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TITLE: Genetically Dissecting Basal Forebrain Circuits Underlying Eating Disorders

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14. ABSTRACT Abnormal eating habits that drive excessive food seeking- or avoidance, can manifest as health-threatening and socially impactful eating disorders. We have previously reported a potent role for basal forebrain cholinergic neurons in regulating feeding behavior, and recently discovered that cell type-specific manipulations of their immediate downstream targets, i.e. Glutamatergic (Glu+) neurons, are capable of directly toggling a spectrum of eating-associated behaviors that range from increased food seeking and excessive food consumption to severe hypophagia, overt food avoidance, and starvation. Continuing on the achievements in the previous report, we have accomplished experiments showing that Glu+ neurons are direct downstream target of cholinergic neurons, the cholinergic receptor types on the Glu+ neurons, Glu+ neuron responses to a variety of external cues including food odors, potent inhibition on feeding behaviors by activation of Glu+ neurons. In addition, we have also made significant progress in other goals including experiments aiming to test the effects of loss of function of forebrain Glu+ neurons, to test the role of LH neurons in mediating the action of forebrain Glu+ neurons in feeding behaviors as well as the effect of the forebrain Glu+ neurons on obesity from leptin deficiency, which laid a solid foundation for the planned goals in year 3. This discovery provides intriguing insight into a previously unknown circuit that links the cholinergic basal forebrain, which is known for its role in attention, wakefulness, and sensory processing, to value based components of eating associated behaviors. Experiments designed in this award will elucidate the mechanisms by which basal forebrain circuits govern food seeking and/or food aversion.					
15. SUBJECT TERMS Basal forebrain, eating disorder, glutamate, acetylcholine, lateral hypothalamus, optogenetics, in vivo Ca2+ imaging, metabolism					
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INTRODUCTION

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

Specifically for this report period, we have accomplished the major goals planned for this period. Regarding completion of major goals stated from the onset of the proposed research, we have made significant progress towards accomplishing major goals associated with major goals 1-6, and have also made significant progress towards the other remaining major goals.

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 and included in the last report.

Major goal 2: map local inputs onto basal forebrain Glu+ neurons; SOW completion data and site: 3-12 months in Site 2 (Dr. Tong). This goal has been delayed because of unavailability of SAD rabies virus during the pandemic. We have generated the required animal models and the rabies virus has just recently arrived, and we will resume the experiment immediately. This goal will be accomplished 1-24 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 partially accomplished. Initial expression analysis has been conducted using FISH, but we are still confirming basal forebrain cell types of expression. This has been delayed due to the pandemic, but is currently underway.

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 6-20 months

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.

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 60%, and is currently a focus of our efforts.

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 accomplished 50%, and is currently underway.

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 accomplished 20%.

Major goal 10: Genetic rescue of obesity; SOW completion date and site: 6-36 months and Site 1 (Dr. Arenkiel). This goal has been accomplished approximately 50%.

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 20%, and is currently a primary focus of both labs.

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 30%, and is currently underway.

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 approximately 20%.

Accomplishment during the reporting period:

In the previous report, we have accomplished major goals 1 and 4. During the reporting period, we have accomplished major goals 2-3 and 5-6, and also made significant progress in major goals 7-8, and 10.

Major goal 2 (Dr. Tong): Here the goal is to determine whether ChAT neurons in the basal forebrain send monosynaptic projections to Glu+ neurons. We plan to use monosynaptic pseudotyped rabies viral 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

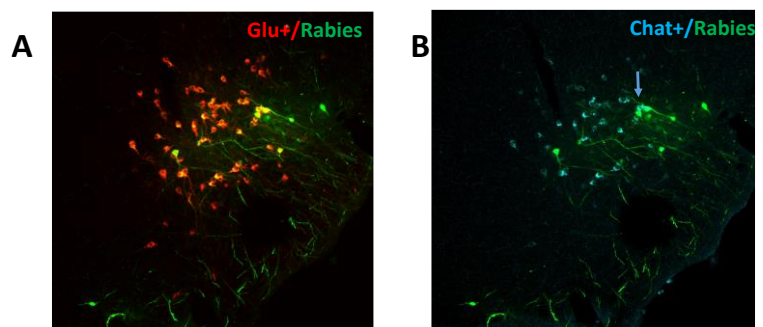
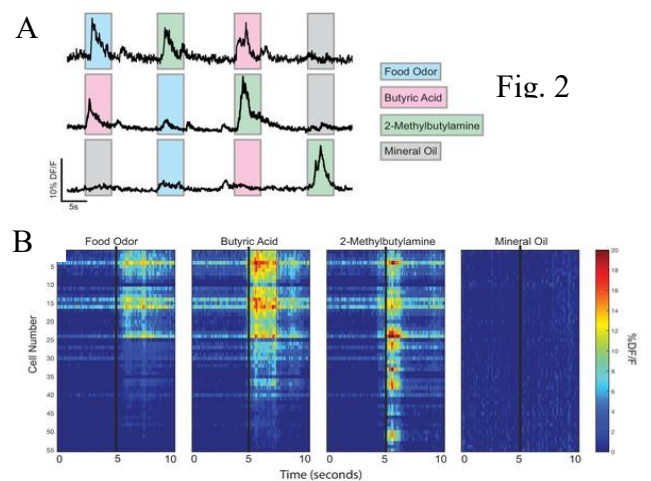


Fig. 1

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.

Major goal 3 (Dr. Arenkiel): Here the goal is to analyze ACH receptors in basal forebrain Glu+ neurons. Toward this, RNA probes have been synthesized, tested for efficacy, and initial expression analysis has been completed. Due to the shutdown we are now just resuming further experimentation to validate the cell type-specific patterns of AChR expression. New approaches towards single cell sequencing of basal forebrain material is 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 (Fig. 2A, traces for Butyric Acid and 2-Methylbutylamine) and found that, interestingly, these neurons responded to different food-related odors, but not to the mineral oil control (Fig. 2A, food odor versus mineral oil). These responses were rapid and reversible (Fig. 2B), suggesting a direct relevance of these neurons to feeding. This goal has been accomplished within the set time frame.



Major goal 6 (Dr. Tong): Here the goal is to determine feeding and body weight 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 (Fig. 3A). We found that NaChBac injected mice exhibited rapid body weight reduction (Fig. 3B and 3C), due to dramatic reduction of feeding (Fig. 3C).

The remaining 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 was not fully rescued (Fig. 3D). This goal has been accomplished within the planned time frame.

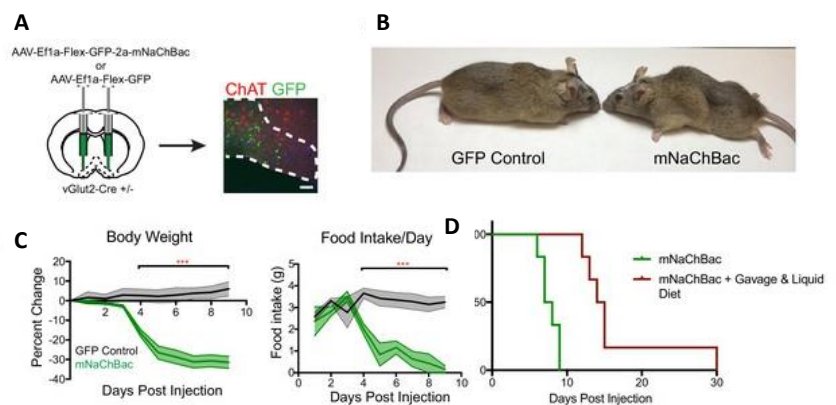


Fig. 3

Major goal 7 (Drs. Arenkiel and Tong): Here we seek to determine the effect of loss of function of basal forebrain Glu+ neurons on 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.

Ongoing efforts are focused on verification of injection and expression, and confirmation of TeNT achieving loss of Glu release. It is projected that the specified goal will be accomplished within the planned time frame.

Major goal 8 (Dr. Tong): Here we set out 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. 4), 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. Currently, we are verifying the anatomy of accurate delivery and expression of the virus, as well as the effect of light delivery. It is estimated that we will accomplish the planned goal within the set time frame.

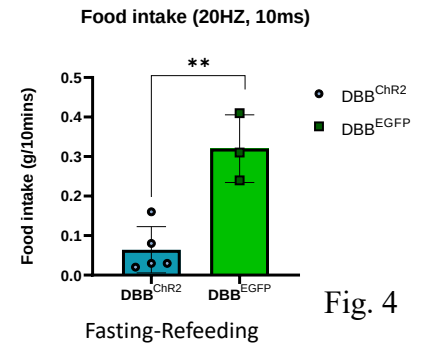


Fig. 4

Major goal 9 (Dr. Tong): Here the goal is 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. Future experiments are planned to evaluate effects of light stimulation on feeding with or without CNO administration. It is estimated that these experiments can be accomplished within the planned time frame.

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

Major goal 11 (Drs. Arenkiel and Tong): Here the goal is to determine whether LH functions as a key downstream target that mediates 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. The mice that received surgery are ready to be assayed for feeding or place preference with light delivery to the LH. It is estimated that this goal will be accomplished within the planned time frame.

Major goal 12 (Dr. Tong): the goal is to determine whether inhibition of basal forebrain Glu+ neuron projections to the LH promote motivation to eat. Delivery of AAV-Flex-eNpHR3.0 virus has been made to bilateral basal forebrain regions of *Vglut2-Cre* and optic fibers have also been implanted targeting the LH. These mice are scheduled to receive training session in a nose poke chamber and then will test wither light delivery will test to measure correct nose pokes and latency to nose poke. It is estimated that the goal will be achieved within the planned time frame.

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

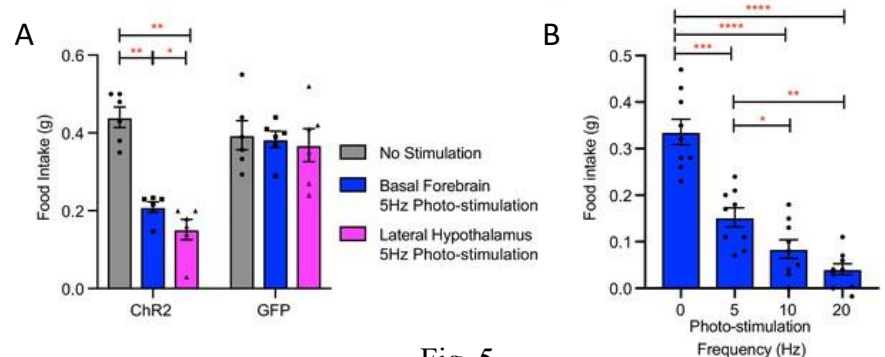


Fig. 5.

local photostimulation in the LH potentially inhibited feeding, the extent of which is similar to that observed with direct activation of basal forebrain Glu+ neurons (Fig. 5A). Of note, the effect on feeding inhibition was dependent on laser frequency (Fig. 5B). Ongoing studies are focused on verification of accurate viral delivery and induction of c-Fos in the LH neurons after photostimulation. It is estimated that the milestone in this goal will be met within the planned time window.

Major goal 14 (Dr. Tong): Here the goal is to determine whether LH Glu+ neurons mediate the effect of basal forebrain Glu+ neurons in feeding. *Vglut2-Cre* mice were prepared and AAV-Flex-ChR2 virus has been delivered to the basal forebrain, while at the same time AAV-Flex-hM4Di-mCherry delivered to the LH. Initial experiments on verification of viral delivery have been confirmed. New mice have been prepared, and studies are ongoing to examine effects on feeding. It is projected that the milestone in this goal will be met within the set time window.

Training and professional development

Dr. Zhiying Jiang, a postdoc and Ms. Jing Cai, a graduate student (Site 2, Dr. Tong) are currently involved in this project, and are supported by this funding. Both of them are required to attend our departmental and center seminar series. The departmental seminar series invites well-known scientists across the US to present their new findings and the center seminar series will mainly presented by postdocs and students from each lab to share their research. Thus, both of them will have the opportunities to be exposed to research outside the campus and to present their own ongoing research. In addition, Ms. Cai will have planned activity and training classed within the graduate school, including required committee meetings in which Dr. Arenkiel is a committee member. We also have a regular lab meeting in which all lab members require to present their own research as well present a journal club on the papers from peers. Importantly, both of them have regular weekly meeting with me. Through these various platforms, they will get trainings in research. With the pandemic, almost all seminar series with the UT and TMC use virtual format, in which they regularly attend. Unfortunately, with the impact of the pandemic, it is not 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 been scheduled to attend this year's SfN meeting in November 2021 at Chicago.

Lab personnel in Arenkiel lab include Ms. Mayuri Patel, 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, 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. 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. However, it might be potential that a significant foreseeable change to the project is that we may need additional time 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. A neural basis for brain leptin action on reducing type 1 diabetic hyperglycemia. Fan S, Xu Y, Lu Y, Jiang Z, Li H, Morrill JC, Cai J, Wu Q, Xu Y, Xue M, Arenkiel BR, Huang C, Tong Q. Nat Commun. 2021 May 11;12(1):2662. doi: 10.1038/s41467-021-22940-4. PMID: 33976218; Acknowledgement of the federal support: Yes.
2. Centrally circulating α -klotho inversely correlates with human obesity and modulates arcuate cell populations in mice. Landry T, Li P, Shookster D, Jiang Z, Li H, Laing BT, Bunner W, Langton T, Tong Q, Huang H. Mol Metab. 2021 Feb;44:101136. doi: 10.1016/j.molmet.2020.101136. Epub 2020 Dec 7. PMID: 33301986; Acknowledgement of the federal support: Yes.
3. Corticotropin Releasing Hormone Signaling in the Bed Nuclei of the Stria Terminalis as a Link to Maladaptive Behaviors. Young CE, Tong Q. Front Neurosci. 2021 Mar 19;15:642379. doi:

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

10.3389/fnins.2021.642379. eCollection 2021. PMID: 33867924 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

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

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.



PI: Qingchun Tong, University of Texas Health Science Center at Houston

Budget: \$1,144,713.00 **Topic Area:** Eating Disorders **Mechanism:** FY 2018 Peer Reviewed Medical Research Program Investigator-Initiated Research Award

Research Area: 1400

Award Status: 01 Sept 2020 – 31 Aug 2021

Study Goals:

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

Specific Aims:

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

Key Accomplishments & Outcomes:

Publications:

1. A neural basis for brain leptin action on reducing type 1 diabetic hyperglycemia. Fan S, Xu Y, Lu Y, Jiang Z, Li H, Morrill JC, Cai J, Wu Q, Xu Y, Xue M, Arenkiel BR, Huang C, Tong Q. Nat Commun. 2021 May 11;12(1):2662. doi: 10.1038/s41467-021-22940-4.
2. Centrally circulating α -klotho inversely correlates with human obesity and modulates arcuate cell populations in mice. Landry T, Li P, Shookster D, Jiang Z, Li H, Laing BT, Bunner W, Langton T, Tong Q, Huang H. Mol Metab. 2021 Feb;44:101136. doi: 10.1016/j.molmet.2020.101136. Epub 2020 Dec 7.
3. Corticotropin Releasing Hormone Signaling in the Bed Nuclei of the Stria Terminalis as a Link to Maladaptive Behaviors. Young CE, Tong Q. Front Neurosci. 2021 Mar 19;15:642379. doi: 10.3389/fnins.2021.642379. eCollection 2021. PMID: 33867924

Patents: Nothing to report

Funding Obtained: Nothing to report

ARTICLE



<https://doi.org/10.1038/s41467-021-22940-4>

OPEN

A neural basis for brain leptin action on reducing type 1 diabetic hyperglycemia

Shengjie Fan^{1,2,9}, Yuanzhong Xu^{2,9}, Yungang Lu², Zhiying Jiang², Hongli Li², Jessie C. Morrill^{2,3}, Jing Cai^{2,3}, Qi Wu⁴, Yong Xu⁴, Mingshan Xue^{5,6}, Benjamin R. Arenkiel⁷, Cheng Huang^{1✉} & Qingchun Tong^{2,3,8✉}

Central leptin action rescues type 1 diabetic (T1D) hyperglycemia; however, the underlying mechanism and the identity of mediating neurons remain elusive. Here, we show that leptin receptor (LepR)-expressing neurons in arcuate (LepR^{Arc}) are selectively activated in T1D. Activation of LepR^{Arc} neurons, Arc GABAergic (GABA^{Arc}) neurons, or arcuate AgRP neurons, is able to reverse the leptin's rescuing effect. Conversely, inhibition of GABA^{Arc} neurons, but not AgRP neurons, produces leptin-mimicking rescuing effects. Further, AgRP neuron function is not required for T1D hyperglycemia or leptin's rescuing effects. Finally, T1D LepR^{Arc} neurons show defective nutrient sensing and signs of cellular energy deprivation, which are both restored by leptin, whereas nutrient deprivation reverses the leptin action. Our results identify aberrant activation of LepR^{Arc} neurons owing to energy deprivation as the neural basis for T1D hyperglycemia and that leptin action is mediated by inhibiting LepR^{Arc} neurons through reversing energy deprivation.

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Patients with type 1 diabetes (T1D) suffer greatly from uncontrolled hyperglycemia, and insulin delivery remains the only treatment option, but is accompanied with obesity development and life-threatening hypoglycemia. Emerging studies have demonstrated that, in addition to its role in body weight regulation, adipocyte-derived leptin is capable of reducing T1D hyperglycemia in an insulin-independent manner^{1,2}. Notably, leptin action in the brain alone is sufficient to restore euglycemia in T1D, while its action in the liver is not required^{3,4}. It is generally accepted that brain leptin action on reducing T1D hyperglycemia is mediated by suppressing counter-regulatory responses (CRR)⁵. The CRR includes glucagon, HPA axis, and sympathetic nerve output, representing a major physiological response against hypoglycemia for glucose homeostasis, but is aberrantly activated in T1D. For brain leptin action on reducing glucose in T1D, a role for β -adrenergic receptor-mediated sympathetic nerve output has been ruled out^{6,7}. Heightened glucagon action has been suggested to be essential⁸, which is later suggested to be dependent on insulin action⁹. The HPA axis has also been suggested to be the mediator^{10,11}, but also is later suggested not to be a sole mediator^{12,13}. It is generally thought that multiple counter-regulatory responses are involved and suppression of one of them may not be sufficient in mediating the leptin action^{5,12}.

Previous studies have shown that leptin receptor (LepR) expression in GABAergic neurons mediates leptin action in reducing hyperglycemia in T1D^{6,14}. In line with this, LepR neurons in the ventromedial hypothalamus (VMH), a major brain region containing mainly glutamatergic neurons and known to regulate glucose homeostasis, are not required for the leptin action¹⁵. However, as both LepR and GABAergic neurons are widely distributed in the brain, the location of GABAergic LepR neurons that mediates leptin's actions on T1D glucose handling remains elusive. Specifically, conflicting results have been obtained regarding leptin action on T1D glucose in the arcuate nucleus (Arc), a region important for glucose homeostasis and known to express abundant LepRs. The melanocortin pathway, which involves proopiomelanocortin (POMC) and agouti-related protein (AgRP) neurons, two well-characterized groups of neurons in the Arc, has been shown to play an important part in mediating leptin action on T1D glucose^{16,17}. Although POMC neuron function contributes only marginally, LepRs in AgRP neurons, a group of GABAergic neurons in the Arc, have been shown to contribute significantly to the leptin action^{6,18}. Consistently, LepR expression in AgRP neurons greatly reduces hyperglycemia-associated leptin deficiency¹⁹. In contrast, chemogenetic manipulations of AgRP neuron activity revealed no effects on the leptin action on T1D glucose²⁰. Importantly, as AgRP neurons only constitute a relatively small number of the Arc neuron population^{21,22}, the relative importance of AgRP versus non-AgRP neurons in mediating leptin action on T1D glucose remains untested.

Despite potent effects of leptin action on reducing T1D hyperglycemia, the underlying cellular mechanism remains unclear. The LepR-STAT3 signaling pathway, known to mediate the major part of leptin action on body weight and glucose homeostasis, is required for leptin action in reducing T1D hyperglycemia⁷, although other signaling pathways may also play a role²³. Leptin either activates or inhibits LepR neurons, depending on the types of neuron and their brain location^{24–26}. Specifically, most LepR^{Arc} neurons are inhibited by leptin^{27,28}, although studies focusing on POMC neurons showed that a subset of POMC neurons are activated by leptin^{25,29}, which may be mediated by direct action or indirectly through leptin inhibition of putative presynaptic inputs onto POMC neurons²¹. It is unknown whether leptin activation or inhibition on LepR neurons mediates its action on T1D glucose. Although leptin action on neuron activation is postulated to be mediated through TRPC

channels^{30,31}, little is known on the cellular mechanisms underlying leptin action on neuronal inhibition related to nutrient supply.

This study shows that LepR^{Arc} neurons are aberrantly activated in T1D models, and that this activation is reversed by leptin. Specific activation of LepR^{Arc}, Arc AgRP neurons, or GABA^{Arc} neurons reverses or abrogates leptin action on T1D glucose handling. Conversely, specific inhibition of GABA^{Arc} neurons causes a leptin-mimicking effect on reducing T1D hyperglycemia, while inhibiting AgRP neurons has a much more attenuated effect. The presence of AgRP neurons is not required for T1D hyperglycemia or for the observed leptin action. Importantly, our results suggest that LepR^{Arc} neurons in T1D are in a state of energy deprivation and not sensitive to nutrient deprivation, both of which are reversed by leptin. Leptin action on reducing T1D hyperglycemia is reversed by nutrient deprivation or activation of nutrient deprivation signaling pathways. These results identify aberrant activation of LepR^{Arc} neurons resulting from loss in response to nutrient supply as one major central mechanism underlying T1D hyperglycemia, and reveal that leptin reduces T1D hyperglycemia through inhibition of these neurons via reversing energy deprivation.

Results

Aberrant activation of LepR^{Arc} neurons in T1D models. T1D exhibits a severe reduction in leptin levels^{1,2}, suggesting a significant contribution of loss of brain leptin action to the pathogenesis of T1D hyperglycemia. Given the known leptin effect on neuron activity, we explored potential changes in LepR neuron activity in T1D and its modulation by leptin. We examined the expression of c-Fos, a well-established indicator for neuron activity. LepR^{Arc} neurons in mice with severe hyperglycemia and insulin deficiency induced by STZ, a commonly used type 1 diabetes model (STZ-T1D), exhibited greatly increased c-Fos expression in the Arc compared to controls (non-T1D conditions) (Fig. 1a, b, d), even fed ad libitum. Of note, STZ-induced c-Fos expression was largely colocalized in LepR-expressing neurons in the Arc (Fig. 1b and Supplementary Fig. 1), whereas negligible colocalization was found in other brain sites with LepR expression (Supplementary Fig. 1). The c-Fos expression in LepR^{Arc} neurons was significantly reduced by i.c.v. infusion of leptin (Fig. 1c, d), suggesting a potent inhibitory action by leptin on these neurons. In addition, we also observed similar increases in c-Fos and responses to leptin treatment in another, nonobesity diabetic (NOD) model (Fig. 1e and Supplementary Fig. 2), suggesting that Arc neuron activation and its reversal by leptin represent a common feature of T1D. Interestingly, we noticed that fasting-induced c-Fos expression in the Arc was also significantly colocalized with LepR neurons (Fig. 1g, i and Supplementary Fig. 1), compared to control-fed conditions (Fig. 1f, i). Strikingly, leptin administration reduced c-Fos expression in the Arc (Fig. 1h, i), in a similar fashion to T1D. Given low leptin as a common feature of both fasting and T1D, these results suggest that LepR^{Arc} neuron activation is due to reduced leptin action. Since leptin action on reducing T1D hyperglycemia is known to be associated with restraining counter-regulatory hormone levels^{10,12}, we tested the effect of leptin i.c.v. infusion on fasting responses. While glucose levels were not different between the fed ad libitum group and the saline treatment group with 8-h fasting (Fig. 1j), glucose levels in the leptin-treated group were rapidly reduced upon food removal, and reached to 2.5 mM (Fig. 1j), at which point the experiment was ended to avoid severe glucopenic complications. Interestingly, compared to the saline-treated control group, glucagon, a typical counter-regulatory hormone, was also reduced by leptin (Fig. 1k). Thus, T1D and fasting share a common mechanism in which heightened activation of LepR^{Arc}

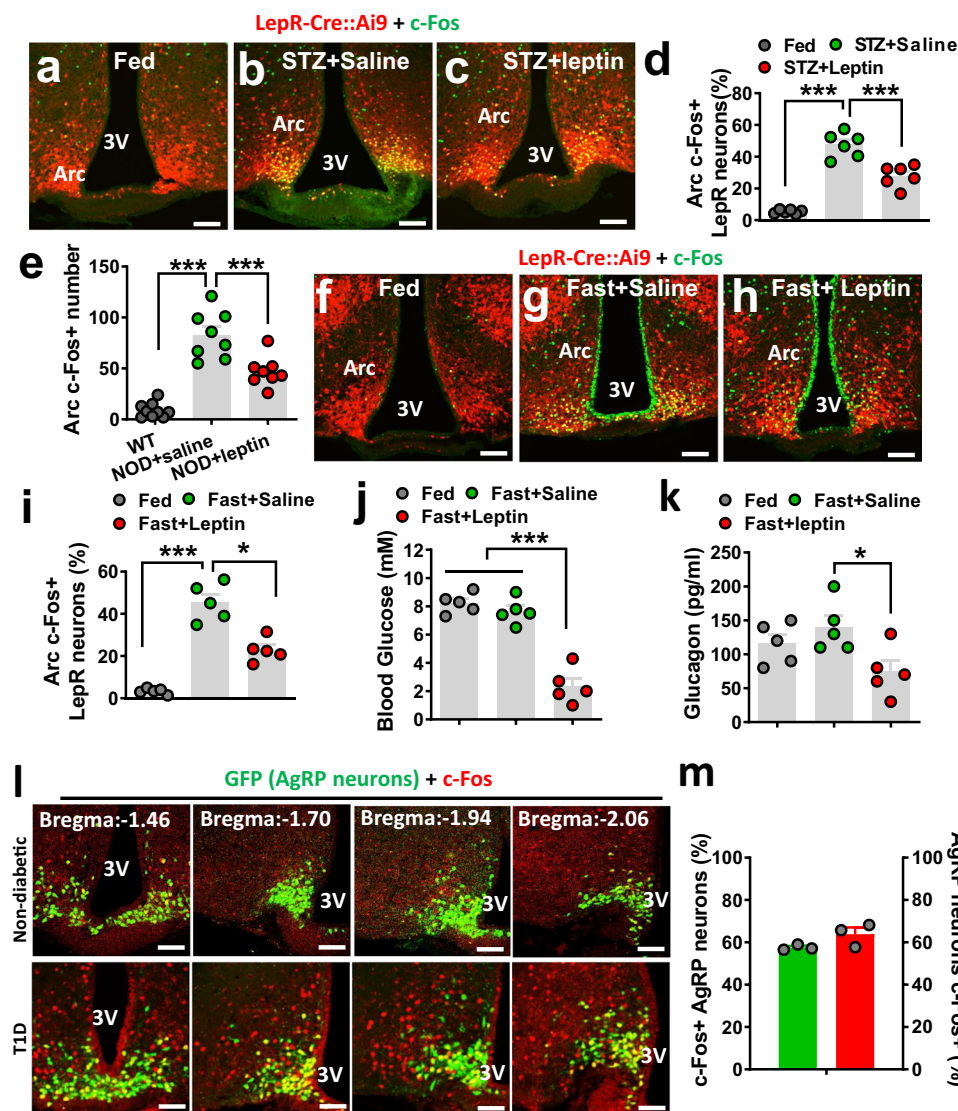


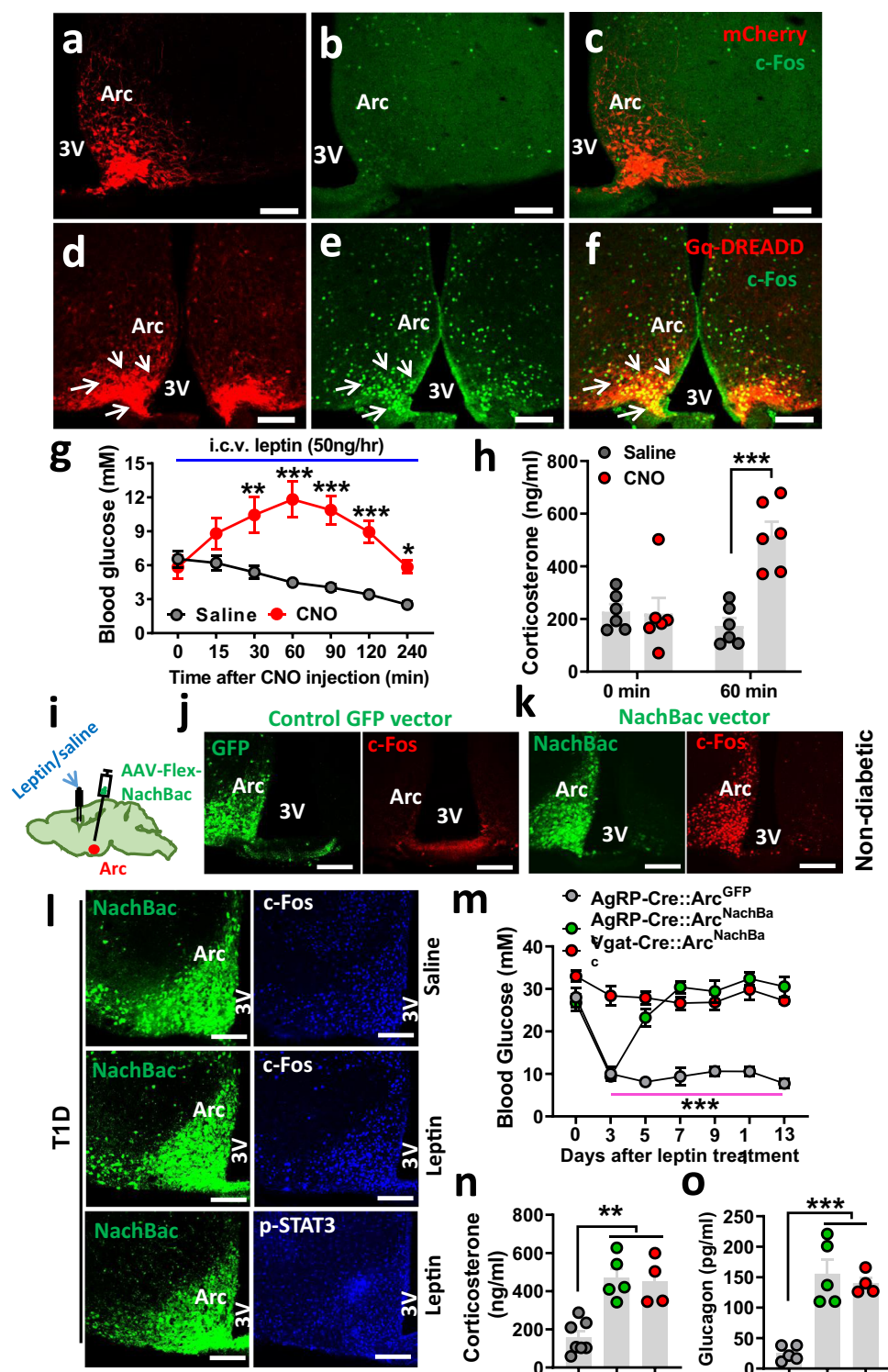
Fig. 1 Leptin suppresses counter-regulatory responses to fasting. **a–i** Leptin-Cre::Ai9 mice (8–10 weeks of age, males) were used as fed controls (**a, f**), made type 1 diabetic induced by STZ administration (**b, c**), subject to overnight fasting (**g, h**), or treated with i.c.v. leptin infusion (**c, h**). LepR neurons were shown by Ai9 reporter expression (red) and immunostaining was performed for c-Fos (green) (**a–c, f–h**). **d–i** Comparison in the percentage of LepR neurons with c-Fos expression among the indicated groups was shown in T1D mice (**d**, one-way ANOVA, $n = 6$ /each, $F(2, 10) = 136.9$, $***P < 0.001$, control vs STZ-saline; $***P = 0.004$, STZ-saline vs STZ-leptin) or controls (**i**, one-way ANOVA, $n = 5$ /each, $F(2, 8) = 44.4$, $***P < 0.0001$, fed vs fast-saline; $*P = 0.0035$, fast-saline vs fast-leptin). **e** Comparison in the number of c-Fos neurons in the Arc in controls, saline, and leptin-treated nonobesity diabetic (NOD) mice (one-way ANOVA, $n = 9$ for WT and $n = 8$ for both NOD-saline and NOD-leptin, $F(2, 22) = 44.69$, $***P < 0.0001$, WT vs NOD-saline; $***P = 0.0006$, NOD-saline vs NOD-leptin). **j, k** In mice presented in panels **f–i**, comparisons were shown in glucose levels between fed and fasting, and between saline and leptin treatment conditions (**j**, one-way ANOVA, $n = 5$ /each, $F(2, 8) = 116.6$, $***P < 0.0001$, fed vs fast-leptin; $***P < 0.0001$, fast-saline vs fast-leptin) and in glucagon levels between saline and leptin-treated groups (**k**, one-way ANOVA, $n = 5$ /each, $F(2, 8) = 4.575$, $P = 0.1745$, fed vs fast-leptin; $*P < 0.0427$, fast-saline vs fast-leptin). Blood glucose was measured between fed and 8-h fast (from early morning to late afternoon) and blood glucagon was measured at 8-h fasting. **l, m** In AgRP-Cre GFP-reporter mice, colocalization of c-Fos induction in STZ-T1D was shown in GFP-positive neurons in a series of sections from rostral to caudal Bregma levels (**l**) and percentage of c-Fos neurons that were colocalized in AgRP and percentage of AgRP neurons were c-Fos positive in T1D mice (**m**, $n = 3$ /each). 3V the third ventricle, Arc arcuate nucleus, scale bar: 100 μ M. All data presented as mean $\pm$ SEM.

neurons owing to reduced leptin action causes augmented counter-regulatory responses.

As AgRP neurons represent a subpopulation of Arc GABAergic (GABA^{Arc}) neurons^{21,22} and have been a research focus on leptin action in T1D glucose, we also examined c-Fos expression patterns in these neurons in the STZ-T1D model. Using AgRP-Cre GFP-reporter mice to visualize AgRP neurons, we found that T1D-induced c-Fos expression was partly colocalized with AgRP neurons but also found in a large number of non-AgRP neurons (Fig. 1l, bottom panels), whereas a negligible number of c-Fos

neurons was observed in control non-T1D mice (Fig. 1l, top panels). Roughly 50% of c-Fos neurons were found in AgRP neurons and >60% AgRP neurons were positive for c-Fos (Fig. 1m). These results suggest potential importance for both Arc AgRP and non-AgRP neurons in mediating the leptin action.

Leptin action on reducing T1D hyperglycemia is reversed by Arc neuron activation. Given the abundant T1D-induced c-Fos expression in the Arc, we tested the effect of direct Arc neuron



activation. We first examined whether acute activation of Arc LepR neurons reverses the leptin action. Toward this, we stereotactically delivered control vectors (Fig. 2a) or AAV-Flex-hM3D(Gq)-mCherry vectors (Gq-DREADD) (Fig. 2d) bilaterally to the Arc of LepR-Cre mice. Selective viral targeting was verified by mCherry expression (Fig. 2a, d), and the function of Gq-DREADD was verified by CNO-induced c-Fos specifically in Arc LepR neurons (Fig. 2b, c, e, f). These mice were first made T1D and i.c.v.-treated with leptin, and then these glucose-normalized mice were divided in two groups, one treated with saline and the other with i.p. CNO. CNO effectively increased glucose compared

to falling glucose levels in controls (Fig. 2g). This glucose-increasing effect lasted for nearly 4 h, within the range of duration in CNO-induced activation of DREADD receptors. CNO-induced glucose increase was accompanied by increased corticosterone (cort) levels (Fig. 2h), an opposite response to leptin treatment effects in T1D. To rule out a potential nonspecific effect of CNO in the observed glucose-increasing effects, we generated a new group of STZ-T1D mice with glucose normalized by i.c.v. leptin treatment (Supplementary Fig. 3a), and found that CNO injections had no effect on the glucose level in these mice (Supplementary Fig. 3b). Thus, acute activation of Arc LepR neurons is

Fig. 2 Activation of Arc neurons reverses leptin action on reducing T1D hyperglycemia. **a–f** DREADD-mediated acute activation of Arc LepR neurons reverses the leptin action. LepR-Cre mice were injected with AAV-Flex-mCherry control vectors (**a–c**) and AAV-DIO-h3MD(Gq)-mCherry (**d–f**) to bilateral Arc, and expression of mCherry (**a, d**) and CNO-induced c-Fos (**b, e**) were confirmed in the Arc. Representative pictures of 6 repeats as shown in **g** and **h**. **g, h** Effects on glucose with single i.p. injection of CNO in T1D LepR-Cre mice with Gq-DREADD delivery to bilateral Arc and euglycemia restoration via i.c.v. leptin infusion (**g**, two-way ANOVA, $n = 6$ /each, $F(1, 70) = 79.47$, $***P < 0.0001$ (at the 60-min time point); $**P = 0.0001$ (at the 120-min time point), and comparison in glucagon levels between saline and leptin-treated groups (**h**, two-way ANOVA, $n = 6$ /each, $F(1, 20) = 13.97$, $***P < 0.0001$, saline vs CNO at the 90-min time point). **i–n** Effects of NachBac-mediated chronic activation of Arc neurons on the leptin action. **i** Diagram showing injections of NachBac/or control GFP vectors in AgRP or GABA^{Arc} neurons through the delivery of respective vectors to the Arc of AgRP or Vgat-Cre mice, and expression of GFP (green) and c-Fos (red) with one side of the Arc receiving vector injections for control GFP vectors (**j**) and NachBac vectors (**k**). **l** In Vgat-Cre mice that were made T1D and received NachBac vector injections to bilateral Arc with i.c.v. pumps infusing saline or leptin as indicated, representative NachBac expression (GFP, left panels), and c-Fos/p-STAT3 (blue, right panels) are shown. Pictures in (**j–l**) are representative of three repeats. **m** Effects on glucose levels in response to i.c.v. infusion of leptin in the indicated groups of mice (two-way ANOVA, $n = 6$ for AgRP-GFP or AgRP-NachBac, and $n = 4$ for Vgat-NachBac, $F(2, 91) = 190.6$, $P = 0.9647$, AgRP-GFP vs AgRP-NachBac at day 3; $***P < 0.0001$, AgRP-GFP vs Vgat-NachBac at day 3). **n, o** Measurements and comparison of cort (**n**, one-way ANOVA, $n = 7$ for AgRP-GFP, $n = 5$ for AgRP-NachBac and $n = 4$ for Vgat-NachBac, $F(2, 13) = 16.52$, $**P = 0.006$, AgRP-GFP vs AgRP-NachBac; $**P = 0.0017$, AgRP-GFP vs Vgat-NachBac) and glucagon (**o**, one-way ANOVA, $n = 5$ for AgRP-GFP and AgRP-NachBac, and $n = 4$ for Vgat-NachBac, $F(2, 11) = 21.89$, $***P = 0.0002$, AgRP-GFP vs AgRP-NachBac; $***P = 0.001$, AgRP-GFP vs Vgat-NachBac) levels in the blood at day 13 of i.c.v. leptin infusion shown in panel **m**. 3V the third ventricle, Arc arcuate nucleus, scale bar: 100 μ m. All data presented as mean $\pm$ SEM.

sufficient to cause a transient blocking effect on leptin in reducing glucose and counter-regulatory responses.

We next examined the effect of chronic activation of Arc neurons on leptin action. Toward this, we bilaterally delivered an AAV-EF1a-Flex-EGFP-P2A-mNachBac vector to the Arc (Fig. 2i). The vector conditionally expresses, in a Cre-dependent manner, a type of sodium channel from bacteria (NachBac) that has a low membrane threshold for action potential firing and slow inactivation kinetics, and has been used to effectively elevate neuron activity without a need of ligand administration^{32–34}. Given the technical difficulty in achieving specific targeting Arc LepR neurons with stereotaxic delivery to the Arc of LepR-Cre mice owing to abundant Cre expression in the nearby regions including VMH, we switched to Vgat-Cre mice, which have been used previously to specifically target Arc neurons and avoid surrounding glutamatergic neurons³². As leptin action on T1D glucose is known to be mediated by GABAergic neurons⁶, using Vgat-Cre will be able to capture Arc neurons that mediate the leptin action. Delivery of NachBac vectors to one side of the Arc of Vgat-Cre mice led to the abundant expression of c-Fos in the injected side compared to the noninjected side (Fig. 2k), where no differences were observed in mice with control GFP vector injections (Fig. 2j), confirming that NachBac expression led to increased neuron activity levels. We then generated STZ-induced T1D in AgRP-Cre or Vgat-Cre mice. After establishing stable T1D hyperglycemia, we delivered NachBac or control vectors bilaterally to the Arc of these mice, and at the same time implanted osmotic minipumps (2-week duration) for i.c.v. infusion of leptin or saline. In contrast to severe obesity demonstrated previously³², NachBac expression in Arc AgRP or Vgat neurons in T1D mice failed to cause body weight changes, which is likely due to already-elevated neuron activity of these neurons in the T1D condition. Consistent with the aforementioned data, in control mice with GFP vector expression, leptin treatment effectively reduced c-Fos induction in the Arc of T1D, which was associated with increased p-STAT3 expression (Supplementary Fig. 4). In contrast, leptin failed to reduce c-Fos in NachBac-expressing GABA^{Arc} neurons (Fig. 2l, middle panels), compared to the saline treatment group (Fig. 2j, top panels). We validated that failure to reduce c-Fos expression was not due to failed delivery of leptin, given that we observed abundant p-STAT3 induction in the Arc (Fig. 2j, bottom rows). Similar results were also observed in NachBac-expressing AgRP neurons (Supplementary Fig. 5). Strikingly, i.c.v. leptin infusion

failed to reduce T1D hyperglycemia in NachBac-injected Vgat-Cre mice, while it maintained its normal effects in reducing glucose in GFP-injected Vgat-Cre mice (Fig. 2m). Interestingly, in NachBac-injected AgRP mice, leptin initially reduced glucose to a normal level, which was reversed around day 5 during the leptin treatment (Fig. 2m). The delayed reversal of the leptin action may reflect a delay in NachBac expression in a small number of Arc neurons (i.e., AgRP neurons) and rapid pharmacological action of leptin. Consistently, leptin infusion failed to reduce blood cort levels in the groups with failed reduction of T1D hyperglycemia (Fig. 2n). The changes in glucose were not caused by changes in body weight as there was no body weight difference among mouse groups (Supplementary Fig. 6a). Consistent with the reversal of T1D-reduced glucose, the control group exhibited a significant reduction in feeding (Supplementary Fig. 6b). Together, these results demonstrate that leptin action on reducing T1D hyperglycemia is mediated through inhibition of GABA^{Arc} neurons, and that aberrant activation of Arc neurons, including AgRP neurons, contributes significantly to both pathogenesis of T1D hyperglycemia and leptin action on reversal of T1D hyperglycemia.

Acute inhibition of Arc neurons on T1D hyperglycemia. Given the aforementioned data that when leptin failed to reduce c-Fos in the Arc it failed to reduce T1D hyperglycemia, we examined whether inhibition of Arc neurons caused a leptin-mimicking effect on reducing T1D hyperglycemia. For this, we delivered AAV-Flex-h4MGi-mCherry (Gi-DREADD) bilaterally to the Arc of AgRP-Cre or Vgat-Cre mice. To verify the effects of Gi-DREADD, we generated STZ-induced T1D in these mice. Abundant c-Fos expression was found in the Arc of control STZ-T1D mice (Fig. 3a). Consistent with differences in the number of AgRP and GABA^{Arc} neurons, in CNO-injected AgRP-Cre mice, there was no obvious colocalization between c-Fos and AgRP neurons (Fig. 3b) and CNO reduced the majority of c-Fos expression in T1D Vgat-Cre mice (Fig. 3c), suggesting an effective action of CNO-induced neuron inhibition. Interestingly, a 3-day injection protocol of CNO effectively reduced T1D hyperglycemia in Vgat-Cre mice, but had no effects on either AgRP-Cre mice or “missed-targeted” mice (Fig. 3d). Notably, the reduction in glucose was transient, and the glucose levels gradually went up to control levels at day 3 after the CNO treatment protocol. No difference in body weight (Supplementary Fig. 6c) or feeding (Supplementary Fig. 6d) was observed in these mice. The

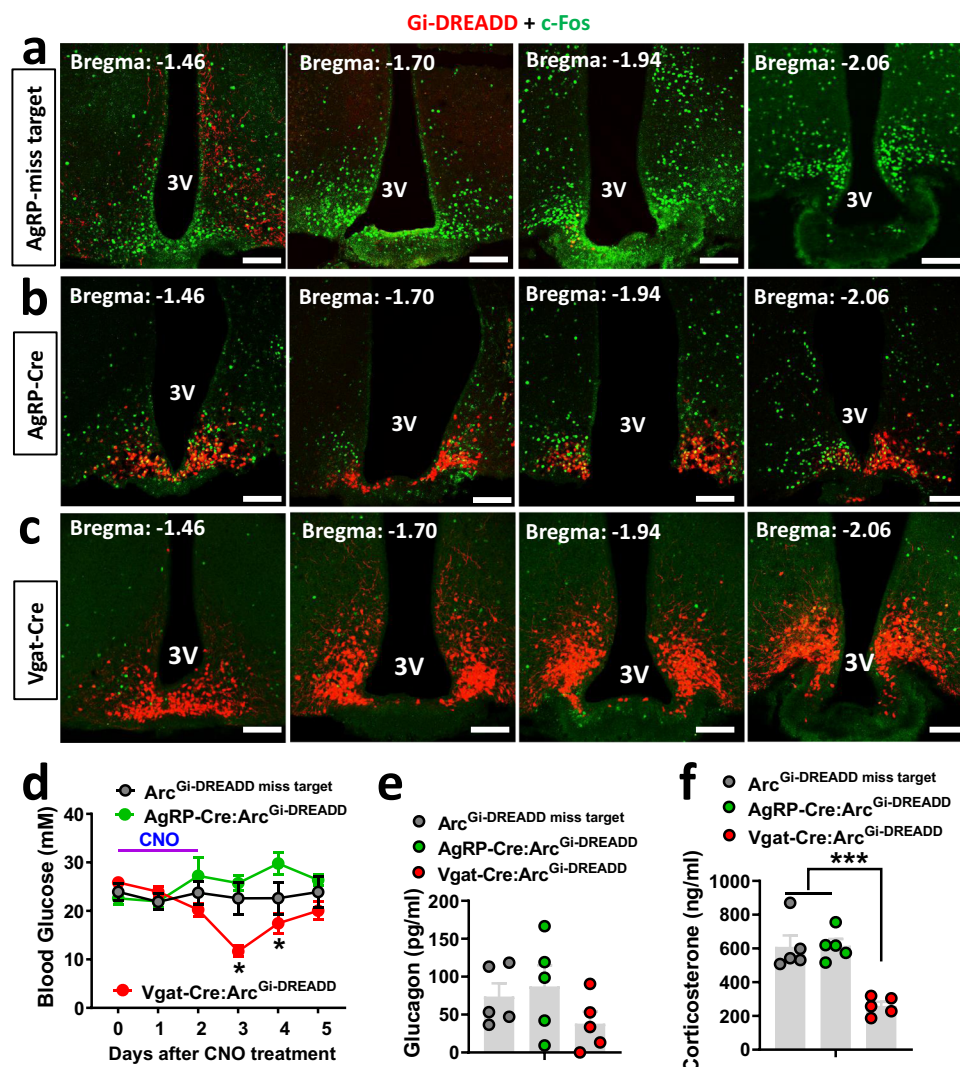


Fig. 3 Acute inhibition of Arc neurons on T1D glucose. AgRP-Cre or Vgat-Cre mice were injected with AAV-DIO-h4MD(Gi)-mCherry vectors to bilateral Arc and then were made STZ-T1D. **a–c** Series of sections spanning the Arc showing specific expression of viral vector expression (red) and c-Fos expression (green) in mistargeted mice (**a**), AgRP-Cre (**b**), and Vgat-Cre mice (**c**). Representative pictures over 3 repeats. **d–f** Comparisons in blood glucose in the indicated groups of mice in response to a 3-day CNO treatment protocol (twice daily for 3 days) (**d**, two-way ANOVA, $n = 5$ /each, $F(2, 75) = 16.41$, $*P < 0.0001$, AgRP-Gi-DREADD vs Vgat-Cre-Gi-DREADD at day 3; $*P < 0.0001$, missed vs Vgat-Cre-Gi-DREADD at day 3), comparisons in corticosterone (**e**, one-way ANOVA, $n = 5$ /each, $F(2, 12) = 1.449$, $P = 0.4774$, missed vs Vgat-Cre-Gi-DREADD at day 3) and glucagon (**f**, one-way ANOVA, $n = 5$ /each, $F(2, 12) = 18.93$, $***P = 0.0005$, Vgat-Gi-DREADD vs missed; $***P = 0.0005$, Vgat-Gi-DREADD vs AgRP-Gi-DREADD) at day 3 after the initiation of CNO treatment. 3V the third ventricle, Arc arcuate nucleus. All data presented as mean $\pm$ SEM. Scale bar: 100 μ m.

reduction in glucose was associated with reduced cortis (Fig. 3d) and a trend in reduced glucagon levels (Fig. 3e). Thus, acute inhibition of Arc GABA⁺ neurons, but not AgRP neurons, is sufficient to cause a transient reduction in both T1D glucose and counter-regulatory responses.

Chronic inhibition of Arc neurons on T1D hyperglycemia.

Brain leptin infusion effectively reduces T1D hyperglycemia during the whole period of leptin infusion, while acute inhibition through CNO-Gi-DREADD only produced a transient reduction in T1D glucose, suggesting chronic inhibition of Arc neurons is required to achieve long-term remission on T1D glucose. To specifically examine this, we used conditional AAV-DIO-Kir2.1-P2A-dTomato (DIO-Kir2.1) vectors expressing a mutated form of the Kir2.1 channel, which is known to effectively reduce neuron activity and excitability^{32–34}. To verify Kir2.1 expression

and function, we first delivered the virus to one side of the Arc (Fig. 4a, left panels) of AgRP-Cre (top panels) or Vgat-Cre mice (bottom panels). In T1D, as expected, the noninjected side exhibited abundant c-Fos, while the injected side showed significantly reduced levels of c-Fos expression (Fig. 4a, middle and right panels). These results suggest that Kir2.1 expression leads to chronic neuron inhibition.

Using STZ-T1D AgRP-Cre or Vgat-Cre mice, we bilaterally delivered either the Kir2.1 or control mCherry viruses to the Arc, and monitored weekly glucose levels following viral injection. While control groups exhibited persistent T1D hyperglycemia, Kir2.1 expression in GABA^{Arc} neurons effectively reduced T1D to a normal level and this effect remained during the whole 8-week monitoring period (Fig. 4b). Interestingly, Kir2.1 expression in AgRP neurons caused an intermediate effect (Fig. 4b). Consistent with the glucose effect, feeding was reduced in both groups of mice (Fig. 4c), but body weight showed no obvious

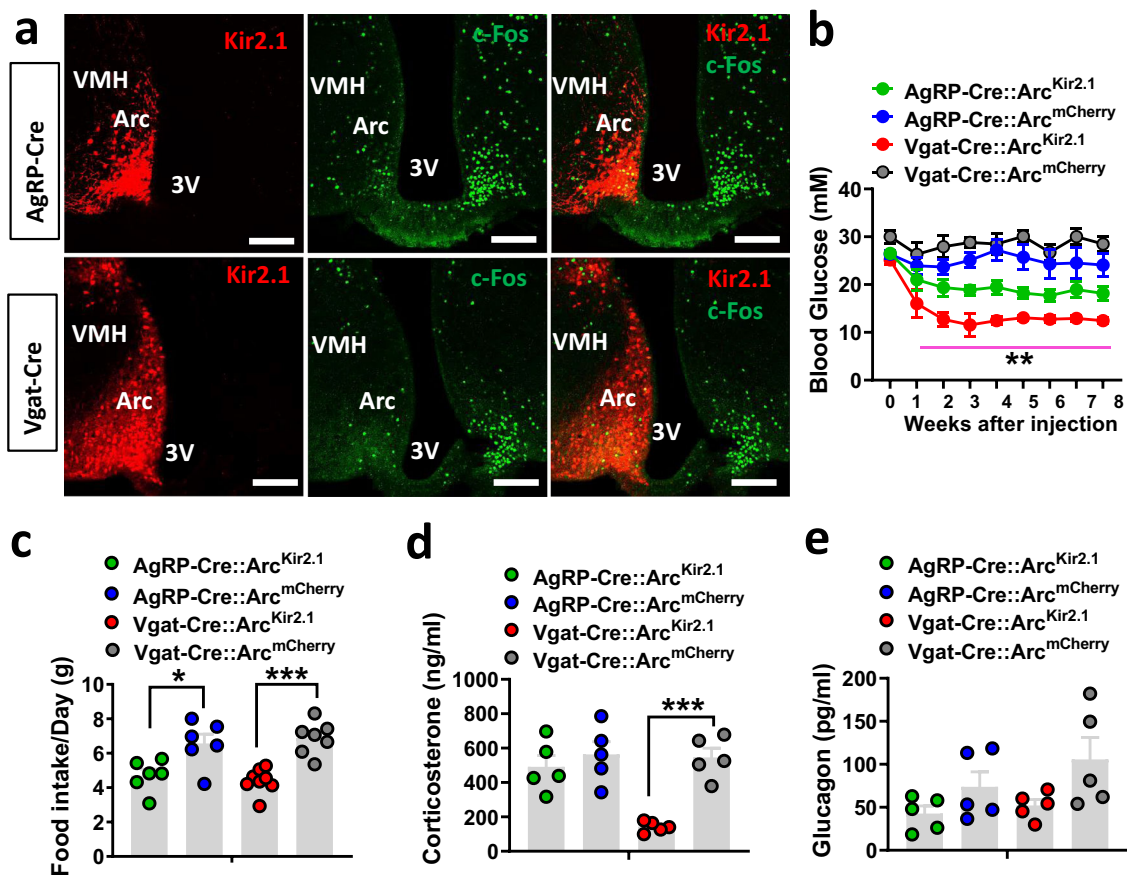


Fig. 4 Chronic inhibition of Arc neurons on T1D glucose. AgRP-Cre or Vgat-Cre mice (8–10 week-old males) were first made STZ-T1D and then injected with AAV-DIO-Kir2.1-P2A-dTomato vectors or control AAV-Flex-mCherry to bilateral Arc. **a** Representative sections showing comparison in the expression of Kir2.1 (red) and c-Fos (green) in the Arc of AgRP-Cre mice (top) and Vgat-Cre (bottom), in which the Kir2.1 vector was delivered to one side of the Arc. Representative pictures from $n = 2$ mice. **b–e** Weekly glucose levels following injections of the viral vectors (**b**, two-way ANOVA, $n = 6$ for AgRP-mCherry, AgRP-Kir2.1, or Vgat-mCherry, and $n = 7$ for Vgat-Kir2.1, $F(3, 189) = 120.1$, $P = 0.0641$, AgRP-mCherry vs AgRP-Kir2.1; $^{***}P < 0.0001$, Vgat-mCherry vs Vgat-Kir2.1), food intake during week 3–4 after viral delivery (**c**, two-way ANOVA, $n = 6$ for both AgRP-mCherry and AgRP-Kir2.1, $n = 9$ for Vgat-mCherry, and $n = 8$ for Vgat-Kir2.1, $F(3, 23) = 11.68$, $^{*}P = 0.0149$, AgRP-mCherry vs AgRP-Kir2.1; $^{***}P = 0.0003$, Vgat-mCherry vs Vgat-Kir2.1) and blood levels of corticosterone (**d**, two-way ANOVA, $n = 5$ /each, $F(3, 16) = 12.16$, $P = 0.8091$, AgRP-mCherry vs AgRP-Kir2.1; $^{***}P = 0.0006$, Vgat-mCherry vs Vgat-Kir2.1) and glucagon (**e**, two-way ANOVA, $n = 5$ /each, $F(3, 6) = 2.904$, $P = 0.06$, Vgat-mCherry vs Vgat-Gi-DREADD at week 8 after viral injection. 3V the third ventricle, Arc arcuate nucleus. All data presented as mean $\pm$ SEM. Scale bar: 100 μ m.

changes (Supplementary Fig. 6e). Cort levels were suppressed by Kir2.1 expression in Vgat-Cre mice but not in AgRP mice (Fig. 4d), while both groups exhibited a trend of reduced glucagon levels (Fig. 4e). These results demonstrate that Kir2.1 expression in GABA^{Arc}, but not AgRP neurons, is sufficient to restore T1D hyperglycemia, mimicking brain leptin action on T1D glucose. Together, these results reveal an important role for both Arc AgRP and non-AgRP neurons in mediating the leptin action.

AgRP neurons are not required for T1D hyperglycemia or for leptin action on reducing T1D glucose. To directly examine the role of Arc non-AgRP neurons in T1D hyperglycemia, we bred Vgat-Cre with the previously established strain AgRP^{DTR} mice, which can be used to lesion AgRP neurons with diphtheria toxin (DTX) administration³⁵. In Vgat-Cre::AgRP^{DTR} mice, DTX treatment at P3 effectively killed AgRP neurons (Fig. 5a). These mice were then treated with STZ at 7–8 weeks of age, and monitored for changes in blood glucose. Interestingly, blood glucose levels were not different between DTX and control groups, both of which exhibited typical glucose levels in T1D (Fig. 5b), suggesting that the presence of AgRP neurons is not required for the development of T1D hyperglycemia. The DTX-

treated T1D mice were then divided into two groups, one treated with saline and the other with i.c.v. leptin infusion. While saline had no effects on T1D hyperglycemia, leptin effectively reduced T1D hyperglycemia to normal levels (Fig. 5c), suggesting that the presence of AgRP neurons is not required for leptin action on reducing T1D glucose. Further measurements showed that i.c.v. leptin indeed reduced c-Fos expression (Fig. 5d), glucagon, (Fig. 5e), and cort (Fig. 5f) associated with T1D development, consistent with brain leptin effects. No changes in body weight (Supplementary Fig. 6f) but reduced feeding were observed in the leptin-treated group (Supplementary Fig. 6g), consistent with the leptin-mediated restoration of euglycemia.

Loss of LepR^{Arc} neurons in nutrient sensing in STZ-T1D and reversal by leptin. LepR^{Arc} neurons become activated upon fasting when glucose is low, suggesting they are glucose-inhibited neurons. However, these neurons in T1D, an uncontrolled hyperglycemic condition, were also activated, suggesting that LepR^{Arc} neurons in T1D may be defective in nutrient sensing. To test this, we examined c-Fos expression in LepR^{Arc} neurons in both control and T1D mice. Compared to fed conditions (Fig. 6a), LepR^{Arc} neurons responded with dramatic c-Fos

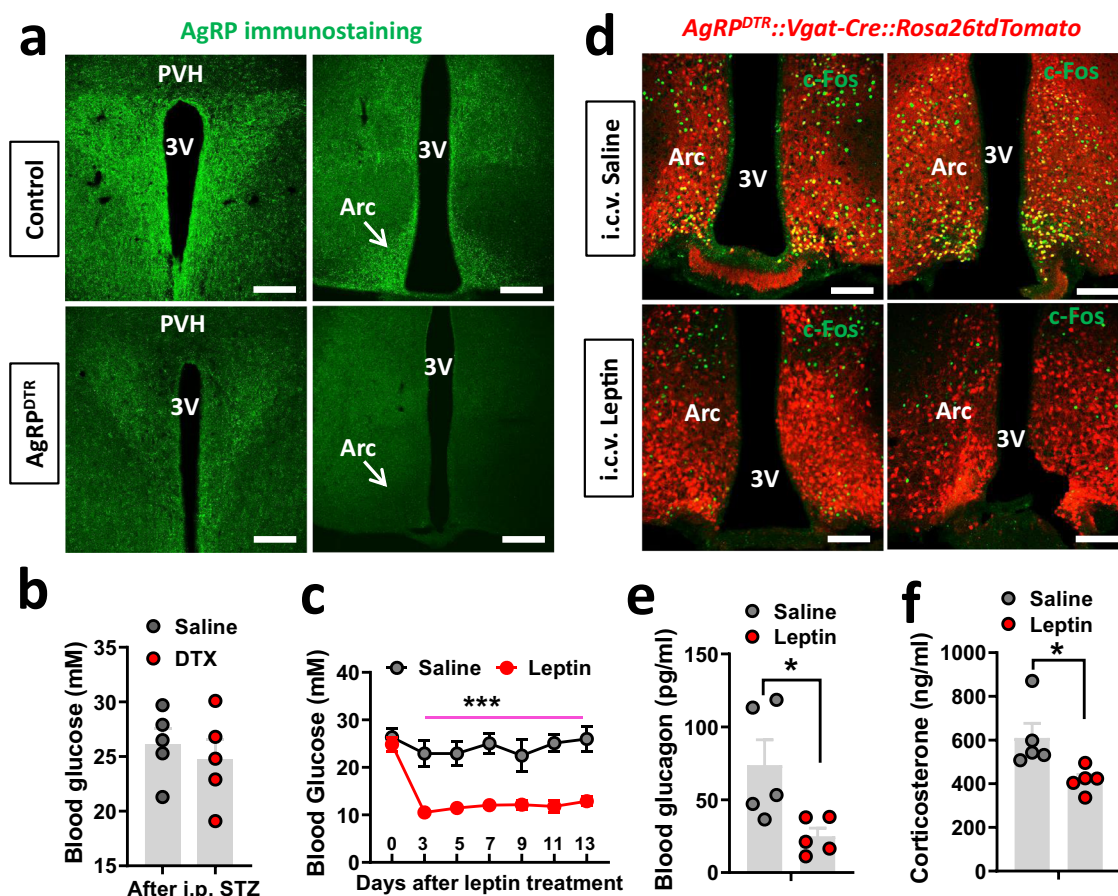


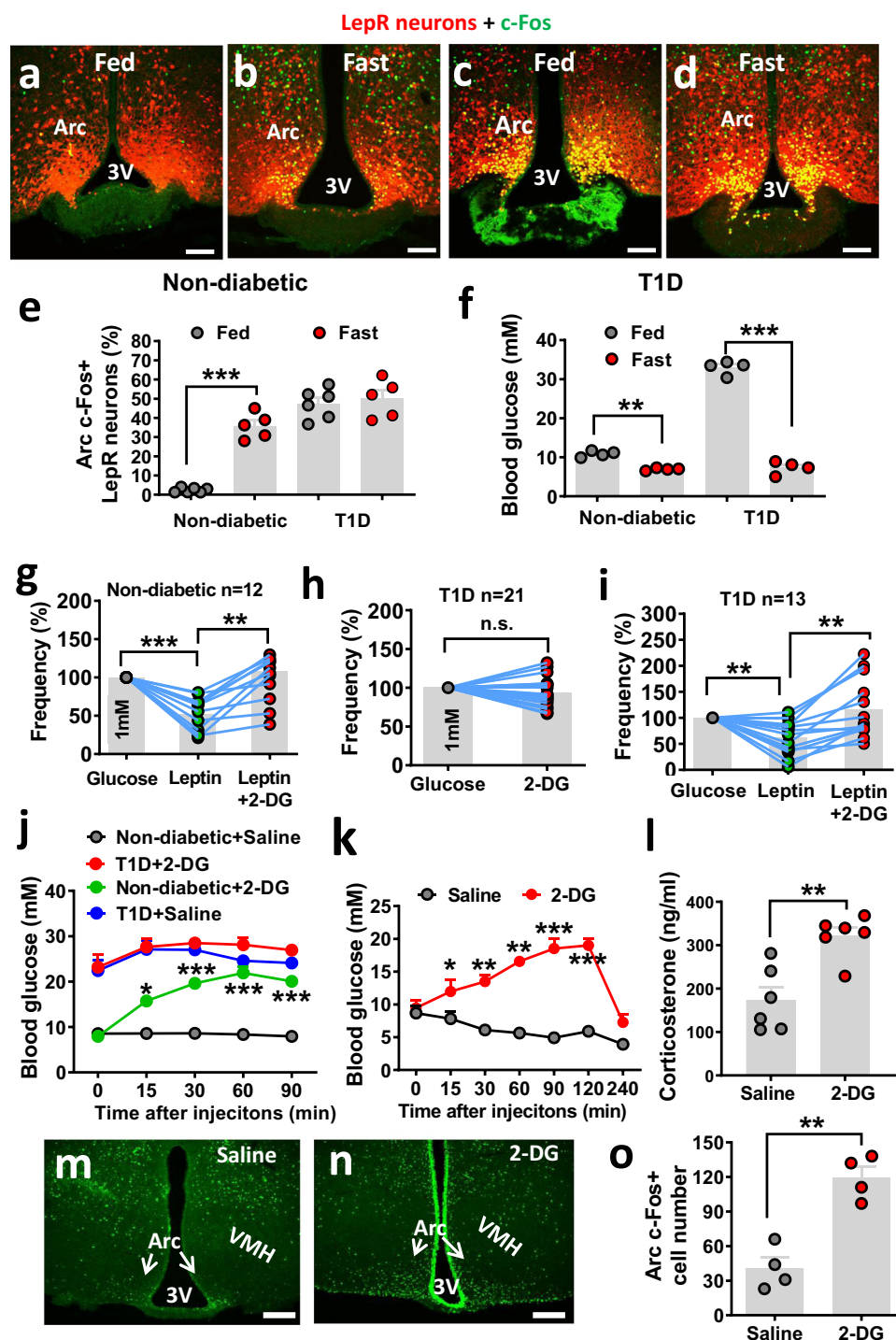
Fig. 5 AgRP neurons are not required for T1D hyperglycemia development and for leptin action in reducing T1D glucose. Vgat-Cre::Ai9::AgRP^{DTR} mice were treated with DTX/saline at P3, made STZ-T1D at 8–9-weeks old, and then implanted with i.c.v. minipump for a 2-week leptin infusion. **a** Representative pictures from *n* = 3 mice showing immunostaining for AgRP in the Arc (right panels) and PVH (left panels) of both saline-treated controls (top) and the DTX-treated group (bottom). **b** Comparisons in blood glucose between saline and DTX-treated groups after STZ treatment (two-tailed unpaired Student's *t* tests, *n* = 5/each, *t* = 0.6019, *df* = 8, *P* = 0.5639). **c** Responses in glucose levels in DTX-treated Vgat-Cre::Ai9::AgRP^{DTR} mice that received i.c.v. treatment of saline or leptin during the 14-day leptin treatment period (two-way ANOVA, *n* = 5/each, *F*(1, 56) = 150.4, ****P* < 0.0001, saline vs leptin at day 13). **d** Representative sections from *n* = 2 mice showing expression of c-Fos (green) in Arc GABA⁺ neurons (red) in i.c.v. saline-treated (left panel) and leptin-treated mice (right panel). **e, f** Comparisons in glucagon (**e**, two-tailed unpaired Student's *t* tests, *n* = 5/each, **P* = 0.0266) and corticosterone (**f**, two-tailed unpaired Student's *t* tests, *n* = 5/each, **P* = 0.027) at day 13 with i.c.v. leptin treatment. 3V the third ventricle, Arc arcuate nucleus. All data presented as mean ± SEM. Scale bar: 100 μm.

induction in response to fasting (Fig. 6b, e). Similar to the aforementioned results, these same neurons in T1D showed abundant c-Fos expression even fed ad libitum (Fig. 6c), and fasting failed to further increase c-Fos expression (Fig. 6d, e). As expected, blood glucose levels in the control group were significantly reduced in fasting (8.1 mM) compared to fed conditions (10.0 mM). Strikingly, glucose levels in T1D mice showed a much greater reduction (30.2–8.2 mM) in T1D mice (Fig. 6f). Thus, in T1D mice, the dramatic reduction in glucose levels (>20 mM) in response to fasting failed to initiate a significant change in c-Fos expression, which is in contrast to a dramatic increase in c-Fos expression in control mice, even with a small reduction in glucose (<2 mM). These contrasting results suggest that LepR^{Arc} neurons lose the ability in nutrient sensing in vivo. To directly test this, we monitored the activity of LepR^{Arc} neurons through electrophysiological recordings. For this, we primarily focused on neurons with observable changes in neuronal firing in response to altered levels of glucose and/or leptin (Supplementary Fig. 7a). With recordings from 89 Arc LepR neurons, we noted a clear effect in firing with changes in glucose and leptin (Supplementary Fig. 7b). Among them, most of them were inhibited by leptin or

glucose, and ~67% of these neurons were inhibited by both leptin and glucose (Supplementary Fig. 7b). Given our data described above on potential effects of leptin and glucose inhibition on Arc neurons in T1D glucose levels, we next chose to focus on those neurons that were inhibited by glucose or leptin.

To explore the mechanism of leptin action on nutrient sensing, we tested the response of LepR^{Arc} neurons to 2-deoxy glucose (2-DG), a glucopenic agent that causes nutrient deprivation. The leptin inhibitory action on LepR^{Arc} neurons was reversed by 2-DG (Fig. 6g), suggesting that the leptin inhibition requires nutrient supply. Interestingly, LepR^{Arc} neurons in T1D were not sensitive to 2-DG (Fig. 6h), consistent with the notion that these neurons lose nutrient sensing. In contrast, in the presence of leptin, LepR^{Arc} neurons in T1D gained inhibitory responses to increased glucose and leptin inhibition was also reversed by 2-DG (Fig. 6i), suggesting that the effect of nutrients, e.g., glucose and 2-DG, requires the presence of leptin. These results suggest that the mechanism underlying leptin action lies in facilitating nutrient sensing of LepR neurons.

To further examine the physiology associated with the reversal effect of 2-DG on leptin inhibitory action, we tested the effect of 2-DG on glucose in both control and STZ-induced T1D mice. As



expected, i.p. 2-DG induced a significant increase in glucose levels in controls (Fig. 6j). However, it failed to further increase glucose levels in T1D mice (Fig. 6j). However, it could not be ruled out that the failure in increasing glucose levels might be due to a ceiling effect. To further address this and specifically examine the effect of 2-DG in the brain, we tested the effect of i.c.v. injection of 2-DG on leptin's ability to reduce T1D hyperglycemia. We first generated STZ-induced T1D with glucose normalization by i.c.v. infusion of leptin as described previously⁷. These mice were then randomly divided into two groups, one injected with i.c.v. saline and the other with i.c.v. 2-DG. Both groups had food removed during the testing. While the saline-injected group gradually reduced glucose levels, i.c.v. 2-DG induced a rapid increase in

glucose levels (Fig. 6k), suggesting that 2-DG potentially reversed the leptin action on reducing T1D hyperglycemia. The rapid increase in glucose levels was followed by a gradual drop to baseline levels at 4 h after 2-DG injection (Fig. 6k), which is consistent with a typical duration of hyperglycemia responses normally induced by 2-DG, and confirms that the rapid increase in glucose levels is not due to loss of leptin action. Interestingly, the increase in glucose levels was associated with higher corticosterone levels (Fig. 6l), suggesting that the reversal of glucose was mediated by the reversal of counter-regulatory hormone levels. Moreover, while Arc neurons in saline-treated mice showed negligible c-Fos expression (Fig. 6m, o), those in 2-DG-treated mice showed significantly more c-Fos (Fig. 6n, o).

Fig. 6 **LepR^{Arc} neurons in T1D lose nutrient sensing and restoration by leptin.** **a–d** LepR-Cre::Ai9 reporter mice (8–10 weeks of age, males) were used either as controls (**a, b**) or made STZ-T1D (**c, d**), and were used either at fed (**a** and **c**) or overnight fasting conditions (**b, d**). Representative pictures from $n = 5$ –6 mice showing in **e** in **a–d** showing that c-Fos immunostaining was performed and colocalized with LepR neurons. **e, f** Comparison in the percentage of LepR^{Arc} neurons that are c-Fos positive (**e**, two-way ANOVA, $n = 6$ for non-diabetic fed and T1D fast, and $n = 5$ for non-diabetic fed and T1D fast, $F(1, 12) = 36.21$, $***P < 0.0001$, non-diabetic fed vs fast; $P = 0.7375$, T1D fed vs fast) and blood glucose levels (**f**, two-way ANOVA, $n = 5$ /each, $F(1, 13) = 526.1$, $**P = 0.002$, non-diabetic fed vs fast; $***P < 0.0001$, T1D fed vs fast) between fed and fasting or between controls and STZ groups. **g** The third ventricle, Arc: arcuate nucleus. Scale bar: 100 μ M. **g–i** Electrophysiological recording was performed on LepR^{Arc} neurons on brain sections from LepR-Cre reporter mice (8–10 weeks of age, males), which were used as controls (**g**, one-way ANOVA, $F(11, 22) = 1.101$, $n = 13$ /each, $***P < 0.0001$, glucose vs leptin; $**P = 0.0079$, leptin vs leptin/2-DG) or made T1D (**h**, two-tailed unpaired Student's t tests, $n = 21$ /each, $t = 0.1622$, $df = 40$, $P = 0.1126$, and **i**, one-way ANOVA, $n = 14$ /each, $F(12, 24) = 2.285$, $**P = 0.0026$, glucose vs leptin; $**P = 0.0011$, leptin vs leptin/2-DG) and LepR^{Arc} neurons were recorded for firing frequency in response to leptin and/or 2-DG. Data were expressed relative to baseline firing rates for each recorded neuron. **j** Comparison in glucose levels in nondiabetic controls and T1D mice treated with i.p. injection of either saline or 2-DG during a period of 90 min after the injection (two-way ANOVA, $n = 6$ /each, $F(3, 50) = 159.2$, $***P < 0.0001$, non-diabetes—saline vs non-diabetes-2-DG, $P = 0.4716$, T1D-saline vs T1D-2-DG). **k–o** Groups of wild-type mice (8–10 weeks of age, males) were first made T1D and were then implanted with both i.c.v. minipump for leptin infusion and i.c.v. cannulas for brain injection of 2-DG or saline. Brain injections of 2-DG or saline were performed after leptin-mediated euglycemia restoration. Blood glucose was measured following i.c.v. injection of 2-DG at the indicated time points (**k**, two-way ANOVA, $n = 6$ /each, $F(1, 35) = 240.1$, $***P < 0.0001$, saline vs 2-DG at the 120-min time point) and corticosterone levels were measured at 90 min after 2-DG injections (**l**, two-tailed unpaired Student's t tests, $t = 4.108$, $df = 10$; $n = 6$ /each, $**P = 0.0021$). Brain sections were used for c-Fos immunostaining from i.c.v. saline (**m**) and 2-DG treatments (**n**) at 90 min after i.c.v. 2-DG injection, and neuron number with c-Fos expression was compared between these two groups (**o**, two-tailed unpaired Student's t tests, $t = 5.885$, $df = 6$, $n = 4$ /each, $**P = 0.0011$). Data presented as mean $\pm$ SEM. 3V the third ventricle, Arc arcuate nucleus, VMH ventromedial hypothalamus. Scale bar: 100 μ M.

Taken together, these results suggest that leptin inhibition of Arc LepR neurons in T1D is mediated by replenishing the nutrient supply, which is reversed by 2-DG.

Leptin action on LepR^{Arc} neurons in reducing T1D hyperglycemia involves the AMPK pathway. AMP kinase (AMPK) is known to be a key energy sensor in neurons³⁶. To test a potential involvement of AMPK in leptin-mediated glucose nutrient sensing in LepR^{Arc} neurons, we performed immunohistochemistry for phospho-AMPK (p-AMPK), the activated form of AMPK that is known to be increased during energy deprivation. Compared to controls (Fig. 7a), LepR^{Arc} neurons in T1D exhibited a dramatic increase of p-AMPK (Fig. 7a, b). Notably, p-AMPK expression in T1D was mostly colocalized with LepR neurons. Leptin treatment significantly reduced p-AMPK in LepR neurons (Fig. 7a, b), which was reversed by 2-DG (Fig. 7a, b) indicating a specific role for leptin action in facilitating nutrients to these neurons. Interestingly, p-AMPK and c-Fos were highly colocalized in the Arc (Fig. 7c), suggesting that activation of Arc LepR neurons is due to nutrient deprivation. To examine the role of AMPK activation in T1D hyperglycemia, we treated STZ-induced T1D mice, in which glucose levels were normalized by i.c.v. leptin infusion, with saline or AICAR, a specific agonist of AMPK. While glucose in the saline group gradually reduced during the testing period, AICAR effectively reversed the leptin action and increased glucose levels (Fig. 7d), which was associated with increased glucagon levels (Fig. 7e), consistent with a reversal of suppressing effects on counter-regulatory hormones by leptin action in reducing T1D hyperglycemia. These results suggest that leptin action on Arc LepR neuron activity involves the AMPK pathway, further confirming that leptin action on T1D glucose is mediated through suppressing the activation of LepR neurons owing to energy deprivation.

Discussion

Insulin deficiency is the cause of T1D; however, accumulating evidence suggests an involvement of brain dysfunction in T1D pathogenesis, as demonstrated by central leptin action on euglycemia restoration independent of insulin action³. T1D models exhibit a severe reduction of leptin levels, suggesting a physiologic contribution of leptin loss toward uncontrolled hyperglycemia in T1D. Despite much interest in the brain mechanisms underlying the leptin action, the brain location and the nature of the defects remain largely elusive. Based on

combined mouse genetics, selective brain targeting and pharmacology, electrophysiological recordings, and the investigation of STZ-induced T1D models, our studies collectively reveal the following important insights: (1) GABAergic LepR^{Arc} neurons mediate leptin action on reducing T1D hyperglycemia, (2) Arc GABAergic non-AgRP neurons play a critical role in mediating the leptin action, (3) the presence of AgRP neurons is not required for either T1D development or for leptin action on reducing T1D hyperglycemia, (4) LepR^{Arc} neurons in T1D are in an activated state owing to energy deprivation, and (5) leptin action is mediated by inhibiting the activated LepR^{Arc} neurons in T1D through reversing energy deprivation (Fig. 7f).

This study mainly uses a T1D rodent model induced by STZ to examine the insulin-independent effect of leptin. It is important to note that the term of T1D referred here is specifically for the state of untreated insulinopenic diabetes induced by STZ, which is distinct from the typical type 1 diabetes in humans that receive insulin treatments with largely controlled glucose levels. Our results demonstrate that GABAergic LepR^{Arc} neurons mediate T1D hyperglycemia and leptin action on reducing T1D glucose. Among various LepR neurons in the brain, only LepR^{Arc} neurons were selectively activated in T1D. The physiological importance of this activation is reflected from the striking effects on T1D glucose by direct manipulation of Arc neuron activity. Both acute and chronic activation of GABA^{Arc} neurons reversed leptin action on reducing T1D, while inhibition of these neurons was sufficient to produce leptin-mimicking action on reducing T1D hyperglycemia. Due to technical limitations in using LepR-Cre to target LepR^{Arc} neurons, which caused varied offsite targeting to nearby glutamatergic neurons in the ventromedial hypothalamus (VMH), we instead used Vgat-Cre to selectively target GABA^{Arc} neurons. Since the function of LepR is mediated by GABAergic neurons in both body weight²¹ and T1D glucose⁶, it is most likely that GABAergic LepR^{Arc} neurons mediate the effects we observed in our study. The revealed importance of GABAergic LepR^{Arc} is consistent with previous observations on the noninvolvement of VMH LepR neurons in mediating leptin action on T1D glucose¹⁵. Thus, heightened activation of GABAergic LepR^{Arc} represents one major pathogenic cause of T1D hyperglycemia. Therefore, strategies aiming to inhibit these neurons should represent an effective therapy to achieve leptin-mimicking euglycemia restoration in T1D.

Our results revealed previously unappreciated importance of non-AgRP Arc neurons in T1D pathogenesis and mediating

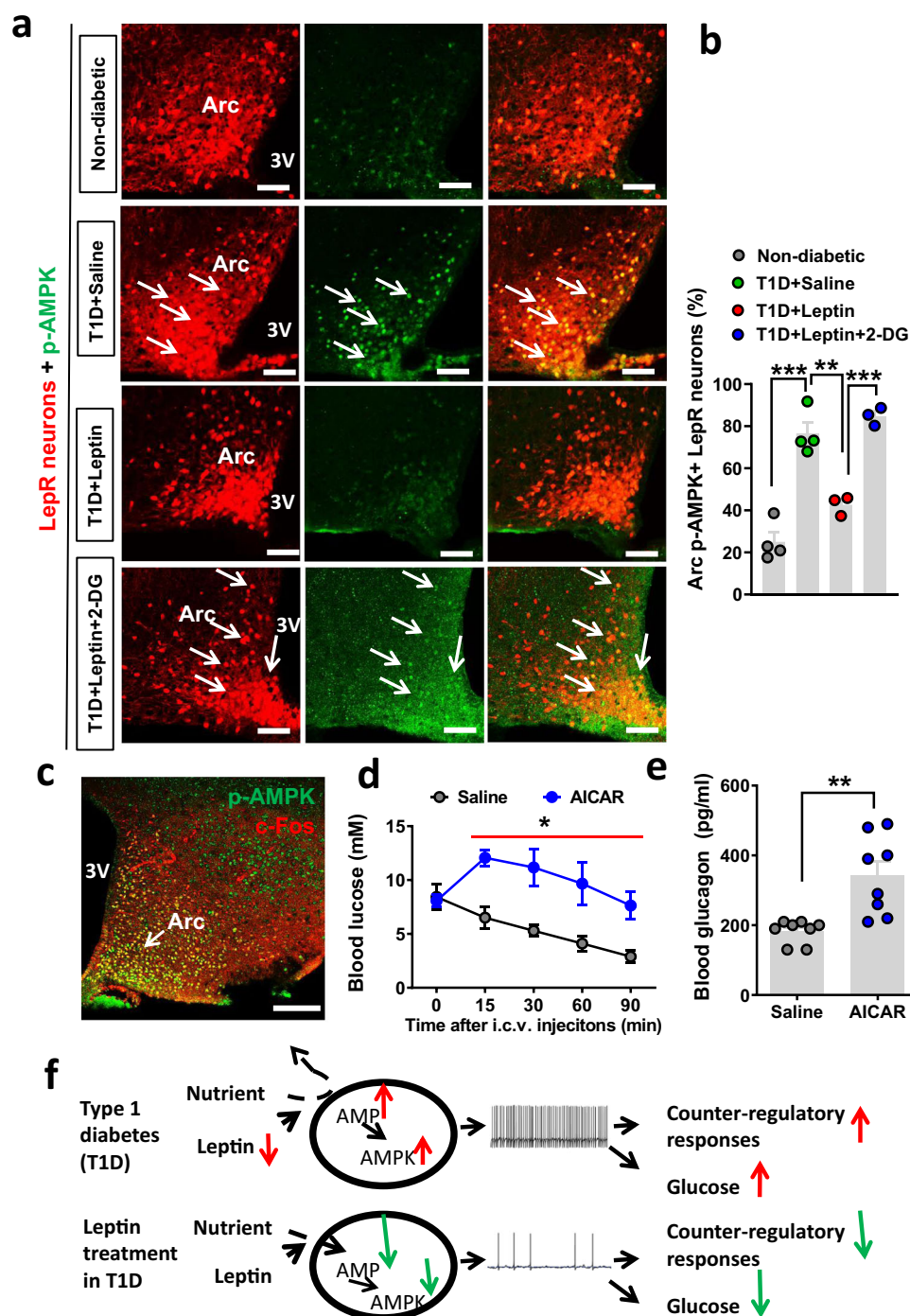


Fig. 7 T1D hyperglycemia and leptin action on reducing T1D hyperglycemia involve the AMPK pathway. **a** Representative immunostaining images from $n = 3$ –4 mice as shown in **b** for p-AMPK in hypothalamic sections were shown from control (**a**, Non-diabetic), T1D with saline treatment (**a**, T1D + saline), T1D with leptin treatment (**a**, T1D + leptin), and T1D with both leptin and 2-DG treatments (T1D + leptin+2-DG) at fed conditions. **b** Comparison in the percentage of LepR^{Arc} neurons with p-AMPK expression among the four groups (one-way ANOVA, $n = 4$ for nondiabetic controls and T1D-saline and $n = 3$ for T1D-leptin and T1D-leptin+2-DG, $F(3, 10) = 42.27$, $***P < 0.0001$, nondiabetic vs T1D-saline; $**P = 0.0051$, T1D-saline vs T1D-leptin; $***P = 0.004$, T1D-leptin vs T1D-leptin+2-DG). **c** Representative pictures from $n = 1$ mouse showing colocalization between p-AMPK (green) and c-Fos (red) in the Arc of T1D LepR-Cre mice. **d**, **e** Wild-type mice (8–9 weeks of age, males) were made T1D and then i.c.v. treated with leptin to restore euglycemia. These mice were then treated with i.c.v. injections of either saline or AICAR, an agonist of AMPK. Comparisons in blood glucose were shown following i.c.v. AICAR (**d**, two-way ANOVA, $n = 3$ /each, $F(1, 20) = 35.91$, $*P = 0.0374$, saline vs AICAR at the 90-min time point) and in blood glucagon at 90 min after AICAR injection (**e**, two-tailed unpaired Student's t tests, $n = 8$ /each, $t = 3.862$, $df = 14$; $n = 9$ /each, $***P = 0.0017$). **f** Diagram depicting hyperglycemia in T1D caused by heightened Arc GABAergic neuron activity due to energy deprivation and low leptin levels (top) and euglycemia restoration by leptin inhibition of neuron activity through reversing energy deprivation (bottom). 3V: the third ventricle and Arc: arcuate nucleus. Data presented as mean $\pm$ SEM. 3V the third ventricle, Arc arcuate nucleus; Scale bar: 100 μ m.

leptin action on T1D glucose. Previous studies suggest the importance of the melanocortin pathway, and especially AgRP neurons, in mediating leptin action^{16–19}. Given the extensive research focus on AgRP neurons, the roles for non-AgRP Arc neurons have largely been neglected. Mice with AgRP neuron lesions developed T1D hyperglycemia in response to STZ and importantly, leptin action in reducing T1D hyperglycemia in these mice remained intact, suggesting that the presence of AgRP neurons is neither required for T1D hyperglycemia nor for mediating leptin action on reducing T1D hyperglycemia. Our results showed that activation of AgRP neurons exhibited a delayed reversal of leptin action, while chronic inhibition of these neurons led to a partial reduction in T1D glucose. These findings contrast a complete reversal of leptin action and leptin-mimicking effect by respective activation and inhibition of GABA^{Arc} neurons, suggesting an important role for Arc GABAergic non-AgRP neurons in mediating the leptin action. A mild role for AgRP neurons in mediating the leptin action is supported by a recent study with DREADD-mediated manipulation of AgRP neuron activity²⁰. Given the demonstrated c-Fos expression in a subset of GABAergic non-AgRP Arc neurons in T1D, these Arc GABAergic non-AgRP neurons may represent an important novel brain site for T1D pathogenesis. Of note, previously identified non-AgRP Trh/Cxcl12 GABAergic neurons in the Arc may represent one such group²². Further delineation of the functional identity of these neurons in their underlying neural pathway may reveal new insights on leptin action on reducing T1D hyperglycemia.

We provided several lines of evidence supporting that LepR^{Arc} neurons are activated by energy deprivation in T1D. LepR^{Arc} neurons were similarly activated in T1D and fasting, a condition with nutritional deprivation. Importantly, brain leptin infusion was potent in reducing circulating glucose in fasting associated with suppressing counter-regulatory hormone levels, all effects mimicking leptin action on reducing T1D hyperglycemia. Supporting this, except for blood glucose levels, T1D and fasting share many common features in blood hormones and physiology, including low insulin and leptin levels, and augmented counter-regulatory responses³⁷. In addition, LepR^{Arc} neurons in T1D show a dramatic increase in the activation of AMPK, which is known to be an intracellular energy status sensor³⁶. The colocalization of p-AMPK and c-Fos in the Arc provides direct evidence supporting that energy deprivation causes LepR neuron activation. Consistently, activation of AMPK also occurs in the hypothalamus during fasting and can be reversed by leptin³⁶. It is striking that the sign of energy deprivation in LepR^{Arc} neurons is accompanied with uncontrolled severe hyperglycemia in T1D. Moreover, LepR^{Arc} neurons failed to respond to dramatic changes in blood glucose. In contrast to abundant induction of c-Fos in control LepR^{Arc} neurons in response to a reduction of <2 mM glucose in controls, no changes were observed in c-Fos in T1D LepR^{Arc} neurons with a change of >20 mM glucose. These lines of evidence collectively support a state of defective nutrient sensitivity of LepR^{Arc} neurons in T1D. Colocalization of c-Fos and p-AMPK in T1D LepR^{Arc} neurons suggests that these neurons are “locked” in a state of energy deprivation, signaling starvation and leading to heightened counter-regulatory responses, hyperglycemia, and other changes associated with starvation, all typical symptoms of T1D.

Our data suggest that leptin action on T1D LepR^{Arc} neuron inhibition and hyperglycemia is mediated by reversal of energy deprivation in T1D LepR^{Arc} neurons. Leptin effectively reduces the heightened activity of LepR^{Arc} neurons in T1D, in a similar fashion to that occurring in fasting states. In addition, this action is accompanied by reversing the activation of AMPK. Importantly, leptin action can be reversed by 2-DG, a nutrient-depriving agent. Conversely, T1D LepR^{Arc} neurons were not sensitive to 2-DG, but

gained sensitivity in the presence of leptin, providing direct supporting evidence that leptin mediates nutrient sensitivity of these neurons. The reversal of energy deprivation by leptin is further corroborated by the effect of 2-DG in reversing leptin action on reducing T1D hyperglycemia, as well as inhibiting the heightened activity of Arc neurons, suggesting that leptin inhibition may be mediated by replenishing energy supply to these neurons. The mechanism underlying the leptin action in reversing energy deprivation is not clear, but may involve increased uptake of nutrient substrates, including blood glucose or lactate from glial cells^{38,39}. Supporting this, previous observations suggest that glucose and leptin exert synergistic effects on intracellular leptin signaling⁴⁰. In line with this, leptin action on presynaptic GABAergic input to POMC neurons is reduced by glucose and increased by the activation of AMP kinase⁴¹, and AMPK plays a role in mediating leptin and glucose effects on LepR neurons^{24,36,40,42}. Increased intracellular nutrient supply may inhibit neuron activity through membrane channels²⁸.

In our recording experiments, we focused on LepR^{Arc} neurons that were inhibited by leptin, as these neurons are presumed to be the most relevant to T1D hyperglycemia due to their heightened activity associated with low leptin levels. The remaining leptin-excited LepR^{Arc} neurons, including POMC neurons, may also be involved in glucose homeostasis²⁶. However, as Arc GABAergic LepR^{Arc} neurons are the primary mediator of the leptin action in T1D, POMC neurons, which are non-GABAergic²¹, may contribute little to T1D hyperglycemia and leptin action in reducing T1D glucose.

Notably, a recent human clinical trial with a combination of insulin and leptin treatment of T1D shows that leptin reduces the dose of insulin for human T1D treatment, but without a dramatic effect on glucose⁴³. Although this failed clinical trial, which is in contrast to the demonstrated leptin effect in rodents, may be due to many reasons, including potential differences between humans and rodents, one key difference in human T1D patients is that they are always on insulin treatment, and the leptin level is only reduced 50%, compared to non-T1D humans^{5,44,45}, presumably due to the reason that insulin is capable of increasing leptin levels^{46,47}. In contrast, rodent T1D models are essentially insulinopenic and as a result, leptin levels are extremely low^{1,2,46,48}. It is thus conceivable that an additional leptin dose on top of the mildly reduced leptin level in human T1D may not be able to provide additional beneficial effects. In other words, the beneficial effect of brain leptin action may have been reflected by the effects of insulin treatments⁴⁹. Further studies are warranted to specifically address this possibility. In summary, our current study reveals that uncontrolled insulinopenic T1D represents a state of chronic fasting in terms of chronic activation of LepR^{Arc} neurons owing to energy deprivation. Together, these data demonstrate that leptin action on reducing T1D hyperglycemia is mediated through reversing energy deprivation in LepR^{Arc} neurons, leading to inhibition of these neurons, and reduced counter-regulatory responses. Thus, understanding the mechanism underlying leptin action on reversal in energy deprivation is imperative for a full understanding of T1D pathogenesis and more efficient treatment strategies against T1D.

Methods

Animals. All mice were housed with ad libitum access to water and food in a temperature-controlled room (21–22 °C) with a 12:12-h light–dark cycle. Animal testing and research follow all relevant ethical regulations. Animal care and procedures were approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston (Protocol # 18-021). LepR-Cre mice were bred to (ROSA)26Sortm9(CAG-tdTomato) (also called Ai9) reporter to generate LepR-Cre::Ai9 mice for electrophysiological recording or colocalization with c-Fos immunohistochemistry. AgRP-Cre and Vgat-Cre mice were obtained from the Jax Laboratory, and these mice were also bred with (ROSA)26Sortm1.1(CAG-cas9*, -EGFP) to allow visualization of AgRP and GABAergic neurons with GFP expression (GFP reporters). All mice that were used for stereotaxic injections were at least 8–10-weeks old. AgRP^{DTR} mice were provided by Dr. Qi Wu of Baylor

College of Medicine and AgRP lesion with the injection of DTX (Sigma, St. Louis, MO). The experiments on the nonobese diabetes model (NOD, Jax lab) were performed in Shanghai University of Traditional Chinese Medicine (SUTCM) and the procedures were approved by the Animal Welfare Committee of SUTCM (Approval number: SZY20160900).

STZ-induced T1D. STZ (Sigma-Aldrich, St. Louis, MO) dissolved in cold-sterile acetate buffer, pH 4.5, was injected intraperitoneally (i.p.) to 6–8-week-old male mice at 150 mg/Kg (two times at 1-week intervals). Nondiabetic control mice received i.p. injection of acetate buffer alone. T1D was defined as nonfasting blood glucose >23 mM on 3 consecutive days.

Icv pump infusion studies. Recombinant mouse leptin (A.F. Parlow, National Hormone and Peptide Program, Torrance, CA) dissolved in phosphate-buffered saline (PBS, pH 8.0) was loaded into 14-day mini-osmotic pumps (DURECT Corporation, Cupertino, CA) that were connected with commercial brain infusion kits (DURECT Corporation). Pumps were surgically implanted in the mouse interscapular cavity 7 days after the establishment of T1D. All mice were anesthetized with ketamine (100 mg/Kg) and xylazine (10 mg/Kg), and then cannulas were stereotactically positioned into the lateral cerebral ventricles using the coordinates: -0.30 mm from the bregma, $\pm$ 1.0 mm lateral, and -2.5 mm of the skull. Leptin was continuously delivered at the speed of 50 ng per hour (0.25 μ L/h) to the ventricles. T1D mice receiving the same amount of icv vehicle infusion were taken as controls. CNO (Sigma, St. Louis, MO) was i.p. injected at the dose of 1 mg/kg.

From day 3 after icv surgery, blood glucose levels, food intake, and body weight of all animals were recorded once every two days. All measurements were performed between 10:00 AM and 12:00 AM. Glucose levels were measured by using tail vein blood with OneTouch Glucometer.

For dual intracerebroventricular cannulation surgery, T1D mice were anesthetized and then cannulas were positioned into the left lateral cerebral ventricles with the coordinates: bregma, -0.5 mm, midline, -1.8 mm, and dorsal surface, -2.2 mm at a -10° angle. Meanwhile, minipumps with leptin (0.1 mg/ml, 100 μ L/pump) were implanted into the right lateral cerebral ventricles with the coordinates: bregma, -0.5 mm, midline, +1.8 mm, and dorsal surface, -2.2 mm with a +10° angle. 2-DG (2 mg/3 μ L/mouse) was i.c.v. injected after 3-day recovery while leptin was pumping into the brain as well and blood glucose levels were measured.

Stereotaxic injections and viral vectors. Stereotaxic surgeries to deliver viral constructs were performed based on a revised previous protocol⁷. Briefly, mice were anesthetized with a ketamine/xylazine cocktail (100 and 10 mg/kg, respectively), and their heads affixed to a stereotaxic apparatus. Viral vectors were delivered through a 0.5- μ L syringe (Neuros Model 7000.5 KH, point style 3, Hamilton, Reno, NV, USA) mounted on a motorized stereotaxic injector (Quintessential Stereotaxic Injector, Stoelting, Wood Dale, IL, USA) at a rate of 30 nL/min. Viral preparations were made through the Baylor NeuroConnectivity Core and Gene Vector Core with either serotype 5, 9, or DJ and titered at more than $\sim 10^{12}$ particles/mL. Viral delivery was targeted to the Arc through four local injections with two each side (200 nL/side, anteroposterior (AP): -1.35 and -1.5 mm, mediolateral (ML): $\pm$ 0.2 mm, and dorsoventral (DV): -5.9 mm). AAV-DIO-hM3D(Gq)-mCherry, AAV-DIO-hM4D(Gi)-mCherry, AAV-EF1a-Flex-EGFP-P2A-mNacBac, or AAV-EF1a-DIO-Kir2.1-P2A-dTomato were delivered bilaterally into the Arc of AgRP-Cre or Vgat-Cre mice. For LepR-Cre, a volume of was injected into bilateral Arc instead. Two mutations (E224G and Y242F) were made in the Kir2.1 construct so that its activity will be less rectified by membrane potentials, thereby more effective in reducing neuron activity⁵⁰. AAV-Flex-GFP or AAV-Flex-mCherry injections were used as a control group.

Blood collection and tissue processing. Blood samples were collected from all mice with deep anesthesia into a chilled tube containing 15 μ L EDTA. Plasma was isolated by centrifugation (3000 $\times$ g at 4°C for 15 min), and then stored at -80°C. After blood collection, all mice were transcardially perfused with 0.9% normal saline and followed by 10% formalin. Brain and pancreas were harvested and cryoprotected in 30% sucrose for further analysis. Plasma glucagon, insulin, and corticosterone were measured by the Vanderbilt University Hormone Assay & Analytical Services Core (Nashville, TN).

Immunohistochemistry (IHC). Brain sections were sliced with microtome (Leica, Germany) at 30- μ m thickness. Phosphor-STAT3 (p-STAT3) was immunostained with rabbit anti-p-STAT3 antibody (1:400, Cell Signaling, Danvers, MA), p-AMPK (Cell Signaling, Danvers, MA) used at 1:1000, and c-Fos antibody (Millipore) used at 1:1000, the specificity of which was confirmed previously⁵¹, or all brain sections were incubated with antibodies using free-floating methods with 24-well plates⁵², and visualized with AlexaFluor 488-conjugated donkey anti-rabbit IgGs (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were photographed under a TCS SP5 confocal microscope (Leica). Neurons with clear immunostaining of c-Fos or p-AMPK were counted from four to five matched sections containing Arc from individual mice ($n = 3$ –4/group) and the number of was averaged and compared using the indicated statistical methods.

Brain slice electrophysiological recordings. Coronal brain slices (250–300 μ m) containing the arcuate nucleus of the hypothalamus (ARC) were cut in ice-cold artificial cerebrospinal fluid (aCSF) containing the following (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25 NaH₂PO₄, 25 NaHCO₃, and 11 D-glucose bubbling with 95% O₂/5% CO₂. Slices containing the ARC were maintained for recovery for at least 1 h at 32–34°C. Individual slices were transferred to a recording chamber mounted on an upright microscope (Olympus BX51WI) and continuously superfused (2 ml/min) with ACSF warmed to 32–34°C by passing it through a feedback-controlled in-line heater (TC-324B, Warner Instruments). Cells were visualized through a $\times 40$ water-immersion objective with differential interference contrast (DIC) optics and infrared illumination. Whole-cell current-clamp recordings were made from neurons with the tdTomato reporter fluorescence within the Arc. Patch pipettes (3–5 M Ω) were filled with a K⁺-based low Cl⁻ an internal solution containing (in mM) 145 K-gluconate, 1 MgCl₂, 10 HEPES, 0.2 EGTA, 4 ATP, 0.3 GTP, and 10 phosphocreatine, pH 7.35, 270–285 mOsm. To test the glucose dose effect on neuronal firing, the osmolarity of the solution was balanced with sucrose. Mouse leptin (Harbor-UCLA Medical Center) at a concentration of 80 nM was perfused at 2 ml/min for up to 3 min to examine the effects of leptin on LepR neurons. To test LepR^{Arc} neurons from T1D mice, brain sections were obtained 1 week after the establishment of T1D conditions with blood-glucose concentration >25 mM. For recording, we used bath 1 mM as a starting glucose concentration to mimicking a low glucose level, aiming for a higher level of baseline activity for leptin inhibitory action. We increased glucose levels from 1 to 10 mM, which grossly mimics high brain glucose levels in T1D, to examine the effect of glucose on neuron activity change.

Hormone measurement. Cheek blood from mice was collected and the serum was stored in a -80°C freezer until measurement. Hormone measurements were performed either by ELISA kits from Millipore or by RIA kits at the Hormone Assay Core Facility of Vanderbilt University.

Statistical analysis. All data were presented as mean $\pm$ SEM. Statistical analyses were performed using Student's *t* tests or ANOVA tests with Tukey post hoc test as specified for each study (GraphPad Prism, GraphPad Software, La Jolla, CA). A *P* value <0.05 was considered significant.

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

Data availability

All data generated or analyzed during this study are included in this published article. Source data are provided with this paper.

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References

- Wang, M. Y. et al. Leptin therapy in insulin-deficient type 1 diabetes. *Proc. Natl Acad. Sci. USA* **107**, 4813–4819 (2010).
- Yu, X., Park, B. H., Wang, M. Y., Wang, Z. V. & Unger, R. H. Making insulin-deficient type 1 diabetic rodents thrive without insulin. *Proc. Natl Acad. Sci. USA* **105**, 14070–14075 (2008).
- Fujikawa, T., Chuang, J. C., Sakata, I., Ramadori, G. & Coppari, R. Leptin therapy improves insulin-deficient type 1 diabetes by CNS-dependent mechanisms in mice. *Proc. Natl Acad. Sci. USA* **107**, 17391–17396 (2010).
- Denroche, H. C. et al. Leptin therapy reverses hyperglycemia in mice with streptozotocin-induced diabetes, independent of hepatic leptin signaling. *Diabetes* **60**, 1414–1423 (2011).
- Oberlin, D. & Buettner, C. How does leptin restore euglycemia in insulin-deficient diabetes? *J. Clin. Invest.* **127**, 450–453 (2017).
- Fujikawa, T. et al. Leptin engages a hypothalamic neurocircuitry to permit survival in the absence of insulin. *Cell Metab.* **18**, 431–444 (2013).
- Xu, Y., Chang, J. T., Myers, M. G. Jr., Xu, Y. & Tong, Q. Euglycemia restoration by central leptin in type 1 diabetes requires STAT3 signaling but not fast-acting neurotransmitter release. *Diabetes* <https://doi.org/10.2337/db15-1160> (2016).
- Lee, Y., Wang, M. Y., Du, X. Q., Charron, M. J. & Unger, R. H. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. *Diabetes* **60**, 391–397 (2011).
- Diamond, N. et al. Blockade of glucagon signaling prevents or reverses diabetes onset only if residual beta-cells persist. *eLife* **5**, <https://doi.org/10.7554/eLife.13828> (2016).
- Perry, R. J. et al. Leptin reverses diabetes by suppression of the hypothalamic-pituitary-adrenal axis. *Nat. Med.* **20**, 759–763 (2014).

11. Perry, R. J., Petersen, K. F. & Shulman, G. I. Pleotropic effects of leptin to reverse insulin resistance and diabetic ketoacidosis. *Diabetologia* **59**, 933–937 (2016).
12. Morton, G. J., Meek, T. H., Matsen, M. E. & Schwartz, M. W. Evidence against hypothalamic-pituitary-adrenal axis suppression in the antidiabetic action of leptin. *J. Clin. Investig.* **125**, 4587–4591 (2015).
13. Meek, T. H. & Morton, G. J. The role of leptin in diabetes: metabolic effects. *Diabetologia* **59**, 928–932 (2016).
14. Singha, A. et al. Leptin receptors in RIP-Cre(25Mgn) neurons mediate anti-dyslipidemia effects of leptin in insulin-deficient mice. *Front. Endocrinol.* **11**, 588447 (2020).
15. Meek, T. H. et al. Leptin action in the ventromedial hypothalamic nucleus is sufficient, but not necessary, to normalize diabetic hyperglycemia. *Endocrinology* **154**, 3067–3076 (2013).
16. Meek, T. H. et al. Role of melanocortin signaling in neuroendocrine and metabolic actions of leptin in male rats with uncontrolled diabetes. *Endocrinology* **155**, 4157–4167 (2014).
17. da Silva, A. A., do Carmo, J. M., Freeman, J. N., Tallam, L. S. & Hall, J. E. A functional melanocortin system may be required for chronic CNS-mediated antidiabetic and cardiovascular actions of leptin. *Diabetes* **58**, 1749–1756 (2009).
18. Xu, J. et al. Genetic identification of leptin neural circuits in energy and glucose homeostases. *Nature* **556**, 505–509 (2018).
19. Goncalves, G. H., Li, W., Garcia, A. V., Figueiredo, M. S. & Bjorbaek, C. Hypothalamic agouti-related peptide neurons and the central melanocortin system are crucial mediators of leptin's antidiabetic actions. *Cell Rep.* **7**, 1093–1103 (2014).
20. Uner, A. G. et al. Role of POMC and AgRP neuronal activities on glycaemia in mice. *Sci. Rep.* **9**, 13068 (2019).
21. Vong, L. et al. Leptin action on GABAergic neurons prevents obesity and reduces inhibitory tone to POMC neurons. *Neuron* **71**, 142–154 (2011).
22. Campbell, J. N. et al. A molecular census of arcuate hypothalamus and median eminence cell types. *Nat. Neurosci.* **20**, 484–496 (2017).
23. Kim, G. H. et al. Leptin recruits Creb-regulated transcriptional coactivator 1 to improve hyperglycemia in insulin-deficient diabetes. *Mol. Metab.* **4**, 227–236 (2015).
24. Mountjoy, P. D., Bailey, S. J. & Rutter, G. A. Inhibition by glucose or leptin of hypothalamic neurons expressing neuropeptide Y requires changes in AMP-activated protein kinase activity. *Diabetologia* **50**, 168–177 (2007).
25. Qiu, J., Fang, Y., Ronnekleiv, O. K. & Kelly, M. J. Leptin excites proopiomelanocortin neurons via activation of TRPC channels. *J. Neurosci.* **30**, 1560–1565 (2010).
26. Berglund, E. D. et al. Direct leptin action on POMC neurons regulates glucose homeostasis and hepatic insulin sensitivity in mice. *J. Clin. Investig.* **122**, 1000–1009 (2012).
27. Djogo, T. et al. Adult NG2-Glia are required for median eminence-mediated leptin sensing and body weight control. *Cell Metab.* **23**, 797–810 (2016).
28. Baver, S. B. et al. Leptin modulates the intrinsic excitability of AgRP/NPY neurons in the arcuate nucleus of the hypothalamus. *J. Neurosci.* **34**, 5486–5496 (2014).
29. Williams, K. W. et al. Segregation of acute leptin and insulin effects in distinct populations of arcuate proopiomelanocortin neurons. *J. Neurosci.* **30**, 2472–2479 (2010).
30. Gao, Y. et al. TrpC5 mediates acute leptin and serotonin effects via pomc neurons. *Cell Rep.* **18**, 583–592 (2017).
31. Qiu, J., Wagner, E. J., Ronnekleiv, O. K. & Kelly, M. J. Insulin and leptin excite anorexigenic pro-opiomelanocortin neurons via activation of TRPC5 channels. *J. Neuroendocrinol.* **30**, <https://doi.org/10.1111/jne.12501> (2018).
32. Zhu, C. et al. Profound and redundant functions of arcuate neurons in obesity development. *Nat. Metab.* **2**, 763–774 (2020).
33. Zhu, C. et al. Disrupted hypothalamic CRH neuron responsiveness contributes to diet-induced obesity. *EMBO Rep.* **21**, e49210 (2020).
34. Kim, E. R. et al. Paraventricular hypothalamus mediates diurnal rhythm of metabolism. *Nat. Commun.* **11**, 3794 (2020).
35. Luquet, S., Perez, F. A., Hnasko, T. S. & Palmiter, R. D. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* **310**, 683–685 (2005).
36. Minokoshi, Y. et al. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* **428**, 569–574 (2004).
37. Xu, Y. & Tong, Q. Central leptin action on euglycemia restoration in type 1 diabetes: restraining responses normally induced by fasting? *Int. J. Biochem. Cell Biol.* <https://doi.org/10.1016/j.biocel.2016.09.027> (2016).
38. Magistretti, P. J. & Allaman, I. Lactate in the brain: from metabolic end-product to signalling molecule. *Nat. Rev. Neurosci.* **19**, 235–249 (2018).
39. Yellen, G. Fueling thought: Management of glycolysis and oxidative phosphorylation in neuronal metabolism. *J. Cell Biol.* **217**, 2235–2246 (2018).
40. Su, H., Jiang, L., Carter-Su, C. & Rui, L. Glucose enhances leptin signaling through modulation of AMPK activity. *PLoS ONE* **7**, e31636 (2012).
41. Lee, D. K., Jeong, J. H., Chun, S. K., Chua, S. Jr. & Jo, Y. H. Interplay between glucose and leptin signalling determines the strength of GABAergic synapses at POMC neurons. *Nat. Commun.* **6**, 6618 (2015).
42. Claret, M. et al. AMPK is essential for energy homeostasis regulation and glucose sensing by POMC and AgRP neurons. *J. Clin. Investig.* **117**, 2325–2336 (2007).
43. Vasandani, C., Clark, G. O., Adams-Huet, B., Quittner, C. & Garg, A. Efficacy and safety of Metreleptin therapy in patients with type 1 diabetes: a pilot study. *Diabetes Care* **40**, 694–697 (2017).
44. Hanaki, K., Becker, D. J. & Arslanian, S. A. Leptin before and after insulin therapy in children with new-onset type 1 diabetes. *J. Clin. Endocrinol. Metab.* **84**, 1524–1526 (1999).
45. Hathout, E. H. et al. Changes in plasma leptin during the treatment of diabetic ketoacidosis. *J. Clin. Endocrinol. Metab.* **84**, 4545–4548 (1999).
46. Havel, P. J. et al. Marked and rapid decreases of circulating leptin in streptozotocin diabetic rats: reversal by insulin. *Am. J. Physiol.* **274**, R1482–R1491 (1998).
47. Sivitz, W. I. et al. Plasma leptin in diabetic and insulin-treated diabetic and normal rats. *Metabolism* **47**, 584–591 (1998).
48. German, J. P. et al. Leptin deficiency causes insulin resistance induced by uncontrolled diabetes. *Diabetes* **59**, 1626–1634 (2010).
49. Zouhar, P. et al. UCP1-independent glucose-lowering effect of leptin in type 1 diabetes: only in conditions of hypoleptinemia. *Am. J. Physiol. Endocrinol. Metab.* **318**, E72–E86 (2020).
50. Xue, M., Atallah, B. V. & Scanziani, M. Equalizing excitation-inhibition ratios across visual cortical neurons. *Nature* **511**, 596–600 (2014).
51. Fan, S. et al. An indirect action contributes to C-Fos induction in paraventricular hypothalamic nucleus by neuropeptide Y. *Sci. Rep.* **6**, 19980 (2016).
52. Xu, Y. et al. Glutamate mediates the function of melanocortin receptor 4 on Sim1 neurons in body weight regulation. *Cell Metab.* **18**, 860–870 (2013).

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Author contributions

S.F. conducted the research with help from Yuanzhong X., Y.L., Z.J., J.C.M., J.C., and H. L.; Q.W., M.X., B.R.A., and Yong X. provided essential reagents; Q.T. and C.H. conceived and designed the experiments, and wrote the paper with significant inputs from Q.W. and Yong X.

Competing interests

The authors declare no competing interests.

Additional information

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Centrally circulating α -klotho inversely correlates with human obesity and modulates arcuate cell populations in mice



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ABSTRACT

Objective: Our laboratory recently identified the centrally circulating α -klotho protein as a novel hypothalamic regulator of food intake and glucose metabolism in mice. The current study aimed to investigate novel molecular effectors of central α -klotho in the arcuate nucleus of the hypothalamus (ARC), while further deciphering its role regulating energy balance in both humans and mice.

Methods: Cerebrospinal fluid (CSF) was collected from 22 adults undergoing lower limb orthopedic surgeries, and correlations between body weight and α -klotho were determined using an α -klotho enzyme-linked immunosorbent assay (ELISA) kit. To investigate the effects of α -klotho on energy expenditure (EE), 2-day intracerebroventricular (ICV) treatment was performed in diet-induced obesity (DIO) mice housed in TSE Phenomaster indirect calorimetry metabolic cages. Immunohistochemical staining for cFOS and patch clamp electrophysiology were used to determine the effects of central α -klotho on proopiomelanocortin (POMC) and tyrosine hydroxylase (TH) neurons. Additional stainings were performed to determine novel roles for central α -klotho to regulate non-neuronal cell populations in the ARC. Lastly, ICV pretreatment with fibroblast growth factor receptor (FGFR) or PI3kinase inhibitors was performed to determine the intracellular signaling involved in α -klotho-mediated regulation of ARC nuclei.

Results: Obese/overweight human subjects had significantly lower CSF α -klotho concentrations compared to lean counterparts ($1,044 \pm 251$ vs. 1616 ± 218 pmol/L, respectively). Additionally, 2 days of ICV α -klotho treatment increased EE in DIO mice. α -Klotho had no effects on TH neuron activity but elicited varied responses in POMC neurons, with 44% experiencing excitatory and 56% experiencing inhibitory effects. Inhibitor experiments identified an α -klotho $\rightarrow$ FGFR $\rightarrow$ PI3kinase signaling mechanism in the regulation of ARC POMC and NPY/AgRP neurons. Acute ICV α -klotho treatment also increased phosphorylated ERK in ARC astrocytes via FGFR signaling.

Conclusion: Our human CSF data provide the first evidence that impaired central α -klotho function may be involved in the pathophysiology of obesity. Furthermore, results in mouse models identify ARC POMC neurons and astrocytes as novel molecular effectors of central α -klotho. Overall, the current study highlights prominent roles of α -klotho $\rightarrow$ FGFR $\rightarrow$ PI3kinase signaling in the homeostatic regulation of ARC neurons and whole-body energy balance.

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Keywords α -Klotho; Metabolism; Obesity; POMC neuron; Fibroblast growth factor; PI3kinase

1. INTRODUCTION

Recent evidence identifies the anti-aging protein α -klotho as a potent yet complex hormonal regulator of whole-body metabolism. α -Klotho can be secreted from the kidney [1] and act on peripheral tissues to regulate insulin sensitivity [2], promote insulin release [3,4], protect pancreatic β cells from oxidative damage [5], increase resting energy expenditure [6], and stimulate lipid oxidation [6]. Furthermore, blood α -klotho concentrations are reduced in patients with diabetes or

obesity [7–11], while experimentally reversing these impairments has been shown to be therapeutic in various rodent models of metabolic disease [3–6,9].

Despite its prominent roles in peripheral metabolism, α -klotho cannot cross the blood brain barrier due to its molecular weight [12,13], resulting in a separate, relatively unexplored, pool of α -klotho secreted from the choroid plexus into the cerebrospinal fluid (CSF) [1,14]. Our laboratory recently provided the first evidence that CSF α -klotho also regulates energy balance and glucose metabolism

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[15]. Intracerebroventricular (ICV) administration of α -klotho resulted in suppressed appetite, reduced body weight, improved insulin release, and reduced hepatic gluconeogenic gene expression in mouse models of Type I and II diabetes. We also observed that α -klotho is a novel negative regulator of neuropeptide Y/agouti-related peptide- (NPY/AgRP)-expressing neurons in the arcuate nucleus (ARC) of the hypothalamus, which are critically involved in inducing hunger [16], reducing energy expenditure [17], and regulating glucose metabolism [18,19]. Mechanistically, α -klotho acts as a scaffolding protein to promote FGFR activity and downstream PI3kinase signaling [20], a phenomenon that was found to be essential to central α -klotho-mediated regulation of energy balance and NPY/AgRP neurons [15].

The ARC contains many other cell types critical to metabolic regulation. Similar to NPY/AgRP neurons, ARC tyrosine hydroxylase-expressing (TH) neurons project to the paraventricular nucleus (PVN) to stimulate food intake [21]. Conversely, ARC proopiomelanocortin- (POMC)-expressing neurons induce satiety, increase energy expenditure, and reduce hepatic glucose output [22–24], in part via PI3kinase signaling [25]. Even non-neuronal ARC astrocytes and tanycytes are essential to nutrient sensing, hormonal transport, and neuronal health [26–28], roles recently found to be dependent on FGFR function [29,30]. Considering these diverse ARC cell types, the current study aimed to identify novel molecular effectors of the α -klotho $\rightarrow$ FGFR $\rightarrow$ PI3kinase signaling axis in the ARC, while further deciphering α -klotho's role in regulating energy balance in both humans and mice.

2. METHODS

2.1. Human cerebrospinal fluid (CSF) collection

All human protocols were approved by the medical ethics committee of Beijing Friendship Hospital, Capital Medical University (Beijing, China). After informed consent was provided, CSF was collected from 22 adults (10 males, 12 females, 18–83 years old) undergoing lower limb orthopedic surgeries at the Beijing Friendship Hospital, Beijing, China. All patients were clear of CNS infections, renal diseases, severe cardiopulmonary diseases, such as myocardial infarction or chronic obstructive pulmonary disease, and malignant tumors. Patients were chosen using systematic sampling in odd sequence on the same day each week; CSF was collected from the first, third, fifth, etc. surgery patients each day. Briefly, 1.0 ml of CSF was collected during administration of spinal block anesthesia. Patients were positioned on their sides with their backs at the edge of the operation bed, curled their shoulders and legs, and arched out their lower backs. The needle was placed past the dura mater into subarachnoid space between lumbar vertebrae, usually between L3 and L4 or L4 and L5 to minimize risk of injury to the spinal cord. All specimens were immediately flash-frozen in liquid nitrogen before storage at -80°C . α -Klotho CSF concentrations were measured by enzyme-linked immunosorbent assay (ELISA) using a human soluble α -klotho assay kit (27,998, IBL).

2.2. Animals

C57BL/6, B6.Tg(NPY-hrGFP)1Low/J (NPY-GFP reporter), and Tg(Pomc1-cre)16Low/J (POMC-Cre) mice were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and experimental protocols were approved by the Institutional Animal Care and Use Committees of East Carolina University or University of Texas Health Science Center as appropriate. Mice were housed at $20\text{--}22^{\circ}\text{C}$ with a 12-h light–dark cycle.

2.3. High-fat diet-induced obesity

Five- to 6-week-old male C57BL/6 mice were given ad libitum access to a high-fat diet with a kilocalorie composition of 58%, 25%, and 17% of fat, carbohydrates, and protein, respectively, for 10 weeks (D12331; Research Diets, New Brunswick, NJ) before undergoing ICV cannulation.

2.4. ICV cannulation and treatment

ICV procedures were performed as previously described [15]. Prior to the procedure, mice were given oral analgesic meloxicam and anesthetized with an isoflurane vaporizer. Mice were placed on a stereotaxic device, and a midline incision was made on the head. A hole was drilled (1.0 mm lateral, -0.5 mm posterior, 2.5 mm deep to the bregma), and a cannula was placed into the lateral ventricle. Another hole was drilled, and a screw was placed approximately at the ipsilateral lambdoid structure to aid in supporting the cannula in the skull with 3M carboxylate dental cement. Mice recovered for 14 days before immunohistochemical experiments and 7 days before all other experiments.

ICV treatments included: 2.0 μg of α -klotho (R&D Systems) [15], 10 min of pretreatment with 25 μg of PD1703074 for FGFR inhibition (Fisher Scientific) [31], and/or 1 h of pretreatment with 10 ng of wortmannin for PI3kinase inhibition (Fisher Scientific) [32]. All ICV treatments, were administered via Hamilton syringe at a volume of 2.0 μL between 6:30 and 7:30 p.m. Validation of cannula placement was performed by post-mortem visualization of cannula path in brain slices [15].

2.5. Energy expenditure and body composition

Energy expenditure was measured using the indirect calorimetry TSE PhenoMaster metabolic cages. Diet-induced obesity (DIO) mice were placed in these cages at 16–17 weeks of age and allowed two days for acclimatization before beginning data analysis (Supplemental Fig. 1). To normalize oxygen consumption and carbon dioxide production to lean body mass, body composition was determined by Echo MRI (Echo Medical Systems, Houston, MA, USA) immediately before placement in the metabolic cages.

2.6. Food intake and body weight measurements

Food intake and body weight were measured daily. Food intake was measured by weighing food (8–9 g) and subtracting from the total food. Bedding was inspected thoroughly for residual bits of food, which were included in measurements.

2.7. Quantitative polymerase chain reaction (PCR)

To assess thermogenic gene expression, brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) was removed and flash frozen from euthanized mice after 2 days of ICV treatment with α -klotho or vehicle. RNA was extracted by Trizol (Thermo Fisher Scientific, Waltham, MA). The expression levels of specific mRNA were analyzed using quantitative real-time PCR (RT-qPCR) (Power SYBR Green PCR Master Mix; Applied Biosystems, Foster City, CA). Reactions were performed in triplicate for each sample, while 18S ribosomal RNA was used as a reference gene for normalization.

2.8. Immunohistochemistry

For immunofluorescent analysis, mice were intracardially perfused with phosphate-buffered saline (PBS) followed by 10% formalin before immunohistochemistry was performed as described previously [33]. Briefly, brains were sliced into 20- μm coronal sections using a freezing microtome (VT1000 S; Leica, Wetzlar, Germany) and

incubated overnight in antibody to phosphorylated ERK^{thr202/tyr204} (1:500; Cell Signaling Technology, Danvers, MA), cFOS (1:250; Santa Cruz Biotechnology, Santa Cruz, CA), POMC (1:3,000; Phoenix Pharmaceuticals), glial fibrillary-acid protein (GFAP) (1:1,000; Millipore), tyrosine hydroxylase (TH) (1:100,000; Millipore), doublecortin (DCX) (1:800; Cell Signaling Technology), phosphorylated STAT3^{ser754} (1:3,000; Cell Signaling Technology) and/or Ki67 (1:500; Abcam), followed by incubation with Alexa-fluorophore secondary antibody for 1 h (Abcam). Stains were photographed using an optical microscope (DM6000; Leica), followed by blind analysis using ImageJ. At least 3 anatomically matched images per mouse were quantified.

2.9. Stereotaxic microinjections

To express green fluorescent protein (GFP) in POMC neurons, POMC-cre mice (7–8 weeks) were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic frame. Adeno-associated viruses (AAVs) were delivered bilaterally to the ARC (200 nl/site at 30 nl/min) using the following coordinates (distances from bregma): 1.4 mm posterior, 1.6 mm lateral $\pm$ 0.2 mm, and 5.8 mm deep, with a 0.5 μ l Hamilton syringe connected to a motorized stereotaxic injector (Quintessential Stereotaxic Injector; Stoelting). The injection needle was maintained in place for 5 min following injections to minimize virus spread up the needle track. The viruses used were AAV-EF1 α -Flex-GFP ($> 1 \times 10^{12}$ GC/ml, UNC Vector Core) and AAV (DJ8)-Flex-mCherry ($> 1 \times 10^{12}$ GC/ml, Optogenetics and Viral Vectors Core at Baylor College of Medicine). Mice recovered for 1–2 weeks before electrophysiological recordings.

2.10. Electrophysiological recordings

Electrophysiological experiments were conducted in acutely prepared hypothalamic slices, as previously described [34,35]. Briefly, 8- to 10-week-old adult mice were deeply anesthetized with a mixture of ketamine/xylazine (intraperitoneally) and transcardially perfused with ice-cold cutting solution containing the following (in mM): 75 sucrose, 73 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 15 glucose, 7 MgCl₂, and 0.5 CaCl₂, saturated with 95% O₂/5% CO₂. The brains were quickly removed and blocked, with the rostral face of the block glued to the specimen plate of the buffer tray then immersed in an ice-cold cutting solution. Coronal slices (280 μ m) containing ARC were sectioned using a Leica VT1000S Vibratome and transferred to a holding chamber with artificial CSF (aCSF) containing the following (in mM): 123 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 10 glucose, 1.3 MgCl₂, and 2.5 CaCl₂ and saturated with 95% O₂/5% CO₂ at 31–33 °C for 30 min, then maintained at room temperature for at least 1 h to allow for recovery before any electrophysiological recordings.

Individual slices were transferred from the holding chamber to a recording chamber, where they were submerged and continuously perfused with oxygenated aCSF at 2 ml/min. GFP-expressing POMC neurons in the ARC were first located under epifluorescence illumination, and whole-cell patch-clamp recordings were performed on 16 POMC neurons from 6 mice (3 male, 3 female) under infrared-differential interference contrast visualization at 30–32 °C on a fixed-stage, upright microscope (model BX51WI, Olympus) equipped with a water-immersion 40 $\times$ objective. Pipettes with a resistance of 3–5 M Ω were pulled from borosilicate glass (outer diameter, 1.5 mm; inner diameter, 1.1 mm; Sutter Instruments) using a horizontal puller (P-97, Sutter) and filled with an internal patch solution containing the following (in mM): 142 K-gluconate, 10 HEPES, 1 EGTA, 2.5 MgCl₂, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na₂-phosphocreatine, adjusted to pH 7.25–7.35, osmolality 295–305 with KOH. After “breaking into” cells, at least 5 min was given for baseline to be established before obtaining

baseline measurements for another 5–9 min. α -Klotho was then administered at 3.65 mM in aCSF. The liquid junction potential was not corrected, and series resistance (Rs) was bridge balanced.

2.11. Statistical analysis

Pearson's correlation coefficients were used to determine associations between CSF α -klotho concentrations and other variables. Unpaired *t*-tests or one-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons were used to determine differences between or among groups, respectively, in CSF α -klotho, oxygen consumption, carbon dioxide production, locomotor activity, gene expression, food intake, body weight, or proteins of interest detected by immunofluorescence. To compare pre-post within-group changes over the course of 2-day experiments, paired *t*-tests were performed. Unpaired or paired *t*-tests were used for between or within-neuron patch clamp electrophysiology experiments, respectively. All analyses were performed using GraphPad Prism statistics software, and *p* < 0.05 was considered statistically significant.

2.12. Data availability statement

The data sets generated during the current study are available from the corresponding author on reasonable request.

3. RESULTS

3.1. Human CSF α -klotho concentrations are reduced in overweight and obese adults

CSF was collected from 22 adults (10 males, 12 females) with an average age and body mass index (BMI) of 52.3 ± 17.1 and 25.7 ± 3.6 , respectively (Supplemental Table 1). CSF α -klotho concentrations exhibited a strong inverse correlation with body weight and BMI (*R* = -0.6823 and -0.7822 respectively) (Figure 1A–B). There were no correlations between CSF α -klotho concentrations and fasting blood glucose, cholesterol, or triglycerides (Figure 1D–F). Subjects were then stratified into lean (BMI < 25) and overweight/obese (BMI > 25) categories (mean BMI's were 22.8 ± 1.7 and 28.6 ± 2.4 , respectively). Although there were no significant differences in age, height, fasting blood glucose, blood triglycerides, total cholesterol, or LDL levels between the groups (Supplemental Table 1), overweight/obese subjects had significantly lower CSF α -klotho concentrations and serum high-density lipoprotein (HDL) levels compared to lean counterparts ($1,044 \pm 251$ vs. $1,616 \pm 218$ pmol/L and 1.2 ± 0.2 vs. 1.0 ± 0.2 mmol/L, respectively) (Figure 1C and Supplemental Table 1). To our knowledge, this is the first study to illustrate a strong negative correlation between CSF α -klotho and body weight in human subjects, suggesting the potential involvement of central α -klotho in the development of obesity.

3.2. Two days of ICV α -klotho treatment increases energy expenditure in DIO mice

To further investigate the role of central α -klotho in energy balance regulation, 2 days of ICV α -klotho treatment was performed in male DIO mice. Consistent with previous results, 2 days of ICV α -klotho treatment significantly decreased daily food intake (20.3%) and resulted in modest reductions in body weight (1.5%) compared to vehicle-treated controls (Supplemental Fig. 2). ICV α -klotho treatment also significantly increased average daily oxygen consumption (10.9%) and carbon dioxide production (11.2%), with no effects on locomotor activity (Figure 2A–F). These data suggest a novel ability of central α -klotho to improve energy balance by increasing daily energy expenditure.

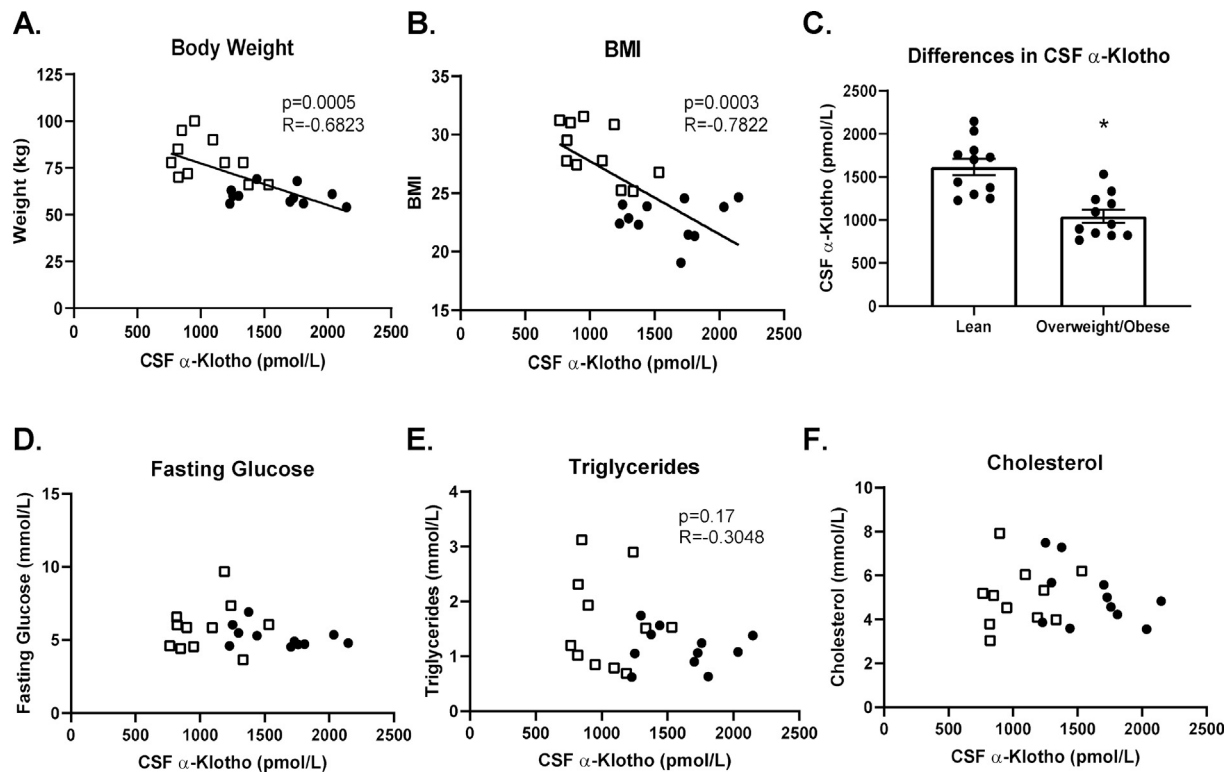


Figure 1: Cerebrospinal fluid α -klotho concentrations are reduced in overweight and obese adults. CSF α -klotho concentrations are inversely correlated with (A) Body weight and (B) BMI ($n = 22$). (C) Comparison of CSF α -klotho concentrations when subjects are stratified into lean vs. overweight/obese categories based on BMI. ($n = 11/\text{group}$). CSF α -klotho concentrations are not correlated with (D) fasting blood glucose, (E) triglycerides, or (F) cholesterol. ● represents lean; □ represents overweight or obese. Data represented as mean $\pm$ SEM. * indicates $p < 0.05$ vs. lean subjects.

UCP1 gene expression also tended to be elevated in BAT and iWAT of α -klotho-treated mice (1.4-fold; $p = 0.07$ and 6.4-fold; $p = 0.11$, respectively), suggesting potentially increased thermogenesis and browning of white adipose tissue (Figure 2G–H). There were also trends toward increased NRF1 gene expression in the BAT of α -klotho-treated mice (1.3-fold; $p = 0.09$), while there were no differences in PRDM16, CIDEA, PGC1 α , or PPAR γ gene expression in either BAT or iWAT.

3.3. Central α -klotho has no obvious effects on TH neuron activity in fasted mice

Previous reports identify direct, inhibitory action of central α -klotho on ARC NPY/AgRP neurons [15]; thus, we aimed to investigate the possibility of additional neuronal targets of central α -klotho in the hypothalamus. For example, TH neurons elicit orexigenic effects in the ARC and regulate thermogenesis in the PVN [21,36]. However, immunofluorescent staining revealed acute ICV α -klotho treatment had no obvious effects on cFOS colocalization with TH neurons in the hypothalamus of fasted male or female mice (Figure 3).

3.4. Central α -klotho regulates ARC POMC neuron activity in fasted mice

POMC neurons are another ARC neuron population involved in reducing hunger and increasing energy expenditure [22–24]. We performed patch clamp electrophysiology experiments and 7 out of 16 POMC neurons analyzed exhibited increased firing rate and membrane potential when treated with α -klotho (0.47 ± 0.13 vs. 1.40 ± 0.28 Hz and -50.52 ± 1.02 vs. -47.57 ± 1.17 mV, respectively) (Figure 4A–

C). Interestingly, all POMC neurons recorded responded to α -klotho, with 9 out of 16 POMC neurons exhibiting decreased firing rate and membrane potential (0.30 ± 0.09 vs. 0.08 ± 0.06 Hz and -47.33 ± 0.73 vs. -52.10 ± 1.76 mV, respectively) (Supplemental Figs. 3A–C). While these data identify POMC neurons as novel neuronal effectors of central α -klotho function, they also highlight the heterogeneity of the POMC-expressing neuron population [37]. For example, POMC neurons stimulated by α -klotho had significantly lower baseline membrane potentials compared to those antagonized by α -klotho, while there were no differences in baseline firing rate (Supplemental Figs. 3D–E).

To further investigate the complex effects of central α -klotho on ARC POMC neuron activity, we performed immunofluorescent staining for cFOS. While acute ICV α -klotho treatment had no effects on ARC POMC neuron activity in fed mice (Supplemental Fig. 4), cFOS colocalization with ARC POMC neurons was significantly elevated in response to acute ICV α -klotho treatment in both fasted male (1.9-fold) and female (1.7-fold) mice (Figure 4D–J). These data highlight potent excitatory effects of α -klotho on ARC POMC neurons in the fasted status, despite the heterogeneous response of POMC neurons observed in electrophysiology experiments.

3.5. Central α -klotho increases ARC POMC neuron activity, at least in part, via FGFR's

Previous studies demonstrate α -klotho's role as a scaffolding protein to facilitate FGF23-FGFR binding and downstream signaling, a phenomenon that was found to be critical to α -klotho-mediated NPY/AgRP neuron inhibition [15,20,38]. To investigate the importance of FGFR's

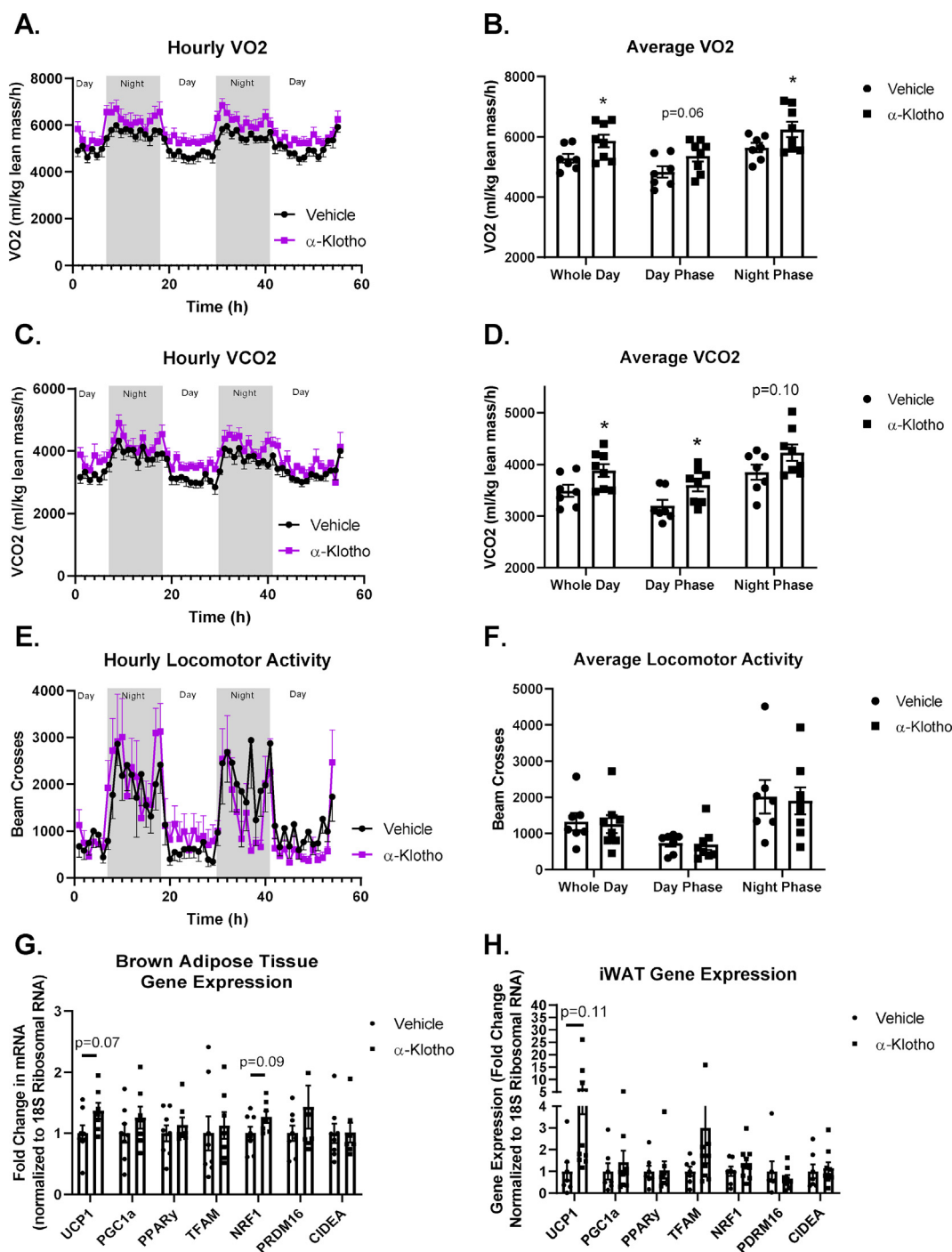


Figure 2: Two days of ICV α -klotho treatment increases energy expenditure in DIO mice. (A–B) Oxygen consumption, (C–D) carbon dioxide production, and (E–F) locomotor activity in 16- to 17-week-old male DIO mice after 2 days ICV α -klotho (2.0 μ g) or vehicle (2.0 μ L) injections ($n = 7$ –8/group). (G) BAT gene expression and (H) iWAT gene expression ($n = 7$ –9/group). Data represented as mean $\pm$ SEM. * indicates $p < 0.05$ vs. vehicle controls.

to α -klotho-mediated POMC activation, ICV pretreatment with the FGFR inhibitor PD173074 was performed. When FGFR activity was inhibited, α -klotho no longer significantly increased POMC neuron activity (1.4-fold increase under FGFR blockade, $p = 0.13$; vs. 2.1-fold increase with vehicle-treated controls, $p < 0.05$) (Figure 5). The blunted effects of α -klotho in response to FGFR inhibition may indicate central α -klotho regulates POMC neurons, at least in part, via FGFR activity (Figure 5).

3.6. α -Klotho/PI3kinase signaling regulates ARC POMC and NPY/AgRP neuron activity

PI3kinase is a prominent signaling mechanism in the regulation of energy balance, ARC neuron activity, and α -klotho-mediated down-regulation of AgRP gene expression [15,25]. When central PI3kinase signaling was impaired using ICV wortmannin treatment, α -klotho no longer significantly increased POMC neuron activity compared to PI3kinase inhibition alone (Figure 6). Past experiments from our lab

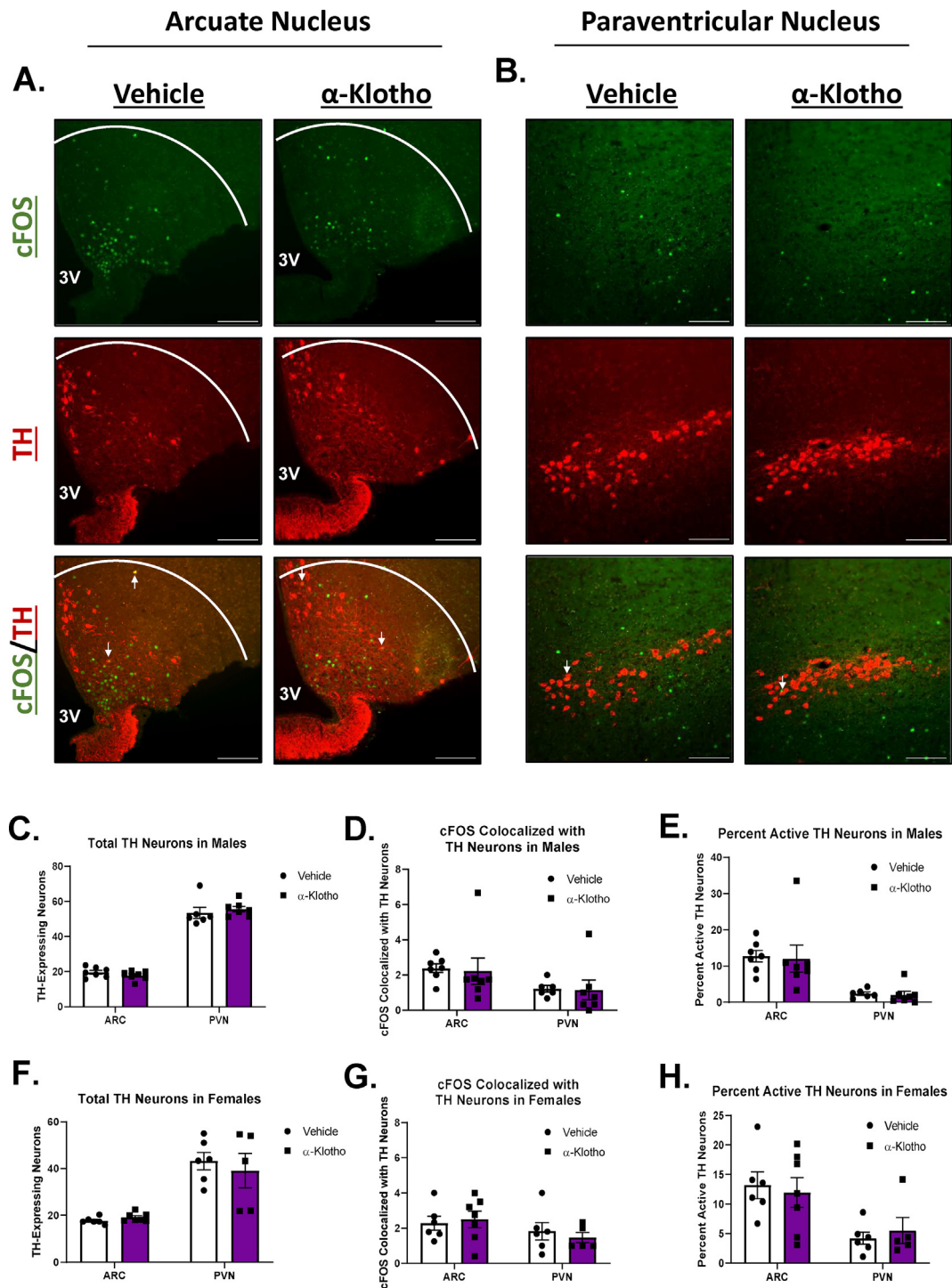


Figure 3: Acute ICV α -klotho treatment has no effects on TH-expressing neuron activity in the ARC or PVN. Representative images of cFOS (green) colocalized with TH-expressing neurons (red) in the (A) ARC and (B) PVN of mice acutely treated with ICV vehicle (2.0 μ L) or α -klotho (2.0 μ g) before an overnight fast. (C) Total TH-expressing neurons, (D) cFOS colocalization, and (E) proportion of active TH-expressing neurons in male mice ($n = 6-7$ /group). (F) Total TH-expressing neurons, (G) cFOS colocalization, and (H) proportion of active TH-expressing neurons in female mice ($n = 5-7$ /group). 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. * indicates $p < 0.05$ vs. vehicle controls.

also indicate α -klotho downregulates AgRP gene expression via PI3kinase signaling [15]. Consistent with previous reports, in the current study, acute ICV α -klotho treatment significantly reduced NPY/AgRP neuron activity (46%) (Figure 7). Furthermore, PI3kinase

inhibition attenuated this α -klotho-mediated suppression of NPY/AgRP neuron activity (Figure 7). Overall, these data suggest PI3kinase signaling is critical to α -klotho-mediated regulation of both NPY/AgRP and POMC neurons.

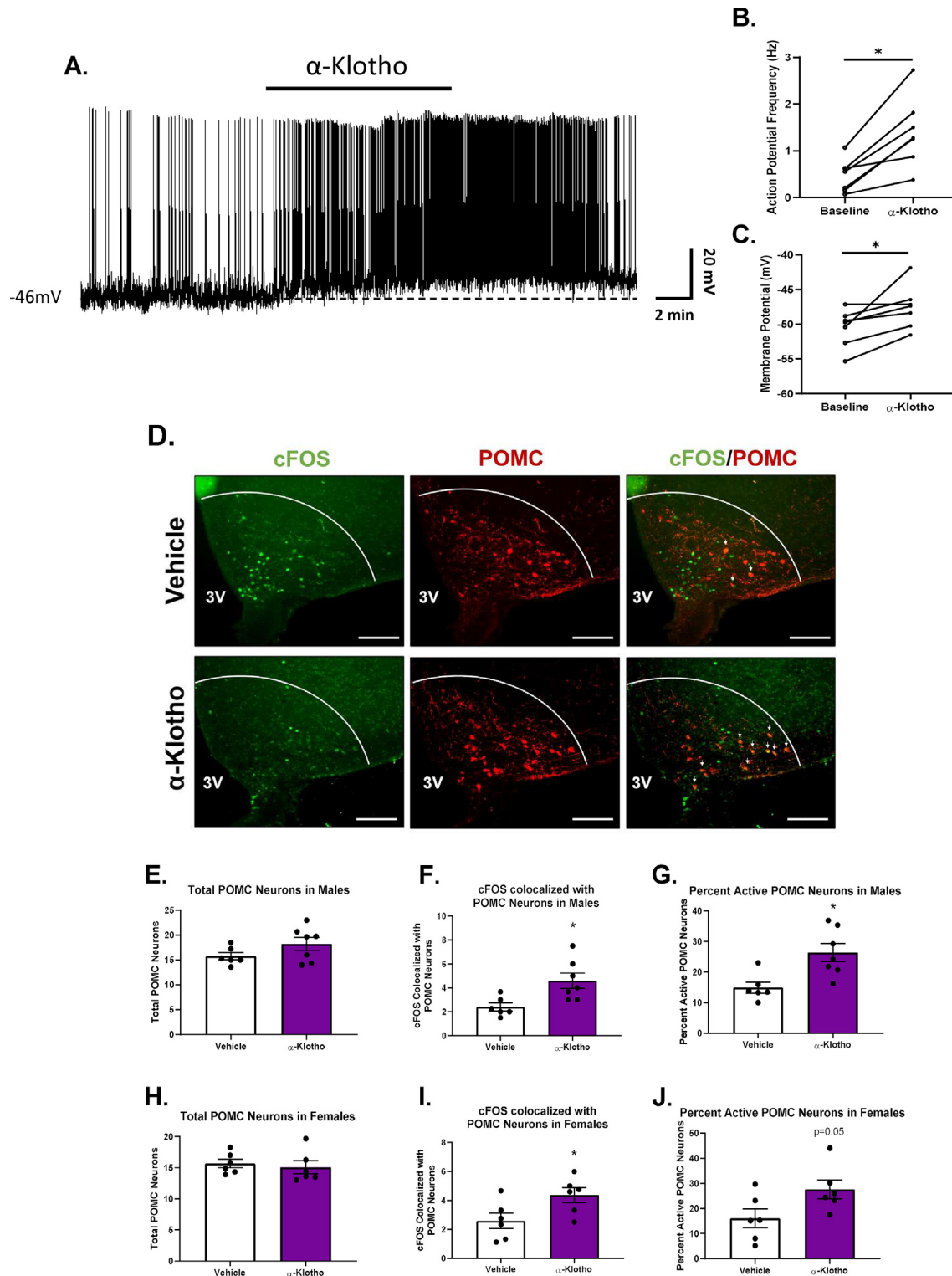


Figure 4: Acute ICV α -klotho treatment increases ARC POMC neuron activity in fasted mice. (A) Representative whole cell patch clamp recording, (B) action potential frequency, and (C) membrane potential of a POMC neuron stimulated by α -klotho treatment ($n = 7$ neurons). (D) Representative images of cFOS (green) colocalized with POMC (red) in the ARC of healthy, male mice acutely treated with ICV α -klotho (2.0 μ g) or vehicle (2.0 μ L) before an overnight fast. (E) Total POMC neurons, (F) cFOS colocalization, (G) and proportion of active POMC neurons in male mice ($n = 6$ –7/group). (H) Total POMC neurons, (I) cFOS colocalization, and (J) proportion of active POMC neurons in female mice ($n = 6$ /group). 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. * indicates $p < 0.05$ vs. vehicle controls.

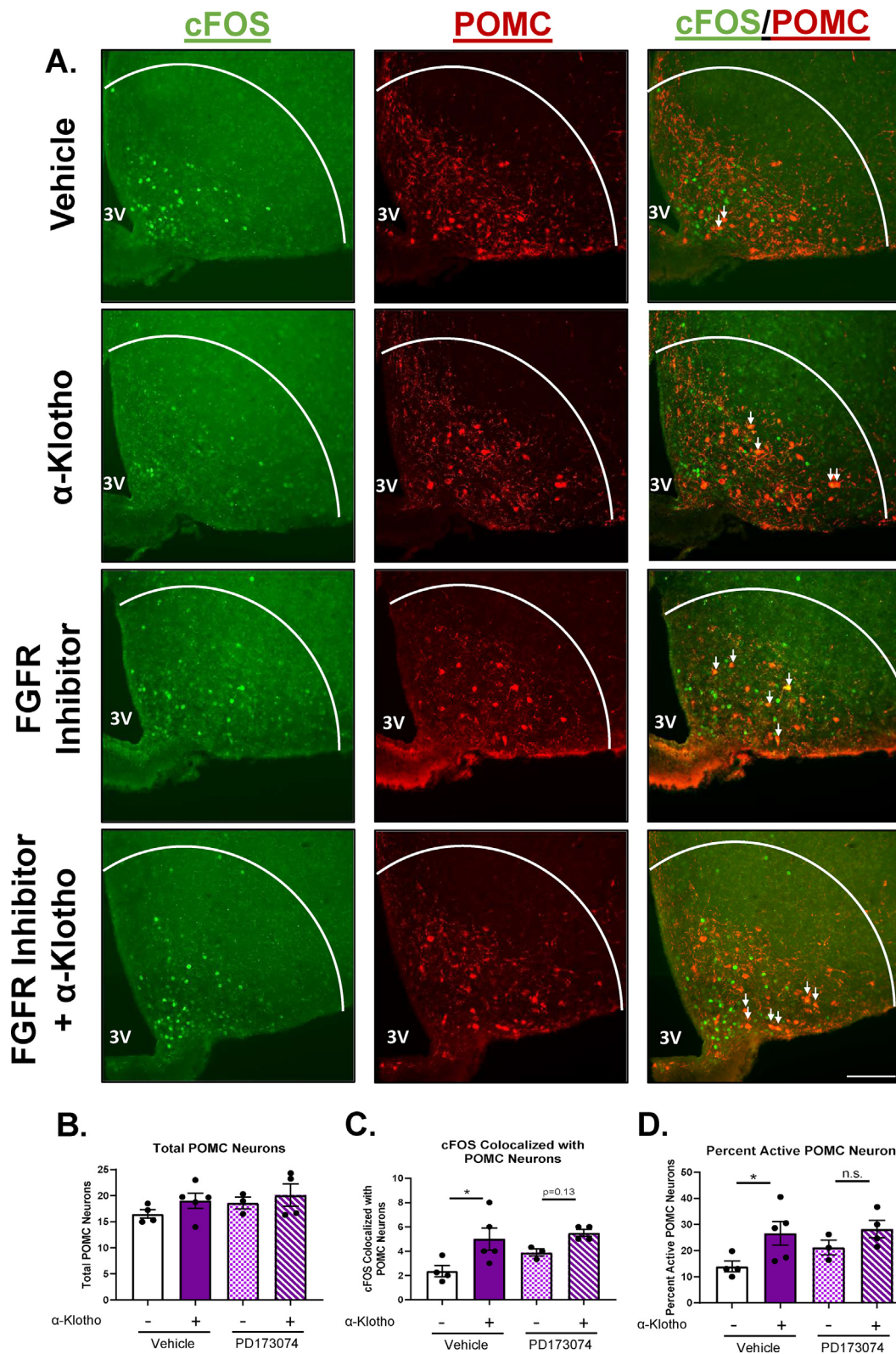


Figure 5: Central α -klotho increases ARC POMC neuron activity, at least in part, via FGFR's. (A) Representative images of cFOS (green) colocalized with POMC (red) in the ARC of healthy, male mice acutely treated with vehicle (2.0 uL), α -klotho (2.0 ug), FGFR inhibitor alone (25 ug), or FGFR inhibitor 10 min before α -klotho before an overnight fast. (B) Total POMC neurons, (C) cFOS colocalization, and (D) proportion of active POMC neurons ($n = 3-5$ /group). 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. n.s = not significant; * indicates $p < 0.05$.

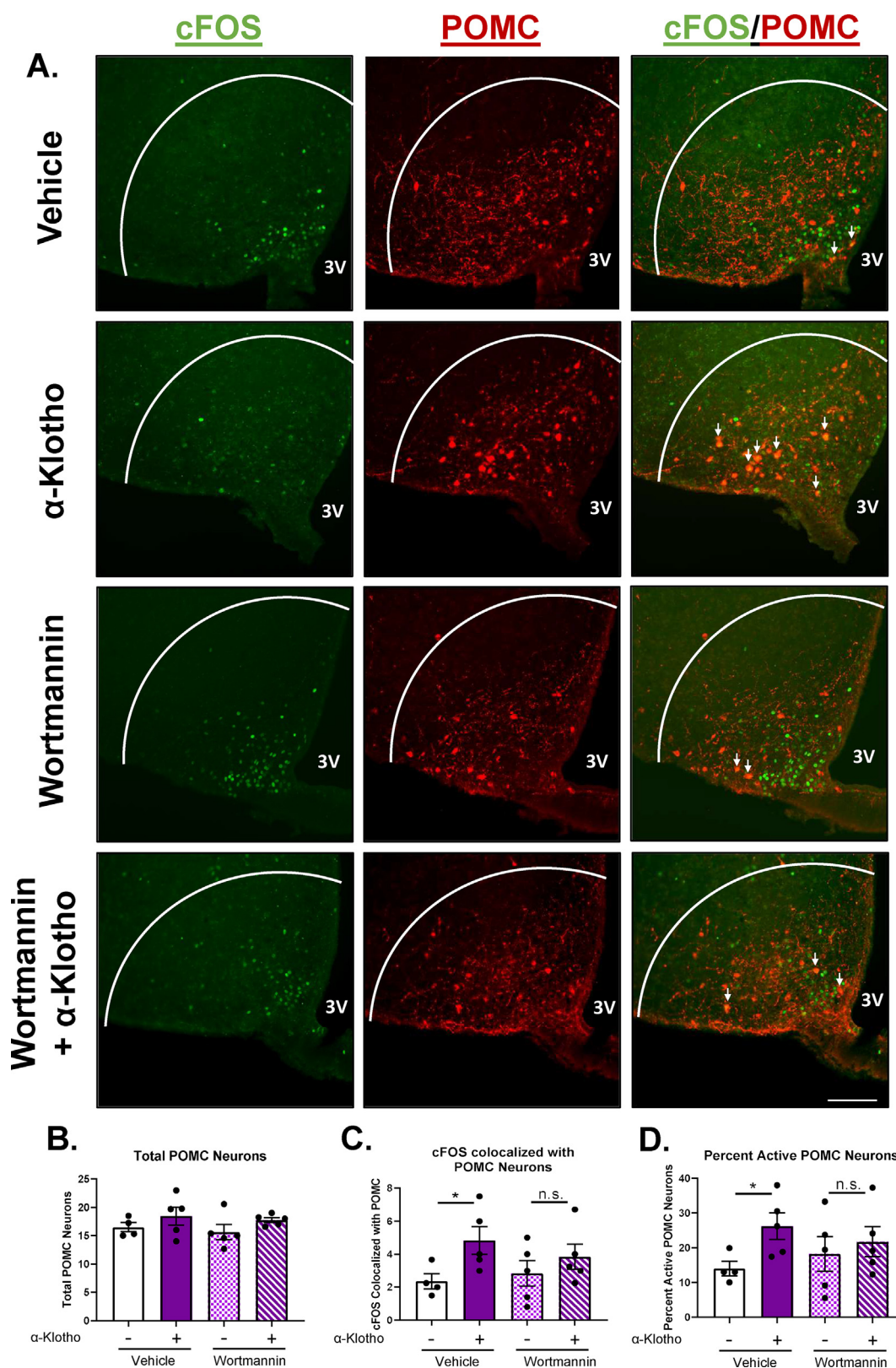


Figure 6: PI3kinase inhibition blunts α -klotho-mediated ARC POMC neuron activation. (A) Representative images of cFOS (green) colocalized with POMC (red) in the ARC of healthy, male mice acutely treated with vehicle (2.0 uL), α -klotho (2.0 ug), wortmannin alone (10 ng), or wortmannin 1 h before α -klotho before an overnight fast. (B) Total POMC neurons, (C) cFOS colocalization, and (D) proportion of active POMC neurons ($n = 4$ –5/group). 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. n.s = not significant; * indicates $p < 0.05$.

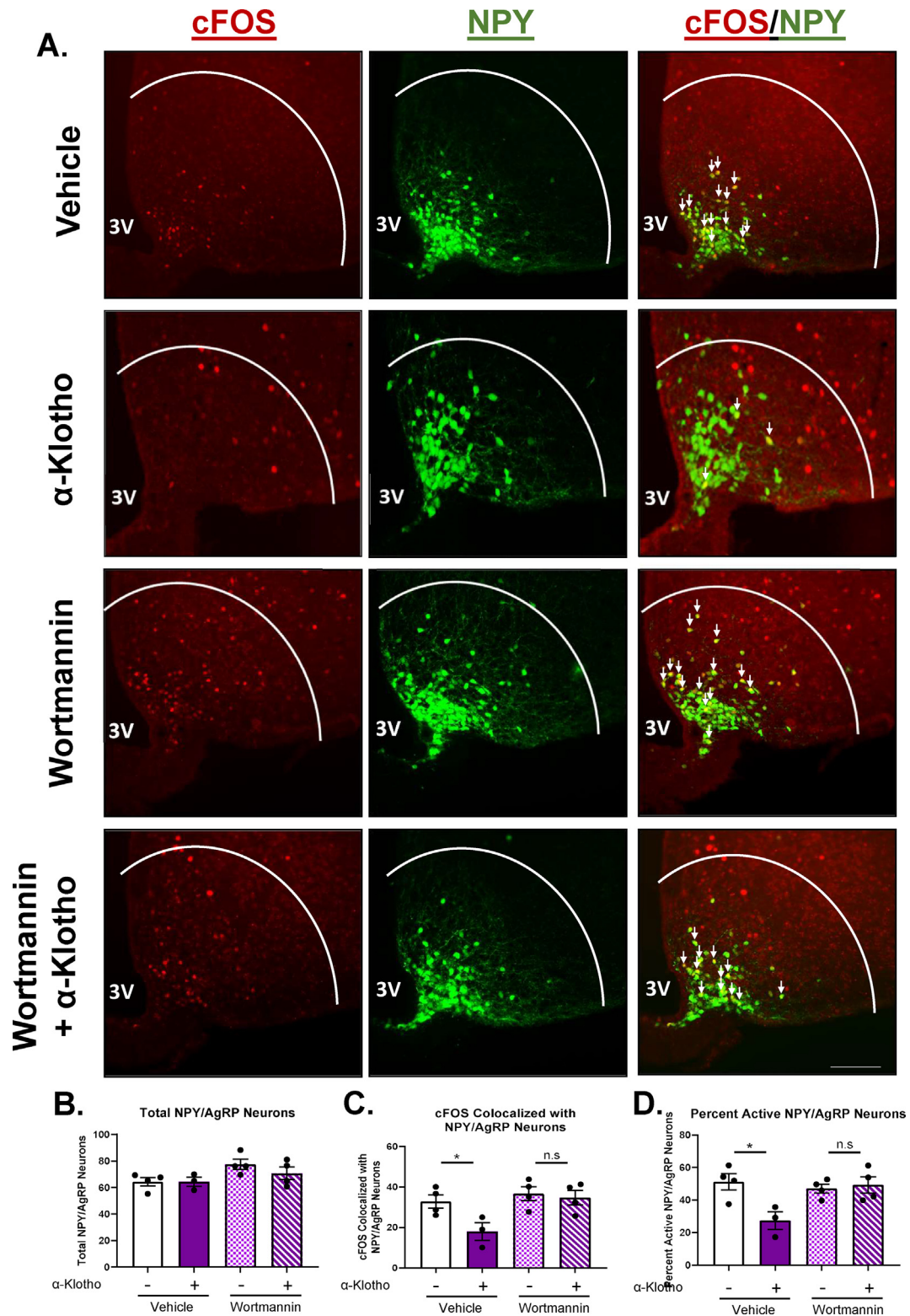


Figure 7: PI3kinase inhibition abolishes α -klotho-mediated ARC NPY/AgRP neuron inhibition. (A) Representative images of cFOS (red) colocalized with NPY/AgRP (green) in the ARC of healthy, male mice acutely treated with vehicle (2.0 uL), α -klotho (2.0 ug), wortmannin alone (10 ng), or wortmannin 1 h before α -klotho before an overnight fast. (B) Total NPY/AgRP neurons, (C) cFOS colocalization, and (D) proportion of active NPY/AgRP neurons ($n = 3-4$ /group). 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. n.s. = not significant; * indicates $p < 0.05$.

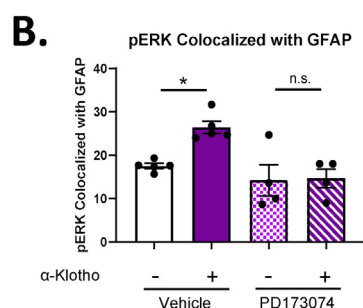
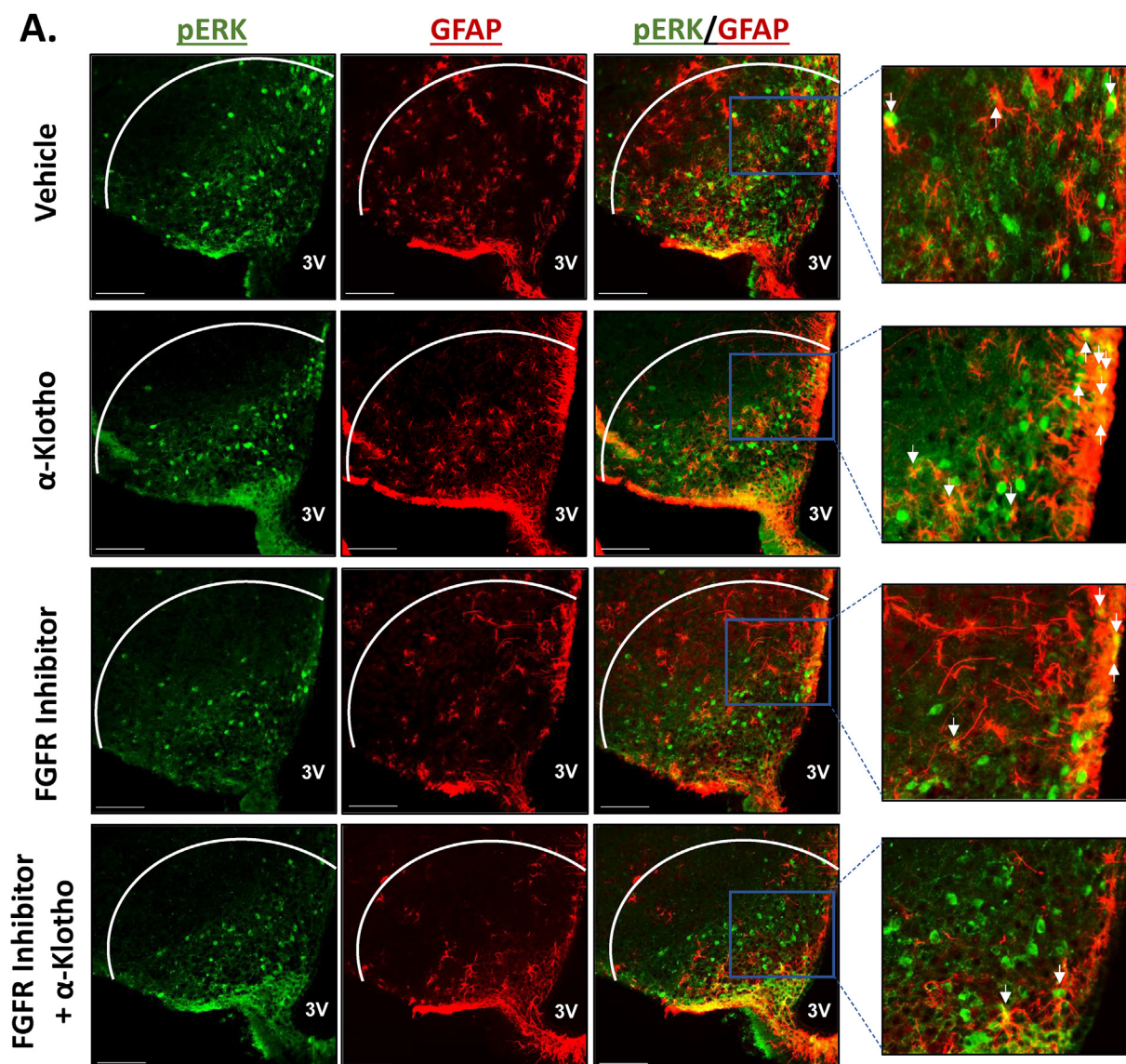


Figure 8: Acute ICV α -klotho treatment stimulates pERK^{thr202/tyr204} in astrocytes. (A) Representative images of pERK^{thr202/tyr204} (green) colocalized with GFAP (red) in the ARC of healthy, male mice acutely treated with vehicle (2.0 μ L), ICV α -klotho (2.0 μ g), FGFR inhibitor alone (25 μ g), or FGFR inhibitor 10 min before α -klotho (2.0 μ g) before an overnight fast. (B) pERK^{thr202/tyr204} colocalized with GFAP. 3V = Third ventricle; scale bar = 50 μ m. Data represented as mean $\pm$ SEM. n.s. = not significant; * indicates $p < 0.05$.

3.7. ICV α -klotho treatment increases phosphorylated ERK^{thr202/tyr204}-in ARC astrocytes

We next aimed to determine whether central α -klotho was involved in the regulation of ARC astrocytes, cells involved in nutrient sensing,

hormonal transport, and neuronal health [26]. ERK signaling is a key regulatory mechanism in astrocyte function and has also been identified as a downstream signaling mechanism of α -klotho in the ARC [15,27,28,39]. There were distinct differences in the localization of

phosphorylated ERK^{thr202/tyr204} in the ARC between the two groups. α -Klotho-treated mice exhibited 1.4-fold higher pERK^{thr202/tyr204} in cells expressing the astrocytic marker GFAP (Figure 8), with qualitatively higher pERK^{thr202/tyr204} concentration in the tanycytic region bordering the third ventricle. Mechanistically, pretreatment with the FGFR inhibitor PD173074 completely abolished this phenomenon (Figure 8). Overall, these data may indicate a novel role for central α -klotho/FGFR signaling to regulate astrocyte function in the ARC, suggesting involvement in supporting other nutrient-sensing and hormonal functions. For example, leptin sensitivity and transport are closely connected to astrocyte function [27,28]. Supporting the hypothesis that α -klotho facilitates hormonal transport and sensitivity, acute ICV α -klotho treatment in fasted mice significantly increased phosphorylated STAT3 2.1-fold in NPY/AgRP neurons, which is an inhibitory signaling mechanism in these neurons and a critical downstream mediator of leptin (Supplemental Fig. 5).

Furthermore, longer duration (12-days) α -klotho treatment increased Ki67 expression 3.0-fold in the ARC (Supplemental Fig. 6), which is an indication of increased proliferating cells. This increase in active progenitor cells is likely non-neuronal, demonstrated by no effects of long-term α -klotho treatment on number of total POMC, NPY/AgRP, and DCX- (a marker of neurogenesis) expressing cells in the ARC (Supplemental Fig. 7). However, the anatomical distribution of Ki67 expression in the ARC suggests that the proliferating cells may be astrocytes or tanycytes, which would indicate that α -klotho not only regulates the function of mature astrocytes, but may also be involved in the generation of new astrocytes.

4. DISCUSSION

A recent study from our laboratory provided promising evidence that central α -klotho may be a critical regulator of hypothalamic neurons and metabolism [15]. Aiming to more comprehensively decipher central α -klotho's metabolic role, the current study identified diverse novel functions of central α -klotho in the regulation of energy balance and ARC cell populations. Our data indicates central α -klotho increases energy expenditure, modulates NPY/AgRP and POMC neuron activity via FGFR $\rightarrow$ PI3kinase signaling, and regulates astrocytes via FGFR $\rightarrow$ ERK signaling. Furthermore, to our knowledge, we provide the first evidence that CSF α -klotho concentrations and body weight exhibit a strong inverse correlation in humans, potentially implicating central α -klotho in the pathology of obesity.

Previous reports have indicated that blood α -klotho concentrations are impaired in humans with diabetes [7,8]. However, there is no correlation between peripheral and central α -klotho concentrations, and regulation of circulating CSF α -klotho levels is poorly understood [40]. The few studies examining CSF α -klotho concentrations have determined no diurnal variations [41], decreased levels in females [40,41], an inverse correlation with age [41], and a positive correlation with FGF23 [40]. Additionally, human CSF α -klotho concentrations are reduced in some neurological disorders, such as Alzheimer's disease [41] and multiple sclerosis [42]. We observed that CSF α -klotho levels exhibit a strong, inverse correlation with body weight and BMI. These data may suggest the involvement of central α -klotho in the pathology of obesity and could possibly identify CSF α -klotho levels as a novel preclinical marker in body weight disorders. Considering previous data showing ICV α -klotho treatment improves hepatic lipid accumulation and central α -klotho inhibition rapidly impairs glucose clearance [15], we also hypothesized that CSF α -klotho may inversely correlate with blood glucose, TG's, or cholesterol. Surprisingly, no such correlations were observed, nor were there significant differences in these

variables between lean and overweight/obese subjects. Notably, blood glucose, TG's, and cholesterol often exhibit a weak relationship with BMI [43–45], and a limitation of the current study is a small sample size. Future studies utilizing a larger sample size may reveal additional correlations between CSF α -klotho concentrations and blood glucose or lipids.

Consistent with previous reports, ICV α -klotho treatment suppressed food intake and resulted in modest body weight reductions in DIO mice [15]. Indirect calorimetry also determined that ICV α -klotho treatment increases resting energy expenditure independent of locomotor activity. Gene expression analysis revealed that increased thermogenesis in BAT and browning of iWAT may be the mechanism underlying increased energy expenditure. While the mechanism for α -klotho-mediated increases in energy expenditure remains unclear, these data highlight a potent role for central α -klotho to improve energy balance by regulating both caloric consumption and utilization.

The observed ICV α -klotho phenotype may be a result of its diverse functions regulating the intricate neurocircuitry in ARC cell populations. For example, α -klotho is an antagonist of ARC NPY/AgRP neurons [15], which, when activated, stimulate food intake, increase circulating glucose levels, decrease energy expenditure, and result in weight gain [16–19]. Here, we identify α -klotho as a novel modulator of ARC POMC neurons, which have opposite metabolic effects compared to NPY/AgRP neurons [24,46]. α -Klotho elicited varied effects on POMC neurons during electrophysiological experiments, with 44% experiencing excitatory and 56% experiencing inhibitory effects. These findings highlight the heterogeneity of the POMC neuron population, with 72% being "canonical", expressing high levels of POMC and very low levels of AgRP, and 28% expressing low levels of POMC and high levels of AgRP [37]. α -Klotho decreases AgRP gene expression and inhibits AgRP-expressing neuron activity [15], which may suggest that α -klotho inhibits the POMC neurons expressing high levels of AgRP. Additionally, POMC neurons have variable receptor and neurotransmitter expression, which likely also alters their response to α -klotho [37,47].

It is also possible α -klotho regulates POMC neurons indirectly via presynaptic inputs. For example, α -klotho's previously documented direct inhibitory role in NPY/AgRP neurons [15] may indirectly stimulate POMC activity via relieved NPY/AgRP-mediated GABAergic inputs. Interestingly, NPY/AgRP $\rightarrow$ POMC connectivity is complex, highlighted by differences in the effects of spontaneous vs. stimulated NPY/AgRP neuron activity. POMC neurons exhibit no change in inhibitory postsynaptic currents (IPSC's) when NPY/AgRP neuron activity or neurotransmitter release is disrupted [48,49]; however, optogenetic stimulation of NPY/AgRP neurons consistently inhibits POMC neurons via direct GABAergic inputs [48,50]. Notably, the current study determined that α -klotho regulates NPY/AgRP and POMC neurons via FGFR's, which are expressed in both populations [51]. As a result, α -klotho can likely elicit direct, postsynaptic regulation of both of these neurons via its role as a scaffolding protein in FGFR's.

Orexigenic ARC TH neurons are also intricately involved in NPY/AgRP and POMC neurocircuitry. For example, TH and NPY/AgRP neurons exhibit a bidirectional synaptic mechanism during which TH neurons stimulate food intake via direct excitatory dopamine action on NPY/AgRP neurons, and NPY/AgRP neurons regulate thermogenesis via Y1 receptors on TH neurons [21,36,52]. Furthermore, both dopamine and GABA released from TH neurons exhibit direct inhibitory action on POMC neurons. Similar to POMC neurons, TH neurons are also a heterogeneous neuron population. TH is the rate-limiting enzyme in dopamine and catecholamine synthesis [53], both of which have varied effects on metabolism. For example, dopamine stimulates food intake [52], and catecholamines induce satiety [54,55]. In the current study,

ICV α -klotho had no significant effects on ARC or PVN TH neuron activity, suggesting TH neurons may not be involved in α -klotho's hypothalamic function. However, one α -klotho-treated mouse appeared to be a statistical outlier (greater than two standard deviations different from the mean). Removal of this animal reveals trends toward α -klotho-mediated TH neuron suppression in males only, highlighting the need for further research into this phenomenon. Overall, while the current study does not identify a specific role of α -klotho to regulate hypothalamic TH neurons, future studies using electrophysiology on single TH neurons would help clarify these complicated findings among different TH subpopulations.

We also aimed to determine whether α -klotho had additional functions in the ARC independent of neuronal activity. In hippocampal cells, α -klotho has been described as a regulator of neurogenesis and astrocyte metabolism [39,56]. While there were no effects of α -klotho on ARC neurogenesis in DIO mice, we did observe an ability of α -klotho to regulate astrocyte function. ARC astrocytes are critically involved in hormonal transport, nutrient sensing, and neuronal maintenance [27], and α -klotho treatment increased phosphorylated ERK specifically in this cell type. ERK signaling in astrocytes is essential to leptin transport through the blood brain barrier [28], suggesting a novel role for central α -klotho to regulate hormonal transport. This hypothesis was supported by increased phosphorylated STAT3, a key downstream mediator of leptin, in NPY/AgRP neurons in response to α -klotho treatment. Considering the diverse functions of central α -klotho in the ARC, deciphering the molecular mechanisms involved is essential to understanding the complex questions surrounding homeostatic and disordered regulation of metabolism.

α -Klotho has a well-documented role as a scaffolding protein in the FGF23 $\rightarrow$ FGFR complex [20], and experimental FGFR activation has recently been shown to elicit therapeutic effects in metabolic disease models [51,57,58]. We determined that α -klotho regulates astrocytes via an FGFR $\rightarrow$ ERK mechanism, similar to the mechanism underlying sustained diabetes remission in response to acute FGF1 microinjection [29]. Interestingly, α -klotho only significantly increased POMC neuron activity when FGFR's were functional. This suggests that α -klotho may regulate POMC neurons via FGFR's, but concurrent receptors are likely involved. Notably, the specific receptor of α -klotho is currently not known, but one potential mechanism could be through transient receptor potential vanilloid-like (TRPV) channels. TRPV channels increase POMC neuron activity to reduce food intake [59], and α -klotho has a well-documented role to hydrolyze these receptor like-channels in renal tubules and pancreatic β cells to increase their membrane localization [3,4,60].

PI3Kinase is another potent regulator of ARC neuron populations and a downstream effector of α -klotho [15,25]. While it has previously been shown that PI3Kinase is essential to α -klotho-mediated AgRP mRNA downregulation, it was unclear whether PI3Kinase was equally important to α -klotho-mediated NPY/AgRP neuron inhibition [15]. The current study showed PI3Kinase inhibition completely abolished α -klotho-mediated NPY/AgRP neuron inhibition and POMC activation. These data indicate a prominent α -klotho $\rightarrow$ FGFR $\rightarrow$ PI3Kinase signaling axis involved in the regulation of ARC neuron populations.

5. CONCLUSIONS

To summarize, this study identified diverse novel functions of α -klotho in the ARC via various molecular mechanisms. Experiments revealed that α -klotho regulates astrocytes via FGFR $\rightarrow$ ERK signaling, as well as NPY/AgRP neurons and POMC neurons via FGFR $\rightarrow$ PI3Kinase

signaling. Furthermore, CSF α -klotho's prominent role in energy balance was demonstrated by strong inverse correlations with body weight and an ability to increase energy expenditure. Further investigation on these novel molecular mechanisms could be integral to deciphering the complex physiology underlying homeostatic and disordered regulation of metabolism.

AUTHOR CONTRIBUTIONS

T.L. conceptualization, methodology, validation, formal analysis, investigation, writing — original draft, writing — review and editing, and visualization; P.L. conceptualization and investigation; D.S. investigation, writing — review and editing; Z.J. investigation; H.L. investigation; B.T.L. conceptualization; W.B. conceptualization, formal analysis; Q.T. supervision, project administration, funding acquisition; H.H. conceptualization, writing — review and editing, supervision, project administration, funding acquisition.

GUARANTOR STATEMENT

Dr. Hu Huang is the guarantor of this work, and, as such, has full access to all the data in the study and takes responsibility for its integrity and the accuracy of the analysis.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2020.101136>.

REFERENCES

- [1] Kuro-o, M., Nabeshima, Y., Matsumura, Y., Aizawa, H., Kawaguchi, H., Suga, T., et al., 1997. Mutation of the mouse klotho gene leads to a syndrome resembling ageing. *Nature* 390:45–51. <https://doi.org/10.1038/36285>.
- [2] Kurosu, H., Yamamoto, M., Clark, J.D., V Pastor, J., Nandi, A., Gurnani, P., et al., 2005. Suppression of aging in mice by the hormone klotho. *Science* 309:1829–1833. <https://doi.org/10.1126/science.1112766>.
- [3] Lin, Y., Sun, Z., 2012. Antiaging gene Klotho enhances glucose-induced insulin secretion by up-regulating plasma membrane levels of TRPV2 in MIN6 β -cells. *Endocrinology* 153:3029–3039. <https://doi.org/10.1210/en.2012-1091>.
- [4] Lin, Y., Sun, Z., 2015. In vivo pancreatic β -cell-specific expression of antiaging gene Klotho: a novel approach for preserving β -cells in type 2 diabetes. *Diabetes* 64:1444–1458. <https://doi.org/10.2337/db14-0632>.

- [5] Lin, Y., Sun, Z., 2015. Antiaging gene *klotho* attenuates pancreatic β -cell apoptosis in type 1 diabetes. *Diabetes* 64:4298–4311. <https://doi.org/10.2337/db15-0066>.
- [6] Rao, Z., Landry, T., Li, P., Bunner, W., Laing, B.T., Yuan, Y., et al., 2019. Administration of alpha *klotho* reduces liver and adipose lipid accumulation in obese mice. *Heliyon* 5:e01494. <https://doi.org/10.1016/j.heliyon.2019.e01494>.
- [7] Devaraj, S., Syed, B., Chien, A., Jialal, I., 2012. Validation of an immunoassay for soluble *klotho* protein. *American Journal of Clinical Pathology* 137:479–485. <https://doi.org/10.1309/AJCPGPMF7SFRB04>.
- [8] Nie, F., Wu, D., Du, H., Yang, X., Yang, M., Pang, X., et al., 2017. Serum *klotho* protein levels and their correlations with the progression of type 2 diabetes mellitus. *Journal of Diabetic Complications* 31:594–598. <https://doi.org/10.1016/j.jdiacomp.2016.11.008>.
- [9] Prud'homme, G.J., Glinka, Y., Kurt, M., Liu, W., Wang, Q., 2020. Systemic *Klotho* therapy protects against insulinitis and enhances beta-cell mass in NOD mice. *Biochemical and Biophysical Research Communications*. <https://doi.org/10.1016/j.bbrc.2020.02.123>.
- [10] Kim, H.J., Lee, J., Chae, D.W., Lee, K.B., Sung, S.A., Yoo, T.H., et al., 2019. Serum *klotho* is inversely associated with metabolic syndrome in chronic kidney disease: results from the KNOW-CKD study. *BMC Nephrology* 20:119. <https://doi.org/10.1186/s12882-019-1297-y>.
- [11] Ji, B., Wei, H., Ding, Y., Liang, H., Yao, L., Wang, H., et al., 2020. Protective potential of *klotho* protein on diabetic retinopathy: evidence from clinical and *in vitro* studies. *J. Diabetes Investig.* 11:162–169. <https://doi.org/10.1111/jdi.13100>.
- [12] Hu, M.C., Shi, M., Zhang, J., Addo, T., Cho, H.J., Barker, S.L., et al., 2016. Renal production, uptake, and handling of circulating α *klotho*. *Journal of the American Society of Nephrology* 27:79–90. <https://doi.org/10.1681/ASN.2014101030>.
- [13] Leon, J., Moreno, A.J., Garay, B.I., Chalkley, R.J., Burlingame, A.L., Wang, D., et al., 2017. Peripheral elevation of a *klotho* fragment enhances brain function and resilience in young, aging, and α -synuclein transgenic mice. *Cell Reports* 20:1360–1371. <https://doi.org/10.1016/j.celrep.2017.07.024>.
- [14] Olason, H., Mencke, R., Hillebrands, J.-L., Larsson, T.E., 2017. Tissue expression and source of circulating α *Klotho*. *Bone* 100:19–35. <https://doi.org/10.1016/j.bone.2017.03.043>.
- [15] Landry, T., Laing, B.T., Li, P., Bunner, W., Rao, Z., Prete, A., et al., 2020. Central α -*klotho* suppresses NPY/AgRP neuron activity and regulates metabolism in mice. *Diabetes* 69:db190941. <https://doi.org/10.2337/db19-0941>.
- [16] Nakajima, K., Cui, Z., Li, C., Meister, J., Cui, Y., Fu, O., et al., 2016. Gs-coupled GPCR signalling in AgRP neurons triggers sustained increase in food intake. *Nature Communications* 7:10268. <https://doi.org/10.1038/ncomms10268>.
- [17] Könnner, A.C., Janoschek, R., Plum, L., Jordan, S.D., Rother, E., Ma, X., et al., 2007. Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. *Cell Metabolism* 5:438–449. <https://doi.org/10.1016/j.cmet.2007.05.004>.
- [18] Wang, Q., Liu, C., Uchida, A., Chuang, J.-C., Walker, A., Liu, T., et al., 2014. Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. *Mol. Metab.* 3:64–72. <https://doi.org/10.1016/j.molmet.2013.10.001>.
- [19] Krashes, M.J., Shah, B.P., Madara, J.C., Olson, D.P., Strohlic, D.E., Garfield, A.S., et al., 2014. An excitatory paraventricular nucleus to AgRP neuron circuit that drives hunger. *Nature* 507:238–242. <https://doi.org/10.1038/nature12956>.
- [20] Chen, G., Liu, Y., Goetz, R., Fu, L., Jayaraman, S., Hu, M.-C., et al., 2018. α -*Klotho* is a non-enzymatic molecular scaffold for FGF23 hormone signalling. *Nature* 553:461–466. <https://doi.org/10.1038/nature25451>.
- [21] Zhang, X., Van Den Pol, A.N., 2016. Hypothalamic arcuate nucleus tyrosine hydroxylase neurons play orexigenic role in energy homeostasis. *Nature Neuroscience* 19:1341–1347. <https://doi.org/10.1038/nn.4372>.
- [22] Varela, L., Horvath, T.L., 2012. Leptin and insulin pathways in POMC and AgRP neurons that modulate energy balance and glucose homeostasis. *EMBO Reports* 13:1079–1086. <https://doi.org/10.1038/embor.2012.174>.
- [23] Wang, D., He, X., Zhao, Z., Feng, Q., Lin, R., Sun, Y., et al., 2015. Whole-brain mapping of the direct inputs and axonal projections of POMC and AgRP neurons. *Frontiers in Neuroanatomy* 9:40. <https://doi.org/10.3389/fnana.2015.00040>.
- [24] Zhan, C., Zhou, J., Feng, Q., Zhang, J. -e., Lin, S., Bao, J., et al., 2013. Acute and long-term suppression of feeding behavior by POMC neurons in the brainstem and hypothalamus, respectively. *Journal of Neuroscience* 33:3624–3632. <https://doi.org/10.1523/JNEUROSCI.2742-12.2013>.
- [25] Al-Qassab, H., Smith, M.A., Irvine, E.E., Guillermet-Guibert, J., Claret, M., Choudhury, A.I., et al., 2009. Dominant role of the p110 β isoform of PI3K over p110 α in energy homeostasis regulation by POMC and AgRP neurons. *Cell Metabolism* 10:343–354. <https://doi.org/10.1016/j.cmet.2009.09.008>.
- [26] García-Cáceres, C., Balland, E., Prevot, V., Luquet, S., Woods, S.C., Koch, M., et al., 2019. Role of astrocytes, microglia, and tanycytes in brain control of systemic metabolism. *Nature Neuroscience* 22:7–14. <https://doi.org/10.1038/s41593-018-0286-y>.
- [27] Yasumoto, Y., Miyazaki, H., Ogata, M., Kagawa, Y., Yamamoto, Y., Islam, A., et al., 2018. Glial fatty acid-binding protein 7 (FABP7) regulates neuronal leptin sensitivity in the hypothalamic arcuate nucleus. *Molecular Neurobiology* 55:9016–9028. <https://doi.org/10.1007/s12035-018-1033-9>.
- [28] Balland, E., Dam, J., Langlet, F., Caron, E., Steculorum, S., Messina, A., et al., 2014. Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain. *Cell Metabolism* 19:293–301. <https://doi.org/10.1016/j.cmet.2013.12.015>.
- [29] Brown, J.M., Scarlett, J.M., Matsen, M.E., Nguyen, H.T., Secher, A., Jorgensen, R., et al., 2019. The hypothalamic arcuate nucleus-median eminence is a target for sustained diabetes remission induced by fibroblast growth factor 1. *Diabetes* 68:db190025. <https://doi.org/10.2337/DB19-0025>.
- [30] Robins, S.C., Stewart, I., McNay, D.E., Taylor, V., Giachino, C., Goetz, M., et al., 2013. α -Tanycytes of the adult hypothalamic third ventricle include distinct populations of FGF-responsive neural progenitors. *Nature Communications* 4:1–13. <https://doi.org/10.1038/ncomms3049>.
- [31] Morton, G.J., Matsen, M.E., Bracy, D.P., Meek, T.H., Nguyen, H.T., Stefanovski, D., et al., 2013. FGF19 action in the brain induces insulin-independent glucose lowering. *Journal of Clinical Investigation* 123:4799–4808. <https://doi.org/10.1172/JCI70710>.
- [32] Rahmouni, K., Haynes, W.G., Morgan, D.A., Mark, A.L., 2003. Intracellular mechanisms involved in leptin regulation of sympathetic outflow. *Hypertension* 41:763–767. <https://doi.org/10.1161/01.HYP.0000048342.54392.40>.
- [33] Laing, B.T., Huang, H., 2016. Exercise improves hypothalamic function induced by high-fat diet. *Immunoenocrinology* 3. <https://doi.org/10.14800/ie.1446>.
- [34] Jiang, Z., Rajamanickam, S., Justice, N.J., 2018. Local corticotropin-releasing factor signaling in the hypothalamic paraventricular nucleus. *Journal of Neuroscience* 38:1874–1890. <https://doi.org/10.1523/JNEUROSCI.1492-17.2017>.
- [35] Zhu, C., Jiang, Z., Xu, Y., Cai, Z.L., Jiang, Q., Xu, Y., et al., 2020. Profound and redundant functions of arcuate neurons in obesity development. *Nat. Metab.* 1–12. <https://doi.org/10.1038/s42255-020-0229-2>.
- [36] Shi, Y.C., Lau, J., Lin, Z., Zhang, H., Zhai, L., Sperk, G., et al., 2013. Arcuate NPY controls sympathetic output and BAT function via a relay of tyrosine hydroxylase neurons in the PVN. *Cell Metabolism* 17:236–248. <https://doi.org/10.1016/j.cmet.2013.01.006>.
- [37] Lam, B.Y.H., Cimino, I., Polex-Wolf, J., Nicole Kohnke, S., Rimmington, D., Iyemere, V., et al., 2017. Heterogeneity of hypothalamic pro-opiomelanocortin-expressing neurons revealed by single-cell RNA sequencing. *Mol. Metab.* 6:383–392. <https://doi.org/10.1016/j.molmet.2017.02.007>.

- [38] Kurosu, H., Ogawa, Y., Miyoshi, M., Yamamoto, M., Nandi, A., Rosenblatt, K.P., et al., 2006. Regulation of fibroblast growth factor-23 signaling by klotho. *Journal of Biological Chemistry* 281:6120–6123. <https://doi.org/10.1074/jbc.C500457200>.
- [39] Mazucanti, C.H., Kawamoto, E.M., Mattson, M.P., Scavone, C., Camandola, S., 2019. Activity-dependent neuronal Klotho enhances astrocytic aerobic glycolysis. *Journal of Cerebral Blood Flow and Metabolism* 39:1544–1556. <https://doi.org/10.1177/0271678X18762700>.
- [40] Kunert, S.K., Hartmann, H., Haffner, D., Leifheit-Nestler, M., 2017. Klotho and fibroblast growth factor 23 in cerebrospinal fluid in children. *Journal of Bone and Mineral Metabolism* 35:215–226. <https://doi.org/10.1007/s00774-016-0746-y>.
- [41] Semba, R.D., Moghekar, A.R., Hu, J., Sun, K., Turner, R., Ferrucci, L., et al., 2014. Klotho in the cerebrospinal fluid of adults with and without Alzheimer's disease. *Neuroscience Letters* 558:37–40. <https://doi.org/10.1016/j.neulet.2013.10.058>.
- [42] Emami Aleagha, M.S., Siroos, B., Ahmadi, M., Balood, M., Palangi, A., Haghighi, A.N., et al., 2015. Decreased concentration of Klotho in the cerebrospinal fluid of patients with relapsing-remitting multiple sclerosis. *Journal of Neuroimmunology* 281:5–8. <https://doi.org/10.1016/j.jneuroim.2015.02.004>.
- [43] Hussain, A., Ali, I., Kaleem, W.A., Yasmeen, F., 2019. Correlation between body mass index and lipid profile in patients with type 2 diabetes attending a tertiary care hospital in Peshawar. *Pakistan J. Med. Sci.* 35:591–597. <https://doi.org/10.12669/pjms.35.3.7>.
- [44] Y.-N. Kim, S. Kim, Y.-O. Cho, Body mass index was positively correlated with blood triglyceride and total cholesterol levels, *The FASEB Journal*. 26 (2012) 1b356–1b356. doi:10.1096/FASEBJ.26.1_SUPPLEMENT.LB356.
- [45] Bakari, A.G., Onyemelukwe, C., Sani, B.G., Aliyu, S., Hassan, S.S., Aliyu, T.M., 2007. Relationship between casual blood sugar and body mass index in a suburban northern Nigerian population: a short communication. *Nigerian Journal of Medicine* 16:77–78. <https://doi.org/10.4314/njm.v16i1.37287>.
- [46] Huo, L., Gamber, K., Greeley, S., Silva, J., Huntoon, N., Leng, X.H., et al., 2009. Leptin-dependent control of glucose balance and locomotor activity by POMC neurons. *Cell Metabolism* 9:537–547. <https://doi.org/10.1016/j.cmet.2009.05.003>.
- [47] Hentges, S.T., Otero-Corchon, V., Pennock, R.L., King, C.M., Low, M.J., 2009. Proopiomelanocortin expression in both GABA and glutamate neurons. *Journal of Neuroscience* 29:13684–13690. <https://doi.org/10.1523/JNEUROSCI.3770-09.2009>.
- [48] Rau, A.R., Hentges, S.T., 2017. The relevance of AgRP neuron-derived GABA inputs to POMC neurons differs for spontaneous and evoked release. *Journal of Neuroscience* 37:7362–7372. <https://doi.org/10.1523/JNEUROSCI.0647-17.2017>.
- [49] Tong, Q., Ye, C.-P., Jones, J.E., Elmquist, J.K., Lowell, B.B., 2008. Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nature Neuroscience* 11:998–1000. <https://doi.org/10.1038/nn.2167>.
- [50] Atasoy, D., Nicholas Betley, J., Su, H.H., Sternson, S.M., 2012. Deconstruction of a neural circuit for hunger. *Nature* 488:172–177. <https://doi.org/10.1038/nature11270>.
- [51] Marcelin, G., Jo, Y.-H., Li, X., Schwartz, G.J., Zhang, Y., Dun, N.J., et al., 2014. Central action of FGF19 reduces hypothalamic AGRP/NPY neuron activity and improves glucose metabolism. *Mol. Metab.* 3:19–28. <https://doi.org/10.1016/j.molmet.2013.10.002>.
- [52] Szczypka, M.S., Mandel, R.J., Donahue, B.A., Snyder, R.O., Leff, S.E., Palmiter, R.D., 1999. Viral gene delivery selectively restores feeding and prevents lethality of dopamine-deficient mice. *Neuron* 22:167–178. [https://doi.org/10.1016/S0896-6273\(00\)80688-1](https://doi.org/10.1016/S0896-6273(00)80688-1).
- [53] Daubner, S.C., Le, T., Wang, S., 2011. Tyrosine hydroxylase and regulation of dopamine synthesis. *Archives of Biochemistry and Biophysics* 508:1–12. <https://doi.org/10.1016/j.abb.2010.12.017>.
- [54] Satoh, N., Ogawa, Y., Katsuura, G., Numata, Y., Tsuji, T., Hayase, M., et al., 1999. Sympathetic activation of leptin via the ventromedial hypothalamus: leptin-induced increase in catecholamine secretion. *Diabetes* 48:1787–1793. <https://doi.org/10.2337/diabetes.48.9.1787>.
- [55] Wang, Q., Zhang, M., Ning, G., Gu, W., Su, T., Xu, M., et al., 2011. Brown adipose tissue in humans is activated by elevated plasma catecholamines levels and is inversely related to central obesity. *PLoS One* 6:e21006. <https://doi.org/10.1371/journal.pone.0021006>.
- [56] Laszczyk, A.M., Fox-Quick, S., Vo, H.T., Nettles, D., Pugh, P.C., Overstreet-Wadiche, L., et al., 2017. Klotho regulates postnatal neurogenesis and protects against age-related spatial memory loss. *Neurobiology of Aging* 59:41–54. <https://doi.org/10.1016/j.neurobiolaging.2017.07.008>.
- [57] D.A. Sarruf, J.P. Thaler, G.J. Morton, J. German, J.D. Fischer, K. Ogimoto, et al, Fibroblast growth factor 21 action in the brain increases energy expenditure and insulin sensitivity in obese rats, (n.d.). doi:10.2337/db09-1878.
- [58] Rojas, J.M., Matsen, M.E., Munding, T.O., Morton, G.J., Stefanovski, D., Bergman, R.N., et al., 2015. Glucose intolerance induced by blockade of central FGF receptors is linked to an acute stress response. *Mol. Metab.* 4: 561–568. <https://doi.org/10.1016/J.MOLMET.2015.05.005>.
- [59] Jeong, J.H., Lee, D.K., Liu, S.M., Chua, S.C., Schwartz, G.J., Jo, Y.H., 2018. Activation of temperature-sensitive TRPV1-like receptors in ARC POMC neurons reduces food intake. *PLoS Biology* 16. <https://doi.org/10.1371/journal.pbio.2004399>.
- [60] Chang, Q., Hoefs, S., van der Kemp, A.W., Topala, C.N., Bindels, R.J., Hoenderop, J.G., 2005. The β -glucuronidase klotho hydrolyzes and activates the TRPV5 channel. *Science*(80-):310.



Corticotropin Releasing Hormone Signaling in the Bed Nuclei of the Stria Terminalis as a Link to Maladaptive Behaviors

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The bed nuclei of the stria terminalis (BST) is a limbic region in the extended amygdala that is heavily implicated in anxiety processing and hypothalamic-adrenal-pituitary (HPA) axis activation. The BST is complex, with many nuclei expressing different neurotransmitters and receptors involved in a variety of signaling pathways. One neurotransmitter that helps link its functions is corticotropin releasing hormone (CRH). BST CRH neuron activation may cause both anxiogenic and anxiolytic effects in rodents, and CRH neurons interact with other neuron types to influence anxiety-like responses as well as alcohol and drug-seeking behavior. This review covers the link between BST CRH neurons and thirteen other neurotransmitters and receptors and analyzes their effect on rodent behavior. Additionally, it covers the translational potential of targeting CRH signaling pathways for the treatment of human mental health disorders. Given the massive impact of anxiety, mood, and substance use disorders on our society, further research into BST CRH signaling is critical to alleviate the social and economic burdens of those disorders.

Keywords: BNST, CRH, CRHR1, extended amygdala, anxiety, addiction

INTRODUCTION

Approximately one in three people in the United States will develop an anxiety disorder during their lifetime (Craske et al., 2017). The bed nuclei of the stria terminalis (BST; also known as BNST) is a small, sexually dimorphic, heterogeneous region that in the past 20 years, has been highlighted for its role in stress and anxiety responses (for review, see Lebow and Chen, 2016). While traditionally BST research has focused on anxiety processing, in recent years the BST has been increasingly implicated in dysregulated signaling patterns consistent with many mental health disorders in both animal models and humans (Gungor and Paré, 2016; Lebow and Chen, 2016; Luyten et al., 2016; Blomstedt et al., 2017; Knight and Depue, 2019). Given the economic and social burden of those disorders on our society today, further study of the BST is a key step in the development of more effective treatments.

The BST circuitry is complex, involving neural connections both between the BST and other brain regions, and within the different nuclei of the BST. The BST connectivity has been discussed

extensively elsewhere (see Bota et al., 2012; Vranjkovic et al., 2017; Ch'ng et al., 2018) and will not be elaborated upon in detail here. In brief, the BST is commonly grouped with the amygdala as part of the extended amygdala because of their proximity and similar cellular composition and immunohistochemistry (Alheid and Heimer, 1988). The two regions are also connected through signaling circuits like that of corticotropin releasing hormone (CRH; also known as CRF) between the central amygdala (CEA) and anteromedial BST (BSTam) (Pomrenze et al., 2019). More recent studies have demonstrated a role for the BST in anxiety processing independent of amygdalar inputs, and they underscore the need for more research specifically focusing on the BST.

Preclinical models are important for advancing the understanding of mental health disorders because they allow for the precise modification of animal brains. Previous research has demonstrated that the BST is involved in the processing of anxiety-like behavior in animal models, characterized by sustained fear due to conditioned or unpredictable cues (Walker and Davis, 1997; Walker et al., 2003; Daldrup et al., 2016), while the amygdala is more involved in the processing of phasic fear induced by short, discrete cues (Davis et al., 2010). The distinction that animals display anxiety-like behavior, and not anxiety, is necessary since their behavior is measured using metrics that are assumed to represent anxiety in animals, such as time spent in the center of an open field (OF) test (Carola et al., 2002), but cannot be confirmed to truly represent anxiety since anxiety as traditionally conceptualized is a subjective state (LeDoux and Pine, 2016). The term “anxiety-like” acknowledges that attributing behaviors to anxiety in animals is a form of anthropomorphism and may not be accurate.

An additional nuance to BST research is the distinction between anxiety and fear. The terms “fear” and “anxiety” are often used interchangeably, both in popular culture and by scientists and medical professionals, and Shackman and Fox (2016) argue that saying “fear and anxiety” together in reference to either will allow for less confusion about the term while focusing on the threat context. However, included in the threat context is the threat imminence, and other researchers such as Gungor and Paré (2016) argue that fear can be categorized as a defensive response to an imminent threat, while anxiety is a response to a diffuse, or not imminent threat. The majority of papers referenced in this review focus on what would be classically considered as anxiety-like responses. While early models created a sharp distinction between the CEA and BST in fear and anxiety processing, more recent research has demonstrated that both regions have a role in processing both uncertain and certain threat contexts, and thus may influence fear and anxiety processing (Shackman and Fox, 2016).

The BST is involved in the development of other maladaptive behaviors beyond anxiety disorders. It has been implicated in binge drinking and alcohol use disorders (AUDs), particularly the stress-induced reinstatement of drug-seeking behaviors (Silberman and Winder, 2013; Nentwig et al., 2019; Snyder et al., 2019). In this model of the addiction cycle, withdrawal produces negative affect and dysregulated BST CRH signaling. Stress can then induce the reinstatement of drug-seeking behavior (for

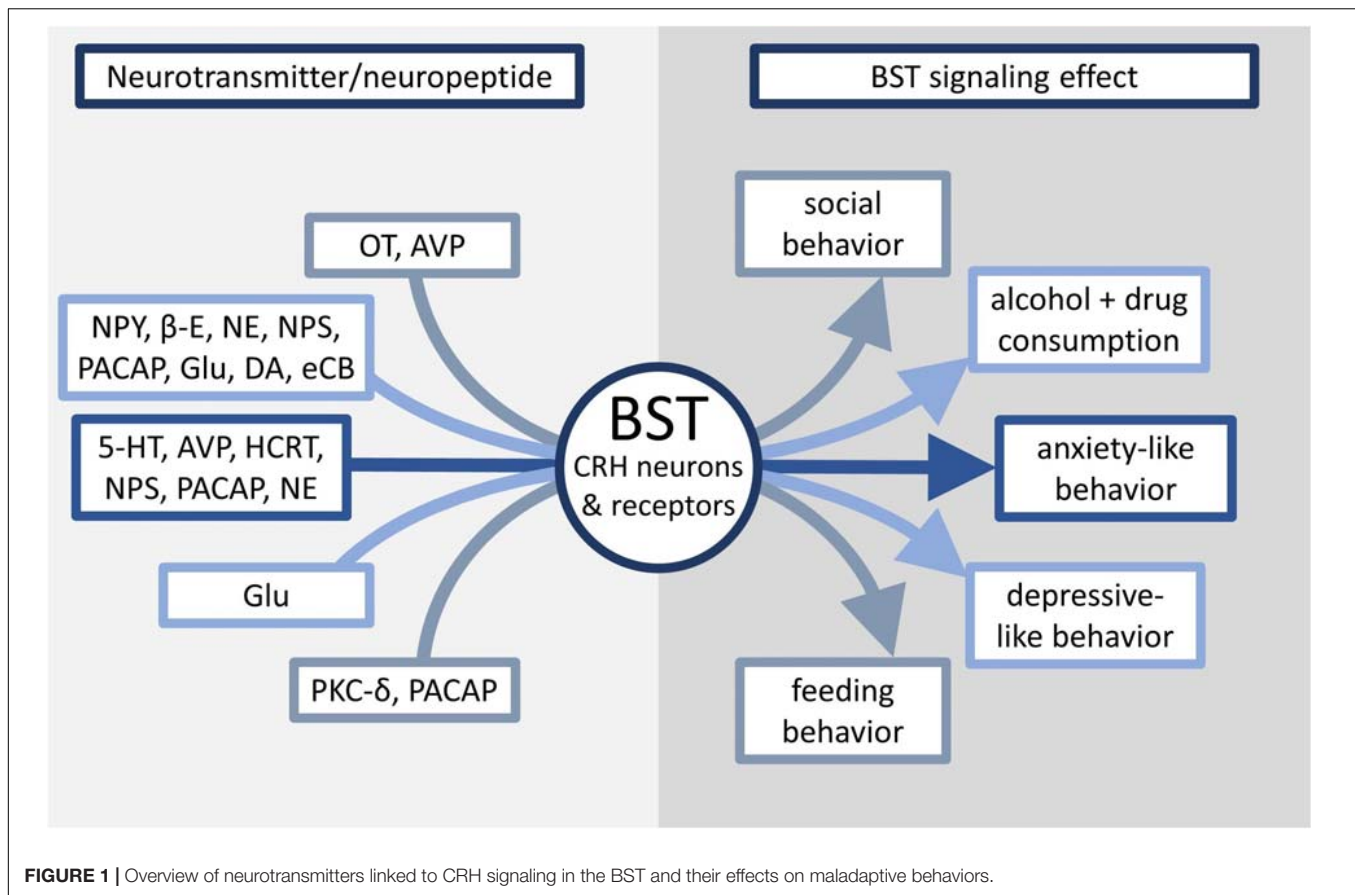
review, see Silberman and Winder, 2013). The BST is also involved in the regulation of depressive-like behaviors, an effect that is CRH-dependent (Salimando et al., 2020). Depressive-like behavior is typically quantified in preclinical models by tests such as the forced swim (FS) and tail suspension tests that measure parameters like helplessness and anhedonia (Krishnan and Nestler, 2011). Finally, several results discussed in this review implicate BST circuits in the regulation of feeding behavior. In humans, feeding behavior is psychologically complex and not driven solely by the demands of energy metabolism, and thus may be difficult to investigate in preclinical models, particularly in relation to maladaptive behaviors (Ellacott et al., 2010). The link between the BST and maladaptive behaviors beyond anxiety is an emerging field of research, and CRH signaling within the BST presents new treatment targets for these behaviors.

Because of the demonstrated impact of CRH on rodent behavior and its interaction with many other neurotransmitters and neuron types, this review will examine stress processing in the BST through the lens of CRH signaling and how it affects animal behavior, as well as possible implications of BST manipulation on the treatment of human mental health disorders. **Figure 1** and **Table 1** provide an overview of the effects discussed in this review.

BST NOMENCLATURE AND ANATOMY

Papers vary in their usage of BST or BNST, as well as nucleus or nuclei in the BST name. Here, usage of the BST acronym and the word “nuclei” instead of “nucleus” in the BST name is to remain consistent with atlas nomenclature (Swanson, 2004; Lein et al., 2007; Shackman and Fox, 2016) and acknowledge the different functions of the BST nuclei. While the accepted number of BST nuclei varies (Gungor and Paré, 2016), this review will use the standard nomenclature system established by the Swanson (2004) rat brain atlas, which includes sixteen different regions, defined mostly by cytoarchitecture (Ju and Swanson, 1989). This is also the nomenclature used by the Allen Reference Atlases, with minor differences in hierarchical structure that include the removal of the cell sparse zone (BSTsz) and premedullary nucleus (BSTpm) as BST nuclei (Lein et al., 2007). Both the Swanson (2004) rat brain atlas and the Allen Mouse Brain Atlas (2004) divide the BST into two major subparts—the anterior and posterior divisions—with most research on stress and anxiety focusing on the anterior division (Kim et al., 2013). Important subdivisions for stress processing include the BSTam, oval nucleus BST (BSTov), dorsomedial nucleus BST (BSTdm), ventral nucleus BST (BSTv), and the anterolateral BST (BSTal) from the anterior division, and the dorsal BST (BSTd) from the posterior division.

Despite the existence of this standard nomenclature system, BST nomenclature is highly inconsistent across papers. This makes it difficult to compare data from paper to paper (Shackman and Fox, 2016), and because of the large number of nuclei in the BST (Gungor and Paré, 2016), accurate labeling and nomenclature is critical for reproducible and easily understood results. For example, if different papers call the anteromedial BST



the anterodorsal, anteroventral, or dorsolateral BST, it is difficult for readers to determine if the papers focus on the same area. When discussing results from various papers, this review uses the same nuclei classification as used by the original authors. **Figure 2** provides an illustration of the BST nuclei, as well as different nomenclatures used by the papers referenced in this review.

OVERVIEW OF CRH SIGNALING IN THE BST

Classically, CRH neurons are thought of in their role in hypothalamic-pituitary-adrenal (HPA) axis activation. In this role, there are at least two different populations of distinct CRH neurons—those in the BST and those in the paraventricular nucleus of the hypothalamus (PVH) (Dabrowska et al., 2013). The BST has been shown to contain mostly GABAergic neurons and a small amount of glutamatergic neurons (Moga and Saper, 1994). Similarly, CRH neurons in the BST are primarily GABAergic (Veinante et al., 1997), with Dabrowska et al. (2013) finding that 95% of CRH neurons in the BSTov colocalized with glutamic acid decarboxylase 67, a marker of GABAergic neurons, and had low colocalization with vesicular glutamate transporter 2, a marker of glutamatergic neurons. Conversely, PVH CRH neurons are primarily glutamatergic (Dabrowska et al., 2013), suggesting a unique role for each of the two populations in HPA

axis activation. BST CRH neurons are concentrated in the BSTov (Ju et al., 1989), which is critical for the regulation of anxiety states (Kim et al., 2013) and may have a role as the master controller of the BST (Ch'ng et al., 2018).

Corticotropin releasing hormone signaling in the BST includes CRH neurons as well as the CRH receptors, which are widely distributed in the BST (Van Pett et al., 2000). CRH binding to CRH receptor type 1 (CRHR1) or CRH receptor type 2 (CRHR2) causes the receptor to couple to a $G_{s\alpha}$ protein, which then stimulates cAMP production through adenylyl cyclase activation. cAMP activates protein kinase A (PKA), which phosphorylates downstream targets to carry out the effects of CRH signaling (for review, see Hillhouse and Grammatopoulos, 2006). Despite the prevalence of CRH neurons and receptors in the BST, in the anterolateral cell group of the BST (BSTalg), Dabrowska et al. (2013) found virtually no colocalization between CRH neurons and CRHR1 or CRHR2-expressing neurons.

CRH infusion to the BST was found to increase anxiety-like behavior in rats in the elevated plus maze (EPM) test, an effect mediated through CRHR1s (Sahuque et al., 2006). BST CRH infusion was also found to induce the reinstatement of cocaine-seeking behavior (Erb and Stewart, 1999), demonstrating the effect of CRH signaling on multiple forms of maladaptive behaviors. An important source of CRH in the BST comes from CRH projections from the CEA to the dorsolateral BST (BSTdl), and those projections are necessary for the induction

TABLE 1 | Summary of BST-CRH signaling studies.

Neurotransmitter/ Neuropeptide	Target receptor	Target region	Treatment	Effect
5-HT	5-HT _{1A}	BSTd	Activation of 5-HT DRD projections	↓ Anxiety-like behavior (Garcia-Garcia et al., 2018)
	5-HT _{2c}	BST	Activation of 5-HT DRD projections	↑ Anxiety-like behavior (Marcinkiewicz et al., 2016)
OT	OTR	BSTam	OTR antagonist administration	↓ Stress-induced social avoidance in female mice (Duque-Wilckens et al., 2018)
AVP	V1aR	BSTmv	V1aR antagonist administration	↑ Sex and context-specific anxiety-like behavior (Duque-Wilckens et al., 2016)
	V1bR	Pituitary	Immobilization stress	↑ HPA axis activation (Dinan and Scott, 2005)
HCRT	—	BSTadl	BST-CRH activation of HCRT neurons	↑ Anxiety-like behavior and arousal (Giardino et al., 2018)
NPS	NPSR	—	i.c.v. NPS administration	↓ Anxiety-like behavior, ↑ arousal (Xu et al., 2004)
		lateral ventricle	CRHR1 antagonist and NPS administration	No increase in self-administered cocaine use (Pañeda et al., 2009)
PACAP	PAC ₁ R	BSTov	Chronic variable mild stress paradigm	↑ Number of PACAP expressing cells in the BSTov (Hu et al., 2020)
		BST	i.c.v. PACAP infusion	↑ Anxiety-like behavior (Hammack et al., 2009a)
		BST	PAC ₁ R antagonist administration	Blocked excessive alcohol consumption in dependent rats (Ferragud et al., 2020)
		BSTp	PACAP agonist administration	↑ Weight loss in rats (Kocho-Schellenberg et al., 2014)
PKC- δ Glutamate	—	BSTov	Optogenetic activation of BSTov PKC- δ neurons	↓ Feeding behavior (Wang et al., 2019)
	NMDAR	BSTdl	Deletion of GluN2D subunit	↑ Depressive-like behavior (Salimando et al., 2020)
	AMPA	BSTdl	Adolescent intermittent ethanol vapor exposure	↓ GluA2 subunit expression (Kasten et al., 2020)
NPY	Y1R	BST	Y1R activation	↓ Binge alcohol drinking (Pleil et al., 2015)
	Y2R	Lateral ventricle	Y2R antagonism	↓ Alcohol consumption (Thorsell et al., 2002)
β -E	μ and δ opioid receptors	BST	β -E deficient mice	↑ Alcohol consumption in female mice (Nentwig et al., 2019)
NE	α_{2a} -AR	BSTd	α_{2a} -AR agonist administration	↓ Anxiety-like behavior (Schweimer et al., 2005; Harris et al., 2018)
	β -AR	BST	NE administration	↑ CRH neuron activation (Snyder et al., 2019)
DA	D1R, D2R	BSTdl	DA administration	↑ glutamatergic transmission (Kash et al., 2008)
eCB	CB1R	BSTd	MAG lipase inhibitor administration	Normalized BST CRH neuron activation following alcohol withdrawal (Centanni et al., 2019)

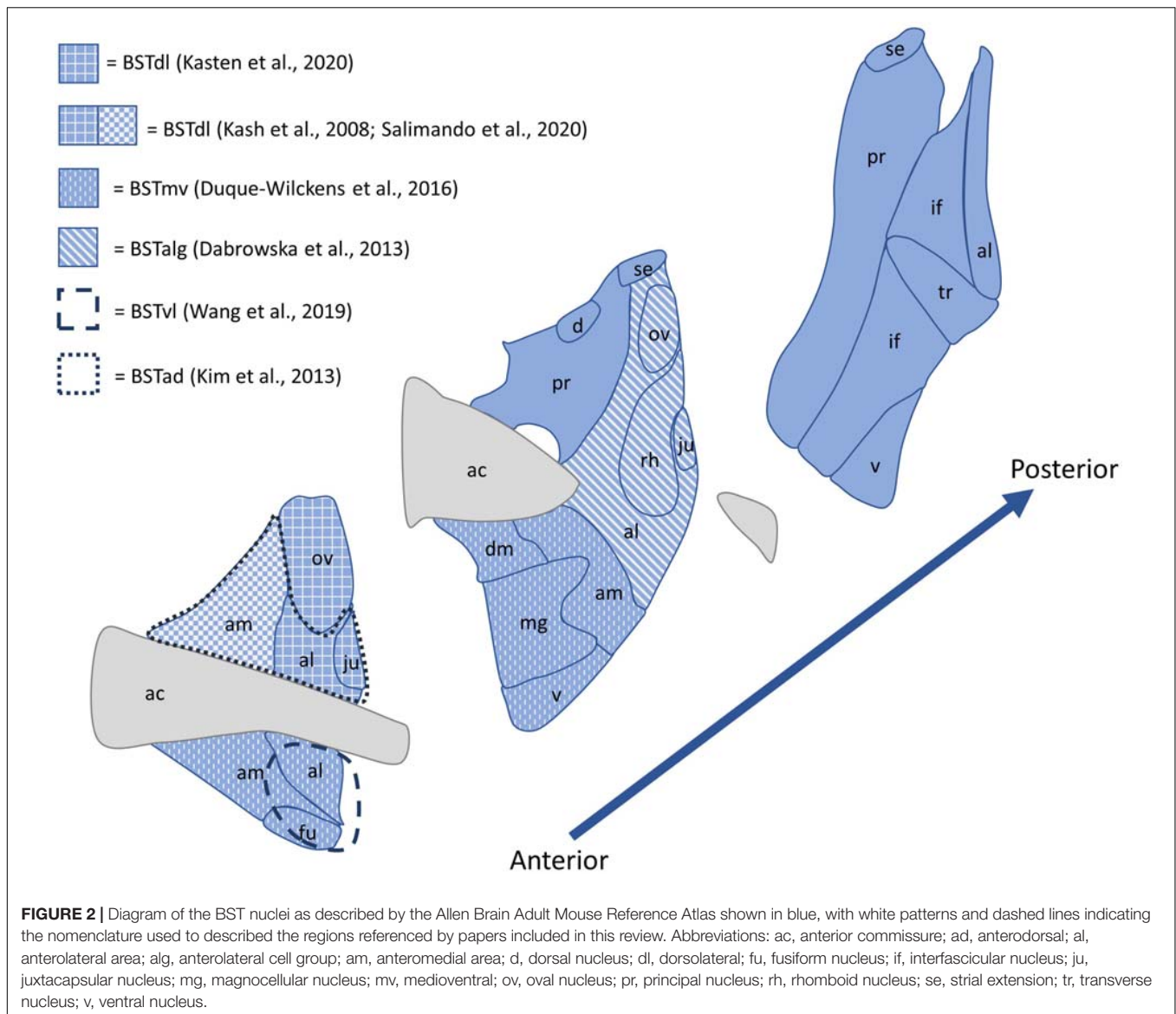
—Indicates value not specified by cited paper.

of stress-induced anxiety (Pomrenze et al., 2019). In support of this conclusion, Ventura-Silva et al. (2020) found that CRH knockdown in the CEA reduced stress-induced anxiety-like behavior in rats and had no effect on the results of a fear-potentiated startle paradigm. This reinforces the role of CEA-BST circuit signaling in mediating chronic, and not acute, stress processing.

Hu et al. (2020) found that a chronic variable mild stress paradigm resulted in increased phosphorylated PKA in the anterior dorsolateral BST (BSTadl). Infusion of mice with a PKA-selective antagonist, H89, attenuated the anxiogenic effects

caused by the stress paradigm, as measured by the EPM, OF, novelty suppressed feeding (NSF) tests. This suggests that PKA is necessary for transmitting anxiogenic signals caused by CRHR1 activation in the BSTov.

While many studies have linked BST activity and CRH signaling to anxiogenic activity, especially anxiogenic activity induced by CRHR1 activation (Bale and Vale, 2004), Henckens et al. (2017) found that CRHR2 activation is necessary for attenuating the stress response and promoting stress recovery. CRHR2 is highly expressed in the posterior BST (BSTp) (Van Pett et al., 2000), and optogenetic activation of CRHR2 neurons



in the BSTp reduced anxiety-like behaviors in mice in the EPM and OF tests, while inhibition increased anxiety-like behaviors (Henckens et al., 2017). This contributes to the heterogeneity of the BST and also has implications for the treatment of PTSD and other stress-related disorders (Lebow and Chen, 2016; Henckens et al., 2017), especially considering that BST-CRHR2 levels can influence susceptibility to PTSD (Lebow et al., 2012).

As stated before, changes to BST CRH signaling have been implicated in many forms of maladaptive behaviors. Its effects are often contradictory (Lebow and Chen, 2016), potentially due to the different electrophysiological profiles of BST neurons, leading to anxiogenic or anxiolytic behaviors. Hammack et al. (2007) found three distinct populations of neurons in the BSTalg, Type I-III, defined by the neuronal electrophysiological response. Moreover, all three types have the potential to contain CRH and express different neurotransmitters and receptors, resulting in CRH neurons that fire under different neurochemical conditions

(Dabrowska et al., 2013). This review will examine individually the effects of different neurotransmitters on CRH signaling in the BST and associated disorders.

SEROTONIN

Serotonin (5-hydroxytryptamine; 5-HT) plays a major role in many psychiatric disorders and is a common treatment target in the form of selective serotonin reuptake inhibitors (SSRIs) (Craske et al., 2017). Its effects are mediated through seven families of 5-HT receptors, 5-HT₁₋₇. Especially important for anxiety processing are the G_{i/o} protein-coupled 5-HT₁ and G_q protein-coupled 5-HT₂ receptor families (for review, see Hammack et al., 2009b). The BST, specifically the BSTdl and BSTvl, receive 5-HT projections from the dorsomedial dorsal raphe nucleus (DRD) (Commons et al., 2003). Levita et al.

(2004) found that activation of the BSTal by DRD 5-HT inputs caused hyperpolarization and inhibition with anxiolytic effects, measured by the acoustic startle reflex. DRD 5-HT neurons are activated by CRH (Lowry et al., 2000), and because of the high density of CRH in the BST, it was questioned if 5-HT and CRH signaling between the DRD and BST formed a feedback loop.

In support of this, Donner et al. (2018) found that rat *Tph2*, the rate-limiting enzyme of 5-HT synthesis, and *tph2* mRNA expression in the DRD were increased after stress exposure. Infusion of the BST with urocortin-1 (*Ucn-1*), a CRHR1 and CRHR2 agonist (Vaughan et al., 1995), was shown by Donner et al. (2020) to increase DRD *tph2* mRNA, suggesting that DRD serotonergic neurons are regulated by BST-CRH neurons and help promote anxiety-like states. Optogenetic activation of 5-HT projections from the DRD to the BSTd resulted in reduced *c-fos* expression and anxiolytic effects on behavior in the EPM, OF, and NSF tests, while inhibition had anxiogenic effects, mediated through 5-HT_{1A} receptors in the BSTd (Garcia-Garcia et al., 2018).

Marcinkiewicz et al. (2016) found that activation of DRD projections to the BST, operating through 5-HT_{2C} receptors, was anxiogenic in the EPM and NSF tests and silenced anxiolytic BST outputs to the ventral tegmental area (VTA) and lateral hypothalamus (LH). The different 5-HT receptor families might explain the opposing effects found by Donner et al. (2020) and Garcia-Garcia et al. (2018). However, a knockdown model of the 5-HT_{1A} receptor yielded no effect on anxiety-like behavior, suggesting that without activation, the receptor may not be in great enough abundance to yield a physiologically relevant effect on behavior (Marcinkiewicz et al., 2019). More research is therefore necessary to further elucidate the role of 5-HT_{1A} receptors in regulating anxiety-like behavior, especially considering the implications of the 5-HT_{1A} receptor on SSRI treatment success (Quentin et al., 2018).

OXYTOCIN

Oxytocin (OT), beyond acting as a hormone, plays a role in the regulation of anxiety processing in the central nervous system and has been the subject of several thorough reviews (Janeček and Dabrowska, 2019; Steinman et al., 2019). OT is primarily synthesized in the PVH and supraoptic nuclei of the hypothalamus, and while some of those neurons project to the BST and act as a source of OT, OT is also synthesized in the BST itself (Lee et al., 2009). Intraperitoneal OT administration has been found to have anxiolytic effects in rats, measured by the emergence and social interaction (SI) tests, as well as decrease alcohol consumption (Bowen et al., 2011). The G_q protein-coupled OT receptors (OTRs) in the BSTam have also been linked to social avoidance in female, but not male, mice, again highlighting the sexual dimorphism of the BST (Duque-Wilckens et al., 2018). More specifically, Dabrowska et al. (2011) found that OT-immunoreactive (ir) fibers in the BSTov colocalized with CRHR2-ir fibers and had perisomatic contact with CRH-ir neurons. These neurons project to the magnocellular portion of the PVH (Dong et al., 2001), forming a reciprocal feedback loop to regulate activation of the HPA axis (Dabrowska et al., 2011).

ARGININE VASOPRESSIN

Like OT, changes to arginine vasopressin (AVP) signaling in the medioventral BST (BSTmv), including expression of AVP and its receptor, the G_q protein-coupled vasopressin V1a receptor (V1aR), have been implicated in sex-specific responses to social defeat and stress (Duque-Wilckens et al., 2016). The BST is a major extrahypothalamic source of AVP, and BST AVP neurons project to many forebrain and midbrain structures (Leeuwen and Caffé, 1983; de Vries and Miller, 1999). Administration of a V1aR antagonist to the BSTmv had anxiogenic effects in both male and female mice in the SI test, but in female mice, the effect only occurred in social contexts (Duque-Wilckens et al., 2016). AVP works with CRH to regulate the HPA axis (Kormos and Gaszner, 2013), and sustained AVP levels due to chronic stress, operating through G_q protein-coupled V1b receptors (V1bRs; also known as V3R), lead to increased HPA activation through the stimulation of adrenocorticotropin (ACTH) release (Dinan and Scott, 2005). Given the role of CRH neurons in HPA axis activation and the presence of V1bRs in the BST (Hernando et al., 2001), it is possible that BST AVP signaling, mediated through CRH signaling, is involved in multiple pathways, including the processing of anxiogenic stimuli and HPA axis activation. Clinically, elevated AVP levels have been seen in depressed patients, and V1bR antagonists are being investigated in preclinical models for their antidepressant and anxiolytic effects (for review, see Alldredge, 2010).

HYPOCRETIN

Hypocretin (HCRT; orexin) is expressed in a subset of LH neurons and is implicated in the regulation of emotional behavior (Soya and Sakurai, 2020). HCRT-LH neurons receive inputs from CRH-BST neurons, and HCRT activation via CRH-BST neurons is associated with negative valence and behavioral avoidance in the real-time place test (Giardino et al., 2018). HCRT is closely associated with wakefulness and narcolepsy (Soya and Sakurai, 2020) because of its role in the regulation of vigilance and arousal states (Boutrel and de Lecea, 2008), so it raises the question of if the BST-HCRT-LH pathway can help explain the sleep disruptions commonly associated with anxiety (American Psychiatric Association, 2013). It is possible that upregulation of CRH neurons in the BST and subsequent activation of HCRT-LH neurons may therefore contribute to the increased arousal states associated with anxiety and other altered mood states, as well as contribute to the role of the BST in regulating addiction (Snyder et al., 2019).

NEUROPEPTIDE S

Neuropeptide S (NPS) and its receptor, the G_s/G_q protein-coupled NPS receptor (NPSR; also known as GPR154), make up a deorphanized GPCR system implicated in the regulation of anxiety, arousal, and wakefulness (Xu et al., 2004), as well as drug and alcohol-related behaviors (Cannella et al., 2009;

Pañeda et al., 2009). While NPS is expressed in only a few regions of the rat brain, notably in a group of neurons between the locus coeruleus and Barrington's nucleus (Xu et al., 2007), the NPSR is more widely distributed and is found in the BSTv (Leonard and Ring, 2011). Administration of NPS in rodents resulted in increased arousal, determined by locomotor activity, and decreased anxiety-like behavior, determined by the EPM, OF, and light-dark box tests (Xu et al., 2004), similar to the effect of nicotine (Koob and Greenwell, 2004). Pañeda et al. (2009) found that in mice, i.c.v. administration of NPS to the lateral ventricle induced cocaine-seeking behavior in a CRHR1-dependent manner. When they either administered the CRHR1 antagonist antalarmin or used a CRHR1 knockout model, NPS administration failed to increase self-administered cocaine use. Following morphine withdrawal, NPSR transcript prevalence decreased in the BST, and NPS administration decreased anxiety-like behavior associated with withdrawal (Ghazal et al., 2013). NPS has also been linked to LH-HCRT neurons (Cannella et al., 2009), further linking the BST and CRH signaling to addiction pathways as discussed in the previous HCRT section.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE

Pituitary adenylate cyclase-activating polypeptide (PACAP), operating through the G_s/G_q protein-coupled PAC_1 receptor (PAC_1R) (Pisegna and Wank, 1996), is an upstream regulator of CRH that innervates CRH neurons in the BSTov, where it contributes to increased anxiety states and can activate the HPA axis (for review, see Lebow and Chen, 2016). Major sources of PACAP to the BST include the PVH and dorsal vagal complex (Kozicz et al., 1998). Hu et al. (2020) found that subjecting male mice to a chronic variable mild stress paradigm significantly increased the number of PACAP expressing cells in the BSTov, as well as *Pacap* mRNA expression in the BSTadl. Effects on anxiety-like behavior in male rats following PACAP infusion into the BST, measured by the light-enhanced startle test, are long lasting, occurring for at least 1 week post infusion (Hammack et al., 2009a). The anterodorsal BST (BSTad) had been previously shown to have anxiolytic effects (Kim et al., 2013), highlighting the heterogeneity and complexity of the BST.

While classically PACAP has been thought of in terms of its regulation of anxiety-like behavior, recently it has also been implicated in the regulation of alcohol consumption. Ferragud et al. (2020) demonstrated that exposure of rats to chronic intermittent ethanol vapors increased PACAP levels in the BST, and that administration of a PAC_1R antagonist to the BST blocked excessive alcohol consumption in ethanol-dependent rats. This research highlights the diverse natures of neurotransmitters in the BST and how they may have multifaceted roles in behavioral regulation.

Additionally, PACAP has been implicated in feeding regulation, with Kocho-Schellenberg et al. (2014) finding that infusion of the BSTp with a PACAP agonist resulted in a dose-dependent weight loss for the 24 h following the infusion in both male and female rats.

Susceptibility to the anxiogenic effects of PACAP depends on previous stress exposure, as well as sex (King et al., 2017). Female rats did not display the same increase in startle reflex as male rats when infused with PACAP in the BST following chronic stress exposure (King et al., 2017). More research is needed to investigate the effects of PACAP expression in female rodents. This is especially important considering that PACAP has been implicated as a potential treatment target for PTSD (Lebow and Chen, 2016), and that women are more than twice as likely as men to develop PTSD following a traumatic event (King et al., 2017).

PROTEIN KINASE C DELTA

The BSTov contains protein kinase C delta (PKC- δ) neurons, which help mediate feeding behaviors through a novel circuit proposed by Wang et al. (2019). The PKC family is composed of ten different isoforms that are sorted into three subfamilies, with PKC- δ belonging to the novel PKC subfamily (Sanchez-Bautista and Nicolas, 2013). PKC- δ is activated when CRH binds to the CRHR1 or CRHR2 receptor, which can then couple to a G_q protein and activate phospholipase C (PLC). PLC then catalyzes the production of inositol trisphosphate (IP3) and diacylglycerol (DAG), which activates PKC- δ . Activating BSTov PKC- δ neurons suppressed feeding in mice, while silencing those neurons increased feeding (Wang et al., 2019). BSTov PKC- δ neurons innervate and inhibit ventrolateral BST (BSTvl) neurons, demonstrating the interconnectivity of the BST subnuclei (Wang et al., 2019). Those BSTvl neurons then project to the LH to promote feeding behaviors, which explains how activating BSTov PKC- δ neurons suppresses feeding by inhibiting the BSTvl neurons that are necessary to activate the LH neurons (Wang et al., 2019). PKC- δ and CRH signaling may therefore link anxiety-like and feeding behaviors—in support of this conclusion, Hu et al. (2020) found that chronically stressed mice had decreased weight gain compared to controls.

GLUTAMATE

Glutamate operates through receptors that can be classified as either ionotropic, composed of *N*-methyl-D-aspartate receptors (NMDARs) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), or metabotropic, and BST neurons contain both (Gracy and Pickel, 1995; Grueter and Winder, 2005; Grueter et al., 2006). Specifically, BST CRH neurons express NMDARs containing the GluN2D subunit (Salimando et al., 2020). NMDARs have been previously implicated in mental health disorders and their treatments (Ghasemi et al., 2014), and consistent with that, Salimando et al. (2020) found that deletion of the BST-GluN2D subunit led to increased depressive-like behaviors in mice as measured by the FS test, potentially because of decreased BST excitatory synaptic potentiation and subsequent increased excitatory drive onto BST-CRH neurons. There was no change in anxiety-like behavior, measured by the OF and elevated zero maze tests. GluN2D receptors were found to be localized in the BST to

the BSTdl (Salimando et al., 2020), possibly contributing to the role of the BSTd in negative affect and valence processing (Kim et al., 2013). This research demonstrates the role of the BST in regulating emotional behavior beyond chronic fear responses and connects it to the regulation of mood disorders, such as major depressive disorder (MDD). Showing the translational potential between pre-clinical models and human studies, NMDAR antagonists are being investigated in clinical trials for the treatment of MDD and bipolar disorder (Ghasemi et al., 2014), and in 2019, the United States Food and Drug Administration (FDA) approved Spravato (esketamine), a NMDAR antagonist, in a nasal spray format for use in conjunction with antidepressants for the treatment of treatment-resistant depression (Daly et al., 2019; Popova et al., 2019; US Food and Drug Administration, 2019). However, concerns have been raised about the treatment length and long-term effects of intranasal esketamine treatment (Schatzberg, 2019; Zheng et al., 2020), and additional clinical trials will be key for improving MDD treatment using esketamine.

Bed nuclei of the stria terminalis neurons also contain AMPARs, a type of excitatory ionotropic glutamate receptor made up of four different subunits, GluA1-GluA4 (for review, see Chater and Goda, 2014). Kasten et al. (2020) found that adolescent intermittent ethanol vapor exposure (AIE) reduced the GluA2 AMPAR subunit expression in the BSTdl. They also found that the mGluR1 antagonist DHPG decreased long term depression in female, but not male, mice treated with AIE. CRHR1 activation resulting in PKA activation in the BST has been shown to cause the phosphorylation of GluR1 AMPAR subunits (Hu et al., 2020), so while the glutamate receptor research discussed above was not directly linked to CRH neurons, considering the previous connections made between CRH neurons in the BST with both AUDs and MDD, it is possible that CRH neurons influence this phenomenon and remains an area for future exploration.

NEUROPEPTIDE Y

Neuropeptide Y (NPY) signaling has been implicated in alcohol abuse disorders (Pleil et al., 2015), feeding regulation (Higuchi, 2012), and fear memory (Bartsch et al., 2020). The majority of NPY input to the BST comes to the BSTa from agouti-related protein (AgRP) neurons in the arcuate nucleus (ARC) that co-release NPY, although the BST also contains NPY interneurons (Betley et al., 2013; Kash et al., 2015). The $G_{i/o}$ protein-coupled NPY receptor (NPYR) family has five receptors, although only four are active in humans, and NPYR activation frequently results in inhibition of adenylyl cyclase and cAMP accumulation (Pedragosa Badia et al., 2013). Since CRH causes cAMP production, NPY and CRH often have opposing effects on behavior. In fact, while CRH signaling in the BST promotes alcohol seeking-behaviors (Nentwig et al., 2019), Pleil et al. (2015) found that activation of the Y1 NPY receptor (Y1R) reduced binge alcohol drinking in mice without modifying anxiety-like behavior in the OF test. They demonstrated that Y1R activation inhibited PKA activity and increased the frequency of

inhibitory postsynaptic currents in BST CRH neurons, suggesting that Y1R activation results in the inhibition of CRH signaling. However, signaling through the Y2 NPY receptor (Y2R), which is also coupled to a G_q protein (Misra et al., 2004), has been demonstrated to have the opposite effect, with Thorsell et al. (2002) finding that administration of a Y2R antagonist to the lateral ventricle in rats decreased alcohol consumption. For further discussion about NPY's role in alcohol consumption, see Ch'ng et al. (2018).

β -ENDORPHIN

β -endorphin (β -E) has been demonstrated to have a role in AUDs and binge drinking behaviors, and targets μ -opioid receptors in the VTA or κ -opioid receptors in the NAc (Herz, 1997). Alcohol (EtOH) activates these receptors, which are part of the dopaminergic reward system, potentially starting an addiction cycle (Herz, 1997). Specifically, the BST has been linked to the withdrawal and relapse phases of addiction (Avery et al., 2016). Like CRH, β -E can regulate the HPA axis. CRH upregulates the release of β -E, which binds to G_i protein-coupled μ - and δ -opioid receptors to inhibit the release of CRH in the PVH through a negative feedback loop (for review, see Al-Hasani and Bruchas, 2011; Crews et al., 2015). Nentwig et al. (2019) found sex-specific differences in the effects of β -E on binge drinking behavior, with β -E-deficient female, but not male, mice consuming more alcohol than both β -E-deficient male mice and control female mice. β -E-deficient mice were found to have greater basal *Crh* expression in the BST, which is reduced upon alcohol consumption to control levels. Increased alcohol consumption in mice with increased *Crh* expression suggests that the behavior is driven, at least partially, by a desire to reduce stress (Nentwig et al., 2019).

NOREPINEPHRINE

Norepinephrine (NE), acting through α - and β -adrenergic receptors (ARs), plays a key role in BST CRH neuron regulation. The main sources of NE input to the BST primarily innervate the BSTv and come from the A1, A2, and A5 brainstem nuclei (Crestani et al., 2013). In the AR family of GPCRs, there are three different types— α_1 coupled to G_q proteins, α_2 coupled to $G_{i/o}$ proteins, and β coupled to G_s proteins—with each type being further divided into three subtypes (Bylund, 2013). Specifically, administering α_{2a} -ARs agonists to the BST have been shown to reduce anxiety-like responses in rats in fear-potentiated and light-enhanced startle tests (Schweimer et al., 2005) through inhibiting NE signaling (Buffalari et al., 2012). Harris et al. (2018) found this effect to be specific to the BSTd. α_{2a} -AR agonists administered during restraint stress decreased CRH activation in the BST, with sex-specific effects (Fetterly et al., 2019). While α -ARs mediate glutamatergic inputs into BST CRH neurons in an inhibitory manner (Fetterly et al., 2019), β -ARs have been shown to depolarize BST CRH neurons when treated with NE, leading to greater neuronal activation (Snyder et al., 2019). β -AR

activation occurs during both stress and EtOH administration, further tying the BST to AUDs (Snyder et al., 2019). Snyder et al. (2019) then go on to propose that NE can alter BST CRH neuron excitability through two distinct pathways: one causing excitatory signaling through β -ARs that is sensitive to stress and EtOH exposure, and one causing inhibitory signaling through α -ARs that is not. This directly opposes findings from Fetterly et al. (2019) that administration of an α_{2a} -AR agonist decreased BST CRH cFos expression during restraint stress, and considering the large overlap of authors between the two papers, this suggests a high level of variability in these stress assays and the need to further delineate the distinct roles of different AR types in response to stress.

DOPAMINE

Dopamine (DA) signaling in the BST has been linked to dysregulated CRH signaling and reinstatement of drug-seeking behavior (Silberman and Winder, 2013). Many drugs have been found to increase basal DA levels in the BST (Carboni et al., 2000). Main sources of DA to the BST, and in particular the BSTdl, include the VTA, dorsal raphe nucleus, and periaqueductal area (Park et al., 2012). Kash et al. (2008) found that DA application to the BSTdl operated through D1 and D2-like receptors, and enhanced glutamatergic transmission of BST neurons in a CRHR1-dependent manner. More specifically, based on the location of D1 mRNA, DA release may upregulate local CRH release in the BSTov (Daniel and Rainnie, 2016). This demonstrates the importance of BST CRH neuron activation in areas beyond the regulation of anxiety-like behavior, and CRHR1 antagonists remain a potential avenue for the development of therapeutics that help prevent the reinstatement of drug-seeking behavior.

ENDOCANNABINOIDS

Endocannabinoids (eCBs), primarily 2-arachidonylglycerol (2-AG) and *N*-arachidonylethanolamine (AEA), are lipid ligands that signal through two different types of $G_{i/o}$ protein-coupled cannabinoid receptors (CBRs), CB1R and CB2R, to help regulate the stress response (for review, see Morena et al., 2016). CB1Rs are widely distributed throughout the brain, and are highly expressed in the BSTd (Puente et al., 2010). Centanni et al. (2019) found greater activation of BSTd CRH neurons following alcohol abstinence in female mice. Treatment with a monoacylglycerol (MAG) lipase inhibitor, which enhances endogenous 2-AG levels, prevented the increase in neuronal activation in the BSTd, specifically through an insular-BSTd circuit (Centanni et al., 2019). Because of the importance of BSTd signaling in the regulation of maladaptive behaviors (Lebow and Chen, 2016), targeting CB1R receptors in that region could comprise a new therapeutic target for the treatment of human mental health disorders (Centanni et al., 2019). However, current research into the link between CRH neuron activation and eCBs in the BST is limited and remains an ongoing area of study.

THERAPEUTIC AND TRANSLATIONAL POTENTIAL

Current Applications in Deep Brain Stimulation Therapy in Humans

The role of the BST in sustained fear processing is not limited to rodents. Heightened levels of BST activity in humans have been demonstrated in patients with general anxiety disorder, PTSD, panic disorder, and specific phobias (for review, see Knight and Depue, 2019; Awasthi et al., 2020). While more research into the human BST is needed, there has already been success in treating certain disorders using deep brain stimulation (DBS) to the BST. Luyten et al. (2016) demonstrated in a double-blind crossover study that DBS using electrodes implanted in the BST caused a significant reduction in obsessive-compulsive disorder (OCD) symptoms. While the BST has traditionally been considered outside the scope of OCD neurocircuitry, the success in DBS treatment suggests adaptations to the current model are needed (Luyten, 2020).

Bed nuclei of the stria terminalis DBS was also used to treat a patient with severe MDD and comorbid anorexia nervosa, resulting in significantly reduced scores on depression indices and a reduction in anxiety concerning food and eating behaviors (Blomstedt et al., 2017). In another study, Raymaekers et al. (2017) also found positive effects for the treatment of MDD using BST stimulation. However, sample sizes were limited and more research is needed to more definitely conclude the efficacy of BST DBS for MDD treatment.

Clinical Trials of CRHR1 Antagonists

Based on the plethora of preclinical evidence implicating the CRH signaling pathway in maladaptive behaviors, several clinical studies have investigated CRHR1 antagonists as potential therapeutic targets for the treatment of anxiety, PTSD, and AUDs. However, they had limited success. Two studies have examined the effects of CRHR1 antagonist verucerfont (formerly GSK561679) on alcohol craving (Schwandt et al., 2016) and PTSD (Dunlop et al., 2017), with both studies finding little effect. However, a study of the CRHR1 antagonist pexacerfont found that the effect of the drug on reducing food cravings was worth further study—although it should be noted that this study was stopped early for legal reasons unrelated to the study safety or outcome (Epstein et al., 2016). While these results might suggest reduced translational potential of CRHR1 antagonists for the treatment of anxiety and other mental health disorders, it is important to consider that the drug administration in humans was not specific to a particular brain region, which is a notable difference from preclinical models. Because injecting drugs directly into the human BST is limited in feasibility due to patient burden and the small size of the BST (Knight and Depue, 2019), it is critical to develop CRHR1 antagonists that are specific to the systems they are targeting. Another limitation of clinical trials is that the experimental design might not exactly match what was being tested in the preclinical model, such as the phase of the addiction model in AUDs (Pomrenze et al., 2017). Careful recruiting of patients to match the conditions of the

preclinical model as closely as possible may yield better results. In addition, there could be other factors in human neurobiology that influence CRHR1 antagonist effects that are not found in preclinical models. That is why Grillon et al. (2019) recommend using a combination of preclinical models and experimental psychopathology using healthy human volunteers to aid the process of drug discovery and validation, going so far as to recommend adding a new testing criteria between phase I and phase II of clinical trials based on anxiety-potentiated startle (Grillon and Ernst, 2020).

CONCLUSION AND FUTURE DIRECTIONS

The BST serves as a hub for the regulation of chronic fear responses and other behaviors that can lead to anxiety and mood disorders. The BSTp has mostly anxiolytic effects, largely mediated through CRHR2s. In contrast, the BSTa, particularly the BSTov, has been shown to mediate anxiety-like behavior and is also implicated in mood disorders and drug and alcohol use. In the BSTa, changes to CRH signaling are critical for these effects. Activation by a wide variety of neurotransmitters and receptors were all demonstrated to affect maladaptive behavior in pre-clinical models. Due to the complicated nature of CRH interaction with these neurotransmitters, careful experimental design using standardized testing procedures and nuclei classifications will aid in the interpretation of results and enhance reproducibility across labs. Because there are so many neurotransmitters and receptors that are connected to CRH signaling, targeting CRH and the CRHRs presents a potential

avenue to cover a broad array of pathways in the treatment of anxiety, mood, and substance use disorders. Considering the high impact those disorders have on society and the economy, and the demonstrated impact of BST treatment on some of these disorders, further research is needed to advance the understanding of how changes in CRH signaling impacts these disorders. In particular, considering the sexually dimorphic nature of the BST, and the gender bias in pre-clinical research (Ciccocioppo, 2017), more research is needed involving both male and female rodents to help elucidate sex-specific differences in maladaptive behaviors. While previous clinical trials for CRHR1 antagonists have not been successful for the treatment of cravings, given the complexity of BST CRH signaling, there is still room to investigate its role in other disorders, as well as treatments targeting different receptors or pathways linked to CRH signaling.

AUTHOR CONTRIBUTIONS

CEY wrote and revised the manuscript. QT provided critical feedback regarding content and structure of the manuscript. Both authors contributed to the article and approved the submitted version.

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REFERENCES

- Al-Hasani, R., and Bruchas, M. R. (2011). Molecular mechanisms of opioid receptor-dependent signaling and behavior. *Anesthesiology* 115, 1363–1381. doi: 10.1097/ALN.0b013e318238bba6
- Alheid, G. F., and Heimer, L. (1988). New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: The striatopallidal, amygdaloid, and corticopetal components of substantia innominata. *Neuroscience* 27, 1–39. doi: 10.1016/0306-4522(88)90217-5
- Allredge, B. (2010). Pathogenic involvement of neuropeptides in anxiety and depression. *Neuropeptides* 44, 215–224. doi: 10.1016/j.npep.2009.12.014
- American Psychiatric Association (2013). *Diagnostic and Statistical Manual of Mental Disorders*, 5th Edn. Washington, DC: APA Publishing.
- Avery, S. N., Clauss, J. A., and Blackford, J. U. (2016). The Human BNST: Functional Role in Anxiety and Addiction. *Neuropsychopharmacology* 41, 126–141. doi: 10.1038/npp.2015.185
- Awasthi, S., Pan, H., LeDoux, J. E., Cloitre, M., Altemus, M., McEwen, B., et al. (2020). The bed nucleus of the stria terminalis and functionally linked neurocircuitry modulate emotion processing and HPA axis dysfunction in posttraumatic stress disorder. *NeuroImage Clin.* 28:102442. doi: 10.1016/j.nicl.2020.102442
- Bale, T. L., and Vale, W. W. (2004). CRF and CRF Receptors: Role in Stress Responsivity and Other Behaviors. *Annu. Rev. Pharmacol. Toxicol.* 44, 525–557. doi: 10.1146/annurev.pharmtox.44.101802.121410
- Bartsch, J. C., Jamil, S., Remmes, J., Verma, D., and Pape, H.-C. (2020). Functional deletion of neuropeptide Y receptors type 2 in local synaptic networks of anteroventral BNST facilitates recall and increases return of fear. *Mol. Psychiatr.* Preprint. doi: 10.1038/s41380-020-0846-x
- Betley, J. N., Cao, Z. F. H., Ritola, K. D., and Sternson, S. M. (2013). Parallel, redundant circuit organization for homeostatic control of feeding behavior. *Cell* 155, 1337–1350. doi: 10.1016/j.cell.2013.11.002
- Blomstedt, P., Naesström, M., and Bodlund, O. (2017). Deep brain stimulation in the bed nucleus of the stria terminalis and medial forebrain bundle in a patient with major depressive disorder and anorexia nervosa. *Clin. Case Rep.* 5, 679–684. doi: 10.1002/ccr3.856
- Bota, M., Sporns, O., and Swanson, L. W. (2012). Neuroinformatics analysis of molecular expression patterns and neuron populations in gray matter regions: The rat BST as a rich exemplar. *Brain Res.* 1450, 174–193. doi: 10.1016/j.brainres.2012.02.034
- Boutrel, B., and de Lecea, L. (2008). Addiction and arousal: The hypocretin connection. *Physiol. Behav.* 93, 947–951. doi: 10.1016/j.physbeh.2007.11.022
- Bowen, M. T., Carson, D. S., Spiro, A., Arnold, J. C., and McGregor, I. S. (2011). Adolescent Oxytocin Exposure Causes Persistent Reductions in Anxiety and Alcohol Consumption and Enhances Sociability in Rats. *PLoS One* 6:e27237. doi: 10.1371/journal.pone.0027237
- Buffalari, D. M., Baldwin, C. K., and See, R. E. (2012). Treatment of cocaine withdrawal anxiety with guanfacine: relationships to cocaine intake and reinstatement of cocaine seeking in rats. *Psychopharmacology* 223, 179–190. doi: 10.1007/s00213-012-2705-1
- Bylund, D. B. (2013). “Adrenergic Receptors,” in *Encyclopedia of Biological Chemistry*, 2nd Edn, eds W. J. Lennarz and M. D. Lane (Waltham: Academic Press), 57–60.
- Cannella, N., Economidou, D., Kallupi, M., Stopponi, S., Heilig, M., Massi, M., et al. (2009). Persistent Increase of Alcohol-Seeking Evoked by Neuropeptide S: an Effect Mediated by the Hypothalamic Hypocretin System. *Neuropsychopharmacology* 34, 2125–2134. doi: 10.1038/npp.2009.37

- Carboni, E., Silvagni, A., Rolando, M. T. P., and Di Chiara, G. (2000). Stimulation of α -& In Vivo α -Dopamine Transmission in the Bed Nucleus of Stria Terminalis by Reinforcing Drugs. *J. Neurosci.* 20:RC102. doi: 10.1523/JNEUROSCI.20-20-j0002.2000
- Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., and Renzi, P. (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behaviour. Brain Res.* 134, 49–57. doi: 10.1016/S0166-4328(01)00452-1
- Centanni, S. W., Morris, B. D., Luchsinger, J. R., Bedse, G., Fetterly, T. L., Patel, S., et al. (2019). Endocannabinoid control of the insular-bed nucleus of the stria terminalis circuit regulates negative affective behavior associated with alcohol abstinence. *Neuropsychopharmacology* 44, 526–537. doi: 10.1038/s41386-018-0257-8
- Chater, T. E., and Goda, Y. (2014). The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity. *Front. Cell. Neurosci.* 8:401. doi: 10.3389/fncel.2014.00401
- Ch'ng, S., Fu, J., Brown, R. M., McDougall, S. J., and Lawrence, A. J. (2018). The intersection of stress and reward: BNST modulation of aversive and appetitive states. *Prog. Neuro Psychopharmacol. Biol. Psychiatr.* 87, 108–125. doi: 10.1016/j.pnpbp.2018.01.005
- Ciccocioppo, R. (2017). Grand Challenge in Psychopharmacology: Setting Priorities to Shape a Bright Future. *Front. Psychiatr.* 8:15. doi: 10.3389/fpsyt.2017.00015
- Commons, K. G., Connolly, K. R., and Valentino, R. J. (2003). A Neurochemically Distinct Dorsal Raphe-Limbic Circuit with a Potential Role in Affective Disorders. *Neuropsychopharmacology* 28, 206–215. doi: 10.1038/sj.npp.1300045
- Craske, M. G., Stein, M. B., Eley, T. C., Milad, M. R., Holmes, A., Rapee, R. M., et al. (2017). Anxiety disorders. *Nat. Rev. Dis. Primers* 3:17024. doi: 10.1038/nrdp.2017.24
- Crestani, C. C., Alves, F. H., Gomes, F. V., Resstel, L. B., Correa, F. M., and Herman, J. P. (2013). Mechanisms in the bed nucleus of the stria terminalis involved in control of autonomic and neuroendocrine functions: a review. *Curr. Neuropharmacol.* 11, 141–159. doi: 10.2174/1570159X11311020002
- Crews, F. T., Sarkar, D. K., Qin, L., Zou, J., Boyadjieva, N., and Vetreno, R. P. (2015). Neuroimmune Function and the Consequences of Alcohol Exposure. *Alcohol Res. Curr. Rev.* 37, 331–351.
- Dabrowska, J., Hazra, R., Ahern, T. H., Guo, J.-D., McDonald, A. J., Mascagni, F., et al. (2011). Neuroanatomical evidence for reciprocal regulation of the corticotrophin-releasing factor and oxytocin systems in the hypothalamus and the bed nucleus of the stria terminalis of the rat: Implications for balancing stress and affect. *Psychoneuroendocrinology* 36, 1312–1326. doi: 10.1016/j.psyneuen.2011.03.003
- Dabrowska, J., Hazra, R., Guo, J., DeWitt, S., and Rainnie, D. (2013). Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. *Front. Neurosci.* 7:156. doi: 10.3389/fnins.2013.00156
- Daldrup, T., Lesting, J., Meuth, P., Seidenbecher, T., and Pape, H.-C. (2016). Neuronal correlates of sustained fear in the anterolateral part of the bed nucleus of stria terminalis. *Neurobiol. Learning Memory* 131, 137–146. doi: 10.1016/j.nlm.2016.03.020
- Daly, E. J., Trivedi, M. H., Janik, A., Li, H., Zhang, Y., Li, X., et al. (2019). Efficacy of Esketamine Nasal Spray Plus Oral Antidepressant Treatment for Relapse Prevention in Patients With Treatment-Resistant Depression: A Randomized Clinical Trial. *JAMA Psychiatr.* 76, 893–903. doi: 10.1001/jamapsychiatry.2019.1189
- Daniel, S. E., and Rainnie, D. G. (2016). Stress Modulation of Opposing Circuits in the Bed Nucleus of the Stria Terminalis. *Neuropsychopharmacol. Offic. Publicat. Am. College Neuropsychopharmacol.* 41, 103–125. doi: 10.1038/npp.2015.178
- Davis, M., Walker, D. L., Miles, L., and Grillon, C. (2010). Phasic vs Sustained Fear in Rats and Humans: Role of the Extended Amygdala in Fear vs Anxiety. *Neuropsychopharmacology* 35, 105–135. doi: 10.1038/npp.2009.109
- de Vries, G. J., and Miller, M. A. (1999). "Chapter 1.1 Anatomy and function of extrahypothalamic vasopressin systems in the brain," in *Progress in Brain Research*, eds I. J. A. Urban, J. P. H. Burbach, and D. De Wied (Elsevier), 3–20.
- Dinan, T. G., and Scott, L. V. (2005). Anatomy of melancholia: focus on hypothalamic-pituitary-adrenal axis overactivity and the role of vasopressin. *J. Anatomy* 207, 259–264. doi: 10.1111/j.1469-7580.2005.00443.x
- Dong, H.-W., Petrovich, G. D., Watts, A. G., and Swanson, L. W. (2001). Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *J. Comparat. Neurol.* 436, 430–455. doi: 10.1002/cne.1079
- Donner, N. C., Davies, S. M., Fitz, S. D., Kienzle, D. M., Shekhar, A., and Lowry, C. A. (2020). Crh receptor priming in the bed nucleus of the stria terminalis (BNST) induces tph2 gene expression in the dorsomedial dorsal raphe nucleus and chronic anxiety. *Prog. Neuro Psychopharmacol. Biol. Psychiatr.* 96:109730. doi: 10.1016/j.pnpbp.2019.109730
- Donner, N. C., Kubala, K. H., Hassell, J. E. Jr., Lieb, M. W., Nguyen, K. T., Heinze, J. D., et al. (2018). Two models of inescapable stress increase tph2 mRNA expression in the anxiety-related dorsomedial part of the dorsal raphe nucleus. *Neurobiol. Stress* 8, 68–81. doi: 10.1016/j.ynstr.2018.01.003
- Dunlop, B. W., Binder, E. B., Isifescu, D., Mathew, S. J., Neylan, T. C., Pape, J. C., et al. (2017). Corticotropin-Releasing Factor Receptor 1 Antagonism Is Ineffective for Women With Posttraumatic Stress Disorder. *Biol. Psychiatr.* 82, 866–874. doi: 10.1016/j.biopsych.2017.06.024
- Duque-Wilckens, N., Steinman, M. Q., Busnelli, M., Chini, B., Yokoyama, S., Pham, M., et al. (2018). Oxytocin Receptors in the Anteromedial Bed Nucleus of the Stria Terminalis Promote Stress-Induced Social Avoidance in Female California Mice. *Biol. Psychiatr.* 83, 203–213. doi: 10.1016/j.biopsych.2017.08.024
- Duque-Wilckens, N., Steinman, M. Q., Laredo, S. A., Hao, R., Perkeybile, A. M., Bales, K. L., et al. (2016). Inhibition of vasopressin V1a receptors in the medioventral bed nucleus of the stria terminalis has sex- and context-specific anxiogenic effects. *Neuropharmacology* 110, 59–68. doi: 10.1016/j.neuropharm.2016.07.018
- Ellacott, K. L. J., Morton, G. J., Woods, S. C., Tso, P., and Schwartz, M. W. (2010). Assessment of feeding behavior in laboratory mice. *Cell Metab.* 12, 10–17. doi: 10.1016/j.cmet.2010.06.001
- Epstein, D. H., Kennedy, A. P., Furnari, M., Heilig, M., Shaham, Y., Phillips, K. A., et al. (2016). Effect of the CRF1-receptor antagonist pexacerfont on stress-induced eating and food craving. *Psychopharmacology* 233, 3921–3932. doi: 10.1007/s00213-016-4424-5
- Erb, S., and Stewart, J. (1999). A Role for the Bed Nucleus of the Stria Terminalis, But Not the Amygdala, in the Effects of Corticotropin-Releasing Factor on Stress-Induced Reinstatement of Cocaine Seeking. *J. Neurosci.* 19:RC35. doi: 10.1523/JNEUROSCI.19-20-j0006.1999
- Ferragud, A., Velazquez-Sanchez, C., Minnig, M. A., Sabino, V., and Cottone, P. (2020). Pituitary adenylate cyclase-activating polypeptide (PACAP) modulates dependence-induced alcohol drinking and anxiety-like behavior in male rats. *Neuropsychopharmacology* 46, 509–518. doi: 10.1038/s41386-020-00904-4
- Fetterly, T. L., Basu, A., Nabit, B. P., Awad, E., Williford, K. M., Centanni, S. W., et al. (2019). α_{2A} -Adrenergic Receptor Activation Decreases Parabrachial Nucleus Excitatory Drive onto BNST CRF Neurons and Reduces Their Activity In Vivo. *J. Neurosci.* 39, 472–484. doi: 10.1523/jneurosci.1035-18.2018
- Garcia-Garcia, A. L., Canetta, S., Stujenske, J. M., Burghardt, N. S., Ansorge, M. S., Dranovsky, A., et al. (2018). Serotonin inputs to the dorsal BNST modulate anxiety in a 5-HT1A receptor-dependent manner. *Mol. Psychiatr.* 23, 1990–1997. doi: 10.1038/mp.2017.165
- Ghasemi, M., Phillips, C., Trillo, L., De Miguel, Z., Das, D., and Salehi, A. (2014). The role of NMDA receptors in the pathophysiology and treatment of mood disorders. *Neurosci. Biobehav. Rev.* 47, 336–358. doi: 10.1016/j.neubiorev.2014.08.017
- Ghazal, P., Ciccocioppo, R., and Ubaldi, M. (2013). Morphine dependence is associated with changes in neuropeptide S receptor expression and function in rat brain. *Peptides* 46, 6–12. doi: 10.1016/j.peptides.2013.05.001
- Giardino, W. J., Eban-Rothschild, A., Christoffel, D. J., Li, S.-B., Malenka, R. C., and de Lecea, L. (2018). Parallel circuits from the bed nuclei of stria terminalis to the lateral hypothalamus drive opposing emotional states. *Nat. Neurosci.* 21, 1084–1095. doi: 10.1038/s41593-018-0198-x
- Gracy, K. N., and Pickel, V. M. (1995). Comparative ultrastructural localization of the NMDAR1 glutamate receptor in the rat basolateral amygdala and bed nucleus of the stria terminalis. *J. Comparat. Neurol.* 362, 71–85. doi: 10.1002/cne.903620105
- Grillon, C., and Ernst, M. (2020). A way forward for anxiolytic drug development: Testing candidate anxiolytics with anxiety-potentiated startle in healthy humans. *Neurosci. Biobehav. Rev.* 119, 348–354. doi: 10.1016/j.neubiorev.2020.09.024

- Grillon, C., Robinson, O. J., Cornwell, B., and Ernst, M. (2019). Modeling anxiety in healthy humans: a key intermediate bridge between basic and clinical sciences. *Neuropsychopharmacology* 44, 1999–2010. doi: 10.1038/s41386-019-0445-1
- Grueter, B. A., and Winder, D. G. (2005). Group II and III Metabotropic Glutamate Receptors Suppress Excitatory Synaptic Transmission in the Dorsolateral Bed Nucleus of the Stria Terminalis. *Neuropsychopharmacology* 30, 1302–1311. doi: 10.1038/sj.npp.1300672
- Grueter, B. A., Gosnell, H. B., Olsen, C. M., Schramm-Sapota, N. L., Nekrasova, T., Landreth, G. E., et al. (2006). Extracellular-Signal Regulated Kinase 1-Dependent Metabotropic Glutamate Receptor 5-Induced Long-Term Depression in the Bed Nucleus of the Stria Terminalis Is Disrupted by Cocaine Administration. *J. Neurosci.* 26, 3210–3219. doi: 10.1523/jneurosci.0170-06.2006
- Gungor, N. Z., and Paré, D. (2016). Functional Heterogeneity in the Bed Nucleus of the Stria Terminalis. *J. Neurosci.* 36:8038. doi: 10.1523/JNEUROSCI.0856-16.2016
- Hammack, S. E., Cheung, J., Rhodes, K. M., Schutz, K. C., Falls, W. A., Braas, K. M., et al. (2009a). Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of the stria terminalis (BNST): Roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology* 34, 833–843. doi: 10.1016/j.psyneuen.2008.12.013
- Hammack, S. E., Guo, J.-D., Hazra, R., Dabrowska, J., Myers, K. M., and Rainnie, D. G. (2009b). The response of neurons in the bed nucleus of the stria terminalis to serotonin: Implications for anxiety. *Prog. Neuro Psychopharmacol. Biol. Psychiatry* 33, 1309–1320. doi: 10.1016/j.pnpbp.2009.05.013
- Hammack, S. E., Mania, I., and Rainnie, D. G. (2007). Differential expression of intrinsic membrane currents in defined cell types of the anterolateral bed nucleus of the stria terminalis. *J. Neurophysiol.* 98, 638–656. doi: 10.1152/jn.00382.2007
- Harris, N. A., Isaac, A. T., Günther, A., Merkel, K., Melchior, J., Xu, M., et al. (2018). Dorsal BNST α_{2A} -Adrenergic Receptors Produce HCN-Dependent Excitatory Actions That Initiate Anxiogenic Behaviors. *J. Neurosci.* 38, 8922–8942. doi: 10.1523/jneurosci.0963-18.2018
- Henckens, M. J. A. G., Printz, Y., Shamgar, U., Dine, J., Lebow, M., Drori, Y., et al. (2017). CRF receptor type 2 neurons in the posterior bed nucleus of the stria terminalis critically contribute to stress recovery. *Mol. Psychiatry* 22, 1691–1700. doi: 10.1038/mp.2016.133
- Hernando, F., Schoots, O., Lolait, S. J., and Burbach, J. P. (2001). Immunohistochemical localization of the vasopressin V1b receptor in the rat brain and pituitary gland: anatomical support for its involvement in the central effects of vasopressin. *Endocrinology* 142, 1659–1668. doi: 10.1210/endo.142.4.8067
- Herz, A. (1997). Endogenous opioid systems and alcohol addiction. *Psychopharmacology* 129, 99–111. doi: 10.1007/s002130050169
- Higuchi, H. (2012). Molecular analysis of central feeding regulation by neuropeptide Y (NPY) neurons with NPY receptor small interfering RNAs (siRNAs). *Neurochem. Int.* 61, 936–941. doi: 10.1016/j.neuint.2012.02.029
- Hillhouse, E. W., and Grammatopoulos, D. K. (2006). The Molecular Mechanisms Underlying the Regulation of the Biological Activity of Corticotropin-Releasing Hormone Receptors: Implications for Physiology and Pathophysiology. *Endocr. Rev.* 27, 260–286. doi: 10.1210/er.2005-0034
- Hu, P., Liu, J., Maita, I., Kwok, C., Gu, E., Gergues, M. M., et al. (2020). Chronic Stress Induces Maladaptive Behaviors by Activating Corticotropin-Releasing Hormone Signaling in the Mouse Oval Bed Nucleus of the Stria Terminalis. *J. Neurosci.* 40, 2519–2537. doi: 10.1523/jneurosci.2410-19.2020
- Janeček, M., and Dabrowska, J. (2019). Oxytocin facilitates adaptive fear and attenuates anxiety responses in animal models and human studies—potential interaction with the corticotropin-releasing factor (CRF) system in the bed nucleus of the stria terminalis (BNST). *Cell Tissue Res.* 375, 143–172. doi: 10.1007/s00441-018-2889-8
- Ju, G., and Swanson, L. W. (1989). Studies on the cellular architecture of the bed nuclei of the stria terminalis in the rat: I. cytoarchitecture. *J. Comparat. Neurol.* 280, 587–602. doi: 10.1002/cne.902800409
- Ju, G., Swanson, L. W., and Simerly, R. B. (1989). Studies on the cellular architecture of the bed nuclei of the stria terminalis in the rat: II. chemoarchitecture. *J. Comparat. Neurol.* 280, 603–621. doi: 10.1002/cne.902800410
- Kash, T. L., Nobis, W. P., Matthews, R. T., and Winder, D. G. (2008). Dopamine Enhances Fast Excitatory Synaptic Transmission in the Extended Amygdala by a CRF-R1-Dependent Process. *J. Neurosci.* 28:13856. doi: 10.1523/JNEUROSCI.4715-08.2008
- Kash, T. L., Pleil, K. E., Marcinkiewicz, C. A., Lowery-Gionta, E. G., Crowley, N., Mazzone, C., et al. (2015). Neuropeptide regulation of signaling and behavior in the BNST. *Mol. Cells* 38, 1–13. doi: 10.14348/molcells.2015.2261
- Kasten, C. R., Carzoli, K. L., Sharfman, N. M., Henderson, T., Holmgren, E. B., Lerner, M. R., et al. (2020). Adolescent alcohol exposure produces sex differences in negative affect-like behavior and group I mGluR BNST plasticity. *Neuropsychopharmacology* 45, 1306–1315. doi: 10.1038/s41386-020-0670-7
- Kim, S.-Y., Adhikari, A., Lee, S. Y., Marshel, J. H., Kim, C. K., Mallory, C. S., et al. (2013). Diverging neural pathways assemble a behavioural state from separable features in anxiety. *Nature* 496, 219–223. doi: 10.1038/nature12018
- King, S. B., Lezak, K. R., O'Reilly, M., Toufexis, D. J., Falls, W. A., Braas, K., et al. (2017). The Effects of Prior Stress on Anxiety-Like Responding to Intra-BNST Pituitary Adenylate Cyclase Activating Polypeptide in Male and Female Rats. *Neuropsychopharmacology* 42, 1679–1687. doi: 10.1038/npp.2017.16
- Knight, L. K., and Depue, B. E. (2019). New Frontiers in Anxiety Research: The Translational Potential of the Bed Nucleus of the Stria Terminalis. *Front. Psychiatr.* 10:510. doi: 10.3389/fpsyt.2019.00510
- Kocho-Schellenberg, M., Lezak, K. R., Harris, O. M., Roelke, E., Gick, N., Choi, I., et al. (2014). PACAP in the BNST Produces Anorexia and Weight Loss in Male and Female Rats. *Neuropsychopharmacology* 39, 1614–1623. doi: 10.1038/npp.2014.8
- Koob, G. F., and Greenwell, T. N. (2004). Neuropeptide S: A Novel Activating Anxiolytic? *Neuron* 43, 441–442. doi: 10.1016/j.neuron.2004.08.007
- Kormos, V., and Gaszner, B. (2013). Role of neuropeptides in anxiety, stress, and depression: From animals to humans. *Neuropeptides* 47, 401–419. doi: 10.1016/j.npep.2013.10.014
- Kozicz, T., Vigh, S., and Arimura, A. (1998). The source of origin of PACAP- and VIP-immunoreactive fibers in the laterodorsal division of the bed nucleus of the stria terminalis in the rat. *Brain Res.* 810, 211–219. doi: 10.1016/S0006-8993(98)00692-1
- Krishnan, V., and Nestler, E. J. (2011). Animal models of depression: molecular perspectives. *Curr. Topics Behav. Neurosci.* 7, 121–147. doi: 10.1007/7854_2010_108
- Lebow, M. A., and Chen, A. (2016). Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol. Psychiatr.* 21, 450–463. doi: 10.1038/mp.2016.1
- Lebow, M., Neufeld-Cohen, A., Kuperman, Y., Tsoory, M., Gil, S., and Chen, A. (2012). Susceptibility to PTSD-Like Behavior Is Mediated by Corticotropin-Releasing Factor Receptor Type 2 Levels in the Bed Nucleus of the Stria Terminalis. *J. Neurosci.* 32, 6906–6916. doi: 10.1523/jneurosci.4012-11.2012
- LeDoux, J. E., and Pine, D. S. (2016). Using Neuroscience to Help Understand Fear and Anxiety: A Two-System Framework. *Am. J. Psychiatry* 173, 1083–1093. doi: 10.1176/appi.ajp.2016.16030353
- Lee, H.-J., Macbeth, A. H., Pagani, J. H., and Young, W. S. III (2009). Oxytocin: the great facilitator of life. *Prog. Neurobiol.* 88, 127–151. doi: 10.1016/j.pneurobio.2009.04.001
- Leeuwen, F., and Caffé, R. (1983). Immunoreactive vasopressin cell bodies in the rat bed nucleus of the stria terminalis. *Cell Tissue Res.* 228, 525–534. doi: 10.1007/BF00211473
- Lein, E. S., Hawrylycz, M. J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., et al. (2007). Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445, 168–176. doi: 10.1038/nature05453
- Leonard, S. K., and Ring, R. H. (2011). Immunohistochemical localization of the neuropeptide S receptor in the rat central nervous system. *Neuroscience* 172, 153–163. doi: 10.1016/j.neuroscience.2010.10.020
- Levita, L., Hammack, S. E., Mania, I., Li, X. Y., Davis, M., and Rainnie, D. G. (2004). 5-hydroxytryptamine_{1A}-likereceptor activation in the bed nucleus of the stria terminalis: Electrophysiological and behavioral studies. *Neuroscience* 128, 583–596. doi: 10.1016/j.neuroscience.2004.06.037
- Lowry, C. A., Rodda, J. E., Lightman, S. L., and Ingram, C. D. (2000). Corticotropin-Releasing Factor Increases *In Vitro* Firing Rates of Serotonergic Neurons in the Rat Dorsal Raphe Nucleus: Evidence for Activation of a Topographically Organized Mesolimbocortical Serotonergic System. *J. Neurosci.* 20, 7728–7736. doi: 10.1523/jneurosci.20-07728.2000

- Luyten, L. (2020). The Bed Nucleus of the Stria Terminalis: Translational Deep Brain Stimulation to Reduce Anxiety. *Neuroscientist* 26, 278–284. doi: 10.1177/1073858419898381
- Luyten, L., Hendrickx, S., Raymaekers, S., Gabriëls, L., and Nuttin, B. (2016). Electrical stimulation in the bed nucleus of the stria terminalis alleviates severe obsessive-compulsive disorder. *Mol. Psychiatry* 21, 1272–1280. doi: 10.1038/mp.2015.124
- Marcinkiewicz, C. A., Bierlein-De, La Rosa, G., Dorrier, C. E., McKnight, M., DiBerto, J. F., et al. (2019). Sex-Dependent Modulation of Anxiety and Fear by 5-HT_{1A} Receptors in the Bed Nucleus of the Stria Terminalis. *ACS Chem. Neurosci.* 10, 3154–3166. doi: 10.1021/acschemneuro.8b00594
- Marcinkiewicz, C. A., Mazzone, C. M., D'Agostino, G., Halladay, L. R., Hardaway, J. A., DiBerto, J. F., et al. (2016). Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala. *Nature* 537, 97–101. doi: 10.1038/nature19318
- Misra, S., Murthy, K. S., Zhou, H., and Grider, J. R. (2004). Coexpression of Y₁, Y₂, and Y₄ Receptors in Smooth Muscle Coupled to Distinct Signaling Pathways. *J. Pharmacol. Exp. Therapeut.* 311, 1154–1162. doi: 10.1124/jpet.104.071415
- Moga, M. M., and Saper, C. B. (1994). Neuropeptide-immunoreactive neurons projecting to the paraventricular hypothalamic nucleus in the rat. *J. Comparat. Neurol.* 346, 137–150. doi: 10.1002/cne.903460110
- Morena, M., Patel, S., Bains, J. S., and Hill, M. N. (2016). Neurobiological Interactions Between Stress and the Endocannabinoid System. *Neuropsychopharmacology* 41, 80–102. doi: 10.1038/npp.2015.166
- Nentwig, T. B., Wilson, D. E., Rhinehart, E. M., and Grisel, J. E. (2019). Sex differences in binge-like EtOH drinking, corticotropin-releasing hormone and corticosterone: effects of β -endorphin. *Addict. Biol.* 24, 447–457. doi: 10.1111/adb.12610
- Pañeda, C., Huitron-Resendiz, S., Frago, L. M., Chowen, J. A., Picetti, R., de Lecea, L., et al. (2009). Neuropeptide S reinstates cocaine-seeking behavior and increases locomotor activity through corticotropin-releasing factor receptor 1 in mice. *J. Neurosci.* 29, 4155–4161. doi: 10.1523/JNEUROSCI.5256-08.2009
- Park, J., Wheeler, R. A., Fontillas, K., Keithley, R. B., Carelli, R. M., and Wightman, R. M. (2012). Catecholamines in the Bed Nucleus of the Stria Terminalis Reciprocally Respond to Reward and Aversion. *Biol. Psychiatry* 71, 327–334. doi: 10.1016/j.biopsych.2011.10.017
- Pedragosa Badia, X., Stichel, J., and Beck-Sickinger, A. (2013). Neuropeptide Y receptors: how to get subtype selectivity. *Front. Endocrinol.* 4:5. doi: 10.3389/fendo.2013.00005
- Pisegna, J. R., and Wank, S. A. (1996). Cloning and Characterization of the Signal Transduction of Four Splice Variants of the Human Pituitary Adenylate Cyclase Activating Polypeptide Receptor: EVIDENCE FOR DUAL COUPLING TO ADENYLATE CYCLASE AND PHOSPHOLIPASE C. *J. Biol. Chem.* 271, 17267–17274.
- Pleil, K. E., Rinker, J. A., Lowery-Gionta, E. G., Mazzone, C. M., McCall, N. M., Kendra, A. M., et al. (2015). NPY signaling inhibits extended amygdala CRF neurons to suppress binge alcohol drinking. *Nat. Neurosci.* 18, 545–552. doi: 10.1038/nn.3972
- Pomrenze, M. B., Fetterly, T. L., Winder, D. G., and Messing, R. O. (2017). The Corticotropin Releasing Factor Receptor 1 in Alcohol Use Disorder: Still a Valid Drug Target? *Alcohol. Clin. Exp. Res.* 41, 1986–1999. doi: 10.1111/acer.13507
- Pomrenze, M. B., Tovar-Diaz, J., Blasio, A., Maiya, R., Giovannetti, S. M., Lei, K., et al. (2019). A Corticotropin Releasing Factor Network in the Extended Amygdala for Anxiety. *J. Neurosci.* 39, 1030–1043. doi: 10.1523/jneurosci.2143-18.2018
- Popova, V., Daly, E. J., Trivedi, M., Cooper, K., Lane, R., Lim, P., et al. (2019). Efficacy and Safety of Flexibly Dosed Esketamine Nasal Spray Combined With a Newly Initiated Oral Antidepressant in Treatment-Resistant Depression: A Randomized Double-Blind Active-Controlled Study. *Am. J. Psychiatry* 176, 428–438. doi: 10.1176/appi.ajp.2019.19020172
- Puente, N., Elezgarai, I., Lafourcade, M., Reguero, L., Marsicano, G., Georges, F., et al. (2010). Localization and function of the cannabinoid CB₁ receptor in the anterolateral bed nucleus of the stria terminalis. *PLoS One* 5:e8869–e8869. doi: 10.1371/journal.pone.0008869
- Quentin, E., Belmer, A., and Maroteaux, L. (2018). Somato-Dendritic Regulation of Raphe Serotonin Neurons: A Key to Antidepressant Action. *Front. Neurosci.* 12:982. doi: 10.3389/fnins.2018.00982
- Raymaekers, S., Luyten, L., Bervoets, C., Gabriëls, L., and Nuttin, B. (2017). Deep brain stimulation for treatment-resistant major depressive disorder: a comparison of two targets and long-term follow-up. *Translat. Psychiatry* 7, 1251–1251e. doi: 10.1038/tp.2017.66
- Sahuque, L. L., Kullberg, E. F., McGeehan, A. J., Kinder, J. R., Hicks, M. P., Blanton, M. G., et al. (2006). Anxiogenic and aversive effects of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis in the rat: role of CRF receptor subtypes. *Psychopharmacology* 186, 122–132. doi: 10.1007/s00213-006-0362-y
- Salimando, G. J., Hyun, M., Boyt, K. M., and Winder, D. G. (2020). BNST GluN2D-Containing NMDA Receptors Influence Anxiety- and Depressive-like Behaviors and Modulate Cell-Specific Excitatory/Inhibitory Synaptic Balance. *J. Neurosci.* 40, 3949–3968. doi: 10.1523/jneurosci.0270-20.2020
- Sanchez-Bautista, S., and Nicolas, F. E. (2013). Recent patents concerning modulators of protein kinase C. *Recent Pat. DNA Gene Seq.* 7, 74–81. doi: 10.2174/1872215611307010011
- Schatzberg, A. F. (2019). A Word to the Wise About Intranasal Esketamine. *Am. J. Psychiatry* 176, 422–424. doi: 10.1176/appi.ajp.2019.19040423
- Schwandt, M. L., Cortes, C. R., Kwako, L. E., George, D. T., Momenan, R., Sinha, R., et al. (2016). The CRF1 Antagonist Vercurfont in Anxious Alcohol-Dependent Women: Translation of Neuroendocrine, But not of Anti-Craving Effects. *Neuropsychopharmacology* 41, 2818–2829. doi: 10.1038/npp.2016.61
- Schweimer, J., Fendt, M., and Schnitzler, H.-U. (2005). Effects of clonidine injections into the bed nucleus of the stria terminalis on fear and anxiety behavior in rats. *Eur. J. Pharmacol.* 507, 117–124. doi: 10.1016/j.ejphar.2004.11.044
- Shackman, A. J., and Fox, A. S. (2016). Contributions of the Central Extended Amygdala to Fear and Anxiety. *J. Neurosci.* 36, 8050–8063. doi: 10.1523/jneurosci.0982-16.2016
- Silberman, Y., and Winder, D. (2013). Emerging Role for Corticotropin Releasing Factor Signaling in the Bed Nucleus of the Stria Terminalis at the Intersection of Stress and Reward. *Front. Psychiatry* 4:42. doi: 10.3389/fpsy.2013.00042
- Snyder, A. E., Salimando, G. J., Winder, D. G., and Silberman, Y. (2019). Chronic Intermittent Ethanol and Acute Stress Similarly Modulate BNST CRF Neuron Activity via Noradrenergic Signaling. *Alcoholism Clin. Exp. Res.* 43, 1695–1701. doi: 10.1111/acer.14118
- Soya, S., and Sakurai, T. (2020). Evolution of Orexin Neuropeptide System: Structure and Function. *Front. Neurosci.* 14:691. doi: 10.3389/fnins.2020.00691
- Steinman, M. Q., Duque-Wilckens, N., and Trainor, B. C. (2019). Complementary Neural Circuits for Divergent Effects of Oxytocin: Social Approach Versus Social Anxiety. *Biol. Psychiatry* 85, 792–801. doi: 10.1016/j.biopsych.2018.10.008
- Swanson, L. W. (2004). *Brain Maps: Structure of the Rat Brain, A Laboratory Guide with Printed and Electronic Templates for Data, Models and Schematics*. Amsterdam: Elsevier.
- Thorsell, A., Rimondini, R., and Heilig, M. (2002). Blockade of central neuropeptide Y (NPY) Y₂ receptors reduces ethanol self-administration in rats. *Neurosci. Lett.* 332, 1–4. doi: 10.1016/S0304-3940(02)00904-7
- US Food and Drug Administration (2019). *FDA approves new nasal spray medication for treatment-resistant depression; available only at a certified doctor's office or clinic*. Washington, D.C.: US Food and Drug Administration.
- Van Pett, K., Viau, V., Bittencourt, J. C., Chan, R. K. W., Li, H.-Y., Arias, C., et al. (2000). Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J. Comparat. Neurol.* 428, 191–212. doi: 10.1002/1096-9861(20001211)428:2<191::AID-CNE1<3.0.CO;2-U
- Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M. H., Lewis, K., Sutton, S., et al. (1995). Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* 378, 287–292. doi: 10.1038/378287a0
- Veinante, P., Stoessel, M. E., and Freund-Mercier, M. J. (1997). GABA- and peptide-immunoreactivities co-localize in the rat central extended amygdala. *Neuroreport* 8, 2985–2989. doi: 10.1097/00001756-199709080-00035
- Ventura-Silva, A. P., Borges, S., Sousa, N., Rodrigues, A. J., and Pêgo, J. M. (2020). Amygdalar corticotropin-releasing factor mediates stress-induced anxiety. *Brain Res.* 1729:146622. doi: 10.1016/j.brainres.2019.146622
- Vranjkovic, O., Pina, M., Kash, T. L., and Winder, D. G. (2017). The bed nucleus of the stria terminalis in drug-associated behavior and affect: A circuit-based

- perspective. *Neuropharmacology* 122, 100–106. doi: 10.1016/j.neuropharm.2017.03.028
- Walker, D. L., and Davis, M. (1997). Double Dissociation between the Involvement of the Bed Nucleus of the Stria Terminalis and the Central Nucleus of the Amygdala in Startle Increases Produced by Conditioned versus Unconditioned Fear. *J. Neurosci.* 17, 9375–9383. doi: 10.1523/jneurosci.17-23-09375.1997
- Walker, D. L., Toufexis, D. J., and Davis, M. (2003). Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety. *Eur. J. Pharmacol.* 463, 199–216. doi: 10.1016/S0014-2999(03)01282-2
- Wang, Y., Kim, J., Schmit, M. B., Cho, T. S., Fang, C., and Cai, H. (2019). A bed nucleus of stria terminalis microcircuit regulating inflammation-associated modulation of feeding. *Nat. Commun.* 10:2769. doi: 10.1038/s41467-019-10715-x
- Xu, Y.-L., Gall, C. M., Jackson, V. R., Civelli, O., and Reinscheid, R. K. (2007). Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S-expressing neurons in the rat brain. *J. Comparat. Neurol.* 500, 84–102. doi: 10.1002/cne.21159
- Xu, Y.-L., Reinscheid, R. K., Huitron-Resendiz, S., Clark, S. D., Wang, Z., Lin, S. H., et al. (2004). Neuropeptide S: A Neuropeptide Promoting Arousal and Anxiolytic-like Effects. *Neuron* 43, 487–497. doi: 10.1016/j.neuron.2004.08.005
- Zheng, W., Cai, D.-B., Xiang, Y.-Q., Zheng, W., Jiang, W.-L., Sim, K., et al. (2020). Adjunctive intranasal esketamine for major depressive disorder: A systematic review of randomized double-blind controlled-placebo studies. *J. Affect. Disord.* 265, 63–70. doi: 10.1016/j.jad.2020.01.002

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.

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