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None.

14. ABSTRACT

Abnormal eating habits that drive excessive food seeking- or avoidance can manifest as health-threatening and socially impactful eating disorders. The scope of research includes 3 main aims: 1) to determine how basal forebrain ACh+ and Glu+ neurons are connected; 2) to determine how Glu+ neurons in the basal forebrain influence feeding behavior; and 3) to identify downstream targets of basal forebrain Glu+ neurons that govern feeding behavior. Specific results from this reporting period have identified that specific activation of DBB Glu+ neurons severely reduced feeding, and importantly, specific activation of DBB Glu+ fibers in the lateral hypothalamus (LH) also induced similar reduction in feeding, suggesting LH as one major downstream target mediating the feeding effect. However, specific inhibition of DBB Glu+ fibers in LH failed to increase feeding, suggesting a sufficient but not necessary role for LH neurons in mediating the feeding effect. The remaining planned experiments including the examination of local DBB circuits between cholinergic and glutamatergic neurons, the genetic interaction between DBB Glu+ neurons and the ob gene and the effect of DBB Glu+ neurons in motivation feeding will be accomplished in the next year. Overall, the results of the supported work will provide insights on how basal forebrain ACh+ and Glu+ neurons are connected, how Glu+ neurons in the basal forebrain influence feeding behavior and related behaviors and identify the downstream targets that mediate basal forebrain Glu+ neurons in regulating eating and related behaviors.

15. SUBJECT TERMS

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

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INTRODUCTION

Abnormal eating habits that drive excessive food seeking- or avoidance readily 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 within the hypothalamus, a key brain region involved in feeding behavior. Although signaling from extra-hypothalamic brain regions has also been implicated in regulating nutrient metabolism, appetite, and satiety, it is unclear which brain regions and pathways are involved. The scope of this research has focused on 3 main aims: 1) to determine how basal forebrain ACh+ and Glu+ neurons are connected; 2) to determine how Glu+ neurons in the basal forebrain influence feeding behavior; and 3) to identify downstream targets of basal forebrain Glu+ neurons that govern feeding behavior. Specific results from this work have identified that selective activation of DBB Glu+ neurons severely reduced feeding, and importantly, targeted activation of DBB Glu+ fibers in the lateral hypothalamus (LH) also reduced feeding, suggesting that the LH as a major downstream target that mediates DBB Glu+ neuron feeding effects. However, specific inhibition of DBB Glu+ fibers in LH failed to increase feeding, suggesting a sufficient but not necessary role for LH neurons in feeding behavior. In this report, we have completed the remaining planned experiments including the examination of local DBB circuits between cholinergic and glutamatergic neurons, the genetic interaction between DBB Glu+ neurons and the ob gene, and the effect of DBB Glu+ neurons in motivational feeding. Overall, the results of the supported work have provided important insights on how basal forebrain ACh+ and Glu+ neurons are connected, how Glu+ neurons in the basal forebrain influence feeding behaviors, and identify the downstream targets that mediate basal forebrain Glu+ neurons in regulating eating and related behaviors.

KEYWORDS

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

ACCOMPLISHMENTS

Major goals

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

Major goal 2: map local inputs onto basal forebrain Glu+ neurons; SOW completion data and site: 3-12 months in Site 2 (Dr. Tong). This goal has been delayed because of unavailability of SAD rabies virus during the pandemic. We have generated the required animal models. However, we found some issues with the use of the SAD rabies virus in local circuit tracing. We have adopted a new retrograde tracing method by using the AAV-DIO-TTC-GFP vector. As such, the goal was modified but completed.

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 accomplished. Initial expression analysis has been conducted using FISH, but we are still confirming basal forebrain cell types of expression. This was initially delayed due to the pandemic, but has recently also been completed. In addition, we have also added the detailed profiling of ACh receptor subtypes in DBB Glu+ neuron targets.

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

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

Major goal 6: Modulate basal forebrain Glu+ neuron activity and assess impact on feeding and physiology; SOW completion date and site: 6-24 months and Site 2 (Dr. Tong). This goal has been accomplished 6-24 months and included in previous reports.

Major goal 7: Inhibition of basal forebrain Glu+ neurons and impact on feeding; SOW completion date and site: 12-28 months and both Sites 1 and 2 (Drs. Arenkiel and Tong). This goal has been accomplished 12-36month and included in previous reports.

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

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 was initially delayed due to issues encountered with animal models. We have used alternative approaches, and this goal has now been accomplished.

Major goal 10: Genetic rescue of obesity; SOW completion date and site: 6-36 months and Site 1 (Dr. Arenkiel). This goal was initially delayed due to breeding issues. The original goal has been modified due to unanticipated findings in which ob/ob mice could not be rescued with originally proposed methods, and as such the overall goal is accomplished.

Major goal 11: Evaluate downstream targets of basal forebrain Glu+ neurons that govern feeding behavior; SOW completion date and site: 18-36 months and both Sites 1 and 2 (Drs. Arenkiel and Tong). This goal has been accomplished 18-36 months and described in previous reports.

Major goal 12: Decipher basal forebrain Glu+ neuron to LH neuron projections in feeding motivation; SOW completion date and site: 12-30 months and Site 2 (Dr. Tong). This goal has been accomplished.

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

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

Accomplishment during the reporting period:

In the final report, we have successfully completed all proposed goals and milestone, which are briefly in the following.

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

Major goal 2 (Dr. Tong): Here the goal was to determine whether ChAT neurons in the basal forebrain send monosynaptic projections to Glu+ neurons. Towards this we initially planned to use a monosynaptic pseudotyped rabies viral tracing approach to trace monosynaptic upstream neurons. For this, we generated *Vglut2-Cre* models, in which AAV-Flex-TVA-mCherry and AAV-Flex-G viral vectors were injected into the basal forebrains of these mice. One week later, SADdeltaG-GFP virus was to be injected to the same location. Two weeks later, mice would then 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. Although we rebounded and accomplished this experiment in year 2, we only identified a limited number of neurons that were presynaptically traced. The low efficiency of this pseudotyped rabies monosynaptic viral tracing has been reported previously. Instead, we implemented a new method based on the tracing ability of the heavy chain of tetanus toxin for much more effective tracing from neuromodulatory cell types (i.e. AAV-DIO-TTC-GFP). Using this new viral method, we observed a similar result (Fig. 2). This result may suggest 1) ChAT neurons send limited projections to Glutamatergic neurons, or 2) there is limitation in the method we are using. The goal of the proposed experiments has been achieved. However, further experiments will be required to test this possibility.

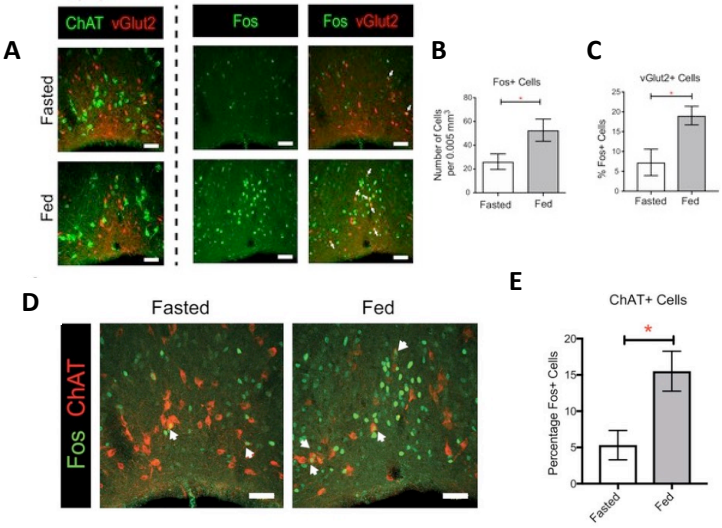

Fig 1

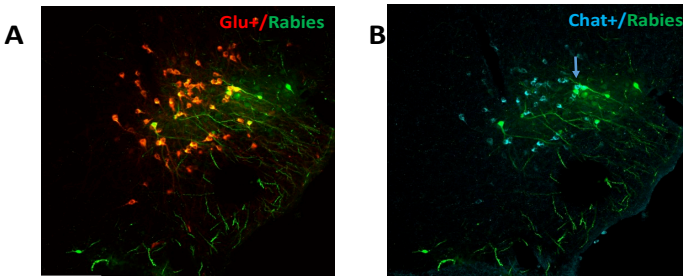

Fig. 2

Major goal 3 (Dr. Arenkiel): Here the goal was to analyze ACh receptors in basal forebrain Glu+ neurons. Toward this, RNA probes were synthesized, tested for efficacy, and initial expression analysis has been completed. Unfortunately, the resolution at the cellular level between different subtypes of receptors was inadequate to resolve by the originally proposed methods. Instead, receptor expression and/or response profiles were obtained by visually guided whole cell patch clamp recordings. See goal 4 and Figure 3.

Major goal 4 (Dr. Arenkiel): Here the goal was to determine the nature of neurotransmission between basal forebrain ACh+ neurons and Glu+ neurons. Towards this we generated ChAT-ChR2::Vglut2-Cre mice and delivered AAV-Flex-mRuby2 virus to the basal forebrains of these mice. Acute sections containing the basal forebrain were used to record laser stimulation induced currents in mRuby2-labelled Vglut2 target neurons. Upon stimulation we detected reliable non-glutamatergic and non-GABAergic mediated currents, which were sensitive to nicotinic Acetylcholine receptor antagonists, suggesting a major role for nicotinic AChRs in mediating the cholinergic action between Chat neurons and Glu+ neurons within the basal forebrain. The milestone in this goal has been achieved.

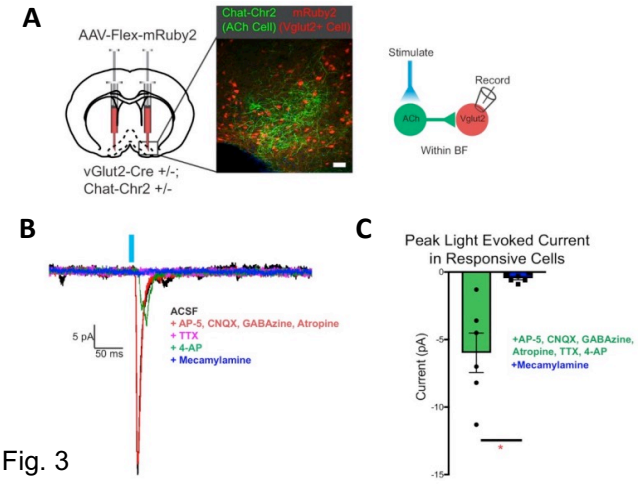

Fig. 3

Major goal 5 (Dr. Arenkiel): Here the goal was to determine the *in vivo* responses of basal forebrain Glu+ neurons to fed, fasting, and food-related odors. For this we generated *Vglut2-Cre* mice, delivered AAV-Flex-GCamp6m virus, and successfully implanted GRIN lenses into these animals. We observed that GCamp6m signaling in the forebrain region responded to various external signals, and found that interestingly, these neurons responded to different food-related odors, but not to the mineral oil controls (Fig. 4). Interestingly, we also found that these same neurons show differential responses to odors with different innate valences, uch as appetitive and/or aversive odor cues. These responses were rapid and reversable, suggesting a direct relevance of these neurons to feeding. This goal has been accomplished.

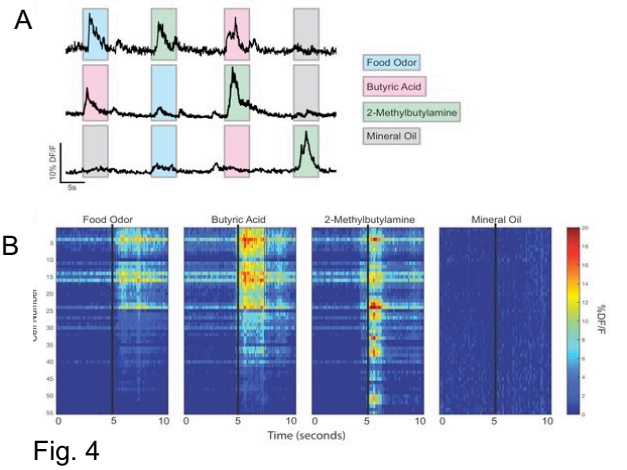

Fig. 4

Major goal 6 (Dr. Tong): Here the goal was to determine feeding and body weight impacts in mice with basal forebrain Glu+ neurons expressing NaChBac, a heterologous sodium channel that causes neurons to fire more action potantials. Previously, we prepared *Vglut2-Cre* mice and delivered AAV-Flex-NaChBac virus or control GFP virus into the basal forebrain. We found that NaChBac injected mice exhibited rapid body weight loss, due to dramatic voluntary reduction of feeding (Fig. 5).

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

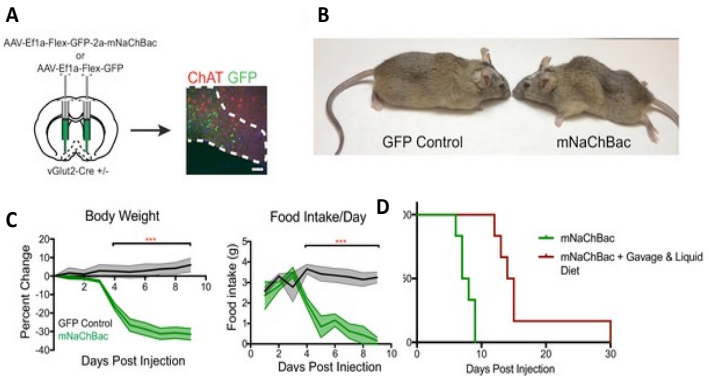

Fig. 5

Major goal 7 (Drs. Arenkiel and Tong): Here we sought to determine how loss-of-function manipulations in basal forebrain Glu+ neurons affects feeding (Dr. Arenkiel) and energy expenditure (Dr. Tong) by eliminating neurotransmitter release via expression of tetanus toxin light chain (TeNT). For this, AAV-Flex-TeNT-p2A-GFP virus was delivered to the forebrains of *Vglut2-Cre* mice, followed by monitoring feeding and body weight. However, we did not observe obvious differences in feeding, body weight, O2 consumption, and/or locomotion, which has been summarized in previous reports. Verification of injection and expression, and confirmation of TeNT achieving loss of Glu release has been completed. To further verify this result, we also performed specific deletion of Vglut2 in the DBB, which is known to be required for glutamate release. Confirmation of Vglut2 deletion by the CRISP-Cas9 approach has been provided (Fig. 6A and B). These mice again showed no changes in body weight (Fig. 6C) or feeding (Fig. 6D), consistent with the negative TeNT data.

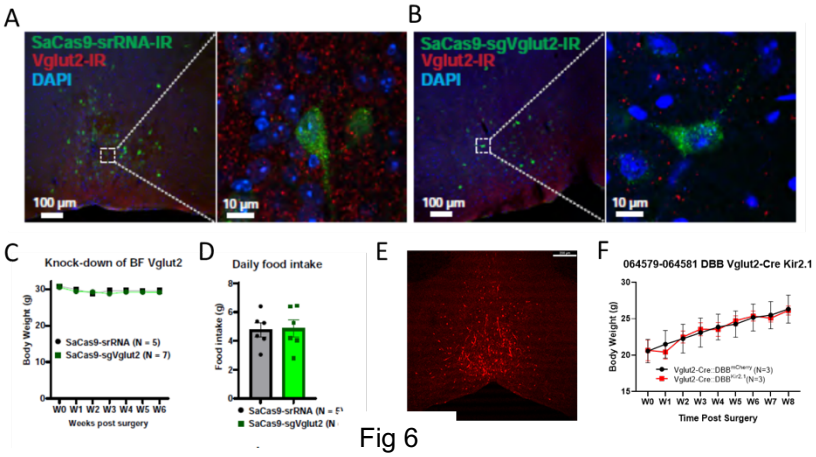

Fig 6

Major goal 8 (Dr. Tong): Here we sought to determine effects on feeding via manipulation of forebrain Glu+ neurons activated with ChR2, or inhibited with eNpHR3.0. Towards this, we delivered AAV-Flex-ChR2-GFP virus to the forebrains of *Vglut2-Cre* mice, and at the same time implanted optic fibers. We found that delivery of blue light significantly reduced fasting-induced refeeding, suggesting an importance of forebrain Glu+ neurons in feeding control (Fig. 7). However, when we delivered AAV-Flex-eNpHR3.0 virus to the same neurons with light stimulation, we failed to observe significant effects on fasting refeeding. All the injections were verified posthoc. However, given the known potential issue with the use of eNPHR3.0, we wanted to verify this negative result by using chemogenetics, i.e. delivering inhibitory hM4Di expression to DBB Glu+ neurons. Interestingly, with this method, we observed a mild increase in food intake in both fasting refeeding and ad limitum feeding (Fig. 8). These results suggest that chemogenetic inhibition may produce more inhibitory effects on this group of neurons. This goal has been accomplished.

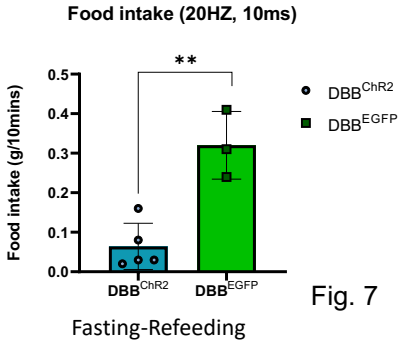

Fig. 7

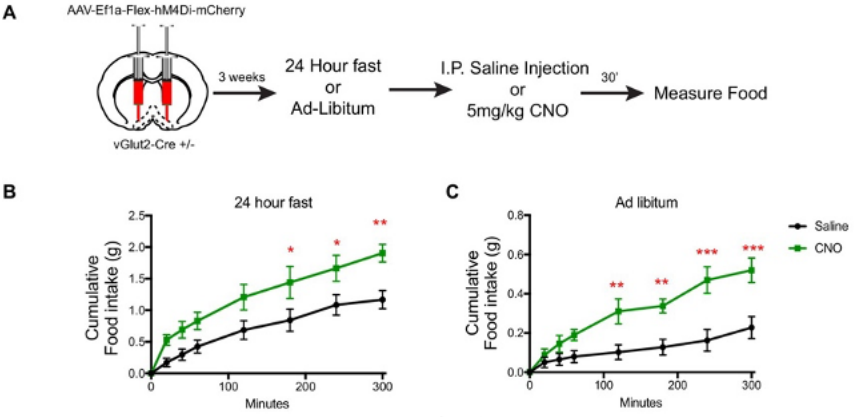

Fig. 8

Major goal 9 (Dr. Tong): Here the goal was to determine whether basal forebrain Glu+ neurons mediate the action of ACh+ neurons in feeding and body weight. Towards this, we 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 basal forebrain. However, we found an issue with Vglut2-Flp mice in that this line did not seem to express Flp in Vglut2 neurons in the DBB area. Given these issues, we have adopted alternative approaches by deleting Vglut2 in the DBB. We found that the deletion has no impact on feeding or body weight, suggesting that DBB Vglut2 expression is not required for feeding (Fig. 6). Thus, the goal of the proposed experiments has been accomplished. However, a caveat is that this deletion represents a chronic manipulation and may not be a robust approach to test acute feeding behavior. Future experiments with a different approach i.e. an acute manipulation, will be explored to confirm this observation.

Major goal 10 (Dr. Arenkiel): Here the goal was 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 measuring weekly body weight and feeding of these mice revealed significant resuces in both feeding and body weight (Fig.9). These results may suggest that activation of DBB Glu+ caused a non-specific reduction in feeding that could override that the known strong hyperphagia in leptin deficient *ob/ob* mice. As such, this experiment has been accomplished.

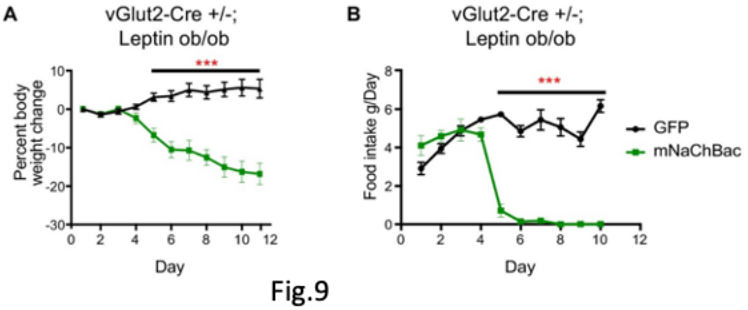

Fig.9

Major goal 11 (Drs. Arenkiel and Tong): Here the goal was to determine whether the LH functions as a key downstream target to mediate hypophagia (Dr. Arenkiel) or aversion (Dr. Tong) induced by forebrain Glu+ neuron activation. Towards this, stereotaxic surgery was performed to deliver AAV-Flex-ChR2 virus to the forebrains of *Vglut2-Cre*

mice, and at the same time, optic fibers were implanted at the LH. Our results showed that stimulation of fibers in LH elicited aversion associated with food odor, which represented a major part of the total aversion induced by the stimulation of DBB Glut+ neurons, suggesting a major role of LH neurons in mediating DBB neuron function in aversion associated with food odor . The goal of this experiment has been accomplished.

Major goal 12 (Dr. Tong): the goal here was to determine whether inhibition of basal forebrain Glu+ neuron projections to the LH promotes motivation to eat. For this, bilateral delivery of AAV-Flex-eNpHR3.0 virus was made to the basal forebrain regions of *Vglut2-Cre* mice and optic fibers were implanted to target the LH. These mice received training in a nose poke chamber and were tested whether light delivery impacted correct nose pokes and latency to nose poke. We have accomplished this experiment and the results showed that inhibition caused no changes in nose poke behaviors, suggesting that this pathway is not required for motivational behaviors. Since the data is largely negative, we didn't attached the figure here.

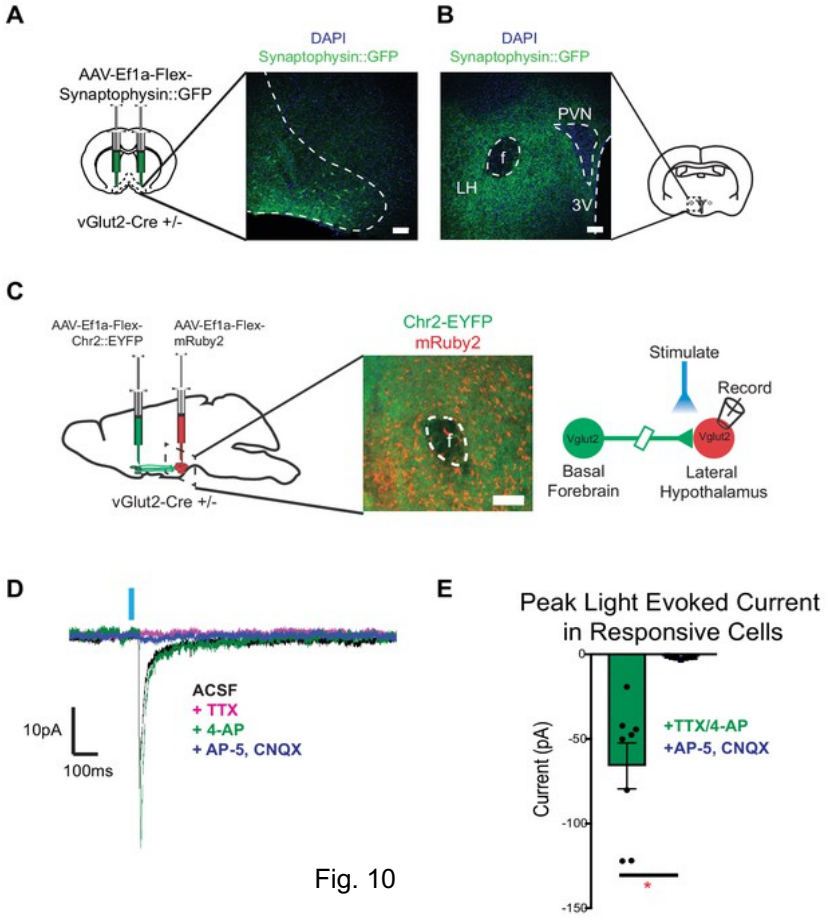

Fig. 10

Major goal 13 (Dr. Arenkiel): Here the goal was to determine whether LH Glu+ neurons are direct downstream targets of basal forebrain Glu+ neurons. Towards this, an AAV-Flex-ChR2-GFP virus was delivered to the basal forebrain, while AAV-Flex-GFP control virus was delivered to the LH of *Vglut2-Cre* mice. The electrophysiological experiments have confirmed that DBB Glu+ neurons send direct monosynaptic projections to LH Glu+ neurons (Fig. 10). We have finished the feeding experiments, where local photostimulation in the LH potently inhibited feeding, the extent of which is similar to that observed with direct activation of basal forebrain Glu+ neurons (Fig. 11). This experiment has been accomplished.

Major goal 14 (Dr. Tong): Here the goal was to determine whether LH Glu+ neurons mediate the effect of basal forebrain Glu+ neurons in feeding. For this, *Vglut2-Cre* mice were prepared and AAV-Flex-ChR2 virus was delivered to the basal forebrain, while at the same time AAV-Flex-hM4Di-mCherry was delivered to the LH. Initial experiments on verification of viral delivery have been confirmed, and thus demonstrated our ability to target and manipulate this region. Following the manipulations, our results showed that inhibition of LH neurons failed to reduce food inhibition effects by DDB Vglut2 neuron activation (Fig. 12), and we reasoned that the results observed might be due to activation of collaterals in other downstream neurons is sufficient to reduce feeding. To further test this possibility, we performed local optogenetic stimulation of DBB Glu+ fibers in the LH, and at the same inhibiting DBB Glu+ neurons with hM4D1-mChery (Fig 13). The result showed that with neuronal soma inhibition, i.e. elimilating potential back-propogation and collateral activation, local stimulation still reduced feeding,

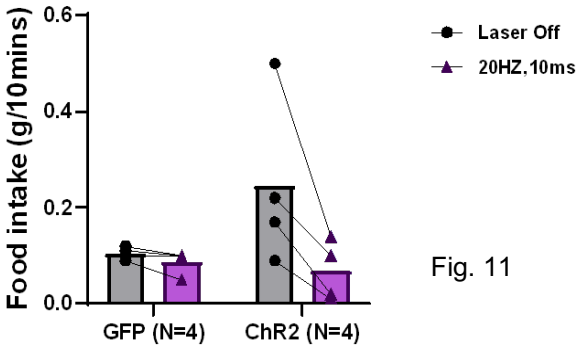

Fig. 11

suggesting LH Glu+ neurons mediate the feeding-inhibiotry action of DBB Glu+ neurons. The goal of this experiment has been accomplished.

Training and professional development

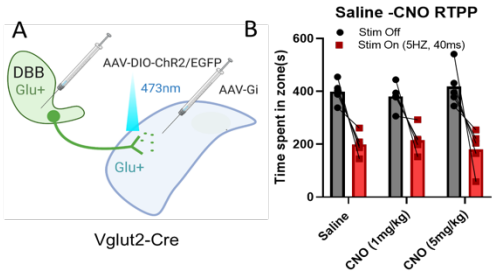

Fig. 12

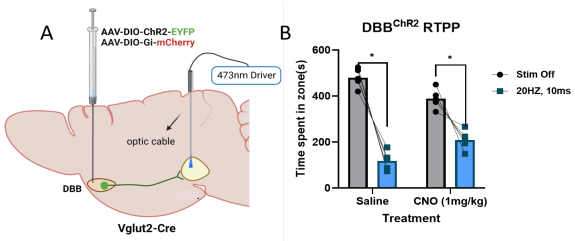

Fig. 13

Dr. Zhiying Jiang (postdoc) and Ms. Jing Cai (graduate student) (Site 2, Dr. Tong) were involved in this project, and supported by this funding. Both of them were required to attend our departmental and center seminar series. The departmental seminar series invites well-known scientists across the US to present their new findings and the center seminar series will mainly presented by postdocs and students from each lab to share their research. Thus, both will have the opportunities to be exposed to research outside the campus and to present their own ongoing research. In addition, Ms. Cai will have planned activity and training classed within the graduate school, including required committee meetings in which Dr. Arenkiel is a committee member. We also have a regular lab meeting in which all lab members are required to present their own research, as well present a journal club on the papers from peers. Importantly, both trainees have regular weekly meetings with me. Through these various platforms, both trainees have ample exposure to extended mentoring and research exposure. With the pandemic, almost all seminar series with the UT and TMC have implemented a virtual format, in which they regularly attend. Unfortunately, with the impact of the pandemic, it is not easy for us to go to local/national meetings and we are trying to participate virtual meetings organized by various research foundations and other institutions, in order to get better exposure to peers. Of note, Ms. Cai and Dr. Tong have attended this year's SfN meeting in November 2022 at San Diego. Importantly, Ms. Cai has just defended her thesis and will move on to her postdoc training at NYU.

Lab personnel in Arenkiel lab include Ms. Mayuri Patel and Ms. Peyshyuan Chin. Both are current graduate students focused on this project as part of their thesis work. Mayuri and Peyshyuan have received direct training in *in vivo* imaging, electrophysiological recordings, and behavioral analysis, and have also been learning imaging and *in vivo* electrophysiological recording methods. They utilized these skills towards the proposed studies. All made excellent progress towards the stated project goals, and have maintained timely committee meeting progress reports. As another training opportunity, both Ms. Patel and Ms. Chin have attended this year's SFN meeting. Also, as partial contributors, Mr. Benjamin Belfort and Mr. Suyang Bao (two new students to the laboratory) served 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 reinitiate new experimentation. Additionally, 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

There were no significant changes in the approach or use of animals. However, due to the prioer pandemic, timelines to accomplish the planned and stated milestones were delayed. Unfortunately, during the initial lockdown, it was mandatory for us to significantly reduce mouse breeding/cages, which caused a major delay in the availability of mouse study subjects. In addition, we operated at a reduced personnel effort to achieve effective social distancing, which also delayed the progress of research and associated research expenditures. Also due to school/daycare shut down with online course at home, the time spent on experiments on lab members with young kids was reduced. Despite facing these adversaries, we have managed to make the planned progress and accomplishments.

PRODUCTS

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

1. AgRP neurons are not indispensable for body weight maintenance in adult mice. Cai J, Chen J, Ortiz-Guzman J, Huang J, Arenkiel BR, Wang Y, Zhang Y, Shi Y, Tong Q, Zhan C. Cell Rep. 2023 Jul 25;42(7):112789. doi: 10.1016/j.celrep.2023.112789. Epub 2023 Jul 8.
   PMID: 37422762. Acknowledgement of the federal support: Yes.

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

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Funding Support	This Award and NIH grants

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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:

4 reprints of publications.