The ventral tegmental area (VTA) located in the midbrain controls diverse behavioral repertoire, including reward processing, aversion, stress modulation, drug addiction, learning, and memory (Haber and Fudge, 1997; Ikemoto, 2007; Arias-Carrion et al., 2010; Polter and Kauer, 2014). The VTA functional diversity is partly reflected by its cellular and circuit heterogeneities. The VTA is composed of ~60% dopaminergic neurons (DA neurons), ~35% GABAergic neurons (GABA neurons), and ~5% glutamate neurons (glutamate neurons) (Nair-Roberts et al., 2008; Yamaguchi et al., 2015). Other than these three classical neurotransmitters, VTA neurons also release peptides, including cholecystokinin, neurotensin, corticotropin-releasing factor, brain-derived neurotrophic factor, and calbindin (Seroogy et al., 1988; Jayaraman et al., 1990; Hyman et al., 1994; Liang et al., 1996; Grieder et al., 2014). Co-release of classical neurotransmitters and neuropeptides increases the cellular heterogeneity of VTA neurons. At the circuit level, these diverse VTA neurons make direct synaptic contacts with multiple brain regions, including the prefrontal cortex (PFC), the nucleus accumbens (NACc), the pedunculopontine tegmentum (PPTg), the laterodorsal tegmentum nucleus (LDTg), the lateral habenula (LHb), the periaqueductal gray (PAG), the bed nucleus of the stria terminalis (BNST), the lateral hypothalamus (LH), the ventral pallidum (VP), and the dorsal raphe nucleus (DRN) (Morales and Margolis, 2017).

Intricate interactions among different VTA neuron populations and their diverse input and output projections mediate the behavioral repertoire of VTA function.

The DA neurons in the VTA have been a hot topic and are considered the major therapeutic target for treating reward-related disorders, such as drug addictions and mood disorders due to their key roles in directing reward-related responses (Price and Drevets, 2010; Polter and Kauer, 2014). Other than VTA DA neurons, increasing studies suggest that VTA GABA neurons are also important for behavioral regulation by forming local synapses onto DA neurons or sending projections to remote brain sites. VTA GABA neurons have been found to modulate reward consumption, depression, stress, and sleep by altering DA release from neighboring DA neurons (Van Zessen et al., 2012; Bouarab et al., 2019; Chowdhury et al., 2019; Yu et al., 2019, 2021; Galaj et al., 2020). Long-range connections of GABA neurons also intertwine with VTA dopaminergic circuits (Beier et al., 2019; Bouarab et al., 2019), suggesting that the function of VTA GABA neurons is at least partially dependent on DA release. Relatively less has been focused on VTA glutamate signaling due to their rare existence in the VTA. However, recent studies suggest that VTA glutamate neurons regulate reward reinforcement, aversive behaviors, wakefulness, and defensive behaviors (Mangieri et al., 2019; Yu et al., 2019; Barbano et al., 2020; Zell et al., 2020), emphasizing the importance of VTA glutamate release. This review aims to give a general overview of VTA glutamate neurons in the aspects of cellular features, neural connections, and behavioral functions that can be DA dependent or independent.

MOLECULAR AND ELECTROPHYSIOLOGICAL FEATURES OF VENTRAL TEGMENTAL AREA GLUTAMATE NEURONS

In the brain, glutamate is synthesized from glutamine by glutaminase and then packaged into vesicles by vesicular glutamate transporters (Vgluts) for its synaptic release (Takamori et al., 2000). Glutamate neurons in the VTA mainly express Vglut2 but not Vglut1 or Vglut3 (Yamaguchi et al., 2007, 2011). Vglut2-expressing glutamate neurons are mostly located in the anterior and middle line of the VTA, where these neurons outnumber DA neurons (Yamaguchi et al., 2007). In addition, Vglut2-expressing neurons form asymmetry synapses and release glutamate to downstream targets once activated, confirming that VTA Vglut2-expressing neurons release glutamate and form excitatory synapse (Chuhma et al., 2004; Hnasko et al., 2012). In addition, about 35% of NAcC-projecting VTA neurons and 66% of PFC-projecting VTA neurons contain Vglut2 (Stuber et al., 2010; Tecuapetla et al., 2010; Yamaguchi et al., 2011; Gorelova et al., 2012), implicating a role for glutamate release from the VTA to both NAcC and PFC in mediating the VTA function.

The VTA glutamate neurons are heterogeneous. A subset of these neurons only releases glutamate, while others also express tyrosine hydroxylase (TH) and may co-release DA

(Hnasko et al., 2010, 2012; Stuber et al., 2010; Tecuapetla et al., 2010; Morales and Root, 2014; Root et al., 2016). Surprisingly, about 50% of Vglut2⁺ TH⁺ expressing neurons do not express vesicular monoamine transporter 2 (Vmat2), DA transporter (DAT), or DA receptor 2 (D2) (Li et al., 2013), which are either required for DA release or normally expressed in VTA DA neurons, indicating that this subset of Vglut2⁺ TH⁺ expressing neurons may solely release glutamate despite expressing TH. The release of glutamate from VTA DA neurons is speculated to be responsible for fast responses (Chuhma et al., 2004; Lavin et al., 2005). Interestingly, there appears to be a development requirement for Vglut2 expression in the VTA. The knockout of Vglut2 from midbrain DA neurons in the neonatal stage results in morphological abnormalities of these neurons (Fortin et al., 2012; Papathanou et al., 2018) and alterations in risk-taking behaviors as well as DA responses to amphetamine (Birgner et al., 2010; Alsio et al., 2011; Berube-Carriere et al., 2012). However, the knockout of Vglut2 from DA neurons in the adult stage had minimal effects on either morphology or behaviors (Papathanou et al., 2018). These contrasting observations indicate an important function of developmentally transient Vglut2 expression in VTA DA neurons. The transient expression of Vglut2 during development in a subset of VTA-DA neurons is supported by a reduced proportion of VTA Vglut2⁺ TH⁺ neurons in adulthood compared to neonates (Berube-Carriere et al., 2009, 2012; Moss et al., 2011). In line with this, neonatal knockout of Vmat2 from Vglut2 expressing neurons also leads to almost complete deletion of Vmat2 expression in the VTA and results in developmental abnormalities and death (Cai et al., 2021), reminiscent phenotypes of knockout of Vmat2 at the whole body (Wang et al., 1997), suggesting extensive transient co-localization between Vmat2 and Vglut2 in the VTA during development. It is interesting to note that the off-expression of Vglut2 in adulthood is an integral part of normal VTA DA neuron development and function as over-expression of Vglut2 in these neurons in adulthood causes neuronal death (Steinkellner et al., 2018). These observations suggest an importance of developmental Vglut2 expression, potentially *via* glutamate release, in the normal development of VTA DA neurons. Thus, it appears that VTA Vglut2⁺ TH⁺ neurons in adulthood may represent only a small subset of Vglut2⁺ TH⁺ neurons in neonates, in which residual Vglut2 expression remains.

A small subset of VTA glutamate neurons co-expresses vesicular GABA transporter (Vgat) and co-releases GABA (Root et al., 2014; Yoo et al., 2016). Most VTA Vglut2⁺ Vgat⁺ neurons are located in the anterior portion of the interfascicular nucleus (IF) (Root et al., 2020). These neurons can establish adjacent asymmetric and symmetric synapses on downstream neurons (Root et al., 2014), suggesting an intriguing co-existence of both excitatory glutamatergic and inhibitory GABAergic synapses formed by these individual presynaptic neurons. Consistently, activation of these neurons is able to induce both fast excitation and inhibition in downstream neurons, the net responses of which seem to be projection-specific (Root et al., 2014; Yoo et al., 2016). Another small subset of Vglut2⁺ neurons expresses both TH and glutamic acid decarboxylases (GADs) (Root et al., 2014),

another marker for GABAergic neurons. However, whether these neurons co-release all three neurotransmitters remains to be demonstrated. Although VTA neurons are known to co-express abundant neuropeptides (Seroogy et al., 1988; Jayaraman et al., 1990; Hyman et al., 1994; Liang et al., 1996; Grieder et al., 2014), relatively less is known about whether VTA glutamate neurons also co-release peptides (Wise and Morales, 2010).

The VTA glutamate neurons exhibit a unique electric property. The hyperpolarization-activated cation current (I_h) mediated by hyperpolarization-activated cyclic nucleotide-gated channels is an important contributor to both resting membrane potential and dendritic integration (Pape, 1996; Robinson and Siegelbaum, 2003). Similar to medial, but contrary to lateral VTA DA neurons, VTA glutamate neurons have a small or no I_h , which leads to a lower resting membrane potential (Hnasko et al., 2012). In addition, compared to lateral DA neurons, VTA glutamate neurons also present a shallower afterhyperpolarization (AHP) and consequent higher firing rate (Hnasko et al., 2012). In addition, *ex vivo* electrophysiological recordings reveal that VTA glutamate neurons present a relatively more hyperpolarized resting membrane potential, greater rheobase, and lower spontaneous firing frequency compared to GABA neurons (Miranda-Barrientos et al., 2021). These studies suggest that VTA glutamate neurons are more excitable than other VTA neurons.

NEURAL CONNECTIONS OF VENTRAL TEGMENTAL AREA GLUTAMATE NEURONS

Intrinsic Connections

Accumulating data support an existence of strong intrinsic connections among VTA neurons. Anatomical and electrophysiological data reveal that VTA GABA neurons form local synapses onto other VTA neurons (Omelchenko and Sesack, 2009; Matsui and Williams, 2011). For DA neurons, the inhibitory input from local GABA neurons appears to be weaker than long-range inhibitory ones (Omelchenko and Sesack, 2009). However, disrupting local VTA GABA release causes major malfunctions in stress and anxiety modulation, which can be rescued by restoring GABAergic control on DA neurons (Van Zessen et al., 2012; Bouarab et al., 2019; Chowdhury et al., 2019; Yu et al., 2021), suggesting the importance of intrinsic GABAergic action in controlling VTA DA neurons and maintaining normal behavioral responses.

Ultrastructural studies suggest that ~50% of VTA fibers forming local synaptic contacts contain Vglut2, and these glutamatergic fibers establish asymmetric synapses on both DA and non-DA neurons (Dobi et al., 2010). Optical stimulation of VTA glutamate neurons elicits AMPA/NMDA receptor-dependent firing in NAcC-projecting DA neurons *via* monosynaptic connections, suggesting a local excitatory synapse connection between glutamate and DA neurons (Wang et al., 2015). This local excitatory connection is important as VTA glutamate neuron activation leads to changes in reward-related responses (Wang et al., 2015; Yoo et al., 2016). Interestingly,

the D2 receptor has been detected in VTA glutamate neurons, and the application of D2 agonists is able to hyperpolarize these neurons (Hnasko et al., 2012), suggesting a reciprocal connection between VTA DA and glutamate neurons. VTA glutamate neurons also receive local inhibitory input from GABA neurons, thereby allowing GABA neurons to restrain neuronal activities of VTA glutamate neurons (Yu et al., 2019). Although none has been reported, it is conceivable that VTA glutamate neurons send local excitatory inputs to VTA GABA neurons.

Long-Range Connections

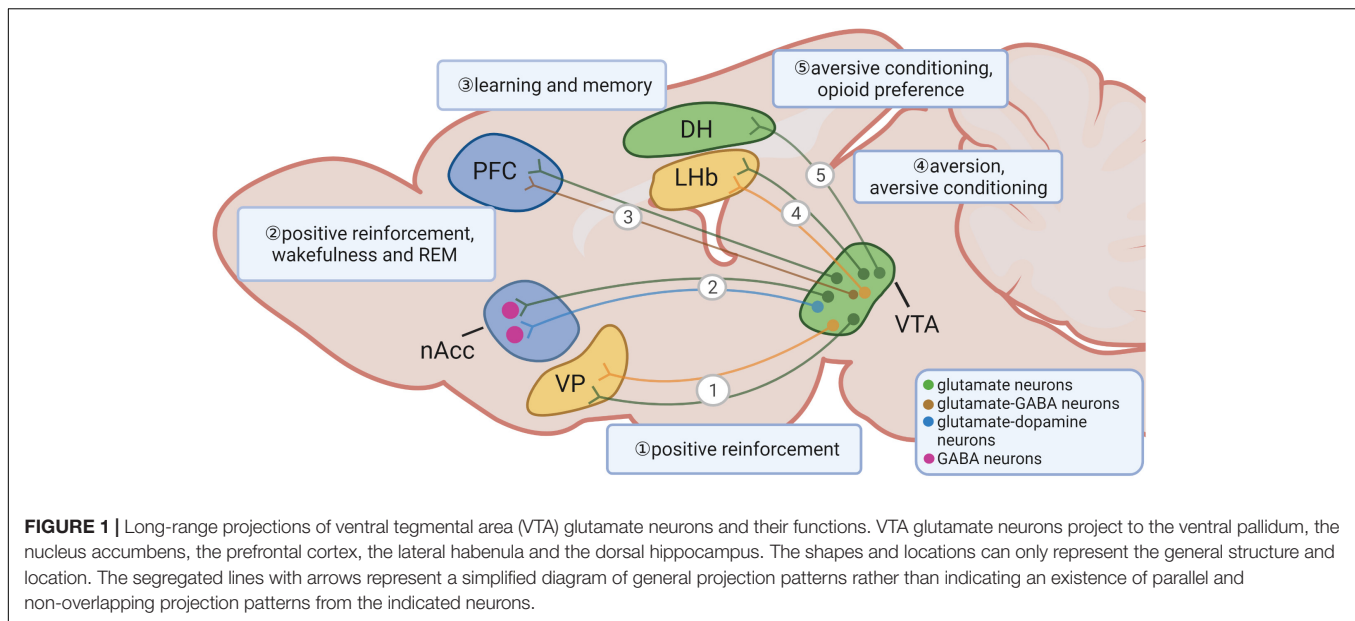
Given the known heterogeneity of VTA neurons, it is important to investigate the differential remote connectivity of these neurons. VTA DA neurons mainly form synaptic contacts with limbic and cortical regions, including the NAcC, amygdala, and PFC (Hnasko et al., 2012; Taylor et al., 2014; Morales and Margolis, 2017). Ventral tegmental area glutamate neurons establish long-range connections either in parallel with or distinct from those of VTA DA neurons. An input–output map has been drawn using a monosynaptic viral tracing approach. In general, VTA glutamate neurons have a grossly similar input–output pattern to VTA DA neurons but with some distinct features (Beier et al., 2015, 2019). For example, similar to VTA DA neurons, VTA glutamate neurons that project to the NAcC receive more inputs from the striatal site and fewer projections from the DRN. Yet, distinct from VTA DA neurons, glutamate neurons that project to the PFC and the amygdala receive more inputs from the VP and the LH (Beier et al., 2019).

A) Afferent inputs

Monosynaptic rabies virus tracing results show that VTA glutamate neurons receive strong upstream projections from the LH, DRN, cortical regions, VP, LDTg, and PAG (Faget et al., 2016). Similar to VTA DA neurons, VTA glutamate neurons receive heavy inputs from the LH and the DRN. However, VTA glutamate neurons receive proportionally more projections from cortical regions, while DA neurons receive more projections from striatal regions (Faget et al., 2016). Cortical inputs to VTA glutamate neurons are from a variety of subregions, mainly the somatosensory motor, insular, and cingulate cortices (Faget et al., 2016). Although the general afferent projections to VTA glutamate neurons are clear, specific upstream neuronal subtypes and their detailed mechanisms in the regulation of VTA glutamate neuron activities are largely underexplored.

B) Efferent projections

The VTA glutamate neurons send projections that are parallel with VTA DA neurons, such as NAcC and PFC, as well as to regions with few VTA dopaminergic inputs, such as LHb and VP (Hnasko et al., 2012; Morales and Root, 2014). As mentioned above, VTA glutamate neurons send abundant projections to the ventral striatum, especially the medial shell of the NAcC (**Figure 1**) (Yamaguchi et al., 2011; Hnasko et al., 2012). TH is present in the majority of VTA glutamate neurons that project to the NAcC, and more than half of TH expressing neurons projecting to the NAcC co-release glutamate



(Stuber et al., 2010; Tecuapetla et al., 2010; Yamaguchi et al., 2011; Hnasko et al., 2012; Mongia et al., 2019), VTA glutamate neurons can drive positive reinforcement by releasing glutamate in the NACc even in the absence of DA release (Zell et al., 2020). VTA glutamatergic projections in the NACc are also sufficient to promote wakefulness and rapid eye movement (REM) independent of DA release (Yu et al., 2019).

Electrophysiological data suggest that glutamate release from the VTA depolarizes PFC neurons and evokes monosynaptic excitatory postsynaptic potentials (EPSPs) (Lavin et al., 2005; Yamaguchi et al., 2011; Gorelova et al., 2012; Hnasko et al., 2012). In addition, VTA glutamate neuron-induced EPSPs in the PFC can be eliminated by glutamate but not DA antagonists (Hnasko et al., 2012). Ultrastructure studies show that ~60% of PFC projecting VTA neurons contain Vglut2, half of which are TH positive (Yamaguchi et al., 2011; Gorelova et al., 2012), suggesting that Vglut2 positive neurons compose a large portion of mesocortical neurons.

As previously mentioned, VTA glutamate neurons also send abundant projections to structures that receive less DA inputs, such as the LHB and the VP (**Figure 1**) (Hnasko et al., 2012; Root et al., 2014; Taylor et al., 2014; Yoo et al., 2016). The majority of Vglut2 positive fibers in the LHB lack TH, and DA release is not detected after optical activation of TH⁺ fibers from the VTA (Hnasko et al., 2012; Taylor et al., 2014). Surprisingly, axons from most mesohabenular neurons are found to co-express Vglut2 and markers of GABAergic neurons, and form both symmetric and asymmetric synapses onto LHB neurons (Root et al., 2014, 2018b; Yoo et al., 2016). Selective *ex vivo* activation of mesohabenular axons evokes GABA_A-mediated outward and AMPA-mediated inward currents on individual neurons (Root et al., 2014). While these neurons co-release two neurotransmitters, glutamate and GABA are packaged and released from distinct pools of vesicles and synapses, suggesting an independent mechanism in accumulation, release, and recycling of each neurotransmitter

(Root et al., 2018b). Interestingly, *ex vivo* train activation of glutamatergic projections to the LHB produces a net inhibitory effect, leading to a persistent decrease in the firing rate of postsynaptic cells (Yoo et al., 2016), suggesting a dominant role of GABA over glutamate release.

The VTA Vglut2-expressing fibers are present at the rostra-caudal “finger-like” extent of the VP. Similar to VTA Vglut2-expressing fibers in the LHB, the majority of these fibers lack TH expression (Hnasko et al., 2012). Optogenetic stimulation of these fibers elicits both AMPAR- and NMDAR-mediated currents, suggesting the existence of functional excitatory glutamatergic terminals (Hnasko et al., 2012; Yoo et al., 2016). Interestingly, optical activation induces gabazine-sensitive inhibitory postsynaptic currents (IPSCs) in the VP (Yoo et al., 2016), suggesting that VTA Vglut2 neurons projecting to the VP also co-release GABA. Unlike the net inhibitory effect from activating VTA glutamatergic fibers in the LHB, activation of VTA Vglut2 expressing terminals in the VP elicits a consistent increase of firing in postsynaptic neurons, suggesting that, contrary to what has been found in the LHB, the effect by glutamate release from VTA glutamatergic fibers in the VP dominates over the effect by GABA release (Yoo et al., 2016).

The VTA dopaminergic inputs to the dorsal hippocampus (DH) have long been proposed to be involved in memory modulation (Lisman and Grace, 2005; Mcnamara et al., 2014; Rosen et al., 2015). However, this idea has been challenged due to the scarcity of dopaminergic projections found in the DH (Kempadoo et al., 2016; Takeuchi et al., 2016). A recent study shows that VTA glutamate neurons send one-way projections to the DH (Ntambi and Luscher, 2016; Adeniyi et al., 2020; Han et al., 2020). In addition, these DH-projecting glutamate neurons are TH negative and are mostly located in rostral VTA (Han et al., 2020). Interestingly, postsynaptic currents in the granule cell layer of the dentate gyrus elicited by monosynaptic VTA projections are sensitive to both GABA and AMPA receptor

antagonists, suggesting that the upstream VTA neurons co-release GABA and glutamate (Ntamat and Luscher, 2016). Thus, VTA glutamate neurons share parallel projections to those of VTA DA neurons but also exhibit distinct projections that may function independently of DA action.

BEHAVIORAL REGULATION BY VENTRAL TEGMENTAL AREA GLUTAMATE NEURONS

Behaviors Associated With Changes in Valence

Extensive research has been focused on the functional roles of VTA DA neurons, and these neurons are known to play distinct roles in both positive and negative reinforcement, resulting in preference and avoidance behaviors, respectively (Adcock et al., 2006; Brischox et al., 2009; Bromberg-Martin et al., 2010). VTA DA neurons respond with increased activities to both rewarding and aversive stimuli (Salamone and Correa, 2012), suggesting physiological implications of these neurons in response to diverse and even conflicting environmental settings. Despite complicated DA neuron responses to seemingly conflicting cues, acute activation of these neurons leads to positive reinforcement and behavioral preference (Tsai et al., 2009; Adamantidis et al., 2011; Witten et al., 2011).

Compared to VTA DA neurons, the role of VTA glutamate neurons seems to be more complicated with inconsistent responses toward rewarding and aversive cues and may induce both preference and avoidance behaviors (Root et al., 2018a). *In vivo* Ca²⁺ + recording data reveal that VTA glutamate neurons exhibit increased overall firing to both aversive and rewarding stimuli (Montardy et al., 2019; McGovern et al., 2021). Interestingly, a small population of VTA glutamate neurons increases firing to both aversive and rewarding stimulus (Root et al., 2018a), which might be explained by the functional heterogeneity of different VTA glutamate neuron populations. For instance, an *in vivo* Ca²⁺ + recording study reveals that Vglut2⁺ Vgat⁻ neurons signal cues predicting reward and Vglut2⁺ Vgat⁺ neurons signal unconditioned rewarding and aversive outcomes (Root et al., 2020). However, *in vivo* electrophysiology recording shows that the majority of VTA glutamate neurons decrease their firing, while a small subset shows no change in firing, in response to reward stimulus (Root et al., 2018a, 2020). This inconsistency might be due to the insensitivity of Ca²⁺ + indicator toward the decrease of neuronal activity (Chen et al., 2013). Nevertheless, these observations implicate VTA glutamate neurons functioning in physiological responses to various environmental cues.

Despite responses of VTA glutamate neurons to both rewarding and aversive cues, direct optical activation of VTA glutamate neurons induces conditioned place preference and appetitive instrumental conditioning (Wang et al., 2015; Yoo et al., 2016). The rewarding effects of VTA glutamate neurons are suggested to be mediated through the activation of VTA DA neurons that project to the NAc (Wang et al., 2015). However, the anatomical connection between VTA glutamate

and DA neurons in mediating the rewarding effects remains to be verified. Interestingly, VTA glutamate neurons could induce reinforcement in the absence of DA release (Zell et al., 2020), suggesting a DA-independent effect on rewarding behaviors. However, sustained stimulation of VTA glutamate neurons is less preferred and could even manifest as apparent behavioral avoidance (Yoo et al., 2016). Similarly, in contrast to the previous discussion on inducing rewarding effects, optical stimulation of VTA glutamate neurons may induce aversive escape behaviors (Barbano et al., 2020). The reasons underlying these contrasting observations are unknown but may involve different subsets of VTA glutamate neurons or different stimulation protocols, which may cause different and sometimes opposite effects on neuron activity (Wang et al., 2015; Yoo et al., 2016; Root et al., 2020; Zell et al., 2020). Therefore, VTA glutamate neurons signal both rewarding and aversive stimuli and may induce rewarding or aversive effects.

Reminiscent of conflicting behavioral outcomes by activating VTA glutamate neurons in remote projecting sites, acute activation of VTA long-range glutamatergic terminals also produces diverse and conflicting behaviors. The mesolimbic pathway is important for aversive conditioning (Pezze and Feldon, 2004; Zweifel et al., 2011). Optogenetic activation of VTA Vglut2→NACc projections induces aversion through glutamate-mediated action on local GABAergic interneurons (Qi et al., 2016). The LHB is a brain region known for its function in conditioning aversion and reward (Lammel et al., 2012; Stopper and Floresco, 2014). Acute optogenetic activation of VTA glutamatergic fibers in the LHB elicits aversion and produces aversive conditioning (Root et al., 2014). Acute activation of VTA glutamatergic terminals in the LHB, VP, and NACc induces self-stimulation (Yoo et al., 2016; Zell et al., 2020). VTA glutamatergic projections to the DH are significant for the formation and retrieval of context memories with aversive stimulus, suggesting that VTA Vglut2→DH projections represent negative valence (Han et al., 2020). The reason underlying these apparent discrepancies within VTA glutamatergic projections to different downstream regions is unknown due to the distinct roles of different subsets of VTA glutamate neurons.

Drug Addiction

The μ -opioid receptors (MORs) are well-known to mediate the rewarding analgesic effects of commonly prescribed and abused opioids (Kieffer and Gaveriaux-Ruff, 2002). It is well established that MORs modulate opioid reward through inhibition of GABA transmission and subsequent DA neuron disinhibition (Steffensen et al., 2006; Hjelmstad et al., 2013; Matsui et al., 2014; Fields and Margolis, 2015). Recent studies suggest that MORs also inhibit glutamatergic transmission onto general Vglut2 expressing synapses, suggesting a role of glutamate neurons in opioid effects (Reeves et al., 2021). In addition, opioid administration brings an immediate effect of increasing cerebral blood flow in the anterior cingulate cortex, thalamus, and amygdala (Schlaepfer et al., 1998; Kosel et al., 2008), followed by long-lasting euphoric effects (Denier et al., 2013), further indicating that glutamate transmission plays a potential role in the “rush” after opioid administration. Histological data

reveal the presence of functional MORs in VTA glutamate neurons (Miranda-Barrientos et al., 2021). Opioids, such as morphine, have been shown to activate VTA DA neurons by inhibiting GABAergic inputs and strengthening glutamatergic inputs (Jalabert et al., 2011; Chen et al., 2015). VTA glutamate transmission also plays an important role in reinstatement of heroin seeking (Bossert et al., 2004). Moreover, optically activating the VTA Vglut2→DH projections promotes opioid preference (Han et al., 2020). Thus, increasing evidence suggests that VTA glutamate neurons mediate opioid effects.

While its use in the United States gains more popularity due to the ongoing legalization process (Schulden et al., 2009), cannabis can also be aversive to certain groups of people (D'souza et al., 2004; Parsons and Hurd, 2015). Similar paradoxical effects have also been observed in experimental animals. Δ^9 -Tetrahydrocannabinol (Δ^9 -THC), the primary psychoactive ingredient of cannabis, is rewarding to squirrel monkeys but not to rhesus monkeys (Mansbach et al., 1994; John et al., 2018). In addition, low doses of Δ^9 -THC facilitate intracranial self-stimulation (ISS), while high doses inhibit ISS (Vlachou et al., 2007; Wiebelhaus et al., 2015). The Δ^9 -THC cannabis reward action is believed to be mediated by activation of the CB1 receptor on VTA GABA neurons and disinhibition of VTA DA neurons (Lupica and Riegel, 2005). Yet, the mechanisms underlying the aversive effects of cannabis are unclear. Mounting evidence demonstrates abundant expression of the CB1 receptor in VTA glutamate neurons (Melis et al., 2000, 2004; Han et al., 2017). It is proposed that the aversive effects of cannabis are due to its dose-dependent inhibition of VTA glutamate neurons. Given its rewarding effects through VTA GABA neurons, the overall effects of cannabis depend on the ratio of CB1 expression levels in the VTA GABA over VTA glutamate neurons (Han et al., 2017).

The VTA is also an important site mediating the nicotine effects. Nicotine addiction is mediated through the nicotinic acetylcholine receptor (nAChR). The expression of $\beta 2$ -containing nAChRs has been detected in VTA DA neurons (Klink et al., 2001; Changeux, 2010), and the nicotine treatment promotes VTA DA release (Mansvelder and McGehee, 2002). In addition, blocking nicotine signaling in the VTA prevents the induction of nicotine preference (Tolu et al., 2013; Durand-De Cuttoli et al., 2018). Recently, nicotinic receptors have been found in VTA glutamate neurons (Yan et al., 2018), and application of nicotine induces glutamatergic excitatory potentiation on VTA DA neurons (Gao et al., 2010; Mao et al., 2011; Yan et al., 2018). In addition, VTA glutamatergic signaling has also been implicated in regulating the reinstatement of cocaine seeking (You et al., 2007; Wise and Morales, 2010; Williams et al., 2014) and benzodiazepine effects (Heikkinen et al., 2009) by regulating VTA DA signaling. These data suggest that VTA glutamatergic inputs to DA neurons might play a role in nicotine addiction.

Other Behaviors

A) Learning and memory

The VTA glutamate neurons are known to form asymmetrical synapses and evoke EPSCs on the PFC and the DH, both of which are known to regulate learning and memory (Miller and

Cohen, 2001; Curtis and D'esposito, 2003; Matus-Amat et al., 2004). Within the PFC, VTA glutamatergic projections function as a signal for rapid behavioral responses, such as prediction error signaling (Gorelova et al., 2012). Within the DH, VTA glutamatergic projections signal negative valence to memory circuits, leading to the formation of fear-inducing context memories and to context-specific reinstatement of fear (Han et al., 2020). Thus, VTA glutamate neurons play a role in memory consolidation, at least through projecting to PFC and DH.

B) Sleep and wakefulness

The VTA DA neurons are known to promote wakefulness through DA D2-like receptors (Eban-Rothschild et al., 2016; Oishi et al., 2017). VTA glutamate neurons increase activity during wakefulness and REM sleep while decreasing firing during non-REM sleep (Yu et al., 2019). Chemogenetic activation of VTA glutamate neurons increases the duration of wakefulness during the inactive phase, and ablation of VTA glutamate neurons reduces wakefulness and increases NREM sleep during the active phase (Yu et al., 2019). It will be interesting to understand the functional anatomy of VTA glutamate neurons with the known brain circuits regulating the sleep-wake cycle.

C) Defensive behaviors

The VTA glutamate neurons encode danger signals and are required for the innate escape behavior in response to threatening stimuli (Barbano et al., 2020). Lateral hypothalamus glutamate neurons are known to increase the activity to predator odors, and optogenetic activation of LH glutamate neurons induces innate defensive behaviors (Lecca et al., 2017; Mangieri et al., 2018; Chen et al., 2020). Ultrastructure and electrophysiology data show that LH glutamate neurons send projections to VTA glutamate neurons (Kempadoo et al., 2013; Barbano et al., 2020). Interestingly, LH glutamate neurons send projections to the paraventricular nucleus (PVH) glutamate neurons, which elicit strong escape jumping behaviors upon activation (Mangieri et al., 2018). PVH glutamate neurons can in turn activate putative VTA glutamate neurons for defensive behaviors (Mangieri et al., 2019). Thus, LH glutamate neurons promote defensive behaviors through both direct projections and indirect projections *via* PVH glutamate neurons to VTA glutamate neurons.

D) Eating behaviors

The VTA neuronal signaling has been found to be negatively correlated with obesity development in human (Baik, 2013; Guo et al., 2014), and specifically, suggesting that patients with obesity have a tendency to consume more calories for compensating the low level of DA signaling. In rodents, VTA neurons have been demonstrated to mediate the function of energy balance-related hormones in feeding and body weight regulation (Fulton et al., 2006; Hommel et al., 2006; Narayanan et al., 2010; Perello and Zigman, 2012; Cone et al., 2014). Although most studies focus on VTA DA neurons, the functional roles of VTA glutamate

neurons have been emerging. Especially, activation of glucagon-like peptide-1 (GLP-1) neurons in the VTA inhibits feeding by increasing AMPA/Kainate signaling in VTA glutamate neuron projections to the NAcC (Mietlicki-Baase et al., 2014), suggesting the role of VTA glutamate neurons in regulating the mediation of GLP-1 from the VTA in feeding behaviors. In addition, the LH and PVH are two important regions for feeding behaviors and body weight homeostasis (Gutierrez et al., 2011; Bonnavion et al., 2016; Krashes et al., 2016; Sutton et al., 2016). Since these two regions send glutamatergic projections or GABAergic projections to VTA glutamate neurons or GABA neurons that regulate VTA glutamate release (Nieh et al., 2016; Tyree and De Lecea, 2017; Mangieri et al., 2019; Barbano et al., 2020), VTA glutamate neurons are well-positioned to modulate homeostatic feeding and body weight balance. Given the known role of VTA in mediating hedonic feeding and VTA glutamate neurons in regulating positive reinforcement (Rossi and Stuber, 2018), these neurons might also be involved in hedonic feeding and contribute to diet-induced obesity. Future studies are warranted to address

the function of these neurons in homeostatic and hedonic feeding as well as body weight regulation.

AUTHOR CONTRIBUTIONS

JC wrote the manuscript with significant inputs from QT. Both authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

We acknowledge the support from NIH R01 DK114279, R21NS108091, R01 DK109934, R01 DK120858, DOD W81XWH-19-1-0429 to QT, and Russell and Diana Hawkins Family Foundation Discovery Fellowship to JC. QT is the holder of the Cullen Chair in Molecular Medicine at McGovern Medical School of UTHealth. **Figure 1** was created with BioRender.com.

REFERENCES

- Adamantidis, A. R., Tsai, H. C., Boutrel, B., Zhang, F., Stuber, G. D., Budygin, E. A., et al. (2011). Optogenetic interrogation of dopaminergic modulation of the multiple phases of reward-seeking behavior. *J. Neurosci.* 31, 10829–10835. doi: 10.1523/JNEUROSCI.2246-11.2011
- Adcock, R. A., Thangavel, A., Whitfield-Gabrieli, S., Knutson, B., and Gabrieli, J. D. (2006). Reward-motivated learning: mesolimbic activation precedes memory formation. *Neuron* 50, 507–517. doi: 10.1016/j.neuron.2006.03.036
- Adeniyi, P. A., Shrestha, A., and Ogundele, O. M. (2020). Distribution of VTA glutamate and dopamine terminals, and their significance in CA1 neural network activity. *Neuroscience* 446, 171–198. doi: 10.1016/j.neuroscience.2020.06.045
- Alsio, J., Nordenankar, K., Arvidsson, E., Birgner, C., Mahmoudi, S., Halbout, B., et al. (2011). Enhanced sucrose and cocaine self-administration and cue-induced drug seeking after loss of VGLUT2 in midbrain dopamine neurons in mice. *J. Neurosci.* 31, 12593–12603. doi: 10.1523/JNEUROSCI.2397-11.2011
- Arias-Carrion, O., Stamelou, M., Murillo-Rodriguez, E., Menendez-Gonzalez, M., and Poppel, E. (2010). Dopaminergic reward system: a short integrative review. *Int. Arch. Med.* 3:24. doi: 10.1186/1755-7682-3-24
- Baik, J. H. (2013). Dopamine signaling in food addiction: role of dopamine D2 receptors. *BMB Rep.* 46, 519–526. doi: 10.5483/bmbrep.2013.46.11.207
- Barbano, M. F., Wang, H. L., Zhang, S., Miranda-Barrientos, J., Estrin, D. J., Figueroa-Gonzalez, A., et al. (2020). VTA Glutamatergic neurons mediate innate defensive behaviors. *Neuron* 107, 368–382.e8. doi: 10.1016/j.neuron.2020.04.024
- Beier, K. T., Gao, X. J., Xie, S., Deloach, K. E., Malenka, R. C., and Luo, L. (2019). Topological organization of ventral tegmental area connectivity revealed by viral-genetic dissection of input-output relations. *Cell Rep.* 26, 159–167.e6. doi: 10.1016/j.celrep.2018.12.040
- Beier, K. T., Steinberg, E. E., Deloach, K. E., Xie, S., Miyamichi, K., Schwarz, L., et al. (2015). Circuit architecture of VTA dopamine neurons revealed by systematic input-output mapping. *Cell* 162, 622–634. doi: 10.1016/j.cell.2015.07.015
- Berube-Carriere, N., Guay, G., Fortin, G. M., Kullander, K., Olson, L., Wallen-Mackenzie, A., et al. (2012). Ultrastructural characterization of the mesostriatal dopamine innervation in mice, including two mouse lines of conditional VGLUT2 knockout in dopamine neurons. *Eur. J. Neurosci.* 35, 527–538. doi: 10.1111/j.1460-9568.2012.07992.x
- Berube-Carriere, N., Riad, M., Dal Bo, G., Levesque, D., Trudeau, L. E., and Descarries, L. (2009). The dual dopamine-glutamate phenotype of growing mesencephalic neurons regresses in mature rat brain. *J. Comp. Neurol.* 517, 873–891. doi: 10.1002/cne.22194
- Birgner, C., Nordenankar, K., Lundblad, M., Mendez, J. A., Smith, C., Le Greves, M., et al. (2010). VGLUT2 in dopamine neurons is required for psychostimulant-induced behavioral activation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 389–394. doi: 10.1073/pnas.0910986107
- Bonnavion, P., Mickelsen, L. E., Fujita, A., De Lecea, L., and Jackson, A. C. (2016). Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. *J. Physiol.* 594, 6443–6462. doi: 10.1113/JP271946
- Bossert, J. M., Liu, S. Y., Lu, L., and Shaham, Y. (2004). A role of ventral tegmental area glutamate in contextual cue-induced relapse to heroin seeking. *J. Neurosci.* 24, 10726–10730. doi: 10.1523/JNEUROSCI.3207-04.2004
- Bouarab, C., Thompson, B., and Polter, A. M. (2019). VTA GABA neurons at the interface of stress and reward. *Front. Neural. Circuits* 13:78. doi: 10.3389/fncir.2019.00078
- Brischoux, F., Chakraborty, S., Brierley, D. I., and Ungless, M. A. (2009). Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4894–4899. doi: 10.1073/pnas.0811507106
- Bromberg-Martin, E. S., Matsumoto, M., and Hikosaka, O. (2010). Dopamine in motivational control: rewarding, aversive, and alerting. *Neuron* 68, 815–834. doi: 10.1016/j.neuron.2010.11.022
- Cai, J., Cassidy, R. M., Morrill, J., and Tong, Q. (2021). “Neurotransmitter co-transmission in brain control of feeding and body weight,” in *Neuronal Signaling in Metabolic Regulation*, 1st Edn, ed. Q. Tong (Boca Raton, FL: CRC Press).
- Changeux, J. P. (2010). Nicotine addiction and nicotinic receptors: lessons from genetically modified mice. *Nat. Rev. Neurosci.* 11, 389–401. doi: 10.1038/nrn2849
- Chen, L., Cai, P., Wang, R. F., Lu, Y. P., Chen, H. Y., Guo, Y. R., et al. (2020). Glutamatergic lateral hypothalamus promotes defensive behaviors. *Neuropharmacology* 178:108239. doi: 10.1016/j.neuropharm.2020.10.8239
- Chen, M., Zhao, Y., Yang, H., Luan, W., Song, J., Cui, D., et al. (2015). Morphine disinhibits glutamatergic input to VTA dopamine neurons and promotes dopamine neuron excitation. *Elife* 4:e09275. doi: 10.7554/eLife.09275
- Chen, T. W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohuan, A., et al. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature* 499, 295–300. doi: 10.1038/nature12354
- Chowdhury, S., Matsubara, T., Miyazaki, T., Ono, D., Fukatsu, N., Abe, M., et al. (2019). GABA neurons in the ventral tegmental area regulate non-rapid eye movement sleep in mice. *Elife* 8:e44928. doi: 10.7554/eLife.44928
- Chuhma, N., Zhang, H., Masson, J., Zhuang, X., Sulzer, D., Hen, R., et al. (2004). Dopamine neurons mediate a fast excitatory signal via their glutamatergic synapses. *J. Neurosci.* 24, 972–981. doi: 10.1523/JNEUROSCI.4317-03.2004

- Cone, J. J., Mccutcheon, J. E., and Roitman, M. F. (2014). Ghrelin acts as an interface between physiological state and phasic dopamine signaling. *J. Neurosci.* 34, 4905–4913. doi: 10.1523/JNEUROSCI.4404-13.2014
- Curtis, C. E., and D'esposito, M. (2003). Persistent activity in the prefrontal cortex during working memory. *Trends Cogn. Sci.* 7, 415–423. doi: 10.1016/s1364-6613(03)00197-9
- D'souza, D. C., Perry, E., Macdougall, L., Ammerman, Y., Cooper, T., Wu, Y. T., et al. (2004). The psychotomimetic effects of intravenous delta-9-tetrahydrocannabinol in healthy individuals: implications for psychosis. *Neuropsychopharmacology* 29, 1558–1572. doi: 10.1038/sj.npp.1300496
- Denier, N., Schmidt, A., Gerber, H., Schmid, O., Riecher-Rossler, A., Wiesbeck, G. A., et al. (2013). Association of frontal gray matter volume and cerebral perfusion in heroin addiction: a multimodal neuroimaging study. *Front. Psychiatry* 4:135. doi: 10.3389/fpsy.2013.00135
- Dobi, A., Margolis, E. B., Wang, H. L., Harvey, B. K., and Morales, M. (2010). Glutamatergic and nonglutamatergic neurons of the ventral tegmental area establish local synaptic contacts with dopaminergic and nondopaminergic neurons. *J. Neurosci.* 30, 218–229. doi: 10.1523/JNEUROSCI.3884-09.2010
- Durand-De Cuttoli, R., Mondoloni, S., Marti, F., Lemoine, D., Nguyen, C., Naude, J., et al. (2018). Manipulating midbrain dopamine neurons and reward-related behaviors with light-controllable nicotinic acetylcholine receptors. *Elife* 7:e37487. doi: 10.7554/eLife.37487
- Eban-Rothschild, A., Rothschild, G., Giardino, W. J., Jones, J. R., and De Lecea, L. (2016). VTA dopaminergic neurons regulate ethologically relevant sleep-wake behaviors. *Nat. Neurosci.* 19, 1356–1366. doi: 10.1038/nn.4377
- Faget, L., Osakada, F., Duan, J., Ressler, R., Johnson, A. B., Proudfoot, J. A., et al. (2016). Afferent inputs to neurotransmitter-defined cell types in the ventral tegmental area. *Cell Rep.* 15, 2796–2808. doi: 10.1016/j.celrep.2016.05.057
- Fields, H. L., and Margolis, E. B. (2015). Understanding opioid reward. *Trends Neurosci.* 38, 217–225. doi: 10.1016/j.tins.2015.01.002
- Fortin, G. M., Bourque, M. J., Mendez, J. A., Leo, D., Nordenankar, K., Birgner, C., et al. (2012). Glutamate corelease promotes growth and survival of midbrain dopamine neurons. *J. Neurosci.* 32, 17477–17491. doi: 10.1523/JNEUROSCI.1939-12.2012
- Fulton, S., Pissios, P., Manchon, R. P., Stiles, L., Frank, L., Pothos, E. N., et al. (2006). Leptin regulation of the mesoaccumbens dopamine pathway. *Neuron* 51, 811–822. doi: 10.1016/j.neuron.2006.09.006
- Galaj, E., Han, X., Shen, H., Jordan, C. J., He, Y., Humburg, B., et al. (2020). Dissecting the role of GABA neurons in the VTA versus SNr in opioid reward. *J. Neurosci.* 40, 8853–8869. doi: 10.1523/JNEUROSCI.0988-20.2020
- Gao, M., Jin, Y., Yang, K., Zhang, D., Lukas, R. J., and Wu, J. (2010). Mechanisms involved in systemic nicotine-induced glutamatergic synaptic plasticity on dopamine neurons in the ventral tegmental area. *J. Neurosci.* 30, 13814–13825. doi: 10.1523/JNEUROSCI.1943-10.2010
- Gorelova, N., Mulholland, P. J., Chandler, L. J., and Seamans, J. K. (2012). The glutamatergic component of the mesocortical pathway emanating from different subregions of the ventral midbrain. *Cereb. Cortex* 22, 327–336. doi: 10.1093/cercor/bhr107
- Grieder, T. E., Herman, M. A., Contet, C., Tan, L. A., Vargas-Perez, H., Cohen, A., et al. (2014). VTA CRF neurons mediate the aversive effects of nicotine withdrawal and promote intake escalation. *Nat. Neurosci.* 17, 1751–1758. doi: 10.1038/nn.3872
- Guo, J., Simmons, W. K., Herscovitch, P., Martin, A., and Hall, K. D. (2014). Striatal dopamine D2-like receptor correlation patterns with human obesity and opportunistic eating behavior. *Mol. Psychiatry* 19, 1078–1084. doi: 10.1038/mp.2014.102
- Gutierrez, R., Lobo, M. K., Zhang, F., and De Lecea, L. (2011). Neural integration of reward, arousal, and feeding: recruitment of VTA, lateral hypothalamus, and ventral striatal neurons. *IUBMB Life* 63, 824–830. doi: 10.1002/iub.539
- Haber, S. N., and Fudge, J. L. (1997). The primate substantia nigra and VTA: integrative circuitry and function. *Crit. Rev. Neurobiol.* 11, 323–342. doi: 10.1615/critrevneurobiol.v11.i4.40
- Han, X., He, Y., Bi, G. H., Zhang, H. Y., Song, R., Liu, Q. R., et al. (2017). CB1 receptor activation on VGLUT2-expressing glutamatergic neurons underlies delta(9)-tetrahydrocannabinol (Delta(9)-THC)-induced aversive effects in mice. *Sci. Rep.* 7:12315. doi: 10.1038/s41598-017-12399-z
- Han, Y., Zhang, Y., Kim, H., Grayson, V. S., Jovasevic, V., Ren, W., et al. (2020). Excitatory VTA to DH projections provide a valence signal to memory circuits. *Nat. Commun.* 11:1466. doi: 10.1038/s41467-020-15035-z
- Heikkinen, A. E., Moykkynen, T. P., and Korpi, E. R. (2009). Long-lasting modulation of glutamatergic transmission in VTA dopamine neurons after a single dose of benzodiazepine agonists. *Neuropsychopharmacology* 34, 290–298. doi: 10.1038/npp.2008.89
- Hjelmstad, G. O., Xia, Y., Margolis, E. B., and Fields, H. L. (2013). Opioid modulation of ventral pallidal afferents to ventral tegmental area neurons. *J. Neurosci.* 33, 6454–6459. doi: 10.1523/JNEUROSCI.0178-13.2013
- Hnasko, T. S., Chuhma, N., Zhang, H., Goh, G. Y., Sulzer, D., Palmiter, R. D., et al. (2010). Vesicular glutamate transport promotes dopamine storage and glutamate corelease in vivo. *Neuron* 65, 643–656. doi: 10.1016/j.neuron.2010.02.012
- Hnasko, T. S., Hjelmstad, G. O., Fields, H. L., and Edwards, R. H. (2012). Ventral tegmental area glutamate neurons: electrophysiological properties and projections. *J. Neurosci.* 32, 15076–15085. doi: 10.1523/JNEUROSCI.3128-12.2012
- Hommel, J. D., Trinko, R., Sears, R. M., Georgescu, D., Liu, Z. W., Gao, X. B., et al. (2006). Leptin receptor signaling in midbrain dopamine neurons regulates feeding. *Neuron* 51, 801–810. doi: 10.1016/j.neuron.2006.08.023
- Hyman, C., Juhasz, M., Jackson, C., Wright, P., Ip, N. Y., and Lindsay, R. M. (1994). Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J. Neurosci.* 14, 335–347. doi: 10.1523/JNEUROSCI.14-01-00335.1994
- Ikemoto, S. (2007). Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res. Rev.* 56, 27–78. doi: 10.1016/j.brainresrev.2007.05.004
- Jalabert, M., Bourdy, R., Courtin, J., Veinante, P., Manzoni, O. J., Barrot, M., et al. (2011). Neuronal circuits underlying acute morphine action on dopamine neurons. *Proc. Natl. Acad. Sci. U.S.A.* 108, 16446–16450. doi: 10.1073/pnas.1105418108
- Jayaraman, A., Nishimori, T., Dobner, P., and Uhl, G. R. (1990). Cholecystokinin and neurotensin mRNAs are differentially expressed in subnuclei of the ventral tegmental area. *J. Comp. Neurol.* 296, 291–302. doi: 10.1002/cne.902960209
- John, W. S., Martin, T. J., Solingapuram Sai, K. K., Nader, S. H., Gage, H. D., Mintz, A., et al. (2018). Chronic delta(9)-THC in rhesus monkeys: effects on cognitive performance and dopamine D2/D3 receptor availability. *J. Pharmacol. Exp. Ther.* 364, 300–310. doi: 10.1124/jpet.117.244194
- Kempadoo, K. A., Mosharov, E. V., Choi, S. J., Sulzer, D., and Kandel, E. R. (2016). Dopamine release from the locus coeruleus to the dorsal hippocampus promotes spatial learning and memory. *Proc. Natl. Acad. Sci. U.S.A.* 113, 14835–14840. doi: 10.1073/pnas.1616515114
- Kempadoo, K. A., Tourino, C., Cho, S. L., Magnani, F., Leininger, G. M., Stuber, G. D., et al. (2013). Hypothalamic neurotensin projections promote reward by enhancing glutamate transmission in the VTA. *J. Neurosci.* 33, 7618–7626. doi: 10.1523/JNEUROSCI.2588-12.2013
- Kieffer, B. L., and Gaveriaux-Ruff, C. (2002). Exploring the opioid system by gene knockout. *Prog. Neurobiol.* 66, 285–306. doi: 10.1016/s0301-0082(02)00008-4
- Klink, R., De Kerchove D'eaerde, A., Zoli, M., and Changeux, J.-P. (2001). Molecular and physiological diversity of nicotinic acetylcholine receptors in the midbrain dopaminergic nuclei. *J. Neurosci.* 21, 1452–1463. doi: 10.1523/JNEUROSCI.21-05-01452.2001
- Kosel, M., Noss, R. S., Hammig, R., Wielepp, P., Bundeli, P., Heidbreder, R., et al. (2008). Cerebral blood flow effects of acute intravenous heroin administration. *Eur. Neuropsychopharmacol.* 18, 278–285. doi: 10.1016/j.euroneuro.2007.11.007
- Krashes, M. J., Lowell, B. B., and Garfield, A. S. (2016). Melanocortin-4 receptor-regulated energy homeostasis. *Nat. Neurosci.* 19, 206–219. doi: 10.1038/nn.4202
- Lammel, S., Lim, B. K., Ran, C., Huang, K. W., Betley, M. J., Tye, K. M., et al. (2012). Input-specific control of reward and aversion in the ventral tegmental area. *Nature* 491, 212–217. doi: 10.1038/nature11527
- Lavin, A., Nogueira, L., Lapish, C. C., Wightman, R. M., Phillips, P. E., and Seamans, J. K. (2005). Mesocortical dopamine neurons operate in distinct

- temporal domains using multimodal signaling. *J. Neurosci.* 25, 5013–5023. doi: 10.1523/JNEUROSCI.0557-05.2005
- Lecca, S., Meyer, F. J., Trusel, M., Tchenio, A., Harris, J., Schwarz, M. K., et al. (2017). Aversive stimuli drive hypothalamus-to-habenula excitation to promote escape behavior. *Elife* 6:e30697. doi: 10.7554/eLife.30697
- Li, X., Qi, J., Yamaguchi, T., Wang, H. L., and Morales, M. (2013). Heterogeneous composition of dopamine neurons of the rat A10 region: molecular evidence for diverse signaling properties. *Brain Struct. Funct.* 218, 1159–1176. doi: 10.1007/s00429-012-0452-z
- Liang, C. L., Sinton, C. M., Sonsalla, P. K., and German, D. C. (1996). Midbrain dopaminergic neurons in the mouse that contain calbindin-D28k exhibit reduced vulnerability to MPTP-induced neurodegeneration. *Neurodegeneration* 5, 313–318. doi: 10.1006/neur.1996.0042
- Lisman, J. E., and Grace, A. A. (2005). The hippocampal-VTA loop: controlling the entry of information into long-term memory. *Neuron* 46, 703–713. doi: 10.1016/j.neuron.2005.05.002
- Lupica, C. R., and Riegel, A. C. (2005). Endocannabinoid release from midbrain dopamine neurons: a potential substrate for cannabinoid receptor antagonist treatment of addiction. *Neuropharmacology* 48, 1105–1116. doi: 10.1016/j.neuropharm.2005.03.016
- Mangieri, L. R., Jiang, Z., Lu, Y., Xu, Y., Cassidy, R. M., Justice, N., et al. (2019). Defensive behaviors driven by a hypothalamic-ventral midbrain circuit. *eNeuro* 6, 1–19. doi: 10.1523/ENEURO.0156-19.2019
- Mangieri, L. R., Lu, Y., Xu, Y., Cassidy, R. M., Xu, Y., Arenkiel, B. R., et al. (2018). A neural basis for antagonistic control of feeding and compulsive behaviors. *Nat. Commun.* 9:52. doi: 10.1038/s41467-017-02534-9
- Mansbach, R. S., Nicholson, K. L., Martin, B. R., and Balster, R. L. (1994). Failure of Delta(9)-tetrahydrocannabinol and CP 55,940 to maintain intravenous self-administration under a fixed-interval schedule in rhesus monkeys. *Behav. Pharmacol.* 5, 219–225. doi: 10.1097/00008877-199404000-00014
- Mansvelder, H. D., and McGehee, D. S. (2002). Cellular and synaptic mechanisms of nicotine addiction. *J. Neurobiol.* 53, 606–617. doi: 10.1002/neu.10148
- Mao, D., Gallagher, K., and McGehee, D. S. (2011). Nicotine potentiation of excitatory inputs to ventral tegmental area dopamine neurons. *J. Neurosci.* 31, 6710–6720. doi: 10.1523/JNEUROSCI.5671-10.2011
- Matsui, A., Jarvie, B. C., Robinson, B. G., Hentges, S. T., and Williams, J. T. (2014). Separate GABA afferents to dopamine neurons mediate acute action of opioids, development of tolerance, and expression of withdrawal. *Neuron* 82, 1346–1356. doi: 10.1016/j.neuron.2014.04.030
- Matsui, A., and Williams, J. T. (2011). Opioid-sensitive GABA inputs from rostromedial tegmental nucleus synapse onto midbrain dopamine neurons. *J. Neurosci.* 31, 17729–17735. doi: 10.1523/JNEUROSCI.4570-11.2011
- Matus-Amat, P., Higgins, E. A., Barrientos, R. M., and Rudy, J. W. (2004). The role of the dorsal hippocampus in the acquisition and retrieval of context memory representations. *J. Neurosci.* 24, 2431–2439. doi: 10.1523/JNEUROSCI.1598-03.2004
- McGovern, D. J., Polter, A. M., and Root, D. H. (2021). Neurochemical signaling of reward and aversion to ventral tegmental area glutamate neurons. *J. Neurosci.* 41, 5471–5486. doi: 10.1523/JNEUROSCI.1419-20.2021
- McNamara, C. G., Tejero-Cantero, A., Trouche, S., Campo-Urriza, N., and Dupret, D. (2014). Dopaminergic neurons promote hippocampal reactivation and spatial memory persistence. *Nat. Neurosci.* 17, 1658–1660. doi: 10.1038/nn.3843
- Melis, M., Gessa, G. L., and Diana, M. (2000). Different mechanisms for dopaminergic excitation induced by opiates and cannabinoids in the rat midbrain. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 24, 993–1006. doi: 10.1016/S0278-5846(00)00119-6
- Melis, M., Perra, S., Muntoni, A. L., Pillolla, G., Lutz, B., Marsicano, G., et al. (2004). Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. *J. Neurosci.* 24, 10707–10715. doi: 10.1523/JNEUROSCI.3502-04.2004
- Mietlicki-Baase, E. G., Ortinski, P. I., Reiner, D. J., Sinon, C. G., Mccutcheon, J. E., Pierce, R. C., et al. (2014). Glucagon-like peptide-1 receptor activation in the nucleus accumbens core suppresses feeding by increasing glutamatergic AMPA/kainate signaling. *J. Neurosci.* 34, 6985–6992. doi: 10.1523/JNEUROSCI.0115-14.2014
- Miller, E. K., and Cohen, J. D. (2001). An integrative theory of prefrontal cortex function. *Annu. Rev. Neurosci.* 24, 167–202. doi: 10.1146/annurev.neuro.24.1.167
- Miranda-Barrientos, J., Chambers, I., Mongia, S., Liu, B., Wang, H. L., Mateo-Semidey, G. E., et al. (2021). Ventral tegmental area GABA, glutamate, and glutamate-GABA neurons are heterogeneous in their electrophysiological and pharmacological properties. *Eur. J. Neurosci.* 54, 4061–4084. doi: 10.1111/ejn.15156
- Mongia, S., Yamaguchi, T., Liu, B., Zhang, S., Wang, H., and Morales, M. (2019). The Ventral tegmental Area has calbindin neurons with the capability to co-release glutamate and dopamine into the nucleus accumbens. *Eur. J. Neurosci.* 50, 3968–3984. doi: 10.1111/ejn.14493
- Montardy, Q., Zhou, Z., Lei, Z., Liu, X., Zeng, P., Chen, C., et al. (2019). Characterization of glutamatergic VTA neural population responses to aversive and rewarding conditioning in freely-moving mice. *Sci. Bull.* 64, 1167–1178. doi: 10.1016/j.scib.2019.05.005
- Morales, M., and Margolis, E. B. (2017). Ventral tegmental area: cellular heterogeneity, connectivity and behaviour. *Nat. Rev. Neurosci.* 18, 73–85. doi: 10.1038/nrn.2016.165
- Morales, M., and Root, D. H. (2014). Glutamate neurons within the midbrain dopamine regions. *Neuroscience* 282, 60–68. doi: 10.1016/j.neuroscience.2014.05.032
- Moss, J., Ungless, M. A., and Bolam, J. P. (2011). Dopaminergic axons in different divisions of the adult rat striatal complex do not express vesicular glutamate transporters. *Eur. J. Neurosci.* 33, 1205–1211. doi: 10.1111/j.1460-9568.2011.07594.x
- Nair-Roberts, R. G., Chatelain-Badie, S. D., Benson, E., White-Cooper, H., Bolam, J. P., and Ungless, M. A. (2008). Stereological estimates of dopaminergic, GABAergic and glutamatergic neurons in the ventral tegmental area, substantia nigra and retrorubral field in the rat. *Neuroscience* 152, 1024–1031. doi: 10.1016/j.neuroscience.2008.01.046
- Narayanan, N. S., Guarnieri, D. J., and Dileone, R. J. (2010). Metabolic hormones, dopamine circuits, and feeding. *Front. Neuroendocrinol.* 31, 104–112. doi: 10.1016/j.yfrne.2009.10.004
- Nieh, E. H., Vander Wee, C. M., Matthews, G. A., Presbrey, K. N., Wichmann, R., Leppla, C. A., et al. (2016). Inhibitory input from the lateral hypothalamus to the ventral tegmental area disinhibits dopamine neurons and promotes behavioral activation. *Neuron* 90, 1286–1298. doi: 10.1016/j.neuron.2016.04.035
- Ntamati, N. R., and Luscher, C. (2016). VTA projection neurons releasing GABA and glutamate in the dentate gyrus. *eNeuro* 3, 1–12. doi: 10.1523/ENEURO.0137-16.2016
- Oishi, Y., Suzuki, Y., Takahashi, K., Yonezawa, T., Kanda, T., Takata, Y., et al. (2017). Activation of ventral tegmental area dopamine neurons produces wakefulness through dopamine D₂-like receptors in mice. *Brain Struct. Funct.* 222, 2907–2915. doi: 10.1007/s00429-017-1365-7
- Omelchenko, N., and Sesack, S. R. (2009). Ultrastructural analysis of local collaterals of rat ventral tegmental area neurons: GABA phenotype and synapses onto dopamine and GABA cells. *Synapse* 63, 895–906. doi: 10.1002/syn.20668
- Papathanou, M., Creed, M., Dorst, M. C., Bimpisidis, Z., Dumas, S., Pettersson, H., et al. (2018). Targeting vglut2 in mature dopamine neurons decreases mesoaccumbal glutamatergic transmission and identifies a role for glutamate co-release in synaptic plasticity by increasing baseline AMPA/NMDA ratio. *Front. Neural. Circuits* 12:64. doi: 10.3389/fncir.2018.00064
- Pape, H. C. (1996). Queer current and pacemaker: the hyperpolarization-activated cation current in neurons. *Annu. Rev. Physiol.* 58, 299–327. doi: 10.1146/annurev.ph.58.030196.001503
- Parsons, L. H., and Hurd, Y. L. (2015). Endocannabinoid signalling in reward and addiction. *Nat. Rev. Neurosci.* 16, 579–594. doi: 10.1038/nrn4004
- Perello, M., and Zigman, J. M. (2012). The role of ghrelin in reward-based eating. *Biol. Psychiatry* 72, 347–353. doi: 10.1016/j.biopsych.2012.02.016
- Pezze, M. A., and Feldon, J. (2004). Mesolimbic dopaminergic pathways in fear conditioning. *Prog. Neurobiol.* 74, 301–320. doi: 10.1016/j.pneurobio.2004.09.004
- Polter, A. M., and Kauer, J. A. (2014). Stress and VTA synapses: implications for addiction and depression. *Eur. J. Neurosci.* 39, 1179–1188. doi: 10.1111/ejn.12490

- Price, J. L., and Drevets, W. C. (2010). Neurocircuitry of mood disorders. *Neuropsychopharmacology* 35, 192–216.
- Qi, J., Zhang, S., Wang, H. L., Barker, D. J., Miranda-Barrientos, J., and Morales, M. (2016). VTA glutamatergic inputs to nucleus accumbens drive aversion by acting on GABAergic interneurons. *Nat. Neurosci.* 19, 725–733. doi: 10.1038/nn.4281
- Reeves, K. C., Kube, M. J., Grecco, G. G., Fritz, B. M., Munoz, B., Yin, F., et al. (2021). Mu opioid receptors on vGluT2-expressing glutamatergic neurons modulate opioid reward. *Addict. Biol.* 26:e12942. doi: 10.1111/adb.12942
- Robinson, R. B., and Siegelbaum, S. A. (2003). Hyperpolarization-activated cation currents: from molecules to physiological function. *Annu. Rev. Physiol.* 65, 453–480. doi: 10.1146/annurev.physiol.65.092101.142734
- Root, D. H., Barker, D. J., Estrin, D. J., Miranda-Barrientos, J. A., Liu, B., Zhang, S., et al. (2020). Distinct signaling by ventral tegmental area glutamate, GABA, and combinatorial glutamate-GABA neurons in motivated behavior. *Cell Rep.* 32:108094. doi: 10.1016/j.celrep.2020.108094
- Root, D. H., Estrin, D. J., and Morales, M. (2018a). Aversion or salience signaling by ventral tegmental area glutamate neurons. *iScience* 2, 51–62. doi: 10.1016/j.isci.2018.03.008
- Root, D. H., Mejias-Aponte, C. A., Zhang, S., Wang, H. L., Hoffman, A. F., Lupica, C. R., et al. (2014). Single rodent mesohabenular axons release glutamate and GABA. *Nat. Neurosci.* 17, 1543–1551. doi: 10.1038/nn.3823
- Root, D. H., Wang, H. L., Liu, B., Barker, D. J., Mod, L., Szocsics, P., et al. (2016). Glutamate neurons are intermixed with midbrain dopamine neurons in nonhuman primates and humans. *Sci. Rep.* 6:30615. doi: 10.1038/srep30615
- Root, D. H., Zhang, S., Barker, D. J., Miranda-Barrientos, J., Liu, B., Wang, H. L., et al. (2018b). Selective brain distribution and distinctive synaptic architecture of dual glutamatergic-GABAergic neurons. *Cell Rep.* 23, 3465–3479. doi: 10.1016/j.celrep.2018.05.063
- Rosen, Z. B., Cheung, S., and Siegelbaum, S. A. (2015). Midbrain dopamine neurons bidirectionally regulate CA3-CA1 synaptic drive. *Nat. Neurosci.* 18, 1763–1771. doi: 10.1038/nn.4152
- Rossi, M. A., and Stuber, G. D. (2018). Overlapping brain circuits for homeostatic and hedonic feeding. *Cell Metab.* 27, 42–56. doi: 10.1016/j.cmet.2017.09.021
- Salamone, J. D., and Correa, M. (2012). The mysterious motivational functions of mesolimbic dopamine. *Neuron* 76, 470–485. doi: 10.1016/j.neuron.2012.10.021
- Schlaepfer, T. E., Strain, E. C., Greenberg, B. D., Preston, K. L., Lancaster, E., Bigelow, G. E., et al. (1998). Site of opioid action in the human brain: mu and kappa agonists' subjective and cerebral blood flow effects. *Am. J. Psychiatry* 155, 470–473. doi: 10.1176/ajp.155.4.470
- Schulden, J. D., Thomas, Y. F., and Compton, W. M. (2009). Substance abuse in the United States: findings from recent epidemiologic studies. *Curr. Psychiatry Rep.* 11, 353–359. doi: 10.1007/s11920-009-0053-6
- Seroogy, K., Ceccatelli, S., Schalling, M., Hokfelt, T., Frey, P., Walsh, J., et al. (1988). A subpopulation of dopaminergic neurons in rat ventral mesencephalon contains both neurotensin and cholecystokinin. *Brain Res.* 455, 88–98. doi: 10.1016/0006-8993(88)90117-5
- Steffensen, S. C., Stobbs, S. H., Colago, E. E., Lee, R. S., Koob, G. F., Gallegos, R. A., et al. (2006). Contingent and non-contingent effects of heroin on mu-opioid receptor-containing ventral tegmental area GABA neurons. *Exp. Neurol.* 202, 139–151. doi: 10.1016/j.expneurol.2006.05.023
- Steinkellner, T., Zell, V., Farino, Z. J., Sonders, M. S., Villeneuve, M., Freyberg, R. J., et al. (2018). Role for VGLUT2 in selective vulnerability of midbrain dopamine neurons. *J. Clin. Invest.* 128, 774–788. doi: 10.1172/JCI95795
- Stopper, C. M., and Floresco, S. B. (2014). What's better for me? Fundamental role for lateral habenula in promoting subjective decision biases. *Nat. Neurosci.* 17, 33–35. doi: 10.1038/nn.3587
- Stuber, G. D., Hnasko, T. S., Britt, J. P., Edwards, R. H., and Bonci, A. (2010). Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. *J. Neurosci.* 30, 8229–8233. doi: 10.1523/JNEUROSCI.1754-10.2010
- Sutton, A. K., Myers, M. G. Jr., and Olson, D. P. (2016). The role of PVH circuits in leptin action and energy balance. *Annu. Rev. Physiol.* 78, 207–221. doi: 10.1146/annurev-physiol-021115-105347
- Takamori, S., Rhee, J. S., Rosenmund, C., and Jahn, R. (2000). Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 407, 189–194. doi: 10.1038/35025070
- Takeuchi, T., Duszkiwicz, A. J., Sonneborn, A., Spooner, P. A., Yamasaki, M., Watanabe, M., et al. (2016). Locus coeruleus and dopaminergic consolidation of everyday memory. *Nature* 537, 357–362. doi: 10.1038/nature19325
- Taylor, S. R., Badurek, S., Dileone, R. J., Nashmi, R., Minichiello, L., and Picciotto, M. R. (2014). GABAergic and glutamatergic efferents of the mouse ventral tegmental area. *J. Comp. Neurol.* 522, 3308–3334. doi: 10.1002/cne.23603
- Tecuapetla, F., Patel, J. C., Xenias, H., English, D., Tadros, I., Shah, F., et al. (2010). Glutamatergic signaling by mesolimbic dopamine neurons in the nucleus accumbens. *J. Neurosci.* 30, 7105–7110. doi: 10.1523/JNEUROSCI.0265-10.2010
- Tolu, S., Eddine, R., Marti, F., David, V., Graupner, M., Pons, S., et al. (2013). Co-activation of VTA DA and GABA neurons mediates nicotine reinforcement. *Mol. Psychiatry* 18, 382–393. doi: 10.1038/mp.2012.83
- Tsai, H. C., Zhang, F., Adamantidis, A., Stuber, G. D., Bonci, A., De Lecea, L., et al. (2009). Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. *Science* 324, 1080–1084. doi: 10.1126/science.1168878
- Tyree, S. M., and De Lecea, L. (2017). Lateral hypothalamic control of the ventral tegmental area: reward evaluation and the driving of motivated behavior. *Front. Syst. Neurosci.* 11:50. doi: 10.3389/fnsys.2017.00050
- Van Zessen, R., Phillips, J. L., Budygin, E. A., and Stuber, G. D. (2012). Activation of VTA GABA neurons disrupts reward consumption. *Neuron* 73, 1184–1194. doi: 10.1016/j.neuron.2012.02.016
- Vlachou, S., Nomikos, G. G., Stephens, D. N., and Panagis, G. (2007). Lack of evidence for appetitive effects of delta 9-tetrahydrocannabinol in the intracranial self-stimulation and conditioned place preference procedures in rodents. *Behav. Pharmacol.* 18, 311–319. doi: 10.1097/FBP.0b013e3282186cf2
- Wang, H. L., Qi, J., Zhang, S., Wang, H., and Morales, M. (2015). Rewarding effects of optical stimulation of ventral tegmental area glutamatergic neurons. *J. Neurosci.* 35, 15948–15954. doi: 10.1523/JNEUROSCI.3428-15.2015
- Wang, Y.-M., Gainetdinov, R. R., Fumagalli, F., Xu, F., Jones, S. R., Bock, C. B., et al. (1997). Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. *Neuron* 19, 1285–1296. doi: 10.1016/s0896-6273(00)80419-5
- Wieland, J. M., Grim, T. W., Owens, R. A., Lazenka, M. F., Sim-Selley, L. J., Abdullah, R. A., et al. (2015). Delta9-tetrahydrocannabinol and endocannabinoid degradative enzyme inhibitors attenuate intracranial self-stimulation in mice. *J. Pharmacol. Exp. Ther.* 352, 195–207. doi: 10.1124/jpet.114.218677
- Williams, C. L., Buchta, W. C., and Riegel, A. C. (2014). CRF-R2 and the heterosynaptic regulation of VTA glutamate during reinstatement of cocaine seeking. *J. Neurosci.* 34, 10402–10414. doi: 10.1523/JNEUROSCI.0911-13.2014
- Wise, R. A., and Morales, M. (2010). A ventral tegmental CRF-glutamate-dopamine interaction in addiction. *Brain Res.* 1314, 38–43. doi: 10.1016/j.brainres.2009.09.101
- Witten, I. B., Steinberg, E. E., Lee, S. Y., Davidson, T. J., Zalocusky, K. A., Brodsky, M., et al. (2011). Recombinase-driver rat lines: tools, techniques, and optogenetic application to dopamine-mediated reinforcement. *Neuron* 72, 721–733. doi: 10.1016/j.neuron.2011.10.028
- Yamaguchi, T., Qi, J., Wang, H. L., Zhang, S., and Morales, M. (2015). Glutamatergic and dopaminergic neurons in the mouse ventral tegmental area. *Eur. J. Neurosci.* 41, 760–772. doi: 10.1111/ejn.12818
- Yamaguchi, T., Sheen, W., and Morales, M. (2007). Glutamatergic neurons are present in the rat ventral tegmental area. *Eur. J. Neurosci.* 25, 106–118. doi: 10.1111/j.1460-9568.2006.05263.x
- Yamaguchi, T., Wang, H. L., Li, X., Ng, T. H., and Morales, M. (2011). Mesocorticolimbic glutamatergic pathway. *J. Neurosci.* 31, 8476–8490. doi: 10.1523/jneurosci.1598-11.2011
- Yan, Y., Peng, C., Arvin, M. C., Jin, X. T., Kim, V. J., Ramsey, M. D., et al. (2018). Nicotinic cholinergic receptors in VTA glutamate neurons modulate excitatory transmission. *Cell Rep.* 23, 2236–2244. doi: 10.1016/j.celrep.2018.04.062
- Yoo, J. H., Zell, V., Gutierrez-Reed, N., Wu, J., Ressler, R., Shenasa, M. A., et al. (2016). Ventral tegmental area glutamate neurons co-release GABA and promote positive reinforcement. *Nat. Commun.* 7:13697. doi: 10.1038/ncomms13697
- You, Z. B., Wang, B., Zitzman, D., Azari, S., and Wise, R. A. (2007). A role for conditioned ventral tegmental glutamate release in cocaine

- seeking. *J. Neurosci.* 27, 10546–10555. doi: 10.1523/JNEUROSCI.2967-07.2007
- Yu, X., Ba, W., Zhao, G., Ma, Y., Harding, E. C., Yin, L., et al. (2021). Dysfunction of ventral tegmental area GABA neurons causes mania-like behavior. *Mol. Psychiatry* 26, 5213–5228. doi: 10.1038/s41380-020-0810-9
- Yu, X., Li, W., Ma, Y., Tossell, K., Harris, J. J., Harding, E. C., et al. (2019). GABA and glutamate neurons in the VTA regulate sleep and wakefulness. *Nat. Neurosci.* 22, 106–119. doi: 10.1038/s41593-018-0288-9
- Zell, V., Steinkellner, T., Hollon, N. G., Warlow, S. M., Souter, E., Faget, L., et al. (2020). VTA glutamate neuron activity drives positive reinforcement absent dopamine co-release. *Neuron* 107, 864–873e4. doi: 10.1016/j.neuron.2020.06.011
- Zweifel, L. S., Fadok, J. P., Argilli, E., Garelick, M. G., Jones, G. L., Dickerson, T. M., et al. (2011). Activation of dopamine neurons is critical for aversive conditioning and prevention of generalized anxiety. *Nat. Neurosci.* 14, 620–626. doi: 10.1038/nn.2808

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Cai and Tong. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

NEUROPHYSIOLOGY

An estrogen-sensitive hypothalamus-midbrain neural circuit controls thermogenesis and physical activity

Hui Ye¹, Bing Feng², Chunmei Wang³, Kenji Saito^{3†}, Yongjie Yang³, Lucas Ibrahimi¹, Sarah Schaul¹, Nirali Patel¹, Leslie Saenz¹, Pei Luo¹, Penghua Lai¹, Valeria Torres¹, Maya Kota¹, Devin Dixit¹, Xing Cai³, Na Qu³, Ilirjana Hyseni³, Kaifan Yu³, Yuwei Jiang⁴, Qingchun Tong⁵, Zheng Sun⁶, Benjamin R. Arenkiel⁷, Yanlin He^{2*}, Pingwen Xu^{1,4*}, Yong Xu^{3,8*‡}

Estrogen receptor- α (ER α) expressed by neurons in the ventrolateral subdivision of the ventromedial hypothalamic nucleus (ER α^{vVMH}) regulates body weight in females, but the downstream neural circuits mediating this biology remain largely unknown. Here we identified a neural circuit mediating the metabolic effects of ER α^{vVMH} neurons. We found that selective activation of ER α^{vVMH} neurons stimulated brown adipose tissue (BAT) thermogenesis, physical activity, and core temperature and that ER α^{vVMH} neurons provide monosynaptic glutamatergic inputs to 5-hydroxytryptamine (5-HT) neurons in the dorsal raphe nucleus (DRN). Notably, the ER α^{vVMH} $\rightarrow$ DRN circuit responds to changes in ambient temperature and nutritional states. We further showed that 5-HT^{DRN} neurons mediate the stimulatory effects of ER α^{vVMH} neurons on BAT thermogenesis and physical activity and that ER α expressed by DRN-projecting ER α^{vVMH} neurons is required for the maintenance of energy balance. Together, these findings support a model that ER α^{vVMH} neurons activate BAT thermogenesis and physical activity through stimulating 5-HT^{DRN} neurons.

INTRODUCTION

Estrogen, an ovarian hormone, has been shown to play a crucial role in body weight control in both animals and humans (1). Estrogen produces essential anti-obesity functions through decreasing food intake (2), increasing physical activity and energy expenditure (3), and modulating fat distribution (4). Estrogen's anti-obesity effects have been suggested to be primarily mediated by estrogen receptor- α (ER α), because genetic deletion of ER α in mice leads to obesity (4).

ER α in the brain plays a vital role in maintaining energy balance and body weight control. Two ER α -expressing neuronal populations essential for estrogenic regulation of body weight were identified in the hypothalamus. ER α expressed by pro-opiomelanocortin neurons in the arcuate hypothalamic nucleus (ARC) regulates food intake (5–7), while ER α in the ventrolateral subdivision of the ventral lateral ventromedial hypothalamic nucleus (vVMH) modulates two components of energy expenditure, spontaneous physical activity and thermogenesis, in females (5, 8). The stimulatory effects of ER α^{vVMH} on energy expenditure have been consistently observed in other studies. For example, stereotaxic delivery of 17 β -estradiol (E2) into the vVMH stimulates brown adipose tissue (BAT) thermogenesis in females (9). Ablation of ER α^{vVMH} neurons induced by

NK2 homeobox transcription factor 1 (NKX2-1) knockout decreases physical activity and increases body weight in females (10). Together, these findings suggest that ER α^{vVMH} neurons play a crucial role in estrogenic regulation of energy expenditure.

In addition to regulating energy expenditure, ER α^{vVMH} neurons also modulate glucose homeostasis in females (11), as well as social investigation, mating, and aggression in both sexes (12–16). Distinct subpopulations of ER α^{vVMH} neurons with nonoverlapping gene expression signatures have been recently identified (17, 18), partially explaining these diverse and sex-specific functions. However, the specific downstream neural circuits that mediate the metabolic functions of ER α^{vVMH} neurons remain unknown.

Here, we aimed to identify the essential downstream neural populations mediating the metabolic effects of ER α^{vVMH} neurons. Using cell type-specific anterograde and retrograde neuronal tracing methods, we identified 5-hydroxytryptamine (5-HT) neurons in the dorsal raphe nucleus (DRN, 5-HT^{DRN}) as one downstream neural population directly innervated by ER α^{vVMH} neurons. Through channelrhodopsin-2 (ChR2) circuit mapping (CRACM) experiments, we confirmed the synaptic neurotransmission of these two neural nodes and further identified glutamate as the essential neurotransmitter at the ER α^{vVMH} $\rightarrow$ 5-HT^{DRN} synapse. We further examined the functions of this glutamatergic ER α^{vVMH} $\rightarrow$ 5-HT^{DRN} circuit in the regulation of thermogenesis and physical activity. Notably, we found that the ER α^{vVMH} $\rightarrow$ DRN circuit also responded dynamically to changes in ambient temperature and nutritional state. Last, we demonstrated that ER α expressed by DRN-projecting ER α^{vVMH} neurons is required for the physiological regulations of energy homeostasis in female mice.

RESULTS

Activation of ER α^{vVMH} neurons stimulates BAT thermogenesis and physical activity

To begin to evaluate the effects of E2 on this circuit, we first tested the electrophysiological responses of identified ER α^{vVMH} neurons

Copyright © 2022
The Authors, some
rights reserved;
exclusive licensee
American Association
for the Advancement
of Science. No claim to
original U.S. Government
Works. Distributed
under a Creative
Commons Attribution
NonCommercial
License 4.0 (CC BY-NC).

Downloaded from https://www.science.org at University of Texas Health Science Center Houston on November 17, 2022

¹Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, The University of Illinois at Chicago, Chicago, IL 60612, USA. ²Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, LA 70808, USA. ³Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine, Houston, TX 77030, USA. ⁴Department of Physiology and Biophysics, The University of Illinois at Chicago, Chicago, IL 60612, USA. ⁵Brown Foundation Institute of Molecular Medicine, University of Texas Health Science Center at Houston, Houston, TX 77030, USA. ⁶Department of Internal Medicine, Baylor College of Medicine, Houston, TX 77030, USA. ⁷Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA. ⁸Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA.

*Corresponding author. Email: yongxu@bcm.edu (Y.X.); pingwenxu@uic.edu (P.X.); yanlin.he@pbrc.edu (Y.H.)

†Present address: Department of Neuroscience and Pharmacology, Roy J. and Lucille A. Carver College of Medicine, The University of Iowa, Iowa City, IA, USA.

‡Lead author.

to E2 in ex vivo brain slices from ER α -ZsGreen mice. In this mouse model, ZsGreen is driven by the mouse ER α promoter and selectively expressed in ER α neurons (19). We started by measuring the effects of E2 on resting membrane potential and firing frequency of these green fluorescent neurons identified in the vVMH (fig. S1, A and B). We found that E2 treatment significantly increased both resting membrane potential and firing frequency of ER α ^{vVMH} neurons (Fig. 1, A to E). To block presynaptic inputs from afferent neurons, we preincubated brain slices with tetrodotoxin (TTX, a potent sodium channel blocker), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a competitive glutamate AMPA receptor antagonist), 2-amino-5-phosphopentanoic acid [D-AP5, a glutamate N-methyl-D-aspartate (NMDA) antagonist], and bicuculline [a competitive γ -aminobutyric acid type A (GABA_A) receptor antagonist]. In the presence of these blockers, E2 (100 nM) treatment still depolarized all recorded ER α ^{vVMH} neurons (fig. S1, C to E), suggesting a direct stimulatory effect of E2 on ER α ^{vVMH} neurons.

On the basis of these initial observations, we next used the designer receptors exclusively activated by designer drugs (DREADD) method to selectively activate ER α ^{vVMH} neurons by stereotaxic injection of adeno-associated virus 2 (AAV2)-DIO-hM3Dq-mCherry into the vVMH of Esr1-Cre mice (Fig. 1F). To exclude possible off-target effects of clozapine N-oxide (CNO) or its metabolites (20), we included wild-type (WT) + hM3Dq/saline, WT + hM3Dq/CNO, and Esr1-Cre + hM3Dq/saline mice as controls. We observed distinct expression of hM3Dq-mCherry in Esr1-Cre + hM3Dq but not in WT + hM3Dq mice (fig. S2A). As expected, CNO treatment increased both resting

membrane potential and firing frequency of hM3Dq-expressed ER α ^{vVMH} neurons (fig. S2, B to D). We found that in females, acute stimulation of ER α ^{vVMH} neurons induced by intraperitoneal injection of CNO significantly increased physical activity, as well as BAT and core temperature, suggesting an increased thermogenesis (Fig. 1, G to J). CNO-induced increases in physical activity lasted for 3 hours, while elevated BAT temperature persisted up to 6 hours following injection (Fig. 1, I and J). Notably, there was no difference in BAT thermogenesis and physical activity between WT + hM3Dq and Esr1-Cre + hM3Dq mice after saline injections (fig. S2, E and F). In addition, repeated activation of ER α ^{vVMH} neurons in ovariectomized (OVX) female mice via daily CNO injections ameliorated high-fat diet (HFD)-induced body weight gain without affecting energy intake (Fig. 1, K and L), suggesting beneficial metabolic effects of ER α ^{vVMH} activation.

We also noted similar observations in male WT + hM3Dq and Esr1-Cre + hM3Dq mice. We found that CNO-induced activation of ER α ^{vVMH} neurons significantly increased both BAT thermogenesis and physical activity, while saline injections did not induce any effects (fig. S2, G to J). These findings indicate that ER α ^{vVMH} neurons have similar metabolic regulatory effects in both females and males.

ER α ^{vVMH} neurons monosynaptically innervate 5-HT^{DRN} neurons

We previously reported that ER α ^{vVMH} neurons send condensed projections to the DRN, which surround 5-HT^{DRN} neurons (11). These findings suggest that ER α ^{vVMH} neurons may synapse on

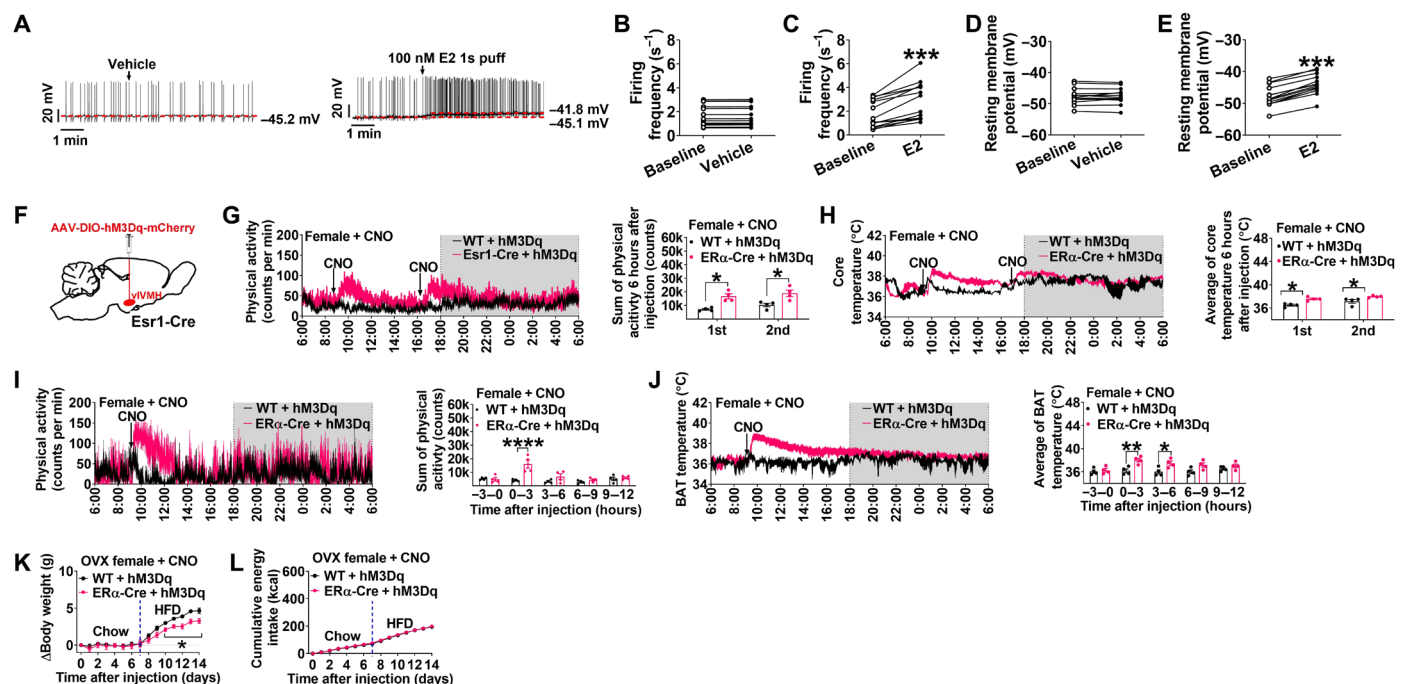


Fig. 1. Chemogenetic activation of ER α ^{vVMH} neurons stimulates physical activity and BAT thermogenesis in females. (See also figs. S1 and S2.) (A) Representative trace of ER α -ZsGreen (+) neurons in the vVMH of female mice treated with vehicle (1% dimethyl sulfoxide in saline) or 17 β -estradiol (E2, 100 nM, 1 s puff). (B to E) Summary of firing frequency (B and C) and resting membrane potential (D and E) ($n = 13$ or 14). (F) Schematic of the experimental strategy. (G and H) Effects of CNO (3 mg/kg) intraperitoneal injection on physical activity (G) and core temperature (H) in female mice with emitter intra-abdominally implanted ($n = 4$). (I and J) Effects of CNO injection on physical activity (I) and BAT temperature (J) in female mice with emitter implanted under BAT ($n = 4$). (K and L) Effects of chronic CNO treatment (twice/day, 3 mg/kg i.p. injection) on body weight gain (K) and energy intake (L) of ovariectomized (OVX) female mice ($n = 3$ or 4). (B to E) Data are presented for each cell. *** $P < 0.001$ in paired t tests. (G to L) Results are shown as means $\pm$ SEM. (G and H) * $P < 0.05$ in unpaired t tests. (I to L) * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ in two-way analysis of variance (ANOVA) analysis followed by post hoc Sidak tests.

5-HT^{DRN} neurons, which play an essential role in BAT thermogenesis (21). To establish the synaptic innervation between ER α ^{vVMH} neurons and 5-HT^{DRN} neurons, we stereotactically injected antero-grad trans-synaptic tracing virus Ad-iN/WED cre-inducible WGA adenoviral vector into the vVMH of Esr1-Cre mice (Fig. 2A). Ad-iN/WED is a Cre-dependent virus that harbors a green fluorescent protein (GFP)-tagged wheat germ agglutinin (WGA) (22) and, following injection, was exclusively expressed in ER α ^{vVMH} neurons (Fig. 2B), and anterogradely translocated to downstream neural populations. Through this anterograde labeling approach, we found abundant WGA-GFP expression in the DRN. Notably, majority of the WGA-GFP (+) neurons in the DRN expressed 5-HT (Fig. 2, C to E). These observations strongly indicate that 5-HT^{DRN} neurons receive synaptic innervation from ER α ^{vVMH} neurons.

WGA has also been reported to exhibit both retrograde trans-synaptic transport (23), and multisynaptic transport of WGA has also been observed (24). Therefore, WGA tracing alone could not fully establish a direct projection from ER α ^{vVMH} neurons to 5-HT^{DRN} neurons. To confirm that ER α ^{vVMH} neurons provide monosynaptic inputs to DRN neural populations, we also used a rabies-mediated retrograde tracing system, including an EnvA-pseudotyped glycoprotein (G)-deleted Rabies-mCherry and Cre-dependent helper AAV virus AAV2-EF1a-FLEX-GT [enhanced GFP (EGFP) +

TVA]. EnvA-pseudotyped rabies virus does not infect neurons in the absence of the avian TVA (the cellular receptor for subgroup A avian leukosis viruses) receptor protein (25). Toward this, we first delivered AAV2-EF1a-FLEX-GT (EGFP + TVA) into the vVMH of Esr1-Cre mice to induce TVA expression exclusively in ER α ^{vVMH} neuron cell bodies and fiber terminals. Subsequently, we introduced EnvA-G-deleted Rabies-mCherry into the downstream DRN brain region, where the fiber terminals of ER α ^{vVMH} neurons are located. The selective expression of TVA in ER α ^{vVMH} neurons allows for rabies infection of ER α ^{vVMH} neuronal fiber terminals and subsequent labeling of the cell bodies within the vVMH (Fig. 2, F to I). We found that Rabies-mCherry expressed and colocalized with TVA-EGFP in the vVMH of the Esr1-Cre mouse (Fig. 2, J to L), confirming monosynaptic inputs from ER α ^{vVMH} neurons to the DRN neural populations.

A similar rabies-mediated retrograde tracing was performed to confirm further that vVMH neurons provide monosynaptic inputs to Tryptophan Hydroxylase 2 (TPH2)^{DRN} neurons (fig. S3A). Specifically, we delivered AAV2-EF1a-FLEX-GTB (EGFP + TVA + rabies B19 glycoprotein) into the DRN of TPH2-iCreER/Rosa26-LSL-tdTOMATO mice to induce TVA and glycoprotein expression exclusively in TPH2^{DRN} neurons (fig. S3, B to D). Subsequently, we introduced EnvA-G-deleted Rabies-mCherry into the same DRN brain region.

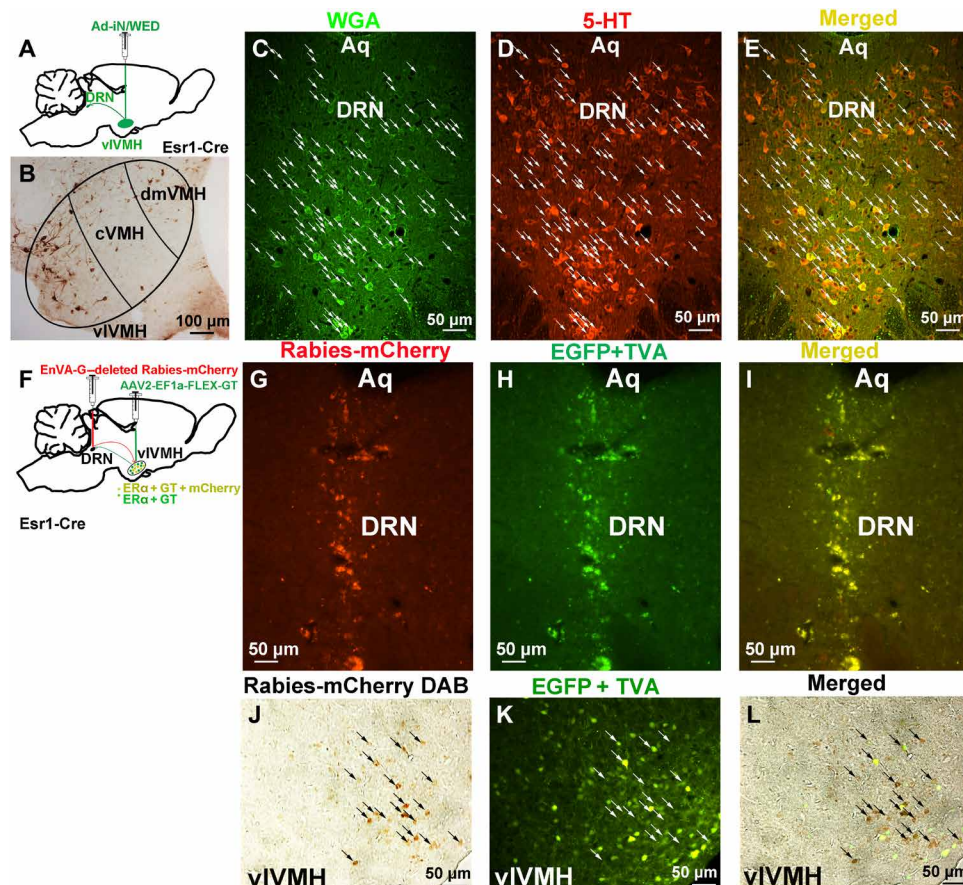


Fig. 2. ER α ^{vVMH} neurons provide monosynaptic innervation to 5-HT^{DRN} neurons. (See also fig. S3.) (A) Schematic of the experimental strategy using the Ad-iN/WED virus as a trans-synaptic anterograde tracer to identify the ER α ^{vVMH} $\rightarrow$ 5-HT^{DRN} circuit. (B) WGA immunoreactivity in the vVMH of female Esr1-Cre mice. (C to E) Immunoreactivity of WGA (C, green), 5-HT (D, red), and merger (E, yellow) in the DRN. White arrows point to yellow dual fluorescent neurons. Aq, aqueduct. (F) Schematic of the experimental strategy using the EnvA-G-deleted Rabies-mCherry virus as a retrograde tracer to identify the ER α ^{vVMH} $\rightarrow$ DRN circuit. (G to I) Fluorescence of Rabies-mCherry (G, red), EGFP + TVA (H, green), and merger (I, yellow) in the DRN. (J to L) Immunoreactivity of Rabies-mCherry (J, brown), EGFP + TVA (K, green), and merger (L) in the vVMH of female Esr1-Cre mice.

Glycoprotein is required for the retrograde trans-synaptic tracing of the rabies virus. The selective expression of TVA and glycoprotein in TPH2^{DRN} neurons allows for rabies infection of TPH2^{DRN} neurons and subsequent labeling of the upstream neurons providing monosynaptic inputs to TPH2^{DRN} neurons. We found that Rabies-mCherry expressed in multiple brain regions, including the ARC, the medial amygdala, the suprachiasmatic nucleus, the medial posterior part of the Arc, and vVMH (fig. S3, E to L), confirming monosynaptic inputs from vVMH neurons to the TPH2^{DRN} neural populations.

ERα^{vVMH} neurons stimulate 5-HT^{DRN} neurons through glutamatergic neurotransmission

We next sought to target both ERα^{vVMH} neurons and 5-HT^{DRN} neurons in the same mice. For the latter population, we used a knock-in TPH2-iCreER mouse allele. TPH2 is an essential enzyme for the synthesis of 5-HT in the brain, and we previously confirmed that TPH2-iCreER drives selective Cre activity in 5-HT neurons within the DRN (26). Using the TPH2-iCreER deliver line, upon tamoxifen induction produced robust Cre activity in the DRN (fig. S4A); no Cre activity was observed in the vVMH (fig. S4B). Notably, Esr1-Cre mice show Cre activity in the DRN as reported by Cre-driven mCherry expression; however, the mCherry-positive neurons in the DRN were identified to be TPH neurons (fig. S4, C to E). Consistent with our previous findings, we confirmed that all ERα-expressing cells within the DRN are 5-HT neurons (26). In other words, the Esr1-Cre did not induce Cre activity in non-5-HT neurons in the DRN. Thus, within the DRN of the compound TPH2-iCreER/Esr1-Cre mice, Cre activity was exclusively induced in 5-HT neurons.

We next generated Esr1-Cre/TPH2-iCreER/Rosa26-LSL-tdTOMATO mice and stereotactically injected both AAV2-DIO-ChR2-EYFP (enhanced yellow fluorescent protein) and Ad-iN/WED into the vVMH (Fig. 3A). In these mice, the blue light-sensitive

photoreceptor ChR2 was expressed in ERα^{vVMH} neurons and their fiber terminals. WGA-GFP labeled ERα^{vVMH} neurons and their downstream neurons (including those in the DRN), and therefore, tdTOMATO(+)WGA-GFP(+) neurons in the DRN were identified as the putative ERα^{vVMH}-innervated 5-HT^{DRN} neurons (fig. S4, F to H). Consistent with the colocalization analysis of WGA-GFP and 5-HT in the DRN, most of the WGA-GFP(+) neurons were also tdTOMATO(+) (Fig. 3, B and C). To characterize the synaptic transmission within the ERα^{vVMH} → 5-HT^{DRN} neural circuit, we used CRACM (27) to record light-induced responses of tdTOMATO(+)WGA-GFP(+) neurons in the DRN following blue light photostimulation of ChR2-expressing ERα^{vVMH} fibers/terminals within the DRN (fig. S4, I to K).

Under the current clamp, we found that photostimulation significantly increased the resting membrane potential of tdTOMATO(+)WGA-GFP(+) neurons in the presence of TTX (Fig. 3, D and E). Together, these data suggest a direct excitatory input from ERα^{vVMH} neurons to 5-HT^{DRN} neurons. Under voltage-clamp conditions, we detected light-evoked excitatory postsynaptic currents (eEPSCs) in most tdTOMATO(+)WGA-GFP(+) neurons tested (70.59% eEPSC versus 29.41% no eEPSC in total 17 neurons; Fig. 3, F and G). Notably, no eEPSCs were detected in tdTOMATO(+)WGA-GFP(-) neurons (Fig. 3H), validating the high fidelity of this WGA-GFP-assisted CRACM approach. After blocking presynaptic inputs by 4-aminopyridine (4-AP) and TTX preincubation, the amplitude and latency of eEPSCs remained unchanged (Fig. 3, I and J). These findings revealed a monosynaptic response induced by blue light, which was consistent with the current-clamp results. We also found that blue light-induced eEPSCs were blocked by incubation of D-AP5 (an NMDA receptor antagonist) + CNQX (an AMPA receptor antagonist), confirming their glutamatergic nature (Fig. 3, F and I). In summary, our results suggest that the ERα^{vVMH} neurons provide monosynaptic glutamatergic inputs to 5-HT^{DRN} neurons.

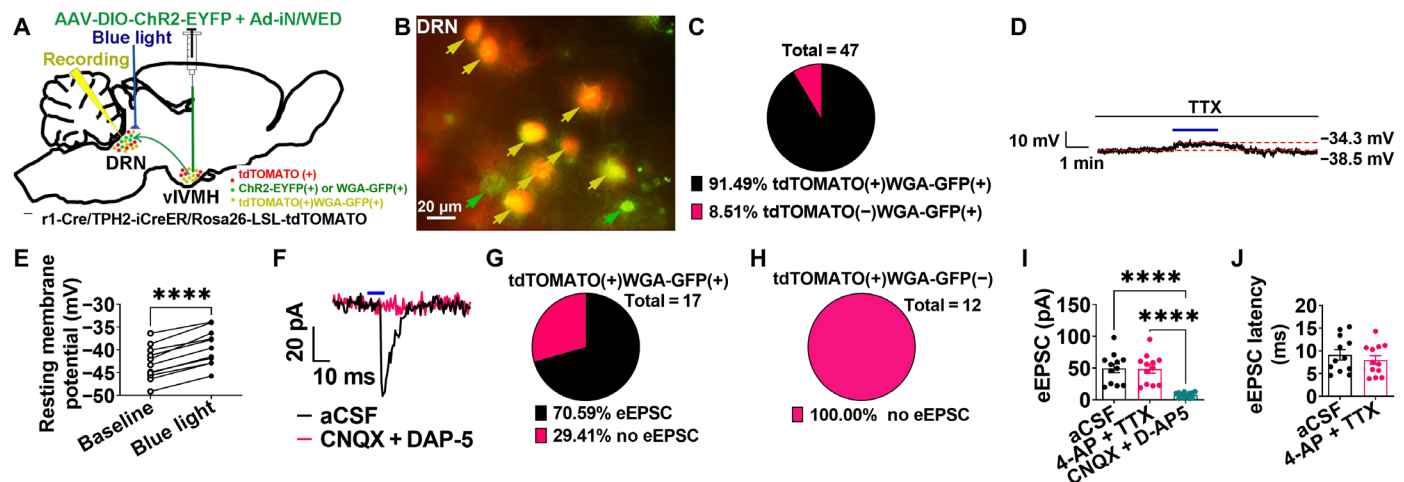


Fig. 3. ERα^{vVMH} → DRN neural circuit stimulates 5-HT^{DRN} neurons through glutamatergic neurotransmission. (See also figs. S4 and S5.) (A) Schematic of the experimental strategy. (B and C) A representative image (B) and a summary (C) of yellow arrow-pointed tdTOMATO(+)WGA-GFP(+) and green arrow-pointed tdTOMATO(-)WGA-GFP(+) neurons in the DRN. (D and E) Representative trace (D) and summary (E) of tdTOMATO(+)WGA-GFP(+) neurons in the DRN responding to blue light photostimulation in the DRN (20 Hz, 10 ms per pulse, and 3 mW for 2 min) in the presence of 1 μM TTX (*n* = 11). (F) Representative eEPSC induced by blue light photostimulation (10 ms, 3-mW stimulation) after preincubation of artificial cerebrospinal fluid (aCSF) or 30 μM CNQX + 30 μM AP-5 in tdTOMATO(+)WGA-GFP(+) neurons in the DRN. (G and H) Summary of eEPSC in tdTOMATO(+)WGA-GFP(+) (G) and tdTOMATO(+)WGA-GFP(-) neurons (H). (I) Summary of eEPSC amplitude in the presence of aCSF, 1 μM 4-aminopyridine (4-AP) + 1 μM TTX, or CNQX + D-AP5 (*n* = 12). (J) Summary of eEPSC latency in the presence of aCSF or 4-AP + TTX (*n* = 12). (E) Data are presented for each cell. *****P* < 0.0001 in paired *t* tests. (I and J) Results are presented as means ± SEM. *****P* < 0.0001 in repeated-measures ANOVA analysis followed by post hoc Dunnett's tests.

To further characterize the synaptic transmission between $ER\alpha^{vVMH}$ and 5-HT(-)^{DRN} downstream neural populations, the same current clamp was also performed in tdTOMATO(-)WGA-GFP(+) neurons. We detected eEPSCs in most tdTOMATO(-)WGA-GFP(+) neurons tested (72.00% eEPSC versus 28.00% no eEPSC in total 25 neurons; fig. S5, A and B). Notably, the amplitude and latency of eEPSCs remained unchanged after 4-AP and TTX preincubation (fig. S5, C and D). However, eEPSCs were blocked by incubation of D-AP5 + CNQX, suggesting glutamatergic transmission (fig. S5, A and C). Collectively, our data support that the $ER\alpha^{vVMH}$ neurons provide monosynaptic glutamatergic inputs to both 5-HT(+) and 5-HT(-) DRN neurons.

$ER\alpha^{vVMH} \rightarrow$ DRN neural circuit responds to changes in ambient temperature and nutritional state

To test the physiological relevance of the $ER\alpha^{vVMH} \rightarrow$ DRN neural circuit, we next recorded the neural dynamics of identified DRN-projecting $ER\alpha^{vVMH}$ neurons during temperature or nutritional changes. Toward this, we injected a retrogradely transported CAV2-Cre virus into the DRN of $ER\alpha$ -ZsGreen/Rosa26-LSL-tdTOMATO mice (Fig. 4A). In this case, CAV2-Cre retrogradely infected

upstream $ER\alpha^{vVMH}$ neurons that project to the DRN and induced tdTOMATO (red) expression. Thus, DRN-projecting $ER\alpha^{vVMH}$ neurons were labeled with tdTOMATO(+)/ZsGreen(+) dual fluorescent colors (fig. S6, A to C). We next performed current-clamp recordings in identified yellow neurons in brain slices that were prepared from mice exposed to different ambient temperatures. We found that the firing frequency of the DRN-projecting $ER\alpha^{vVMH}$ neurons was increased in mice exposed to cold (4°C) and decreased with warm exposure (40°C; Fig. 4, B and C). Resting membrane potential was not significantly altered by the cold exposure but was significantly decreased by warm exposure (Fig. 4D). These results revealed thermosensing capabilities of DRN-projecting $ER\alpha^{vVMH}$ neurons, where low ambient temperatures activate and high temperatures inhibit these neurons. We further investigated the thermosensing properties of DRN-projecting $ER\alpha^{vVMH}$ neurons in OVX female mice with depleted endogenous ovarian hormones. Notably, OVX abolished the responses of firing frequency and resting membrane potential to changes in ambient temperature (Fig. 4, B to D), indicating that ovarian hormones (including estrogen) are required for thermosensing capabilities of DRN-projecting $ER\alpha^{vVMH}$ neurons.

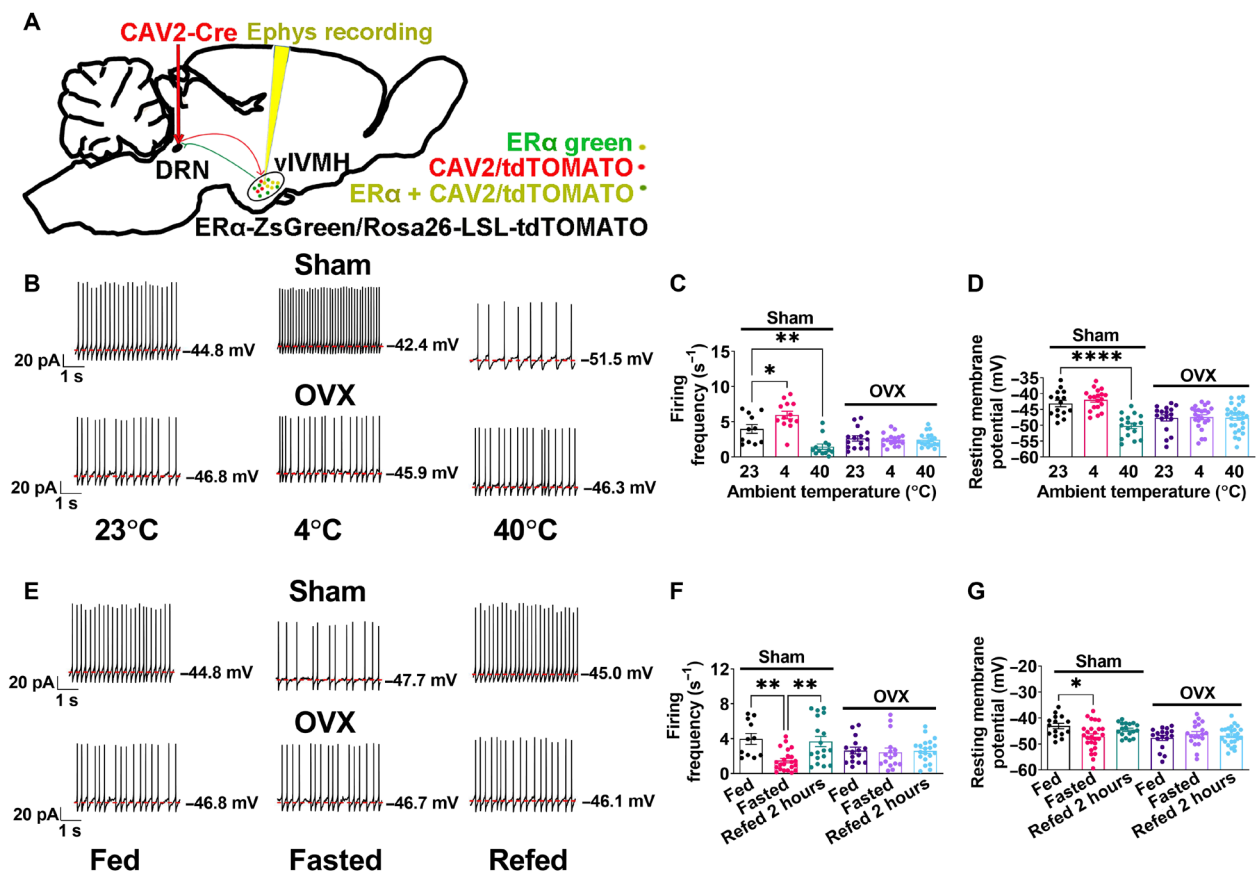


Fig. 4. Neural dynamics of DRN-projecting $ER\alpha^{vVMH}$ neurons. (See also fig. S6.) (A) Schematic of the experimental strategy using the CAV2-Cre virus to label and record the electrophysiological response of DRN-projecting $ER\alpha^{vVMH}$ neurons in Sham or OVX female $ER\alpha$ -ZsGreen/Rosa26-LSL-tdTOMATO mice. (B) Representative responses to the exposure to room temperature (23°C), 4°C, or 40°C for 30 min in DRN-projecting $ER\alpha^{vVMH}$ neurons labeled with dual yellow fluorescent colors (ZsGreen + tdTOMATO) in Sham or OVX female mice. (C and D) Summary of firing frequency (C) and resting membrane potential (D) when exposed to different temperatures ($n = 11, 13, 13, 15, 17, \text{ or } 18$). (E) Representative response to fed condition, 24-hour fasting, or 2-hour refeeding after 24-hour fasting in DRN-projecting $ER\alpha^{vVMH}$ neurons in Sham or OVX female mice. (F and G) Summary data of firing frequency (F) and resting membrane potential (G) under different metabolic conditions ($n = 11, 22, 18, 15, 16, \text{ or } 20$). (C, D, F, and G) Results are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, and **** $P < 0.0001$ in one-way ANOVA analysis followed by post hoc Dunnett's tests.

We then tested responses of DRN-projecting $ER\alpha^{vVMH}$ neurons to fasting and refeeding. We found that the firing frequency of DRN-projecting $ER\alpha^{vVMH}$ neurons was decreased by fasting and increased by refeeding, and the resting membrane potential of these neurons was decreased by fasting (Fig. 4, E to G). These neural responses associated with animals' feeding conditions were also blocked in OVX female mice (Fig. 4, E to G). We next further tested the responses of non-DRN-projecting $ER\alpha^{vVMH}$ tdTOMATO(−) ZsGreen(+) neurons. While DRN-projecting $ER\alpha^{vVMH}$ neurons were sensitive to changes in ambient temperature and nutritional state, these non-DRN-projecting $ER\alpha^{vVMH}$ neurons only showed responses to fasting challenge (fig. S6, D to G).

The stimulatory effects of $ER\alpha^{vVMH} \rightarrow$ DRN circuit on BAT thermogenesis and physical activity are independent of ADRB3

To further test the metabolic effects of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit, we selectively activated this circuit using Chr2-mediated photostimulation. More specifically, we stereotactically injected Cre-dependent AAV2-DIO-ChR2-EYFP into the vVMH of *Esr1-Cre* mice and implanted an optic fiber above the DRN (Fig. 5A and fig. S7A). Blue light stimulation (473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) of the DRN resulted in significant increases in both BAT temperature and physical activity compared to the same mice receiving control yellow light pulses (473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) during the same time of the day (Fig. 5, B, D, E, and G). Notably, post hoc staining of EYFP

and cFOS (Fos proto-oncogene, AP-1 transcription factor subunit) and electrophysiology confirmed accurate injection/implantation and targeted activation of $ER\alpha^{vVMH}$ neurons (fig. S7, B to E). These findings indicate that activation of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit is sufficient to stimulate BAT thermogenesis and physical activity.

To explore the peripheral mechanisms underlying this action, we next intraperitoneally injected mice with SR59230A, a β_3 -adrenergic receptor (ADRB3) antagonist, before photostimulation. As previously reported (28), SR59230A showed inhibitory effects on the baseline BAT thermogenesis and physical activity (fig. S7, E and F). However, pretreatment of SR59230A did not affect photostimulation effects on BAT thermogenesis or physical activity (Fig. 5, C, D, F, and G). Together, these results indicate that the stimulatory effects of the $ER\alpha^{vVMH} \rightarrow$ DRN neural circuit on BAT thermogenesis and physical activity are independent of ADRB3.

Inhibition of $ER\alpha^{vVMH} \rightarrow$ DRN circuit reduces BAT thermogenesis and core temperature

To evaluate the in vivo physiological role of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit, we adopted a selective chloride-conducting channelrhodopsin (iC++) approach to inhibit this circuit. As before, we stereotactically injected AAV-DIO-iC++-EYFP into the vVMH of *Esr1-Cre* mice and implanted optic fibers to target the DRN, followed by blue light stimulation to inhibit the $ER\alpha^{vVMH} \rightarrow$ DRN projection (Fig. 5H). Inhibition of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit by blue light pulses (473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) decreased BAT thermogenesis and core temperature without changing physical

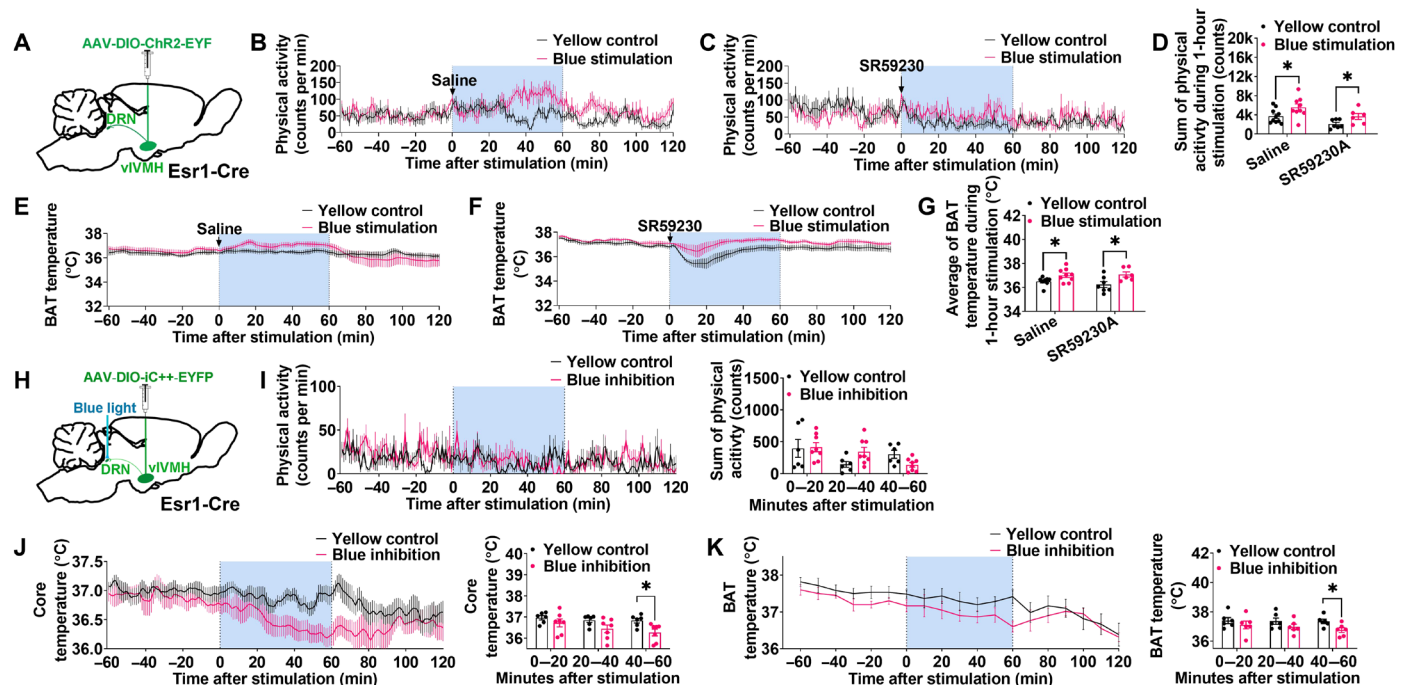


Fig. 5. $ER\alpha^{vVMH} \rightarrow$ 5-HT^{DRN} circuit stimulates both BAT thermogenesis and physical activity. (See also figs. S7 and S8.) (A) Schematic of the experimental strategy. (B to D) Effects of yellow or blue light stimulation (589 or 473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) in the DRN on physical activity. Female mice were intraperitoneally injected with saline ($n = 10$ or 10) or SR59230 (0.5 mg/kg, $n = 7$ or 7) in the beginning of light stimulation. (E to G) Effects of yellow or blue light stimulation in the DRN on BAT temperature ($n = 10$, 10 , 7 , or 7). (H) Schematic of the experimental strategy. (I) Effects of yellow or blue light stimulation (589 or 473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) in the DRN on physical activity ($n = 8$ or 8). (J) Effects of yellow or blue light stimulation in the DRN on core temperature ($n = 6$ or 7). (K) Effects of yellow or blue light stimulation in the DRN on BAT temperature ($n = 6$ or 7). All data are presented as means $\pm$ SEM. * $P < 0.05$ in unpaired t tests.

activity, compared to the same mice receiving yellow light pulses (473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) (Fig. 5, I to K). Notably, the observed inhibitory effects on both BAT and core temperature were observed following 40 min of blue light photostimulation, suggesting that the DRN is an essential downstream brain region for metabolic effects of $ER\alpha^{vVMH}$ neurons. The accuracy of injections and implantations was always validated by IHC of EYFP following experimentation (fig. S8, A and B). We further demonstrated that both resting membrane potentials and firing frequency of $iC++$ -positive $ER\alpha^{vVMH}$ neurons were significantly reduced by blue light exposure at the vVMH (473 nm, 10 ms per pulse, and 20 Hz for 3 min) (fig. S8, C to G). These results indicate that the baseline activity of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit is required for the normal maintenance of BAT thermogenesis and core temperature.

Glutamatergic neurotransmission is required for the stimulatory effects of $ER\alpha^{vVMH} \rightarrow$ DRN circuit on BAT thermogenesis and physical activity

To directly examine the contribution of glutamate signaling on the metabolic effects of $ER\alpha^{vVMH} \rightarrow$ DRN circuit, we injected *Esr1*-Cre female mice with AAV2-DIO-hM3Dq-mCherry into the vVMH and then locally injected CNO with or without the glutamate receptor antagonist D-AP5 and CNQX into the DRN (Fig. 6A). CNO delivery to the DRN stimulated both physical activity and BAT thermogenesis, while coinjecting D-AP5 and CNQX into the DRN completely abolished these effects (Fig. 6, B and C). Therefore, we confirmed that $ER\alpha^{vVMH}$ neurons stimulate BAT thermogenesis and physical activity through the activation of DRN downstream neurons in a glutamate-dependent manner.

The stimulatory effects of $ER\alpha^{vVMH}$ neurons on BAT thermogenesis and physical activity are mediated by activation of 5-HT^{DRN} neurons

To directly test whether 5-HT^{DRN} neurons mediate the metabolic functions of $ER\alpha^{vVMH}$ neurons, we next used dual DREADD technology to stimulate $ER\alpha^{vVMH}$ neurons while simultaneously inhibiting 5-HT^{DRN} neurons (Fig. 6D). Combining *Esr1*-Cre/TPH2-iCreER mice and Cre-dependent AAV vectors, DREADD hM3Dq was targeted for expression in $ER\alpha^{vVMH}$ neurons, while inhibitory DREADD hM4Di was expressed in 5-HT^{DRN} neurons (fig. S9, A to E). After CNO treatment, both resting membrane potential and firing frequency were increased in the $ER\alpha^{vVMH}$ neurons and decreased in 5-HT^{DRN} neurons (fig. S9, F to K), confirming CNO-induced activation of $ER\alpha^{vVMH}$ neurons and inhibition of 5-HT^{DRN} neurons. Possible metabolic contributions of nonspecific effects of CNO were excluded, as demonstrated by unchanged BAT thermogenesis and physical activity in WT + hM3Dq mice after CNO intraperitoneal injections (Fig. 6, E, I, J, and N). In *Esr1*-Cre + hM3Dq mice, CNO injections induced robust increases in BAT thermogenesis and physical activity compared to saline injections in the same mice (Fig. 6, F, I, K, and N). The observed stimulatory effects were significantly attenuated by the inhibition of 5-HT^{DRN} neurons in dual DREADD mice (Fig. 6, H, I, M, and N). Together, these data support that activation of 5-HT^{DRN} neurons is required for increased BAT thermogenesis and physical activity induced by activation of $ER\alpha^{vVMH}$ neurons. Notably, inhibition of 5-HT^{DRN} neurons alone inhibited BAT thermogenesis but not physical activity (Fig. 6, G, I, L, and N).

$ER\alpha$ expressed by DRN-projecting $ER\alpha^{vVMH}$ neurons is required for regulation of BAT thermogenesis, physical activity, and body weight

To further test the physiological functions of $ER\alpha^{vVMH} \rightarrow$ DRN neural circuit, we delivered ΔG Rabies FLPO-dsRedXpress and AAV-hSyn1-frEX-Cre-GFP to the DRN and vVMH, respectively, of female *Esr1^{lox/lox}* mice to retrogradely delete $ER\alpha$ from $ER\alpha^{vVMH} \rightarrow$ DRN neurons. In this setting, retrograde Rabies FLPO-dsRed was taken up by fiber terminals in the DRN and retrogradely transported to the vVMH cell bodies, and AAV-hSyn1-frEX-Cre-GFP virus selectively expresses Cre recombinase only in flpo-expressing cells (see fig. S10, A and B for validation of this vector). Thus, Cre was expressed exclusively in DRN-projecting vVMH neurons, resulting in deletion of $ER\alpha$ selectively in this subset of neurons (Fig. 7A). Notably, the overall number of cells in the vVMH did not change after deletion as indicated by 4',6-diamidino-2-phenylindole staining (fig. S10C), excluding the possibility of rabies-induced cytotoxicity or apoptosis. The specificity of deletion was first validated by dsRed, EGFP, and $ER\alpha$ immunohistochemistry (fig. S10, D to G). We found that $ER\alpha^{vVMH-DRN-KO}$ mice showed significantly decreased $ER\alpha$ -expressing neurons in the vVMH compared to control mice (Fig. 7, B to D). Only a subset of vVMH neurons in $ER\alpha^{vVMH-DRN-KO}$ mice lost $ER\alpha$ expression, consistent with selective deletion of $ER\alpha$ only from the DRN-projecting $ER\alpha^{vVMH}$ neurons. Consistent with the metabolic effects caused by $ER\alpha^{vVMH} \rightarrow$ DRN circuitry manipulations, retrograde deletion of $ER\alpha$ from DRN-projecting $ER\alpha^{vVMH}$ neurons led to decreased BAT thermogenesis and physical activity in female mice (Fig. 7, E and F). Notably, the experimental mice also showed increased body weight gain after 2 weeks of HFD feeding without affecting energy intake (Fig. 7, G and H). These results demonstrate that $ER\alpha$ expressed by DRN-projecting $ER\alpha^{vVMH}$ neurons is physiologically relevant for the regulation of both BAT thermogenesis and physical activity in female mice.

DISCUSSION

Estrogen/ $ER\alpha$ signaling in the vVMH is vital for energy homeostasis and body weight control. The stimulatory effects of $ER\alpha^{vVMH}$ on BAT thermogenesis have been consistently vetted in several studies using transgenic mouse models. Conditional knockout of $ER\alpha$ in the vVMH using SF1-Cre lowers BAT thermogenesis in female mice and yields abdominal obesity, suggesting that $ER\alpha^{vVMH}$ neurons stimulate energy expenditure to decrease adiposity (5). This mechanism of estrogenic control of body weight is further supported by the evidence that vVMH-specific injection of E2 stimulates BAT thermogenesis by inhibiting adenosine monophosphate-activated protein kinase in the vVMH (9). Notably, $ER\alpha^{vVMH}$ neurons have also been shown to stimulate physical activity. For example, short hairpin RNA-induced $ER\alpha$ knockdown in the vVMH decreases not only diet-induced thermogenesis but also physical activity, which results in obesity (8). Consistently, conditional knockout of *Nkx2-1* using SF1-Cre leads to loss of $ER\alpha^{vVMH}$ neurons, decreased physical activity, and obesity (10), suggesting stimulatory effects of $ER\alpha^{vVMH}$ neurons on physical activity. Thus, $ER\alpha^{vVMH}$ neurons represent a unique subpopulation capable of controlling both BAT thermogenesis and physical activity, at least in female animals.

Supporting this notion, a recent study confirmed that acute activation of $ER\alpha^{vVMH}$ neurons enhances energy expenditure by increasing BAT thermogenesis and physical activity (17). We replicated

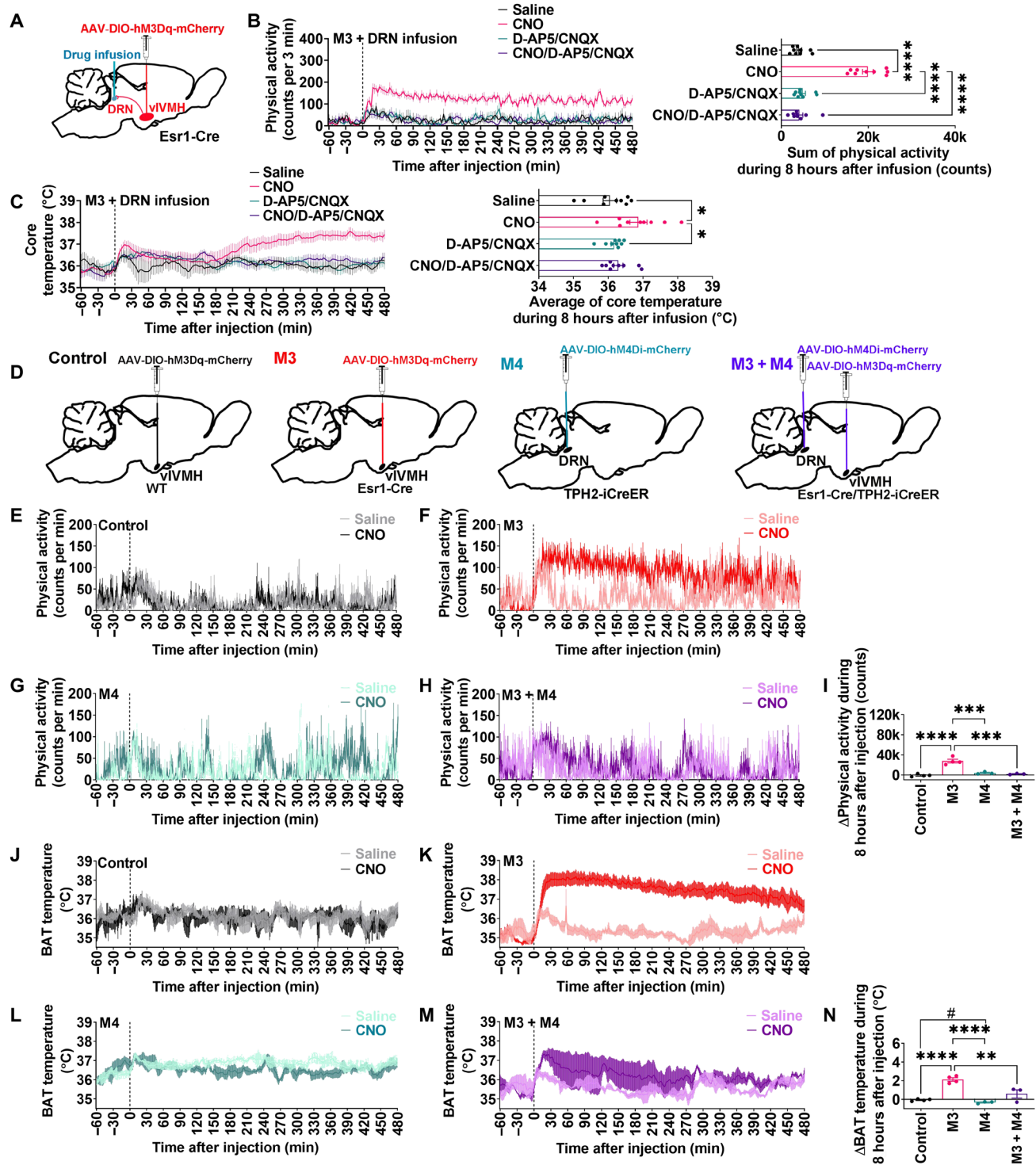


Fig. 6. The stimulatory effects of $ER\alpha^{v1VMH}$ neurons on BAT thermogenesis and physical activity are mediated by activation of 5-HT^{DRN} neurons through glutamatergic transmission. (See also fig. S9.) (A) Schematic of the experimental strategy. (B and C) Effects of 400 nl of DRN-specific infusion of saline, CNO (1 μ g/ μ l), CNO (1 μ g/ μ l) + D-AP5 (6.25 μ g/ μ l) + CNQX (2.5 μ g/ μ l), or D-AP5 (6.25 μ g/ μ l) + CNQX (2.5 μ g/ μ l) on physical activity (B) and core temperature (C) ($n=9$). (D) Schematic of the experimental strategy. (E to I) Effects of saline or CNO (3 mg/kg) intraperitoneal injection on physical activity (E to H) and differences in the sum of physical activity between saline- and CNO-injected mice (I). (J to N) Effects of saline or CNO intraperitoneal injection on BAT temperature (J to M) and differences in the average of BAT temperature between saline- and CNO-injected mice (N) (control, $n=4$; M3, $n=4$; M4, $n=3$; M3 + M4, $n=3$). All data are presented as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ in one-way ANOVA analysis followed by post hoc Tukey tests. # $P < 0.05$ in unpaired t tests.

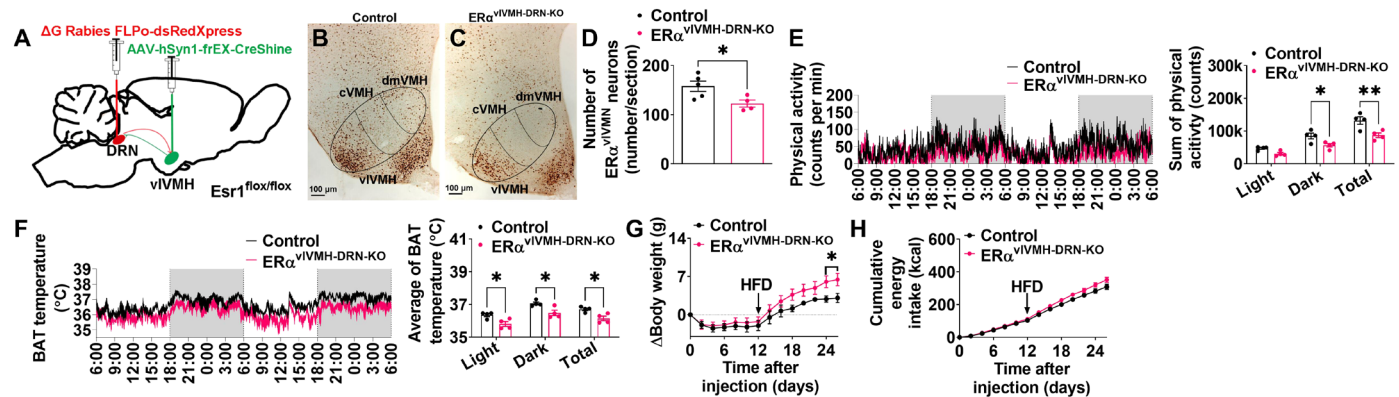


Fig. 7. $ER\alpha$ expressed by $ER\alpha^{vVMH} \rightarrow$ DRN neurons is required for estrogenic stimulation on BAT thermogenesis and physical activity. (See also fig. S10.) (A) Schematic of the experimental strategy using the retrograde ΔG -Rabies-FLPo-dsRedXpress and FLPo-dependent AAV-hSyn1-frEX-Cre-GFP viruses to selectively delete $ER\alpha$ from DRN-projecting $ER\alpha^{vVMH}$ neurons in the female $Esr1^{flox/flox}$ mice ($ER\alpha^{vVMH-DRN-KO}$). Control group is female $Esr1^{flox/flox}$ mice injected with AAV-CMV-GFP virus into the DRN and AAV-hSyn1-frEX-Cre-GFP virus into the vVMH. (B and C) Immunohistochemistry staining of $ER\alpha$ in the vVMH of female control (B) and $ER\alpha^{vVMH-DRN-KO}$ (C) mice. (D) Average of $ER\alpha$ -expressing neurons in the vVMH ($n = 5$ or 4). (E) Physical activity (left) and sum of physical activity during light, dark, or 48 hours recording (right) ($n = 4$). (F) BAT temperature (left) and average of BAT temperature during light, dark, or 48 hours of recording (right) ($n = 4$). (G and H) Body weight gain (G) and cumulative energy intake (H) of female control and $ER\alpha^{vVMH-DRN-KO}$ mice ($n = 5$ or 4). (D to H) Results are presented as means $\pm$ SEM. (D) $*P < 0.05$ in unpaired t tests. (E to H) $*P < 0.05$ and $**P < 0.01$ in two-way ANOVA analysis followed by post hoc Sidak tests.

these observations showing that DREADD activation of $ER\alpha^{vVMH}$ neurons markedly increased BAT thermogenesis and physical activity in both male and female mice. We further expanded this discovery to show that chronic activation of $ER\alpha^{vVMH}$ neurons protected OVX female mice from HFD-induced body weight gain, arguing that activity of these $ER\alpha^{vVMH}$ neurons can have a meaningful impact on the body weight regulation. Combining the observation that E2 directly activates $ER\alpha^{vVMH}$ neurons, our findings support a model that estrogen activates $ER\alpha^{vVMH}$ neurons to stimulate both BAT thermogenesis and physical activity, therefore preventing body weight gain.

In the present study, we further identified that 5-HT^{DRN} neurons represent a key downstream neural node mediator of $ER\alpha^{vVMH}$ actions on BAT thermogenesis and physical activity. We first used a WGA-GFP anterograde tracer to show that $ER\alpha^{vVMH}$ neurons send projections to the DRN, where they exclusively innervate 5-HT neurons. Further, implementing rabies monosynaptic retrograde tracing, as well as the CAV2 retrograde tracing, we provide complementary evidence for these $ER\alpha^{vVMH} \rightarrow$ DRN projections. We used CRACM to functionally confirm that $ER\alpha^{vVMH}$ neurons provide monosynaptic glutamatergic inputs to 5-HT^{DRN} neurons. At the physiological level, we used both ChR2-mediated and DREADD-mediated circuit activation to demonstrate stimulatory effects of the $ER\alpha^{vVMH} \rightarrow$ DRN projections on BAT thermogenesis and physical activity. Notably, infusion of glutamatergic antagonists into the DRN blocked the observed stimulatory effects on BAT thermogenesis and physical activity. These results not only support a key role of glutamatergic neurotransmission in mediating these effects but also exclude potential confounding actions of other collateral projections of DRN-projecting $ER\alpha^{vVMH}$ neurons. We used a dual DREADD approach to show that simultaneous inhibition of 5-HT^{DRN} neurons blocked effects of activation of $ER\alpha^{vVMH}$ neurons, further highlighting the function of the $ER\alpha^{vVMH} \rightarrow$ 5-HT^{DRN} circuit on the regulations of BAT thermogenesis and physical activity.

In supporting the metabolic functions of the $ER\alpha^{vVMH} \rightarrow$ 5-HT^{DRN} circuit, inhibition or ablation of central 5-HT has been previously shown to disrupt temperature control (29, 30). Specifically, chemogenetic activation or inactivation of 5-HT^{DRN} neurons resulted in a significant increase or decrease, respectively, in BAT temperature without affecting food intake (31). In addition, the stimulation of 5-HT^{DRN} neurons has been reported to exert a context-dependent control of physical activity. Optogenetic activation of 5-HT^{DRN} neurons suppressed movement in low and moderate threat environments but induced escape behavior in high threat settings. The movement-related 5-HT^{DRN} neural dynamics are also inverted in low versus high threat environments (32), suggesting a vital role of 5-HT^{DRN} neurons in the regulation of physical activity. These findings indicate that stimulation of 5-HT^{DRN} neurons at least partially recapitulates the increases in thermogenesis and physical activity observed when the $ER\alpha^{vVMH}$ neurons are activated.

Optogenetic and/or chemogenetic approaches are powerful toward illustrating a robust phenotypic outcome when a neural circuit is experimentally manipulated. However, these experimental manipulations may not recapitulate the exact activity of a neural circuit in any physiological condition, and therefore, the physiological relevance of optogenetic/chemogenetic results is often hard to evaluate. To circumvent this issue, we complemented the optogenetic/chemogenetic experiments with a circuit-specific gene deletion approach. In particular, we combined a retrograde flopo vector, a flopo-dependent Cre vector, and a loxP-flanked mouse allele to achieve the deletion of an endogenous gene (encoding $ER\alpha$) in a selectively targeted site (vVMH) with a specific projection (to the DRN). Female mice lacking $ER\alpha$ in this subset of DRN-projecting vVMH neurons showed reduced BAT thermogenesis and physical activity and increased susceptibility to HFD-induced obesity. These results, combined with our observations from optogenetic/chemogenetic manipulations of the $ER\alpha^{vVMH} \rightarrow$ DRN projections, highlight the physiological significance of this estrogen-sensitive vVMH $\rightarrow$ DRN circuit in the regulation of energy homeostasis.

An important question regarding the function of $ER\alpha^{vVMH}$ is whether the stimulatory effects of $ER\alpha^{vVMH}$ on BAT thermogenesis depend on increased physical activity. It is well established that spontaneous physical activity contributes to thermogenesis (33, 34). The observed increased BAT thermogenesis may be a possible secondary effect induced by up-regulation of physical activity. However, our data suggest dissociated regulatory mechanisms. Although DREADD activation of $ER\alpha^{vVMH}$ neurons increased both BAT thermogenesis and physical activity, the time scales of these two responses are different. In both males and females, the up-regulation of physical activity lasted about 3 hours after the CNO injection, whereas the rise of BAT thermogenesis persisted for 6 hours. Consistently, optogenetic inhibition of the $ER\alpha^{vVMH} \rightarrow$ DRN neural circuit decreased BAT thermogenesis but not physical activity during the lights-on period, clearly indicating independent mechanisms. While increased physical activity likely contributes partially to the increased thermogenesis, at least a portion of temperature elevations are mediated through mechanisms independent of physical activity. One potential explanation is the difference in downstream nerve systems/ effectors. The temperature regulation could achieve faster than physical activity because it is a straightforward autonomic response. On the other hand, the physical activity regulation may involve synergistic effects from different inputs on the somatic nervous system and voluntary locomotor activity.

It has been long known that the medial preoptic area (MPOA) of the hypothalamus is a thermoregulatory center. A portion of MPOA neurons can be activated by warm ambient temperature (35, 36), and activation of multiple MPOA neural populations has been shown to markedly reduce body temperature (35, 37–39). Here, we found that DRN-projecting $ER\alpha^{vVMH}$ neurons were activated when animals were exposed to cold, and activation of these cold-sensitive neurons robustly increased BAT thermogenesis and body temperature. While these warm- and cold-sensitive neurons in distinct hypothalamic regions oppositely respond to changes in the ambient temperature, these responses appear to function in concert to provide negative feedback mechanisms to maintain thermal homeostasis. The mechanisms by which DRN-projecting $ER\alpha^{vVMH}$ neurons respond to temperature changes are unclear. Notably, the vVMH receives robust projections from MPOA neurons (37, 40). Thus, it is possible that $ER\alpha^{vVMH}$ neurons respond to changes in ambient temperature through circuitry inputs from warm-sensitive MPOA neurons. In addition, recent single-cell RNA sequencing studies revealed that vVMH neurons express multiple temperature-sensing genes, including *Grik2*, *Trpa1*, and *Trpm8* (18), raising an alternative possibility that vVMH neurons may directly sense temperature changes. Both of these potential mechanisms warrant future investigations.

Another interesting finding from our study was that DRN-projecting $ER\alpha^{vVMH}$ neurons were inhibited by food deprivation. Considering that activation of these neurons increased thermogenesis and physical activity, both of which involve consumption of energy, we suggest that the fasting-induced inhibition of DRN-projecting $ER\alpha^{vVMH}$ neurons represents a protective mechanism for animals to conserve energy when food is not available. Consistent with this idea, we recently reported that DRN-projecting $ER\alpha^{vVMH}$ neurons are inhibited by decreased extracellular glucose concentrations (11). Collectively, DRN-projecting $ER\alpha^{vVMH}$ neurons appear to integrate ambient temperature and nutritional/metabolic states of animals to engage adaptive responses for better survival. Notably,

we show that OVX in female mice abolished responses of these $ER\alpha^{vVMH}$ neurons to cold exposure or to fasting, indicating that endogenous ovarian hormones are required to coordinate the thermoregulatory responses and nutritional states. Of course, it is broadly recognized that VMH neurons also integrate other hormonal cues, including leptin (41), insulin (42), thyroid hormones (43), and glucagon-like peptide-1 (44), to regulate thermogenesis. Considering that these hormones are tightly controlled by animals' nutritional states, it is possible that these signals also regulate the responsiveness of VMH neurons to temperature alterations, which remains to be examined.

BAT thermogenesis is predominantly governed by the sympathetic nervous system (SNS) via the ADRB3 signaling pathway (45). Rabies retrograde tracing studies have shown that multiple brain regions, including the VMH and DRN, send indirect outputs to BAT (46, 47). However, the ADRB3 antagonist failed to block or blunt increased BAT thermogenesis and physical activity induced by optogenetic activation of the $ER\alpha^{vVMH} \rightarrow$ DRN projections, although the ADRB3 blockade itself expectedly reduced the baseline BAT thermogenesis and physical activity. Thus, these findings suggest that the effects of the $ER\alpha^{vVMH} \rightarrow$ DRN circuit on thermogenesis and physical activity involve other neuronal or hormonal pathways independent of the SNS/ADRB3. These alternative pathways may include the vagal afferent (48), adrenocorticotrophic hormones (49), fibroblast growth factor-21 (50), glucagon-like peptide-1 (51), etc. Additional studies are needed to examine the potential contributions of these signals.

In conclusion, we identified the $ER\alpha^{vVMH} \rightarrow$ DRN neural circuit as an estrogen-sensitive pathway to prevent body weight gain by stimulating BAT thermogenesis and physical activity. Notably, this circuit also integrates the nutritional states of the animals with ambient temperature to coordinate appropriate responses for better survival, a mechanism that requires intact ovarian hormones. Our results reveal how $ER\alpha$ neurons in female brains interact with hormonal/neuronal systems and the external environment to maintain homeostatic control of energy balance and thermoregulation. These findings advance our understanding of the neuroendocrine mechanisms underlying female metabolic health and provide a necessary framework for the development of therapeutic interventions for metabolic and/or thermoregulatory dysfunctions in females, especially in those after menopause.

METHODS

Mice

Several transgenic mouse lines were maintained on a C57BL/6J background. These lines include *Esr1*-Cre mice (#017911, The Jackson Laboratory, Bar Harbor, ME), *TPH2*-iCreER (#016584, The Jackson Laboratory), *Rosa26*-LSL-tdTOMATO (#007914, The Jackson Laboratory), *Esr1*^{lox/lox} (52), and *ERα*-ZsGreen (19). *Esr1*-Cre, *TPH2*-iCreER, and *Rosa26*-LSL-tdTOMATO were crossed to generate *Esr1*-Cre/*TPH2*-iCreER and *Esr1*-Cre/*TPH2*-iCreER/*Rosa26*-LSL-tdTOMATO for either dual DREADD or CRACM. *ERα*-ZsGreen and *Rosa26*-LSL-tdTOMATO were crossed to generate *ERα*-ZsGreen/*Rosa26*-LSL-tdTOMATO for electrophysiological recording. Mice were housed in a temperature-controlled environment at 22° to 24°C on a 12-hour light/12-hour dark cycle (6 a.m. and 6 p.m.) or 14-hour light/10-hour dark cycle (5 a.m. and 7 p.m.). Unless otherwise stated, the mice were fed ad libitum with standard mouse chow (6.5% fat; #2920, Harlan-Teklad, Madison, WI) and water.

Neurotracing and histology

To map the downstream neurons of ER α ^{vVMH} neurons, 8-week-old Esr1-Cre mice received stereotaxic injections of Ad-iN/WED to the vVMH [200 nl, −1.5 mm anterior-posterior (AP), ±0.7 mm medial-lateral (ML), and −5.9 mm dorsal-ventral (DV)]. Because Ad-iN/WED is a Cre-dependent virus, WGA-GFP was exclusively expressed in ER α ^{vVMH} neurons and anterogradely traveled along the fibers, past the synapse, and filled the downstream neurons that were innervated by ER α ^{vVMH} neurons terminals. Seven days after injections, mice were perfused, and brains were coronally cut at 25 μ m (five series). The sections were incubated at room temperature in primary goat anti-WGA antibody (1:1000; #AS-2024, VectorLabs, Burlingame, CA, USA) overnight, followed by the secondary donkey anti-goat Alexa Fluor 488 (1:500; #A-11055, Invitrogen, Carlsbad, CA, USA) for 1.5 hours. Subsequently, sections were incubated with a rabbit anti-5-HT antibody (1:10,000; #20080, ImmunoStar, Hudson, WI, USA), followed by secondary goat anti-rabbit Alexa Fluor 594 (1:500; #A-11037, Invitrogen). Fluorescent images were obtained using a Leica DM5500 fluorescence microscope with OptiGrid structured illumination configuration.

To establish the innervation between ER α ^{vVMH} neurons and the DRN neural population, 8-week-old Esr1-Cre female mice were injected with 200 nl of AAV2-EF1a-FLEX-GT into the vVMH. Two weeks after, 400 nl of EnVA-G-deleted Rabies-mCherry was injected in the DRN (−4.65 mm AP, ±0 mm ML, and −3.5 mm DV). One week after EnVA-G-deleted Rabies-mCherry injection, mice were perfused, and brains were coronally sectioned as described above. These brain sections were subjected to mCherry immunohistochemistry. Briefly, brain sections were incubated with rabbit polyclonal DsRed antibody (1:1000; #632496, Clontech, Mountain View, CA, USA) at room temperature overnight, followed by the biotinylated donkey anti-rabbit secondary antibody (1:1000; #711-067-003, Jackson ImmunoResearch) for 2 hours. Sections were then incubated in the avidin-biotin complex (1:1000; PK-6100, Vector Laboratories) and incubated in 0.04% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide. Slides were coverslipped, and both bright-field and fluorescence images were analyzed as described above.

To further establish the innervation between vVMH neurons and the TPH2^{DRN} neural population, 8-week-old TPH2-iCreER/Rosa26-LSL-tdTOMATO female mice were injected with 200 nl of AAV2-EF1a-FLEX-GTB into the DRN. The mice then received a tamoxifen injection 3 days after virus delivery (0.2 mg/g body weight, i.p.). Two weeks after, 200 nl of EnVA-G-deleted Rabies-mCherry was injected into the DRN. Two weeks after EnVA-G-deleted Rabies-mCherry injection, mice were perfused, brains were coronally sectioned, and sections were subjected to mCherry immunohistochemistry as described above.

To examine the Cre activity in the DRN of Esr1-Cre mice, female Esr1-Cre mice were injected with AAV2-DIO-hM3Dq-mCherry into the DRN. Two weeks after surgery, mice were perfused, and brains were coronally cut at 25 μ m (five series). The sections were incubated at room temperature in primary sheep anti-TPH antibody (1:1000; #AB1541, Millipore, Burlington, MA, USA) overnight, followed by the secondary Donkey Anti-Sheep Alexa Fluor 488 (1:500; #713-545-003, Jackson ImmunoResearch, West Grove, PA, USA) for 1.5 hours. Fluorescent images were obtained using a Leica DM5500 fluorescence microscope with OptiGrid structured illumination configuration.

Electrophysiology

The electrophysiological responses of identified ER α neurons in the vVMH to 17 β -estradiol (E2) treatment were investigated in ER α -ZsGreen mice as previously described (26). Briefly, whole-cell patch-clamp recordings were performed on identified green fluorescent neurons in the brain slices containing vVMH from ER α -ZsGreen mice. Six- to twelve-week-old mice were deeply anesthetized with isoflurane and transcardially perfused with an ice-cold, carbogen-saturated (95% O₂ and 5% CO₂) sucrose-based cutting solution (pH 7.3), containing 10 mM NaCl, 25 mM NaHCO₃, 195 mM sucrose, 5 mM glucose, 2.5 mM KCl, 1.25 mM NaH₂PO₄, 2 mM Na pyruvate, 0.5 mM CaCl₂, and 7 mM MgCl₂. The entire brain was removed and coronally cut into slices (250 μ m) with a Microm HM 650V vibratome (Thermo Fisher Scientific, Waltham, MA, USA). Then, the vVMH-containing hypothalamic slices were incubated in oxygenated artificial cerebrospinal fluid (aCSF; adjusted to pH 7.3) containing 126 mM NaCl, 2.5 mM KCl, 2.4 mM CaCl₂, 1.2 mM NaH₂PO₄, 1.2 mM MgCl₂, 11.1 mM glucose, and 21.4 mM NaHCO₃ for 1 hour at 34°C.

Slices were transferred to the recording chamber and perfused at 34°C in oxygenated aCSF at a flow rate of 1.8 to 2 ml/min. ZsGreen-labeled ER α ^{vVMH} neurons were visualized using epifluorescence and infrared-differential interference contrast (DIC) imaging. The intracellular solution (adjusted to pH 7.3) contained the following: 128 mM K gluconate, 10 mM KCl, 10 mM Hepes, 0.1 mM EGTA, 2 mM MgCl₂, 0.05 mM Na-guanosine triphosphate (GTP), and 0.05 mM Mg-adenosine triphosphate (ATP). Recordings were made using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), sampled using Digidata 1440A, and analyzed offline with pClamp 10.3 software (Molecular Devices). Series resistance was monitored during the recording, and the values were generally <10 megohm and were not compensated. The liquid junction potential was +12.5 mV and was corrected after the experiment. Data were excluded if the series resistance increased markedly during the experiment or without overshoot for the action potential. Currents were amplified, filtered at 1 kHz, and digitized at 20 kHz. The current clamp was engaged in testing neuronal firing and resting membrane potential before and after a 1s puff of aCSF containing vehicle or 100 nM E2.

To block the majority of presynaptic inputs, the aCSF solution was supplemented with 1 μ M TTX (a sodium channel blocker; #1078, R&D Systems, Minneapolis, MN, USA) and a cocktail of fast synaptic inhibitors, namely, bicuculline (50 μ M, a GABA receptor antagonist; Tocris Inc., Bristol, UK), D-AP5 (30 μ M, an NMDA receptor antagonist; Tocris Inc.), and CNQX (30 μ M, an AMPA receptor antagonist; Tocris Inc.). D-AP5 and CNQX were used to block glutamatergic inputs, while bicuculline was included to block the γ -aminobutyric acid neuron (GABAergic) inputs. The current clamp was engaged in testing neural firing and resting membrane potential before and after a 1s puff of aCSF containing a vehicle or 100 nM E2 in the presence of TTX, bicuculline, CNQX, and D-AP5.

To assess the effect of CNO on ER α and TPH2 neurons, 8- to 10-week-old Esr1-Cre/TPH2-iCreER mice were simultaneously injected with AAV2-DIO-hM3Dq-mCherry into the vVMH (UNC Vector Core, bilaterally 200 nl; 1.60 mm posterior, 0.70 mm lateral, and 5.90 mm ventral to the bregma, based on Franklin & Paxinos Mouse Brain Atlas) and AAV2-DIO-hM4Di-mCherry into the DRN (UNC Vector Core, 4.65 mm posterior, 0 mm lateral, and 3.60 mm ventral to the bregma) 2 to 3 weeks before recording.

Three days after virus delivery, tamoxifen (0.2 mg/g body weight) was intraperitoneally injected to induce expression of TPH2-iCreER. CNO was applied to the bath solution through perfusion as previously described (53). Effects of CNO (10 μ M) on membrane potential and firing frequency of mCherry-labeled ER α and TPH2 neurons were electrophysiologically recorded.

Photostimulation-induced changes in neuronal firing and membrane potential were recorded with the whole-cell current-clamp mode. Esr1-Cre mice at 8 weeks of age were injected with AAV2-DIO-ChR2-EYFP or AAV-DIO-iC++-EYFP (UNC Vector Core) into vVMH 2 to 3 weeks before recording. To photostimulate ChR2- or iC++-positive fibers, a blue light (473 nm, 10 ms per pulse, 10 pulses per 1 s for 6 min) was focused on the vVMH. Effects of blue light photostimulation on membrane potential and firing frequency of ChR2- or iC++-labeled ER α ^{vVMH} neurons were electrophysiologically recorded.

The electrophysiological responses of identified DRN projecting ER α ^{vVMH} neurons to different environment temperatures were investigated in ER α -ZsGreen/Rosa26-LSL-tdTOMATO mice. Briefly, 8-week-old ER α -ZsGreen/Rosa26-LSL-tdTOMATO mice received stereotaxic injections of 400 nl of CAV2-Cre into the DRN. CAV2-Cre virus retrogradely traveled from the initial injection site to the upstream brain regions projecting to DRN. The Cre recombinase induce red fluorescent tdTOMATO expression in these upstream neurons. One week later, infected mice were exposed to different environmental temperatures including room temperature (23°C), 4°C, and 40°C for 30 min, respectively. In another experiment setting, ER α -ZsGreen mice received stereotaxic injections of 400 nl of red retrobeads into the DRN. The whole-cell patch clamp recordings were performed on identified red (tdTOMATO or retrobeads), green (ER α -ZsGreen), or dual fluorescent neurons (ER α -ZsGreen and tdTOMATO or red retrobeads) in the brain slice containing vVMH to determine the responses of neural firing frequency and membrane potential to the changes of environmental temperature.

CRACM was used to establish a functional connection between ER α ^{vVMH} neurons and 5-HT^{DRN} neurons. Specifically, 8-week-old Esr1-Cre/TPH2-iCreER/Rosa26-LSL-tdTOMATO mice were injected with 200 nl of AAV2-DIO-ChR2-EYFP and 200 nl of Ad-iN/WED (22) into the vVMH. The mice also received tamoxifen injection 3 days after virus delivery (0.2 mg/g body weight, i.p.) to induce expression of TPH2-iCreER, which labeled all TPH2 neurons with red fluorescence (tdTOMATO). Because Ad-iN/WED is a Cre-dependent virus, WGA-GFP was exclusively expressed in ER α ^{vVMH} neurons and anterogradely traveled along the fibers, past the synapse, and filled the downstream neurons that were innervated by ER α ^{vVMH} terminals. Seven days after injections, photostimulation of ChR2-positive fibers was induced by a blue light focused on the DRN. Then, whole-cell patch current clamp recordings were performed on identified dual fluorescent neurons (WGA-GFP and tdTOMATO) in the brain slices containing DRN to determine the responses of neuronal firing frequency and membrane potential to the photostimulation of ER α ^{vVMH} neuronal fibers in the presence of 1 μ M TTX.

In addition, voltage clamping was also performed to record the eEPSC. The intracellular solution for current-clamp recordings contained 125 mM CsCH₃SO₃, 10 mM CsCl, 5 mM NaCl, 2 mM MgCl₂, 1 mM EGTA, 10 mM Hepes, 5 mM (Mg)ATP, and 0.3 mM (Na)GTP (pH 7.3 with NaOH). To block the majority of presynaptic inputs, the aCSF solution also contained 1 μ M TTX, 4-AP (1 μ M,

a potassium channel blocker), and a cocktail of fast synaptic inhibitors, including bicuculline (50 μ M), D-AP5 (30 μ M), and CNQX (30 μ M). The eEPSC in identified dual fluorescent neurons (WGA-GFP and tdTOMATO) was measured with the voltage-clamp mode with a holding potential of -60 mV in the presence of 4-AP and TTX or CNQX and D-AP5.

DREADD manipulation of ER α ^{vVMH} neuron

To examine the metabolic effects of ER α ^{vVMH} neuron acute activation, female Esr1-Cre or WT littermates were bilaterally injected with 200 nl of AAV2-DIO-hM3Dq-mCherry into the vVMH at 8 weeks of age. Simultaneously, a telemetry probe (E-Mitter, Starr Life Sciences Corp, Oakmont, PA, USA) was implanted inside the abdominal cavity. After a 2-week of recovery phase, CNO (3 mg/kg) was intraperitoneally injected in both control (WT + hM3Dq) and mutant mice (Esr1-Cre + hM3Dq) at 9:30 a.m. and 5:00 p.m., respectively. Body core temperature and physical activity were continuously measured by the Emitter Receiver Base during the experimental phase. After a 3-day rest period, both control and mutant mice were subjected to the same procedure but received an intraperitoneal injection of saline. Thirty days after virus injection, all mice were perfused with 10% formalin, and brains were collected. Brains were sectioned and then mounted. The mCherry signals were monitored under a fluorescent microscope for validation of injection accuracy. Only those mice with mCherry signals exclusively in the vVMH were included in analyses for feeding behavior.

In another separate trial, both male and female Esr1-Cre or WT littermates were injected with AAV2-DIO-hM3Dq-mCherry into the vVMH, and Emitter was implanted underneath the BAT to measure BAT temperature. Saline or CNO (3 mg/kg) was intraperitoneally injected at 9:30 a.m. BAT temperature and physical activity were continuously monitored following the same procedure.

To examine the metabolic effects of ER α ^{vVMH} neurons long-term activation, female Esr1-Cre or WT littermates were ovariectomized at 8 weeks of age and simultaneously injected with AAV2-DIO-hM3Dq-mCherry into the vVMH. Two weeks after surgery, both WT and mutant mice received intraperitoneal injection of CNO twice a day (3 mg/kg, 9 a.m. and 5 p.m.) for 2 weeks. All mice were fed normal chow for the first week and changed to HFD for the second week. Food intake and body weight were monitored every day.

DREADD stimulation of DRN-projecting ER α ^{vVMH} neurons

Esr1-Cre female mice at 8 weeks of age were bilaterally injected with 200 nl of AAV2-DIO-hM3Dq-mCherry into the vVMH. Simultaneously, a guide cannula (C315GS-4/SPC with a terminal length of 3.5 mm; P1 Technologies, Roanoke, VA) was implanted to aim the DRN (4.65 mm posterior, 0 mm lateral, and 3.00 mm ventral to the bregma). An internal-cannula (C200IS-5/SPC customized to fit 3.5-mm C200GS-5/SPC with 0.5-mm projection, P1 Technologies) was used for drug infusion. Under the same anesthesia, an emitter was intra-abdominally implanted. Two weeks after surgeries, mice received an intro-DRN injection of 400 nl of saline, 400 nl of CNO (1 μ g/ μ l in saline) (54, 55), 400 nl of CNO (1 μ g/ μ l in saline) + D-AP5 (6.25 μ g/ μ l in saline) + CNQX (2.5 μ g/ μ l in saline) (56), or 400 nl D-AP5 (6.25 μ g/ μ l) + CNQX (2.5 μ g/ μ l). Physical activity and core temperature were continuously recorded before and after infusion. The order of infusion was randomized to avoid “sequence effects,” and each trial was separated by 6 days to ensure “wash out” of the previous infusion.

Dual DREADD

To directly test whether 5-HT^{DRN} neurons mediate the stimulatory effects of ER α ^{vVMH} neurons on BAT thermogenesis and physical activity, the dual DREADD system was applied to simultaneously activate ER α ^{vVMH} neurons and inhibit 5-HT^{DRN} neurons. Specifically, 200 nl of AAV2-DIO-hM3Dq-mCherry was bilaterally injected into vVMH, while 400 nl of AAV2-DIO-hM4Di-mCherry was injected into DRN of 8-week-old Esr1-Cre/TPH2-iCreER mice. An emitter was also implanted under the BAT as described above. The mice then received a tamoxifen injection 3 days after virus delivery (0.2 mg/g body weight, i.p.). Because both viruses are Cre-dependent, hM3Dq was exclusively expressed in ER α ^{vVMH} neurons, while hM4Di was only expressed in 5-HT^{DRN} neurons. Two weeks after surgery, the mice were injected with CNO (3 mg/kg) at 10 a.m. to simultaneously activate ER α ^{vVMH} neurons and inhibit 5-HT^{DRN} neurons. Both BAT temperature and physical activity were continuously recorded before and after CNO injection. Three days later, saline was injected at 10 a.m., and BAT temperature and physical activity were recorded as a baseline. To validate accurate and sufficient infection, all mice were perfused with 10% formalin. Brain sections were obtained at 25 μ m (five series) and subjected to mCherry immunohistochemistry as described above. Only those mice with mCherry signals were included in the analyses.

In another control experiment, 400 nl of AAV2-DIO-hM4Di-mCherry was injected into the DRN of TPH2-iCreER mice. The effects of 5-HT^{DRN} inhibition on BAT thermogenesis and physical activity were determined as described above.

Optogenetic inhibition/activation of ER α ^{vVMH} $\rightarrow$ DRN neurons

Esr1-Cre female mice at 8 weeks of age were bilaterally injected with 200 nl of AAV-DIO-iC++-EYFP into the vVMH. Simultaneously, a light fiber (200-nm-diameter core, numerical aperture 0.22; Thor Laboratories, Newton, NJ, USA) was implanted to target the DRN (4.65 mm posterior, 0.00 mm lateral, and 3.25 mm ventral to the bregma). Under the same anesthesia, an emitter was intra-abdominally implanted, and a temperature transponder (IPTT-300, Bio Medic Data Systems, Seaford, DE, USA) was implanted under the BAT. Two weeks after surgeries, blue light stimulation (473 nm, 10 ms per pulse, 20 Hz, 3-s on, and 2-s off for 1 hour) was used to inhibit ER α ^{vVMH} $\rightarrow$ DRN neurons during the lights-on period between 8 a.m. and 9 a.m. BAT thermogenesis, core temperature, and physical activity were recorded before, during, and 1 hour after stimulation. As a control experiment, yellow light with the same modulation was applied. The intensity of light power exiting the fiber tip corresponds to 5 mW for blue and yellow light.

To determine the primary mediator for the stimulatory effects of ER α ^{vVMH} $\rightarrow$ DRN circuit on BAT thermogenesis and physical activity, AAV-DIO-ChR2-EYFP was bilaterally injected into the vVMH, and a light fiber was implanted into the DRN as described above. After surgery recovery, saline or SR59230A, an ADRB3 antagonist (0.5 mg/kg in saline; #S8688, Sigma-Aldrich, St. Louis, MO, USA) were intraperitoneally injected before blue or yellow light stimulation during lights-on period between 8 a.m. and 9 a.m., respectively. BAT thermogenesis and physical activity were monitored as described above.

As a post hoc validation, mice were perfused 90 min after yellow/blue light photostimulation in the DRN (20 Hz, 10-ms pulses, and 3-mW constant stimulation for 5 min). DRN-specific activation

was validated by immunohistochemistry of cFOS (1:1000; #2250, Cell Signaling) as described above.

Construction of an AAV vector for Flp-dependent expression of Cre recombinase

An AAV carrying Cre recombinase in Flp-dependent double-inverted open reading frame, pAAV-hSyn1-frEX-Cre-GFP, was constructed using pAAV-hSyn1s-DIO-SK3-2A-GFP-bGhPa as a backbone. The following components were arranged in the downstream of left inverted terminal repeat (ITR) of the backbone: human synapsin promoter; frt; F3; inverted Cre-2A-eGFP from Cre Shine (Addgene plasmid, #37404); inverted frt; inverted F3; woodchuck hepatitis virus posttranscriptional regulatory element; bovine growth hormone poly(A) sequence; and right ITR. The AAV-hSyn1-frEX-Cre-GFP vector was packaged into serotype 2/8 through the Neuro-connectivity Core in the Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital. Flp-dependent Cre expression from pAAV-hSyn1-frEX-Cre-GFP was verified in vitro by immunofluorescence imaging for DsRed in infected human embryonic kidney 293 cells transfected with AAV-hSyn1-frEX-Cre-GFP + PGK-LSL-DsRed (Addgene plasmid, #13769) or AAV-hSyn1-frEX-Cre-GFP + CAG-Flpe (Addgene plasmid, #13787) + PGK-LSL-DsRed.

Retrograde deletion

To determine whether ER α expressed by ER α ^{vVMH} $\rightarrow$ DRN neurons is required for estrogenic regulation of body weight control, ER α was retrogradely deleted from ER α ^{vVMH} $\rightarrow$ DRN neurons. Specifically, 8-week-old female Esr1^{flox/flox} mice received a 400-nl Δ G Rabies FLPO-dsRedXpress injection into the DRN and bilateral injections of 200 nl of AAV-hSyn1-frEX-Cre-GFP into vVMH. An emitter was also implanted under the BAT as described above. Because Δ G Rabies FLPO-dsRedXpress is a retrograde virus, Rabies-carried FLPO-dsRed was taken up by the fiber terminals in the DRN and retrograded back to the cell body in the vVMH. Upon injection of flpo-dependent AAV-hSyn1-frEX-Cre-GFP virus, Cre recombinase was expressed exclusively in a subset of vVMH neurons projecting to the DRN and deleted ER α in these neurons. Another group of control Esr1^{flox/flox} mice received AAV2-GFP into the DRN and bilateral injections of AAV-hSyn1-frEX-Cre-GFP into the vVMH. One week after virus injection, all mice were fed with a normal chow diet for 1 week and changed to HFD for 2 weeks. Food intake and body weight were measured once every other day. BAT thermogenesis and physical activity were continuously monitored for the whole experimental period. The specificity of ER α retrograde deletion was first validated by immunohistochemistry of ER α and immunofluorescent staining of dsRed. Briefly, brain sections were incubated with rabbit anti-ER α antibody (1:10,000; catalog no. C1355, Millipore) at room temperature overnight, followed by the biotinylated donkey anti-rabbit secondary antibody (1:1000, #711-067-003, Jackson ImmunoResearch) for 2 hours. Sections were then incubated in the avidin-biotin complex (1:1000; PK-6100, Vector Laboratories) and incubated in 0.04% 3,3'-diaminobenzidine and 0.01% hydrogen peroxide. Subsequently, brain sections were incubated with anti-dsRed-Alexa Fluor 594 antibody (1:200; catalog no. sc-390909 AF594, Santa Cruz Biotechnology) overnight. Both fluorescent and bright-field images were obtained using a Leica DM5500 microscope.

To quantify ER α in the vVMH, another aliquot of brain sections was used for immunohistochemistry of ER α as described above.

After dehydration through graded ethanol, the slides were then immersed in xylene and coverslipped. Bright-field images were analyzed as described before.

Statistics

Statistical analyses were performed using GraphPad Prism. Methods of statistical analyses were chosen on the basis of the design of each experiment and indicated in the figure legends. The data were presented as means $\pm$ SEM. $P \leq 0.05$ was considered to be statistically significant.

Study approval

Care of all animals and procedures were approved by Baylor College of Medicine Institutional and The University of Illinois at Chicago Animal Care and Use Committee.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abk0185>

[View/request a protocol for this paper from Bio-protocol.](#)

REFERENCES AND NOTES

1. F. Mauvais-Jarvis, D. J. Clegg, A. L. Hevener, The role of estrogens in control of energy balance and glucose homeostasis. *Endocr. Rev.* **34**, 309–338 (2013).
2. J. Santollo, M. D. Wiley, L. A. Eckel, Acute activation of ER α decreases food intake, meal size, and body weight in ovariectomized rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **293**, R2194–R2201 (2007).
3. S. Ogawa, J. Chan, J. A. Gustafsson, K. S. Korach, D. W. Pfaff, Estrogen increases locomotor activity in mice through estrogen receptor α : Specificity for the type of activity. *Endocrinology* **144**, 230–239 (2003).
4. P. A. Heine, J. A. Taylor, G. A. Iwamoto, D. B. Lubahn, P. S. Cooke, Increased adipose tissue in male and female estrogen receptor- α knockout mice. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 12729–12734 (2000).
5. Y. Xu, T. P. Nedungadi, L. Zhu, N. Sobhani, B. G. Irani, K. E. Davis, X. Zhang, F. Zou, L. M. Gent, L. D. Hahner, S. A. Khan, C. F. Elias, J. K. Elmquist, D. J. Clegg, Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. *Cell Metab.* **14**, 453–465 (2011).
6. A. W. Smith, M. A. Bosch, E. J. Wagner, O. K. Ronnekleiv, M. J. Kelly, The membrane estrogen receptor ligand STX rapidly enhances GABAergic signaling in NPY/AgRP neurons: Role in mediating the anorexigenic effects of 17 β -estradiol. *Am. J. Physiol. Endocrinol. Metab.* **305**, E632–E640 (2013).
7. G. Pelletier, S. Li, V. Luu-The, F. Labrie, Oestrogenic regulation of pro-opiomelanocortin, neuropeptide Y and corticotrophin-releasing hormone mRNAs in mouse hypothalamus. *J. Neuroendocrinol.* **19**, 426–431 (2007).
8. S. Musatov, W. Chen, D. W. Pfaff, C. V. Mobbs, X. J. Yang, D. J. Clegg, M. G. Kaplitt, S. Ogawa, Silencing of estrogen receptor α in the ventromedial nucleus of hypothalamus leads to metabolic syndrome. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2501–2506 (2007).
9. P. B. Martinez de Morentin, I. Gonzalez-Garcia, L. Martins, R. Lage, D. Fernandez-Mallo, N. Martinez-Sanchez, F. Ruiz-Pino, J. Liu, D. A. Morgan, L. Pinilla, R. Gallego, A. K. Saha, A. Kalsbeek, E. Fliers, P. H. Bisschop, C. Dieguez, R. Nogueiras, K. Rahmouni, M. Tena-Sempere, M. Lopez, Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. *Cell Metab.* **20**, 41–53 (2014).
10. S. M. Correa, D. W. Newstrom, J. P. Warne, P. Flandin, C. C. Cheung, A. T. Lin-Moore, A. A. Pierce, A. W. Xu, J. L. Rubenstein, H. A. Ingraham, An estrogen-responsive module in the ventromedial hypothalamus selectively drives sex-specific activity in females. *Cell Rep.* **10**, 62–74 (2015).
11. Y. He, P. Xu, C. Wang, Y. Xia, M. Yu, Y. Yang, K. Yu, X. Cai, N. Qu, K. Saito, J. Wang, I. Hyseni, M. Robertson, B. Piyaathana, M. Gao, S. A. Khan, F. Liu, R. Chen, C. Coarfa, Z. Zhao, Q. Tong, Z. Sun, Y. Xu, Estrogen receptor- α expressing neurons in the ventrolateral VMH regulate glucose balance. *Nat. Commun.* **11**, 2165 (2020).
12. C. F. Yang, M. C. Chiang, D. C. Gray, M. Prabhakaran, M. Alvarado, S. A. Juntti, E. K. Unger, J. A. Wells, N. M. Shah, Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. *Cell* **153**, 896–909 (2013).
13. H. Lee, D. W. Kim, R. Remedios, T. E. Anthony, A. Chang, L. Madisen, H. Zeng, D. J. Anderson, Scalable control of mounting and attack by Esr1+ neurons in the ventromedial hypothalamus. *Nature* **509**, 627–632 (2014).
14. K. Hashikawa, Y. Hashikawa, R. Tremblay, J. Zhang, J. E. Feng, A. Sabol, W. T. Piper, H. Lee, B. Rudy, D. Lin, Esr1⁺ cells in the ventromedial hypothalamus control female aggression. *Nat. Neurosci.* **20**, 1580–1590 (2017).
15. A. L. Falkner, P. Dollar, P. Perona, D. J. Anderson, D. Lin, Decoding ventromedial hypothalamic neural activity during male mouse aggression. *J. Neurosci.* **34**, 5971–5984 (2014).
16. D. Lin, M. P. Boyle, P. Dollar, H. Lee, E. S. Lein, P. Perona, D. J. Anderson, Functional identification of an aggression locus in the mouse hypothalamus. *Nature* **470**, 221–226 (2011).
17. J. E. van Veen, L. G. Kammel, P. C. Bunda, M. Shum, M. S. Reid, M. G. Massa, D. V. Arneson, J. W. Park, Z. Zhang, A. M. Joseph, H. Hrnir, M. Liesa, A. P. Arnold, X. Yang, S. M. Correa, Hypothalamic oestrogen receptor α establishes a sexually dimorphic regulatory node of energy expenditure. *Nat. Metab.* **2**, 351–363 (2020).
18. D. W. Kim, Z. Yao, L. T. Graybuck, T. K. Kim, T. N. Nguyen, K. A. Smith, O. Fong, L. Yi, N. Kouloula, N. Pierson, S. Shah, L. Lo, A. H. Pool, Y. Oka, L. Pachter, L. Cai, B. Tasic, H. Zeng, D. J. Anderson, Multimodal analysis of cell types in a hypothalamic node controlling social behavior. *Cell* **179**, 713–728.e17 (2019).
19. K. Saito, Y. He, X. Yan, Y. Yang, C. Wang, P. Xu, A. O. Hinton Jr., G. Shu, L. Yu, Q. Tong, Y. Xu, Visualizing estrogen receptor- α -expressing neurons using a new ER α -ZsGreen reporter mouse line. *Metabolism* **65**, 522–532 (2016).
20. J. L. Gomez, J. Bonaventura, W. Lesniak, W. B. Mathews, P. Sysa-Shah, L. A. Rodriguez, R. J. Ellis, C. T. Richie, B. K. Harvey, R. F. Dannals, M. G. Pomper, A. Bonci, M. Michaelides, Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science* **357**, 503–507 (2016).
21. J. M. McGlashan, M. C. Gorecki, A. E. Kozlowski, C. K. Thirnbek, K. R. Markan, K. L. Leslie, M. E. Kotas, M. J. Potthoff, G. B. Richerson, M. P. Gillum, Central serotonergic neurons activate and recruit thermogenic brown and beige fat and regulate glucose and lipid homeostasis. *Cell Metab.* **21**, 692–705 (2015).
22. G. W. Louis, G. M. Leininger, C. J. Rhodes, M. G. Myers Jr., Direct innervation and modulation of orexin neurons by lateral hypothalamic LepRb neurons. *J. Neurosci.* **30**, 11278–11287 (2010).
23. D. A. Steindler, R. H. Bradley, N-[acetyl-3H] wheat germ agglutinin: Anatomical and biochemical studies of a sensitive bidirectionally transported axonal tracer. *Neuroscience* **10**, 219–241 (1983).
24. N. Kinoshita, T. Mizuno, Y. Yoshihara, Adenovirus-mediated WGA gene delivery for transsynaptic labeling of mouse olfactory pathways. *Chem. Senses* **27**, 215–223 (2002).
25. I. R. Wickersham, D. C. Lyon, R. J. Barnard, T. Mori, S. Finke, K. K. Conzelmann, J. A. Young, E. M. Callaway, Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron* **53**, 639–647 (2007).
26. X. Cao, P. Xu, M. G. Oyola, Y. Xia, X. Yan, K. Saito, G. Zou, C. Wang, Y. Yang, A. Hinton Jr., C. Yan, H. Ding, L. Zhu, L. Yu, B. Yang, Y. Feng, D. J. Clegg, S. Khan, R. DiMarchi, S. K. Mani, Q. Tong, Y. Xu, Estrogens stimulate serotonin neurons to inhibit binge-like eating in mice. *J. Clin. Invest.* **124**, 4351–4362 (2014).
27. L. Petreanu, D. Huber, A. Sobczyk, K. Svoboda, Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. *Nat. Neurosci.* **10**, 663–668 (2007).
28. Y. Ootsuka, K. Kulasekara, R. C. de Menezes, W. W. Blessing, SR59230A, a beta-3 adrenoceptor antagonist, inhibits ultradian brown adipose tissue thermogenesis and interrupts associated episodic brain and body heating. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R987–R994 (2011).
29. R. S. Ray, A. E. Corcoran, R. D. Brust, J. C. Kim, G. B. Richerson, E. Nattie, S. M. Dymecki, Impaired respiratory and body temperature control upon acute serotonergic neuron inhibition. *Science* **333**, 637–642 (2011).
30. N. M. Murray, G. F. Buchanan, G. B. Richerson, Insomnia caused by serotonin depletion is due to hypothermia. *Sleep* **38**, 1985–1993 (2015).
31. Y. Han, G. Xia, D. Srisai, F. Meng, Y. He, Y. Ran, Y. He, M. Farias, G. Hoang, I. Toth, M. O. Dietrich, M. H. Chen, Y. Xu, Q. Wu, Deciphering an AgRP-serotonergic neural circuit in distinct control of energy metabolism from feeding. *Nat. Commun.* **12**, 3525 (2021).
32. C. Seo, A. Guru, M. Jin, B. Ito, B. J. Sleezer, Y. Y. Ho, E. Wang, C. Boada, N. A. Krupa, D. S. Kullakanda, C. X. Shen, M. R. Warden, Intense threat switches dorsal raphe serotonin neurons to a paradoxical operational mode. *Science* **363**, 538–542 (2019).
33. A. M. Harris, L. R. Macbride, R. C. Foster, S. K. McCrady, J. A. Levine, Does non-exercise activity thermogenesis contribute to non-shivering thermogenesis? *J. Therm. Biol.* **31**, 634–638 (2006).
34. L. Girardier, M. G. Clark, J. Seydoux, Thermogenesis associated with spontaneous activity: An important component of thermoregulatory needs in rats. *J. Physiol.* **488** (Pt. 3), 779–787 (1995).
35. C. L. Tan, E. K. Cooke, D. E. Leib, Y. C. Lin, G. E. Daly, C. A. Zimmerman, Z. A. Knight, Warm-sensitive neurons that control body temperature. *Cell* **167**, 47–59.e15 (2016).
36. S. R. Kelso, M. N. Perlmuter, J. A. Boulant, Thermosensitive single-unit activity of in vitro hypothalamic slices. *Am. J. Physiol.* **242**, R77–R84 (1982).

37. S. Rodriguez-Cuenca, E. Pujol, R. Justo, M. Frontera, J. Oliver, M. Gianotti, P. Roca, Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue. *J. Biol. Chem.* **277**, 42958–42963 (2002).
38. T. M. Takahashi, G. A. Sunagawa, S. Soya, M. Abe, K. Sakurai, K. Ishikawa, M. Yanagisawa, H. Hama, E. Hasegawa, A. Miyawaki, K. Sakimura, M. Takahashi, T. Sakurai, A discrete neuronal circuit induces a hibernation-like state in rodents. *Nature* **583**, 109–114 (2020).
39. S. Hrvatin, S. Sun, O. F. Wilcox, H. Yao, A. J. Lavin-Peter, M. Cicconet, E. G. Assad, M. E. Palmer, S. Aronson, A. S. Banks, E. C. Griffith, M. E. Greenberg, Neurons that regulate mouse torpor. *Nature* **583**, 115–121 (2020).
40. L. Lo, S. Yao, D. W. Kim, A. Cetin, J. Harris, H. Zeng, D. J. Anderson, B. Weissbourd, Connective architecture of a mouse hypothalamic circuit node controlling social behavior. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 7503–7512 (2019).
41. H. Dhillon, J. M. Zigman, C. Ye, C. E. Lee, R. A. McGovern, V. Tang, C. D. Kenny, L. M. Christiansen, R. D. White, E. A. Edelstein, R. Coppari, N. Balthasar, M. A. Cowley, S. Chua Jr., J. K. Elmquist, B. B. Lowell, Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. *Neuron* **49**, 191–203 (2006).
42. S. Amir, M. Lagiorgia, R. Pollock, Intra-ventromedial hypothalamic injection of insulin suppresses brown fat thermogenesis in the anaesthetized rat. *Brain Res.* **480**, 340–343 (1989).
43. M. Lopez, L. Varela, M. J. Vazquez, S. Rodriguez-Cuenca, C. R. Gonzalez, V. R. Velagapudi, D. A. Morgan, E. Schoenmakers, K. Agassandian, R. Lage, P. B. M. de Morentin, S. Tovar, R. Nogueiras, D. Carling, C. Lelliott, R. Gallego, M. Oresic, K. Chatterjee, A. K. Saha, K. Rahmouni, C. Dieguez, A. Vidal-Puig, Hypothalamic AMPK and fatty acid metabolism mediate thyroid regulation of energy balance. *Nat. Med.* **16**, 1001–1008 (2010).
44. D. Beiroa, M. Imbernon, R. Gallego, A. Senra, D. Herranz, F. Villarroya, M. Serrano, J. Ferno, J. Salvador, J. Escalada, C. Dieguez, M. Lopez, G. Fruhbeck, R. Nogueiras, GLP-1 agonism stimulates brown adipose tissue thermogenesis and browning through hypothalamic AMPK. *Diabetes* **63**, 3346–3358 (2014).
45. W. Zhang, S. Bi, Hypothalamic regulation of brown adipose tissue thermogenesis and energy homeostasis. *Front. Endocrinol.* **6**, 136 (2015).
46. D. Richard, Cognitive and autonomic determinants of energy homeostasis in obesity. *Nat. Rev. Endocrinol.* **11**, 489–501 (2015).
47. V. Ryu, A. G. Watts, B. Xue, T. J. Bartness, Bidirectional crosstalk between the sensory and sympathetic motor systems innervating brown and white adipose tissue in male Siberian hamsters. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **312**, R324–R337 (2017).
48. C. J. Madden, E. P. Santos da Conceicao, S. F. Morrison, Vagal afferent activation decreases brown adipose tissue (BAT) sympathetic nerve activity and BAT thermogenesis. *Temperature* **4**, 89–96 (2017).
49. J. C. van den Beukel, A. Grefhorst, C. Quarta, J. Steenbergen, P. G. Mastroberardino, M. Lombès, P. J. Delhanty, R. Mazza, U. Pagotto, A. J. van der Lely, A. P. N. Themmen, Direct activating effects of adrenocorticotrophic hormone (ACTH) on brown adipose tissue are attenuated by corticosterone. *FASEB J.* **28**, 4857–4867 (2014).
50. E. Hondares, M. Rosell, F. J. Gonzalez, M. Giral, R. Iglesias, F. Villarroya, Hepatic FGF21 expression is induced at birth via PPARalpha in response to milk intake and contributes to thermogenic activation of neonatal brown fat. *Cell Metab.* **11**, 206–212 (2010).
51. S. H. Lockie, K. M. Heppner, N. Chaudhary, J. R. Chabenne, D. A. Morgan, C. Veyrat-Durebex, G. Ananthakrishnan, F. Rohner-Jeanrenaud, D. J. Drucker, R. DiMarchi, K. Rahmouni, B. J. Oldfield, M. H. Tschop, D. Perez-Tilve, Direct control of brown adipose tissue thermogenesis by central nervous system glucagon-like peptide-1 receptor signaling. *Diabetes* **61**, 2753–2762 (2012).
52. Y. Feng, D. Manka, K. U. Wagner, S. A. Khan, Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 14718–14723 (2007).
53. C. Zhu, P. Xu, Y. He, Y. Yuan, T. Wang, X. Cai, L. Yu, L. Yang, J. Wu, L. Wang, X. Zhu, S. Wang, P. Gao, Q. Xi, Y. Zhang, Y. Xu, Q. Jiang, G. Shu, Heparin increases food intake through AgRP neurons. *Cell Rep.* **20**, 2455–2467 (2017).
54. S. X. Luo, J. Huang, Q. Li, H. Mohammad, C.-Y. Lee, K. Krishna, A. M.-Y. Kok, Y. L. Tan, J. Y. Lim, H. Li, L. Y. Yeow, J. Sun, M. He, J. Grandjean, S. Sajikumar, W. Han, Y. Fu, Regulation of feeding by somatostatin neurons in the tuberal nucleus. *Science* **361**, 76–81 (2018).
55. T. J. Stachniak, A. Ghosh, S. M. Sternson, Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus→midbrain pathway for feeding behavior. *Neuron* **82**, 797–808 (2014).
56. D. L. Walker, G. Y. Paschall, M. Davis, Glutamate receptor antagonist infusions into the basolateral and medial amygdala reveal differential contributions to olfactory vs. context fear conditioning and expression. *Learn. Mem.* **12**, 120–129 (2005).

Acknowledgments: We wish to thank the Laboratory of Animal Center of Baylor College of Medicine, Pennington Biomedical Research Center at Louisiana State University, and The University of Illinois at Chicago for invaluable help in mouse colony maintenance. The Ad-IN/WED virus was provided by M. Myers (University of Michigan). **Funding:** This work was supported by grants from NIH (P01 DK113954, R01 DK117281, R01 DK115761, and R01 DK125480 to Y.X.; R01 DK120858 to Y.X. and Q.T.; R00 DK107008 and R01 DK123098 to P.X.; T32 AA026577 to V.T.; K01 DK119471 to C.W.; K01 DK111771 to Y.J.; P20 GM135002 to Y.H.; R01 DK114279 and R21 NS108091 to Q.T.; R01 DK109934 to B.R.A. and Q.T.; R01ES027544 and R03AG070687 to Z.S.), USDA/CRIS (51000-064-015 to Y.X.), DOD (Innovative Grant W81XWH-19-PRMRP-DA to P.X.; DOD W81XWH-19-1-0429 to B.R.A. and Q.T.), DRTC (The Pilot and Feasibility Award DK020595 to P.X.), and American Diabetes Association (1-17-PDF-138 to Y.H.). **Author contributions:** H.Y. is the main contributor to the conduct of the study and data collection; S.S., C.W., Y.Y., L.I., N.P., L.S., Pei L., V. T., Pen. L., M.K., D.D., X.C., N.Q., K.S., I.H., K.Y., and B.F. contributed to the conduct of the study; Y.J., Q.T., Z.S., and B.R.A. contributed to the manuscript writing and data interpretation; and Y.H., P.X., and Y.X. contributed to the study design, data interpretation, and manuscript writing. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. The ERα-ZsGreen mouse line can be provided by Y.X. pending scientific review and a completed material transfer agreement. Requests for the ERα-ZsGreen should be submitted to yongx@bcm.edu. The AAV-hSyn1-frEX-Cre-GFP vector can be provided by Y.X. pending scientific review and a completed material transfer agreement. Requests for the AAV-hSyn1-frEX-Cre-GFP vector should be submitted to yongx@bcm.edu.

Submitted 16 June 2021
 Accepted 23 November 2021
 Published 19 January 2022
 10.1126/sciadv.abk0185

An estrogen-sensitive hypothalamus-midbrain neural circuit controls thermogenesis and physical activity

Hui YeBing FengChunmei WangKenji SaitoYongjie YangLucas IbrahimiSarah SchaulNirali PatelLeslie SaenzPei LuoPenghua LaiValeria TorresMaya KotaDevin DixitXing CaiNa Qullirjana HyseniKaifan YuYuwei JiangQingchun TongZheng SunBenjamin R. ArenkielYanlin HePingwen XuYong Xu

Sci. Adv., 8 (3), eabk0185. • DOI: 10.1126/sciadv.abk0185

View the article online

<https://www.science.org/doi/10.1126/sciadv.abk0185>

Permissions

<https://www.science.org/help/reprints-and-permissions>

Use of this article is subject to the [Terms of service](#)

Science Advances (ISSN) is published by the American Association for the Advancement of Science. 1200 New York Avenue NW, Washington, DC 20005. The title *Science Advances* is a registered trademark of AAAS.

Copyright © 2022 The Authors, some rights reserved; exclusive licensee American Association for the Advancement of Science. No claim to original U.S. Government Works. Distributed under a Creative Commons Attribution NonCommercial License 4.0 (CC BY-NC).

Reviews

Hypothalamic CRH neurons: A crossroad between stress and metabolism

Zhiying Jiang and Qingchun Tong

Abstract

Stress responses have a major impact on whole body physiology, including energy metabolism, but the underlying neural substrates and pathways mediating stress effects on energy metabolism are complex. The corticotropin-releasing hormone neurons in the hypothalamic paraventricular nucleus play a central role in stress response. Recently, accumulating evidence indicates that the PVN CRH neurons are a direct link between stress and obesity. Obesity impairs CRH neurons responsiveness to stress, and perturbations of CRH neurons activity cause obesity. Therefore, CRH neurons in the hypothalamus stand at the intersection of stress response and energy balance regulation. Here, we reviewed recent advances in understanding the regulation of appetite and energy metabolism by PVN CRH neurons, with a focus on the distinct long-term contributions of CRH neuropeptides and glucocorticoids.

Addresses

The Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, McGovern Medical School of the University of Texas Health Science Center at Houston, Houston, TX, 77030, USA

Corresponding author: Tong, Qingchun (Qingchun.Tong@uth.tmc.edu)

 (Tong Q.)

Current Opinion in Endocrine and Metabolic Research 2022, 26:100384

This review comes from a themed issue on **Neuroendocrine Response to Stress**

Edited by **Megan Holmes** and **Ferenc Antoni**

For complete overview of the section, please refer the article collection - [Neuroendocrine Response to Stress](#)

Available online 6 August 2022

<https://doi.org/10.1016/j.coemr.2022.100384>

2451-9650/© 2022 Elsevier Ltd. All rights reserved.

Keywords

Energy homeostasis, Obesity, Hypothalamic-pituitary-adrenal (HPA) axis, Corticotropin-releasing hormone (CRH), Corticotropin-releasing factor (CRF), CRHR1, CRHR2, Glucocorticoids, Circadian rhythms, Paraventricular hypothalamus (PVH), Paraventricular hypothalamic nucleus (PVN).

Introduction

The stress response, coined by Dr. Hans Selye as “the non-specific response of the body to any demand for

change” [1], is a highly conserved and multi-faceted mechanism vital for survival. The corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus of the hypothalamus (PVN) play a crucial role in stress responses. During stress, the stress-responsive brain regions and the hypothalamic-pituitary-adrenal (HPA) axis are rapidly engaged to coordinate behavioral, autonomic, and hormonal outputs that favor survival and later to restore organisms to allostasis. Information about real or perceived threats through both ascending and descending pathways converges on the CRH neurons of the PVN, thereby stimulating CRH release to specific brain regions, including the median eminence. In the brain, CRH activates neural pathways to tune behavioral and autonomic responses. In the median eminence, through the portal circulation, CRH reaches the anterior pituitary, where it stimulates adrenocorticotrophic hormone (ACTH) production and release, thereby eventually leading to corticosteroids synthesis and release from the adrenal cortex. The activation of the HPA axis, through released corticosteroids acting on the mineralocorticoid (MR) and glucocorticoid receptors (GR), is paramount to mobilize energy and coordinate key organs to fuel stress responses and maintain homeostasis.

Stress responses are tightly intertwined with energy homeostasis [2]. Defective stress responses are typical symptoms of psychiatric disorders, such as anxiety and depression. Eating disorders, such as anorexia nervosa and binge eating, are also associated with anxious and depressive phenotypes. This behavioral association supports that stress-responsive brain neurons, including PVN CRH neurons, may lie at the crossroad of stress responses and energy metabolism. In this review, we attempt to untangle the complex interaction of PVN CRH neurons and energy homeostasis, with a focus on long-term perturbations of the HPA axis and the consequences on energy balance.

CRH neurons in the PVN in energy homeostasis

CRH neurons in the PVN are interoceptive to acute hunger and satiety signals. CRH neurons decrease their activity when food and reward cues are present, by rebalancing excitatory and inhibitory synaptic inputs, even before consumption behaviors start [3–5]. Caloric states, such as refeeding after food deprivation, increase

c-fos expression in PVN neurons and CRH neurons [6–8]. Moreover, CRH neurons' responsivity is also shaped by energy states. Long-term intake of high-fat diets (HFD) decreases PVN and CRH neuron responsiveness to stressful stimuli [7,8]. Palatable food, either high in sugar or fat, also dampens the HPA axis function and diminishes ACTH and corticosterone response to restraint stress [9]. On the contrary, chronic variable stress, which is often associated with suppressed body weight gain, sensitizes the HPA axis to a novel stressor [10]. CRH neuronal activity and circulating corticosterone are even affected by gut microbiota [11]. Taking together, CRH neurons activity and responsiveness are impacted by nutritional states.

The responsivity of PVN CRH neurons to nutritional status is likely mediated by upstream network, as CRH neurons are densely innervated by brain regions involved in food intake, reward, and energy expenditure, such as raphe nucleus, bed nucleus of the stria terminalis (BNST), paraventricular nucleus of the thalamus (PVT), parabrachial nucleus (PB), and even PVN itself [4,12,13]. PVN neurons receive extensive inputs from neurons in the arcuate nucleus (ARC) [14,15]. Both AgRP and POMC neurons in the ARC send projections to the PVN, and selective activation of POMC neurons increases neuronal fos expression in the PVN [16,17]. It is conceivable that some neurons in the ARC, both glutamatergic and GABAergic, send direct projections to PVN CRH neurons [4], although functional evidence is still lacking. CRH neurons also receive direct inputs from catecholaminergic neurons from ventrolateral medulla (VLM) and nucleus of the solitary tract (NTS), which are regions important for stress-induced hyperglycemia [18,19]. Furthermore, CRH neurons are robustly innervated by anorexigenic glucagon-like peptide 1 (GLP-1) neurons in the NTS. The activation of GLP-1 neurons releases both GLP-1 and glutamate, which work synergistically to increase AMPAR trafficking to the membrane, and thus boosting glutamate neurotransmission efficacy. Consequently, the loss of GLP-1R signaling from PVN neurons in adult mice leads to obesity [12].

In addition to the evidence that nutritional signals affect CRH neuron activity and responsiveness, direct manipulation of CRH neuron activity alters food intake and energy expenditure. Acute modulation of CRH neurons activity alters food-seeking behavior. Optogenetic activation of CRH neurons induces conditioned place aversion, which is strong enough to overcome the motivation of food seeking in a hungry state [3]. In line with this, acute chemogenetic activation of CRH neurons decreases food intake [12,20]. The effect of acute changes in CRH neuron activity is transient, suggesting a potential contribution of these neurons to feeding inhibition in response to acute stress stimulation. However, acute change in food intake may not always predict body weight changes. As body weight changes,

including obesity development, require accumulation of changes on feeding or energy expenditure, animal models with chronic modulation of PVN CRH neurons are necessary to examine the impact of these neurons on energy balance regulation.

Toward this, we clamped CRH neurons activity at either high or low levels, by expressing NaChBac, a low threshold, slow deactivating sodium channel, or Kir2.1, a mutant potassium channel, selectively in PVN CRH neurons. We found that clamping CRH neuron activities at either high or low levels render mice susceptible to diet-induced obesity. Moreover, these mice showed a flattened diurnal rhythmicity, both in food intake and energy expenditure. Importantly, consistent with their activity being clamped, these neurons show diminished responses to stressors and nutritional signals [7]. These results demonstrate that the responsiveness of PVN CRH neurons, not the absolute activity levels, underscores the susceptibility to obesity development. Although it remains to be tested, chronic clamping CRH neuron activity at high levels may represent the activity state of these neurons during chronic stress conditions in humans, which may constitute a neural basis underlying chronic stress-induced obesity development.

Given the importance of CRH neuron responsiveness in feeding and body weight regulation, it will be imperative for future studies to examine the molecular mechanism underlying CRH neurons responsiveness and the associated changes induced by HFD and stressors.

CRH receptors in energy balance

CRH neurons in the PVN exert their effects on energy balance through both direct neural pathway and indirect neuroendocrine pathway, that is, through the CRH receptors and the HPA axis. It is shown that CRH neurons, at least a subset of CRH neurons, send collateral projections to the lateral hypothalamus and the median eminence [21]. The released CRH binds two G-protein coupled receptors: CRHR1 and CRHR2. CRH has 10-fold higher affinity for CRHR1 than CRHR2. CRHR1 are mainly expressed in the brain [22] and are believed to be the principal activator of the HPA axis and a key mediator of anxiety-like behaviors [23,24]. CRHR2 are expressed in both peripheral tissues and the brain and are shown to play a major role in energy expenditure [22,25].

Compelling evidence established that stress, or central administration of CRH, decreases food intake dose-dependently. This anorexigenic effect is potent and lasts 24 h or longer [26,27]. Consistent with this, CRH overexpression mice exhibit a reduction in fasting-induced food intake [28]. Nevertheless, studies of receptor-specific knockout (KO) mice come up with conflicting results. CRHR1-KO mice exhibit no change or only a mild body weight reduction [29,30], and

CRHR2-KO mice also show normal body weight when fed on chow [25]. Interestingly, both KO mouse strains exhibit resistance to diet-induced obesity. When fed on HFD, CRHR1-KO mice show no change in overall daily food intake, albeit blunted diurnal food intake pattern, and higher locomotion and energy expenditure [30]. CRHR2-KO mice consume more food but are able to maintain similar body weight on HFD, which results from lower body fat content and higher lean mass, indicating higher energy expenditure [25]. CRHR2-KO mice also have a smaller BAT and WAT adipocyte size, increased BAT UCP-1 expression, and manifest elevation in iBAT thermogenesis and sympathetic nervous system outflow [31]. However, the selective deletion of CRH receptors in adult mice results in different phenotypes. In contrast to little effect on energy balance on chow in the global CRHR2-KO model, selective knock-down (KD) of CRHR2 only in the ventromedial hypothalamus (VMH) in adult mice increases body weight gain, due to increased fat mass and increased adipocyte cell size. VMH CRHR2-KD mice also have increased food intake and energy expenditure [32]. Therefore, it is likely that CRH exerts its anorexigenic effect, at least partially, through VMH CRHR2 signaling, in intact animals.

The lack of obesogenic phenotype in CRHR1-KO and CRHR2-KO models may be due to specific expression and functions of CRHR2 in different tissues, but it is more likely due to developmental defects and compensation. It is noteworthy that loss of CRH/CRHR1 during development results in lung dysplasia, adrenal atrophy, and pancreas islet hypoplasia [23,24,30,33], CRHR2-KO mice manifest developmental compensation by CRHR1 [31], and double KO of both CRHR1 and CRHR2 impairs HPA axis at a greater degree than each single KO model [34]. The energy metabolism changes in embryonic KO mice are likely to result from adaptive changes during development. Future studies selectively targeting CRH receptors in specific brain regions in healthy adults are needed to elucidate the real role of CRH receptors in energy homeostasis and their interplay with palatable food.

Besides CRH neuropeptides, CRH neurons in the PVN are capable of releasing glutamate as a neurotransmitter [21]. The action of glutamate release may also contribute to the discordant outcomes between altered CRH neuron activities and deficiency in CRH receptors. Whether glutamate neurotransmission to lateral hypothalamus, a well-known brain region inhibiting feeding behavior [35] or other downstream brain regions, plays a role in regulating energy balance is still a puzzle to be solved.

Glucocorticoids in feeding behavior and energy metabolism

In contrast to the largely neglected roles of PVN CRH release to brain neurons in energy balance, the role of

glucocorticoids (GCs) is well studied. The HPA axis exerts its impact on energy homeostasis through the actions of its end product corticosteroids (cortisol in humans and corticosterone in rodents) on the receptors. Since most MRs are saturated at basal conditions, GRs are the receptors that are responsible for the metabolic effects of corticosteroids. GCs are known to increase food intake in both humans and rodents [36–38], and chronic elevation of GCs also increases fat adiposity, such as in Cushing syndrome, and patients undergoing GCs therapy. Adrenalectomy, which results in hypocortisolemia, decreases food intake and body weight, and the reduction in food intake and body weight are rescued by corticosterone supplementation [39,40].

GCs stimulate both homeostatic and non-homeostatic feeding. Hunger driven by hyperleptinemia and hypoglycemia requires activation of the HPA axis and increase in corticosterone [40]. Study found that corticosterone exerts its orexigenic effect by increasing both excitatory synapses onto orexigenic AgRP/NPY neurons and inhibitory synapses onto anorexigenic POMC neurons in the ARC nucleus [39], as well as directly increasing AgRP/NPY neuron autonomous firing [40]. GCs also influence on non-homeostatic feeding. GCs signaling on dopaminergic neurons in ventral tegmental area (VTA) encodes rewarding value of HFD without affecting energy metabolism; thus, mice lacking GR in dopaminergic neurons lose interest in HFD under calorically restricted conditions [41].

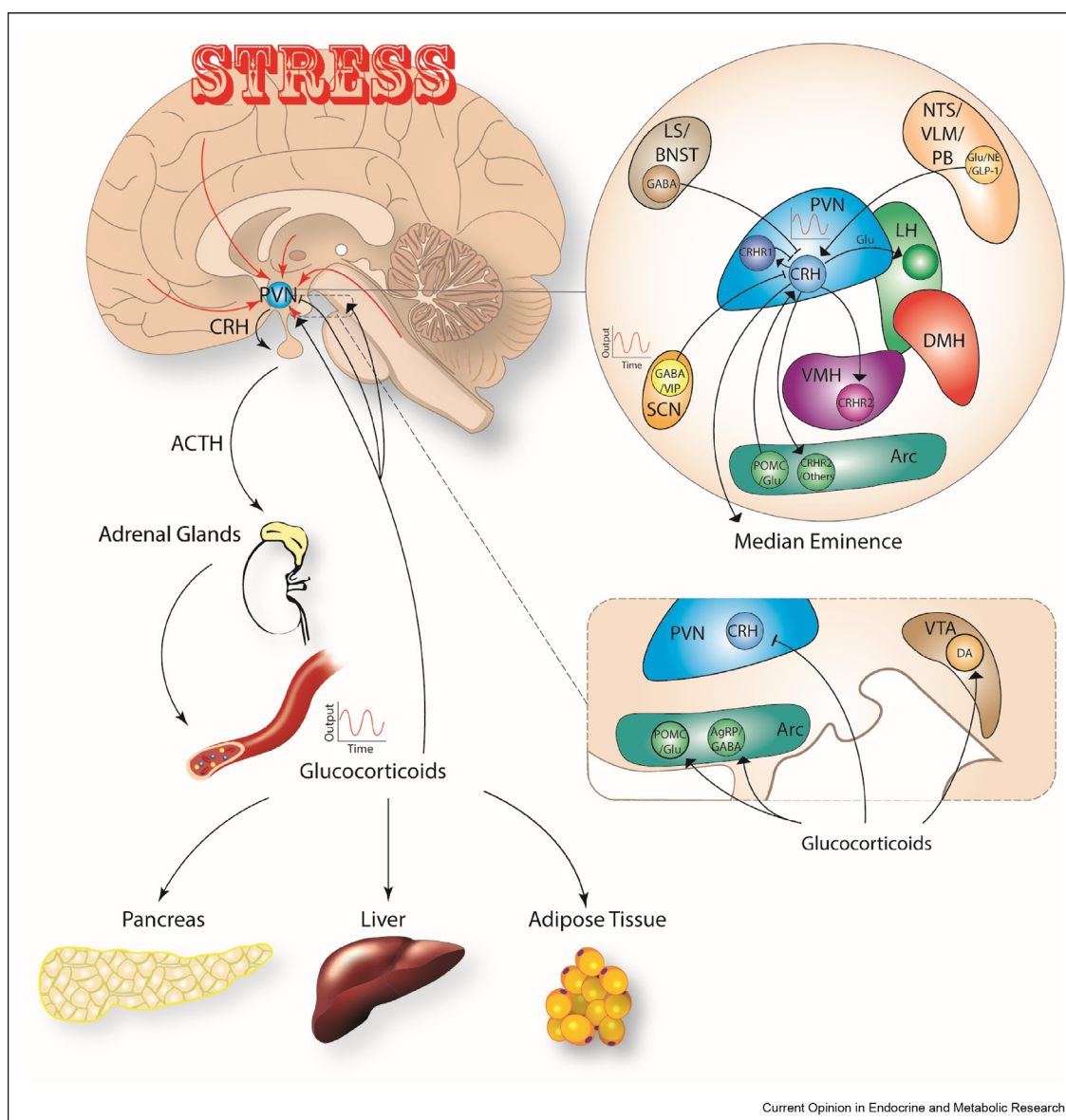
Apart from apparently increasing the motivation to eat food, recent studies found that disruption in circadian rhythmicity of corticosteroids also contributes to obesity development. The circulating GCs show prominent and robust circadian rhythms [42], maintained by coordinated daily oscillations of CRH neurons activity and adrenal glands sensitivity to ACTH. The selective deletion of clock gene *Bmal1* from CRH neurons, which maintains intact response to stress, disrupts the circadian change in CRH neuron activity and dampens the circadian oscillation of corticosteroids [43]. Similarly, we also found that the selective deletion of *Bmal1* gene from PVN neurons reduces diurnal rhythmicity in energy expenditure [8]. Given the previous work involving CRH neurons in diet-induced obesity [44], it will be interesting to know the impact of disrupted CRH neuron activity patterns owing to *Bmal1* deletion on body weight. CRH neurons are entrained by rhythmic GABAergic inputs from suprachiasmatic nucleus (SCN) [43,45], and PVN neurons, likely including CRH neurons, also display diurnal expression of functional GABA_A receptors. These observations argue for a circadian brain pathway for diurnal GC rhythmicity [8]. Therefore, besides stimulation of food intake, patterns of GCs action may play an important role in body weight regulation.

Coordination of CRH and glucocorticoids in feeding and metabolism regulation

Stress-induced activation of the HPA axis controls energy homeostasis through different pathways and at different levels, as summarized in Figure 1. Stress activates the HPA axis to release CRH neuropeptides and eventually increases circulating corticosteroids. Appropriate responsivity and rhythmicity of CRH neurons, along with circulating GCs, are critical for the maintenance of energy balance in ad libitum conditions, and

perturbations of CRH neurons responsivity and rhythmicity disrupt the balance of energy intake and expenditure, leading to obesity. However, as discussed above, it is surprising that, both as indispensable parts of the stress response, CRH and corticosteroids have opposite effects on food intake. It is noteworthy that those two hormones are released at different phases during a stress response [46,47]. During the initial phase, in milliseconds to minutes, immediate activation of CRH neurons activates mainly neural pathways to prioritize “fight or flight”

Figure 1



Pathways that connect the HPA axis to obesity. CRH neurons receive tonic and event-evoked inputs from the central master clock SCN and stress-sensitive brain regions. Upon stress exposure, CRH neuropeptides act on their receptors in brain regions involved in food intake, such as VMH, ARC, and PVN. Later, corticosteroids act on the PVN CRH neurons to shut off the HPA axis and act on the ARC and VTA to increase both homeostatic and hedonic eating.

responses over “rest and digest,” which is beneficial to maximize chances of survival; at a later phase, in hours and even days, organisms need to “shut off” the stress response, restore energy balance, and even prepare the body with more glycogen and fat for future uncertainties, which are achieved through the orexigenic and obesogenic actions of GCs. Hence, precise temporal control of CRH and corticosteroids release allows efficient response to and recovery from stress.

It is common to see increased body weight in humans experiencing chronic stress [48], but most chronic stress rodent models have suppressed body weight gain [10,49,50]. One key determining factor may lie in the alterations in the sensitivity of the HPA axis. Rodent models often use severe stress paradigms, such as chronic restraint, chronic variable stress, and chronic social defeat stress, which cause sensitization of the HPA axis response to novel stressors, similar to diet-resistant rats having higher CRH and corticosterone levels [51]. In contrast, diet-induced obesity reduces CRH neuron responsiveness and blunts HPA rhythmicity. In humans, chronic stress is often caused by mild psychological stressors, and the activity of the HPA axis does not increase with body mass index (BMI) [52]. It is also known that different stressors engage different repertoires of neuronal populations, and thus may differentially influence food intake. Furthermore, humans have easy access to high fat/sucrose “comfort food” [36]. People eat more palatable food when they are stressed [53]. In the short term, it relieves negative feelings, but in the long run, overconsumption of palatable food leads to obesity. To make things worse, overweight and obesity cause psychological stress in humans (weight stigma) and drive them to consume more “comfort food”, which becomes a vicious cycle. When given free food choice, rats also consume a higher proportion of palatable food, and consumption of palatable food diminishes ACTH response to restraint stress [9]. Future studies with free access to different nutrients will be valuable to model stress-induced obesity in humans. CRH neurons also encode stress controllability and regulate behavior selection [54], and the selective activation of a subset of CRH neurons expressing AMPK induces a preference of high carbohydrate diet over HFD after food deprivation [20]. Whether CRH neurons directly control the decision to consume different nutrients based on the contexts of stressors and to minimize the negative emotions associated with stressful experience, remains an interesting topic to explore. A deeper understanding of the interplay of PVN CRH neurons and different nutrients may provide new strategies for the development of effective prevention and treatments for obesity in modern society.

Conflict of interest statement

Nothing declared.

Acknowledgement

The authors acknowledge the support from NIH R01 DK114279, R21NS108091, R01 DK109934, R01 DK120858, and DOD W81XWH-19-1-0429. Q.T. is the holder of the Cullen Chair in Molecular Medicine at McGovern Medical School of UTHealth.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

** of outstanding interest

1. The Stress of Life, Hans Selye MD: *New York, McGraw-hill book company, inc.* 1956.
2. Liu T, Xu Y, Yi C-X, Tong Q, Cai D: **The hypothalamus for whole-body physiology: from metabolism to aging.** *Protein Cell* 2021, <https://doi.org/10.1007/s13238-021-00834-x>.
3. Kim J, et al.: **Rapid, biphasic CRF neuronal responses encode positive and negative valence.** *Nat Neurosci* 2019, **22**:576–585. * This paper demonstrates that PVN CRH neurons show increase in calcium activity in response to stressful stimuli, and decrease in response to food. And selective inhibition of CRH neurons induces conditioned place preference, and selective activation of CRH neurons induces conditioned place aversion, the latter is strong enough to overcome the motivation to seek food in hungry states.
4. Yuan Y, et al.: **Reward inhibits paraventricular CRH neurons to relieve stress.** *Curr Biol* 2019, **29**:1243–1251. e4. * This paper demonstrates that reward decreases PVN CRH neurons activity by rebalancing synaptic inputs. The authors also mapped long range monosynaptic inputs to PVN CRH neurons.
5. Melnick I, Krishtal OA, Colmers WF: **Integration of energy homeostasis and stress by parvocellular neurons in rat hypothalamic paraventricular nucleus.** *J Physiol* 2020, **598**:1073–1092.
6. Li C, et al.: **Defined paraventricular hypothalamic populations exhibit differential responses to food contingent on caloric state.** *Cell Metabol* 2019, **29**:681–694. e5.
7. Zhu C, et al.: **Disrupted hypothalamic CRH neuron responsiveness contributes to diet-induced obesity.** *EMBO Rep* 2020, **21**:e49210. ** This paper demonstrates that clamping PVN CRH neurons activity both at high and low levels makes mice susceptible to diet-induced obesity, which is attributed to blunted circadian rhythms in food intake and energy expenditure.
8. Kim ER, et al.: **Paraventricular hypothalamus mediates diurnal rhythm of metabolism.** *Nat Commun* 2020, **11**:3794. * This paper demonstrates that disruption in clock gene or circadian rhythms of GABA neurotransmission to PVN neurons decreases energy expenditure, and causes obesity.
9. Foster MT, et al.: **Palatable foods, stress, and energy stores sculpt corticotropin-releasing factor, adrenocorticotropin, and corticosterone concentrations after restraint.** *Endocrinology* 2009, **150**:2325–2333.
10. Franco AJ, et al.: **Sensitization of the hypothalamic-pituitary-adrenal Axis in a male rat chronic stress model.** *Endocrinology* 2016, **157**:2346–2355.
11. Wu W-L, et al.: **Microbiota regulate social behaviour via stress response neurons in the brain.** *Nature* 2021, **595**:409–414.
12. Liu J, et al.: **Enhanced AMPA receptor trafficking mediates the anorexic effect of endogenous glucagon-like peptide-1 in the paraventricular hypothalamus.** *Neuron* 2017, **96**:897–909. e5. * This paper demonstrated that PVN CRH neurons receive monosynaptic inputs from anorexigenic GLP-1 neurons in the NTS, and loss of GLP-1 signaling in the PVN causes obesity. GLP-1 suppresses appetite by increasing glutamate transmission efficacy via PKA-dependent AMPA receptor trafficking to the membrane.
13. Jiang Z, Rajamanickam S, Justice NJ: **Local corticotropin-releasing factor signaling in the hypothalamic paraventricular nucleus.** *J Neurosci* 2018, **38**:1874–1890.

14. Krashes MJ, *et al.*: **An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger.** *Nature* 2014, **507**: 238–242.
 15. Atasoy D, Betley JN, Su HH, Sternson SM: **Deconstruction of a neural circuit for hunger.** *Nature* 2012, **488**:172–177.
 16. Betley JN, Cao ZFH, Ritola KD, Sternson SM: **Parallel, redundant circuit organization for homeostatic control of feeding behavior.** *Cell* 2013, **155**:1337–1350.
 17. Wei Q, *et al.*: **Uneven balance of power between hypothalamic peptidergic neurons in the control of feeding.** *Proc. Natl. Acad. Sci. U. S. A.* 2018, **115**:E9489–E9498.
 18. Chen C, *et al.*: **Astrocytes amplify neuronal dendritic volume transmission stimulated by norepinephrine.** *Cell Rep* 2019, **29**:4349–4361. e4.
 19. Zhao Z, *et al.*: **A central catecholaminergic circuit controls blood glucose levels during stress.** *Neuron* 2017, **95**:138–152. e5.
 20. Okamoto S, *et al.*: **Activation of AMPK-regulated CRH neurons in the PVH is sufficient and necessary to induce dietary preference for carbohydrate over fat.** *Cell Rep* 2018, **22**:706–721.
 21. Füzesi T, Daviu N, Wamsteeker Cusulin JI, Bonin RP, Bains JS: **Hypothalamic CRH neurons orchestrate complex behaviours after stress.** *Nat Commun* 2016, **7**:11937.
 22. Van Pett K, *et al.*: **Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse.** *J Comp Neurol* 2000, **428**:191–212.
 23. Smith GW, *et al.*: **Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development.** *Neuron* 1998, **20**:1093–1102.
 24. Timpl P, *et al.*: **Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1.** *Nat Genet* 1998, **19**:162–166.
 25. Bale TL, *et al.*: **Corticotropin-releasing factor receptor-2-deficient mice display abnormal homeostatic responses to challenges of increased dietary fat and cold.** *Endocrinology* 2003, **144**:2580–2587.
 26. Semjonous NM, *et al.*: **Coordinated changes in energy intake and expenditure following hypothalamic administration of neuropeptides involved in energy balance.** *Int J Obes* 2009, **33**:775–785.
 27. Levine AS, Rogers B, Kneip J, Grace M, Morley JE: **Effect of centrally administered corticotropin releasing factor (CRF) on multiple feeding paradigms.** *Neuropharmacology* 1983, **22**: 337–339.
 28. Stengel A, *et al.*: **Corticotropin-releasing factor-overexpressing mice exhibit reduced neuronal activation in the arcuate nucleus and food intake in response to fasting.** *Endocrinology* 2009, **150**:153–160.
 29. Bradbury MJ, McBurnie MI, Denton DA, Lee KF, Vale WW: **Modulation of urocortin-induced hypophagia and weight loss by corticotropin-releasing factor receptor 1 deficiency in mice.** *Endocrinology* 2000, **141**:2715–2724.
 30. Sakamoto R, *et al.*: **Roles for corticotropin-releasing factor receptor type 1 in energy homeostasis in mice.** *Metabolism* 2013, **62**:1739–1748.
 31. Carlin KM, Vale WW, Bale TL: **Vital functions of corticotropin-releasing factor (CRF) pathways in maintenance and regulation of energy homeostasis.** *Proc Natl Acad Sci USA* 2006, **103**: 3462–3467.
 32. Chao H, Digruccio M, Chen P, Li C: **Type 2 corticotropin-releasing factor receptor in the ventromedial nucleus of hypothalamus is critical in regulating feeding and lipid metabolism in white adipose tissue.** *Endocrinology* 2012, **153**:166–176.
 33. Zhang R, *et al.*: **Loss of hypothalamic corticotropin-releasing hormone markedly reduces anxiety behaviors in mice.** *Mol Psychiatr* 2017, **22**:733–744.
 34. Bale TL, *et al.*: **Mice deficient for both corticotropin-releasing factor receptor 1 (CRFR1) and CRFR2 have an impaired stress response and display sexually dichotomous anxiety-like behavior.** *J Neurosci* 2002, **22**:193–199.
 35. Mangieri LR, *et al.*: **A neural basis for antagonistic control of feeding and compulsive behaviors.** *Nat. Commun Now* 2018, **9**:52.
 36. Dallman MF, *et al.*: **Chronic stress and obesity: a new view of 'comfort food'.** *Proc Natl Acad Sci USA* 2003, **100**:11696–11701.
 37. Berthoin BS, MacDonald-Wicks LK, Wood LG: **A systematic review of the effect of oral glucocorticoids on energy intake, appetite, and body weight in humans.** *Nutr Res* 2014, **34**:179–190.
 38. Karatsoreos IN, *et al.*: **Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse.** *Endocrinology* 2010, **151**: 2117–2127.
 39. Gyengesi E, *et al.*: **Corticosterone regulates synaptic input organization of POMC and NPY/AgRP neurons in adult mice.** *Endocrinology* 2010, **151**:5395–5402.
 40. Perry RJ, *et al.*: **Leptin's hunger-suppressing effects are mediated by the hypothalamic-pituitary-adrenocortical axis in rodents.** *Proc Natl Acad Sci USA* 2019, **116**:13670–13679.
- This paper demonstrates that hunger induced by hyperleptinemia and hypoglycemia requires activation of the HPA axis and increase in corticosterone.
41. Mizoguchi A, *et al.*: **Glucocorticoid receptor signaling in ventral tegmental area neurons increases the rewarding value of a high-fat diet in mice.** *Sci Rep* 2021, **11**:12873.
 42. Mendoza J: **Nighttime light hurts mammalian physiology: what diurnal rodent models are telling us.** *Clocks Sleep* 2021, **3**:236–250.
 43. Jones JR, Chaturvedi S, Granados-Fuentes D, Herzog ED: **Circadian neurons in the paraventricular nucleus entrain and sustain daily rhythms in glucocorticoids.** *Nat Commun* 2021, **12**:5763.
- This paper demonstrates that PVN CRH neurons display rhythmicity in clock gene expression and calcium activity. Deletion of clock genes from CRH neurons results in arrhythmic CRH neuron activity and corticosterone release. Discrete activation of inactivation of SCN VIP neurons alter CRH neuron activity and patterns of corticosterone release.
44. Zhu C, *et al.*: **Profound and redundant functions of arcuate neurons in obesity development.** *Nat Metab* 2020, **2**:763–774.
 45. Paul S, *et al.*: **Output from VIP cells of the mammalian central clock regulates daily physiological rhythms.** *Nat Commun* 2020, **11**.
 46. Vahl TP, *et al.*: **Comparative analysis of ACTH and corticosterone sampling methods in rats.** *Am J Physiol Endocrinol Metab* 2005, **289**:E823–E828.
 47. Joëls M, Baram TZ: **The neuro-symphony of stress.** *Nat Rev Neurosci* 2009, **10**:459–466.
 48. Wardle J, Chida Y, Gibson EL, Whitaker KL, Steptoe A: **Stress and adiposity: a meta-analysis of longitudinal studies.** *Obesity* 2011, **19**:771–778.
 49. Pecoraro N, Reyes F, Gomez F, Bhargava A, Dallman MF: **Chronic stress promotes palatable feeding, which reduces signs of stress: feedforward and feedback effects of chronic stress.** *Endocrinology* 2004, **145**:3754–3762.
 50. Balsevich G, *et al.*: **Interplay between diet-induced obesity and chronic stress in mice: potential role of FKBP51.** *J Endocrinol* 2014, **222**:15–26.
 51. Shin AC, *et al.*: **Responsiveness of hypothalamo-pituitary-adrenal axis to leptin is impaired in diet-induced obese rats.** *Nutr Diabetes* 2019, **9**:10.
 52. Tenk J, *et al.*: **Obesity, HPA Axis Activity does not increase with BMI, but declines with aging: a meta-analysis of clinical studies.** *PLoS One* 2016, **11**, e0166842.

53. Tomiyama AJ, Dallman MF, Epel ES: **Comfort food is comforting to those most stressed: evidence of the chronic stress response network in high stress women.** *Psychoneuroendocrinology* 2011, **36**:1513–1519.
54. Daviu N, *et al.*: **Paraventricular nucleus CRH neurons encode stress controllability and regulate defensive behavior selection.** *Nat Neurosci* 2020, **23**:398–410.