Regulation of Feeding Behavior by Optogenetic Activation of Inputs to Lateral Hypothalamic Area

Hassan S. Choudhry, Kuldeep Shrivastava, Vikshar Athreya, Mark A. Rossi

Abstract

The bed nucleus stria terminalis (BNST) and parabrachial nucleus (PBN) are two brain regions in correspondence with the lateral hypothalamic area (LHA) that are responsible for regulating feeding behavior in mice. It is acknowledged that increasing the activity of GABAergic BNST inputs (which release the inhibitory neurotransmitter GABA) to the LHA inhibits LHA glutamate neurons and increases feeding, whereas increasing activity of glutamatergic PBN inputs (which release the excitatory neurotransmitter glutamate) to the LHA excites local glutamate neurons and decreases feeding. Obesity is a prevalent problem in our society, and therefore it is important to understand the reasons behind excessive food intake habits of humans. To address this issue, we tested for effects of activating the BNST-LHA and PBN-LHA pathways in relation to the satiety state of the mice. We stimulated the BNST and PBN inputs to LHA in fasted and fed mice. We tested the effects of Satiety State (fed vs. fasted) and stimulation Frequency (5-40 Hz) on sucrose seeking behavior of two Groups of mice (ChR2 vs YFP controls). The BNST-LHA pathway tended to show a general increase in feeding behaviors, while the PBN-LHA pathway did not show a significant effect of activation. From these results, further studies can be conducted to explore more about these neural pathways and the mechanisms underlying feeding behaviors. On a broader scale, these findings can inform future therapeutics that could help prevent unhealthy eating habits and obesity.

1 Introduction

Overeating and obesity are increasing in prevalence among our society. In fact, the 2022 World Obesity Atlas published by the World Obesity Federation states that by 2030, approximately 1 billion people will be obese (*World Obesity Atlas*, 2022). Previous research has suggested that the recent rise in obesity, especially in the United States, is due to an increase in eating portions, lack of physical activity, and greater consumption of fats, sugars, and grains (*Why are Americans obese*, 2022). In order to understand why obesity prevalence is continuing to increase, further exploration of brain circuits that control feeding is essential.

The mouse genome is approximately 99% similar to the human genome (Vandamme, 2014). Moreover, mice and human brains are also quite similar in terms of structure and function. Due to this, we can use mouse models to inform our understanding of human neurobiology. The lateral hypothalamic area (LHA) is a region of the brain that is responsible for controlling feeding behaviors in rodents and humans (Rossi et al., 2019). The bed nucleus of the stria terminalis (BNST) and the parabrachial nucleus (PBN) are two brain regions that provide inputs to the LHA where they synapse (connect) onto glutamatergic neurons (Jennings et al., 2013; Phua et al., 2021). It has previously been discovered that selective activation of LHA glutamate neurons halts feeding (Rossi et al., 2019). Additionally, activation of inhibitory (GABAergic) inputs from BNST inhibits the LHA glutamate neurons, thereby increasing feeding (Jennings et al., 2013). However, activation of excitatory (glutamatergic) inputs from PBN excites the LHA glutamate neurons, thereby decreasing feeding (Phua et al., 2021). The bidirectional nature of selectively activating LHA glutamate neurons to either increase or decrease feeding was demonstrated previously (Stamatakis et al., 2016; Jennings et al., 2013).

While previous research has been conducted using stimulation of these pathways, this study highlights the relationship between the BNST-LHA/PBN-LHA pathways and satiety state. We are specifically looking at that to see if the activity differentially influences food seeking based on the current energy needs of the mice. This includes understanding the factors that influence the ability of stimulation of LHA inputs for modifying feeding behavior (e.g., hunger, satiety, obesity). Cell-type and projection-specific neuron activation is achieved with Channelrhodopsin-2 (ChR2) proteins. These are light-gated cation channels that open when exposed to blue light (Deisseroth & Hegemann, 2017). In turn, these ChR2 channels allow quick depolarization (influx of positively charged ions into the cell) of neurons, which is important (along with repolarization) for driving action potentials to communicate electrical signals between neurons. Ultimately, understanding the role that these pathways play in regulating feeding behavior can help us understand more of the neurobiology underlying eating disorders and obesity.

In addition to observing the effect of activation of neural pathways on consummatory behaviors, this paper is also interested in exploring how satiety influences the ability of LHA inputs to control eating. We hypothesize that the optogenetic stimulation (activation of ChR2 channels) of PBN inputs to the LHA will excite glutamate neurons and decrease food intake, while the activation of BNST inputs to the LHA will suppress glutamate neurons and increase food intake. Motivational state will influence the effect of stimulation because hypothalamic glutamate neurons will be suppressed when the mice are hungry, but they will be excited when the mice are satiated. It is believed that before obesity, the LHA glut cells act as a brake on feeding (Rossi et al., 2019). Specifically, these cells are excited after a mouse starts eating and the magnitude of their responses is negatively associated with feeding Thus, it is believed that these cells act as a brake on feeding. During diet-induced obesity, these cells become hypoactive, so the removal of that brake may be facilitating overeating (Rossi et al., 2019). This study intends to build upon previous studies by utilizing numerous stimulation frequencies to observe the effect of satiety state on feeding habits of mice and the role that ChR2 plays in these neural pathways compared to when it is absent (YFP).

2 Methodology

Animal Care and Surgeries

The subjects of this experiment are male and female C57BL6J wild type mice for the BNST and Vglut2-Cre mice for the PBN (Jackson Laboratories). At 3 weeks old, the mice were weaned to follow standard animal welfare methods. The mice were set on a 12 hour light-dark cycle with all tests being performed during light hours. All experiments received approval from the Rutgers University Institutional Animal Care and Use Committee. All experiments were also conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

A virus injection surgery was performed for two different viruses on the mice at ~6 weeks of age. To target the BNST-LHA pathway, AAV-hSyn-ChR2-YFP (AAV: adenosine associated virus; hSyn: human synapsin promoter) or AAV-hSyn-YFP were injected into the BNST. To target the PBN-LHA pathway, the Cre-dependent viruses AAV-hSyn-DIO-ChR2-YFP (DIO: double inverted operator) or AAV-hSyn-DIO-YFP were injected into the PBN. The purpose of the control was to provide justification that if there was to be a difference between the results from the two viruses, then that difference would be attributed to the ChR2 (not the AAV virus or external light source from the optogenetic experiment soon to be discussed). The virus was injected into the PBN or BNST (PBN coordinates: AP-5.05mm, ML+/-1.25mm, DV-2.82mm; BNST coordinates: AP+15mm, ML+/-0.90mm, DV-3.82mm) of the mice depending on the targeted area of study: 10 BNST-ChR2 mice (6 males, 4 females); 7 BNST-YFP mice (5 males, 2 females); 5 PBN-ChR2 mice (2 males; 3 females); 2 PBN-YFP mice (1 male, 1 female) - further research will consist of more PBN mice experiments. After the virus was injected, two optic fibers were surgically implanted bilaterally above the LHA region to allow selective activation of axon terminal signals from the BNST and PBN.

Behavioral Paradigms

Three weeks after the surgeries were completed, a custom head-fixed behavior set-up was used to track the mice's consumption of sucrose. This set-up restrained the mouse in a tube attached to a stand to lock the mouse in place, which prevented the mouse from moving but allowed it to voluntarily consume sucrose solutions. Activation of the BNST-LHA or PBN-LHA pathways was made possible by sending 473 nm laser light at different frequencies to the optic fibers placed in the LHA. These different frequencies would reveal the effect of stimulation frequency on how motivated the mice were in licking the sucrose. As a result, the BNST and PBN pathways are activated to test their effects on the consummatory behavior of the mice. Drops of 10% sucrose solution were randomly delivered through a spout positioned directly in front of the mice, and licks at the spout were recorded with a custom lickometer. During each session, a total of 50 trials (sucrose reward deliveries) were performed on each mouse. 25 of these trials included optogenetic stimulation, while 25 of them did not. Optical stimulation was 3 s in duration (5 ms pulses at 5-40 Hz) and occurred either immediately before (BNST group) or after (PBN group) reward delivery. BNST stimulation occurred before reward delivery because since BNST stimulation is hypothesized to increase feeding, stimulation after reward delivery would not produce clear results due to a ceiling effect (already maximized licking). PBN stimulation occurred after reward delivery because since PBN stimulation is hypothesized to decrease feeding, stimulation before reward delivery would not produce clear results due to a floor effect (already minimized licking). The rate of licking during optical stimulation was quantified.

After the behavior experiment was successfully completed, the mice were then transcardially perfused for histological analysis. Mice were euthanized with pentobarbital and 20 mL of phosphate-buffered saline (PBS) and paraformaldehyde (PFA) were perfused through their circulatory system to fix the brain tissue (made rigid in order to eventually splice the brain). The purpose of this was to ensure a clearer image under the confocal microscope when doing histological analysis later in the process.

Histology

After the perfusion was completed, virus expression and fiber placement were confirmed. To do this, we sliced the brains at 35 µm with a cryostat, mounted them on microscope slides, and imaged the fluorescence with confocal microscopy. Both a tile scan and z-stack were performed to create images of the brain to include the BNST, PBN, or LHA. Histological analysis allowed us to confirm that the BNST and PBN showed expression because of ChR2 or YFP, and that the optic fibers were correctly implanted in the LHA. All viruses had the YFP fluorophore, so all virus expression was verified by visualizing YFP in the areas of interest. DAPI was utilized, which is a blue stain that allows us to see all cell nuclei. It is used to visualize the cells/tissue because otherwise the tissue would be invisible with fluorescence-based confocal microscopy.

Data Analysis and Statistics

Head-fixed behavior data was analyzed using custom Python code via Jupyter Notebook. Optically evoked licks were measured in accordance with their respective frequencies (BNST mice: 5Hz, 10Hz, 20Hz, and 40Hz; PBN mice: 10Hz and 20Hz). The frequencies were chosen because they span the range of the natural firing of the BNST cells. Data for the BNST mice were recorded during the pre-reward epoch of 0-3 seconds before stimulation, while PBN data were recorded during the post-reward epoch of 0-3 seconds after stimulation. GraphPad Prism was used to create grouped graphs of the data and for statistical analyses. Two-way ANOVAs with corrections for Sadik post hoc tests were performed to determine significance. A Grubbs' test was performed to test for statistical outliers in the data.

3 Results

Histological Analysis

To validate the viral injections and expression of ChR2 within the BNST and placement of optic fibers in the LHA, we performed histological

analysis with confocal microscopy. The image shows that the surgeries were performed correctly with virus expression in the BNST and the presence of optic fibers in the LHA (Figure 1). While DAPI was used for the BNST and LHA during the experiment, we did not include it in the LHA image below because the background of the green channel (how the YFP is seen) was high enough to visualize fiber placements. In concise terms, the virus was injected correctly into the BNST and the optic fibers were implanted correctly into the LHA.

To validate the viral injections and expression of ChR2 within the PBN and placement of optic fibers in the LHA, we performed histological analysis with confocal microscopy. The image shows that the surgeries were performed correctly with virus expression in the PBN and the presence of optic fibers in the LHA (Figure 1). While DAPI was used for the PBN and LHA during the experiment, we did not include it in the LHA image below because the background of the green channel (how the YFP is seen) was high enough to visualize fiber placements. In concise terms, the virus was injected correctly into the PBN and the optic fibers were implanted correctly into the LHA.

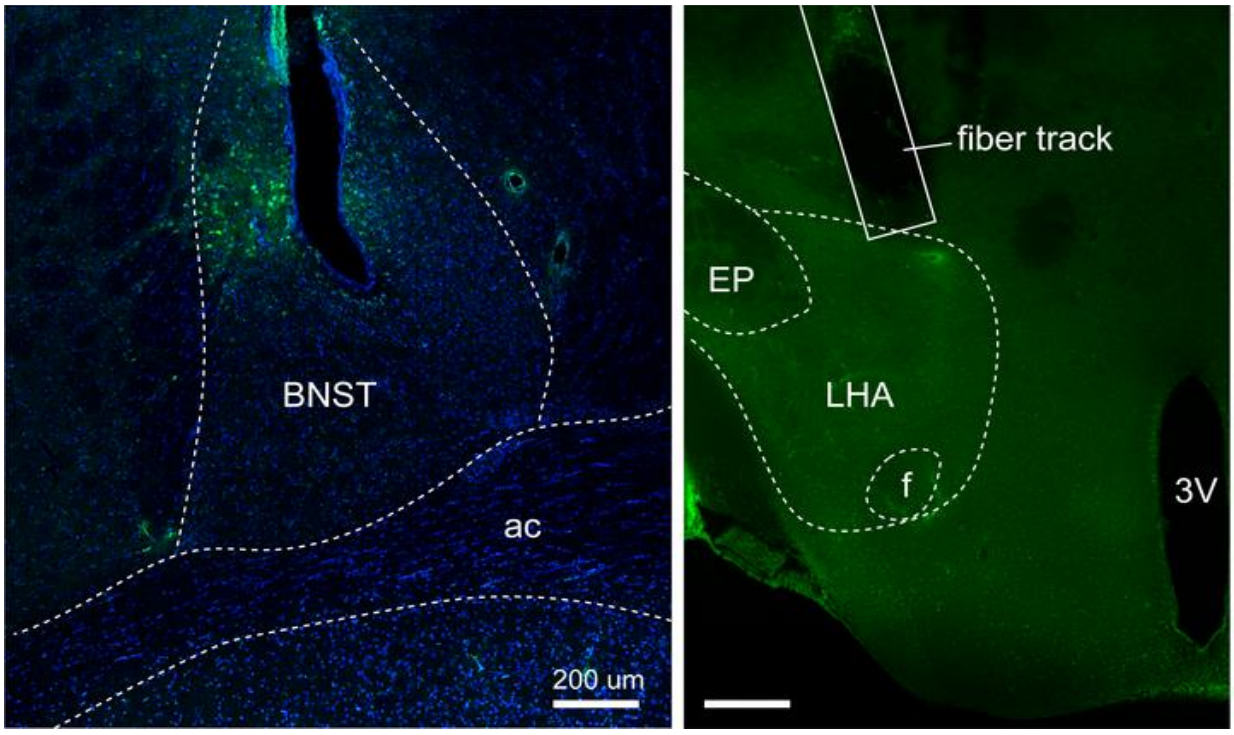

Figure 1: *Confocal Microscope Image of BNST and LHA. Left panel shows the ChR2-eYFP injection site in BNST. Right panel shows optical fiber placement above the LHA. BNST: bed nucleus of the stria terminalis, ac: anterior commissure, EP: entopeduncular nucleus, LHA: lateral hypothalamic area, f: fornix, 3V: third ventricle.*

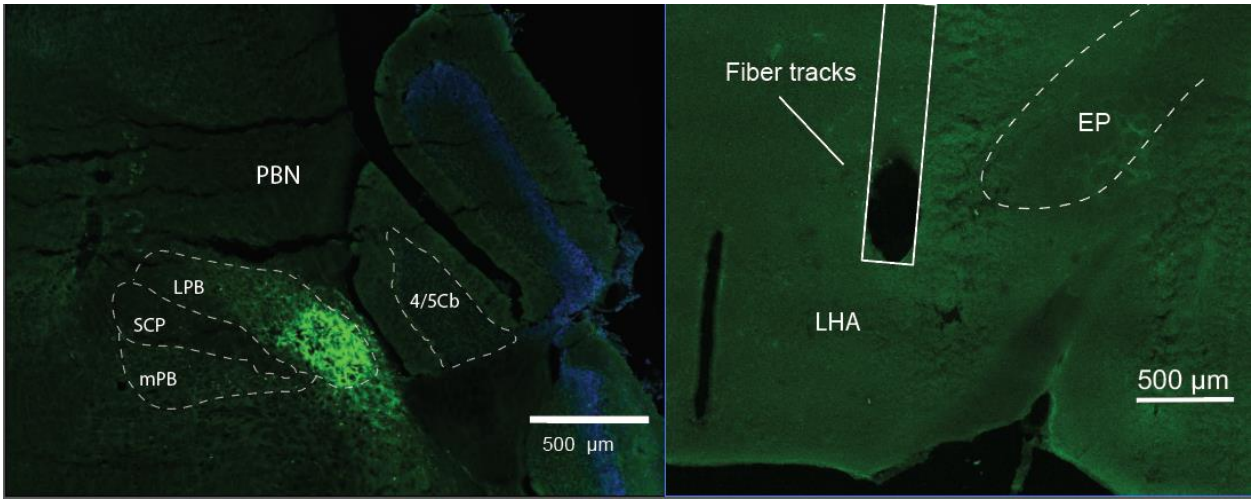

Figure 2: *Confocal Microscope Image of PBN and LHA. Left panel shows the ChR2-eYFP injection site in PBN. Right panel shows optical fiber placement above the LHA. PBN: parabrachial nucleus, LPB: lateral parabrachial nucleus, SCP: superior cerebellar peduncle, mPB: medial parabrachial nucleus, 4/5Cb: lobules 4 & 5 of the cerebellar vermis, EP: entopeduncular nucleus, LHA: lateral hypothalamic area.*

Optogenetic Activation of the BNST-LHA Pathway

To determine if the inputs from the BNST and PBN will affect motivated behavior, we utilized a custom-head fixed behavior set-up. It was not supported that motivation can influence feeding behaviors of mice due to satiety states (Figure 3A, B). Both of these figures did not show a main effect from the Satiety State or an overall interaction between Satiety State and Frequency. The results showed that increasing the frequency made a significant effect on the licking behaviors of the ChR2 mice (Figure 3A). Furthermore, the results show that the ChR2 group licked significantly more due to activation of the BNST pathway in both the fed and fasted states respectively (Figure 3C, D). This supports our hypothesis that activation of the BNST would cause an increase in consummatory behavior. However, increasing the frequency alone did not cause a significant difference in licking for fed mice (Figure 3C), but it did for fasted mice (Figure 3D). Overall, there was an interaction between Group and Frequency for fed mice (Figure 3C), with the Sidak post hoc test confirming a significant difference at 20Hz (Figure 3C). However, the fasted mice did not show an interaction between Group and Frequency (Figure 3D). There were no outliers detected from the statistical analysis. In concise terms, optogenetic activation of the BNST-LHA pathway resulted in an increase in feeding behaviors of the mice.

Optogenetic Activation of the PBN-LHA Pathway

Results were also observed for the PBN pathway (Figure 4). We hypothesized that the satiety state would cause a significant effect on the food intake of mice. This was supported by the observed interaction between Satiety State and Frequency of the ChR2 fasted mice compared to the fed mice (Figure 4A), but there was no interaction for the YFP mice (Figure 4B). Part of our hypothesis, however, was not supported: activation of the PBN pathway failed to decrease food intake in mice. However, there was no significant effect of Group and there was no interaction between Group and Frequency (Figure 4C, D). Additionally, the Sidak post hoc test revealed that when the mice were fed, 20Hz stimulation reduces their licking (Figure 4C). However, when the mice were fasted, there was no significant effect of Frequency (Figure 4D). There were no outliers detected from the statistical analysis. In concise terms, optogenetic activation of the PBN-LHA pathway did not have a significant effect on mice feeding behaviors.

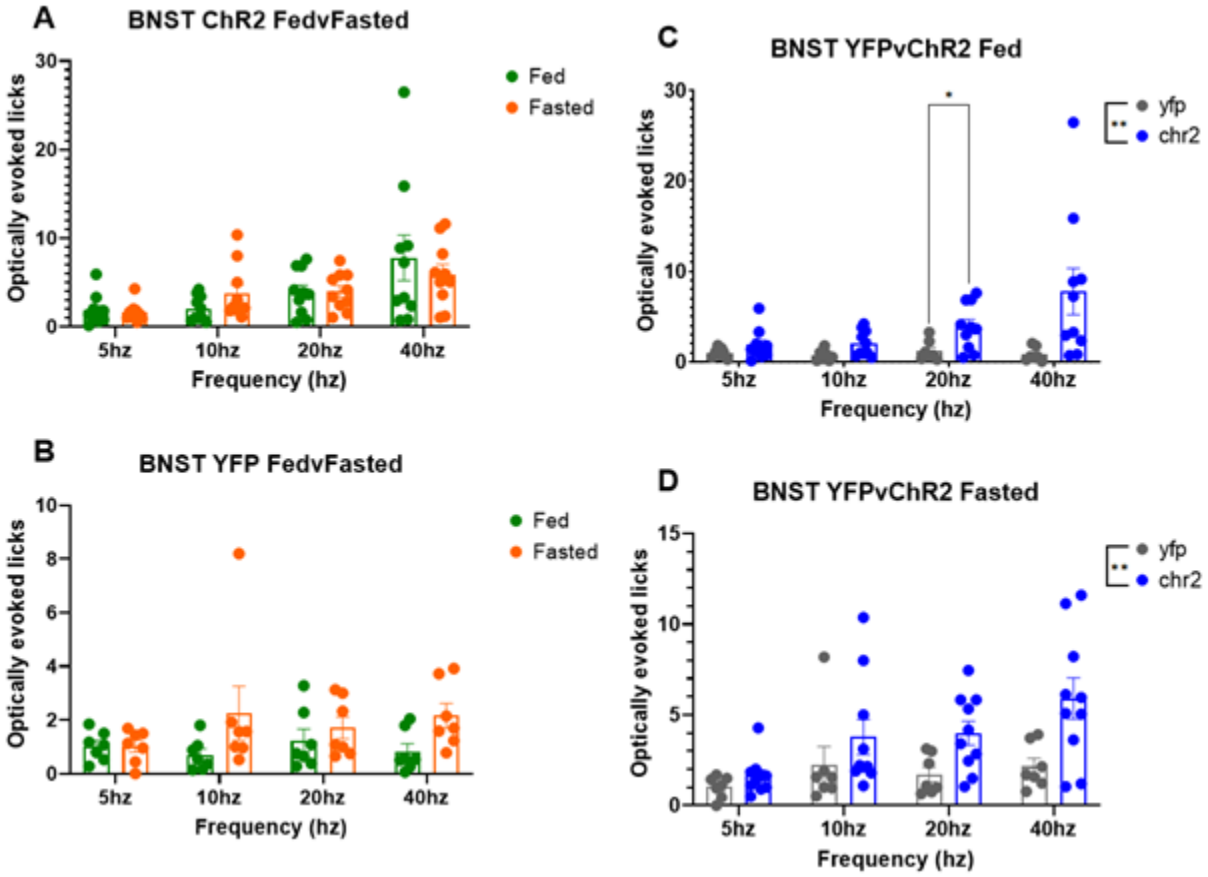

FIGURE 3: *Behavior Data of BNST-LHA Pathway. A) We performed a two-way ANOVA test (Satiety State x Frequency). There is no main effect of Satiety State: F(1.0, 9.0) = 0.01, p = 0.93. There is a main effect of Frequency: F(1.3, 11.6) = 11.16, p = 0.004. There is no interaction between Satiety State and Frequency: F(1.5, 13.7) = 1.00, p = 0.37. B) We performed a two-way ANOVA test (Satiety State x Frequency). There is no main effect of Satiety State: F(1.0, 6.0) = 5.67, p = 0.05. There is no main effect of Frequency: F(1.4, 8.7) = 0.65, p = 0.50. There is no interaction between Satiety State and Frequency: F(1.5, 9.3) = 1.55, p = 0.26. C) We performed a two-way ANOVA test (Group x Frequency). There is a main effect of Group: F(1, 15) = 7.09, p = 0.02. There is no main effect of Frequency: F(1.3, 19.2) = 3.42, p = 0.07. There is an interaction between Group and Frequency: F(3, 45) = 3.54 p = 0.02. Sidak post hoc test revealed a difference at 20Hz. D) We performed a two-way ANOVA test (Group x Frequency). There is a main effect of Group: F(1, 15) = 7.00, p = 0.02. There is an effect of Frequency: F(2.5,37.4)=5.55, p=0.005. There is no interaction between Group and Frequency: F(3,45)=1.97, p=0.13. All groups (A-D) were tested for potential outliers. A Grubbs' test was performed with alpha = 0.05, but no outliers were detected.*

*Note: * Indicates the frequency at which the overall interaction between Group and Frequency occurs in accordance with the Sidak test.*

*Note: ** Indicates a main effect of Group.*

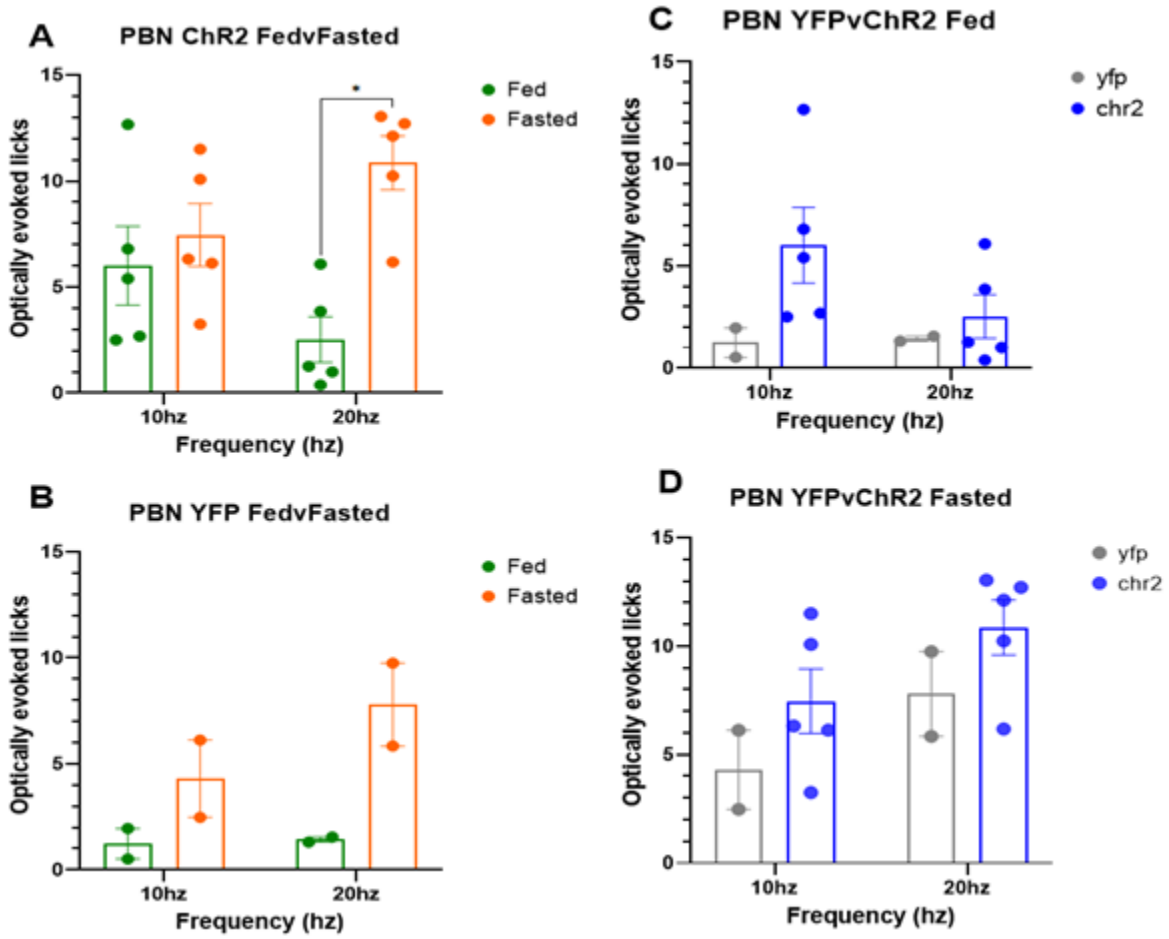

FIGURE 4: *Behavior Data of PBN-LHA Pathway. A) We performed a two-way ANOVA test (Satiety State x Frequency). There is no main effect of Satiety State: F(1, 4) = 6.17, p = 0.07. There is no main effect of Frequency: F(1, 4) = 0.003, p = 0.96. There is an interaction between Satiety State and Frequency: F(1, 4) = 23.11, p = 0.01. Sidak post hoc test revealed a difference at 20Hz. B) We performed a two-way ANOVA test (Satiety State x Frequency). There is no main effect of Satiety State: F(1, 1) = 4.18, p = 0.29. There is no main effect of Frequency: F(1, 1) = 25.28, p = 0.13. There is no interaction between Satiety State and Frequency: F(1, 1) = 50.16, p = 0.09. C) We performed a two-way ANOVA test (Group x Frequency). There is no main effect of Group: F(1, 5) = 1.68, p = 0.26. There is no main effect of Frequency: F(1, 5) = 1.99, p = 0.22. There is no interaction between Group and Frequency: F(1, 5) = 2.49, p = 0.18. D) We performed a two-way ANOVA test (Group x Frequency). There is no main effect of Group: F(1, 5) = 1.64, p = 0.26. There is a main effect of Frequency: F(1, 5) = 27.79, p = 0.003. There is no interaction between Group and Frequency: F(1, 5) = 0.005, p = 0.95. All groups (A-D) were tested for potential outliers. A Grubbs' test was performed with alpha = 0.05, but no outliers were detected.*

*Note: * Indicates the frequency at which the overall interaction between Satiety State and Frequency occurs in accordance with the Sidak test.*

4 DISCUSSION

Overall, the results showed that the PBN inputs to the LHA may not be as relevant for feeding, but activation of BNST inputs to the LHA did influence consummatory licking. Specifically, activation of the BNST-LHA pathway caused a general increase in licking, which is similar to what was found previously (Jennings et al., 2013). While previous literature showed activation of the PBN-LHA pathway to cause a decrease in feeding (Phua et al., 2021), we did not observe an effect of stimulation on licking. FIGURE 5 shows a schematic representation of the cell projections from the BNST and PBN to the LHA, while also showing the GABAergic and glutamatergic inputs from the BNST and PBN regions respectively.

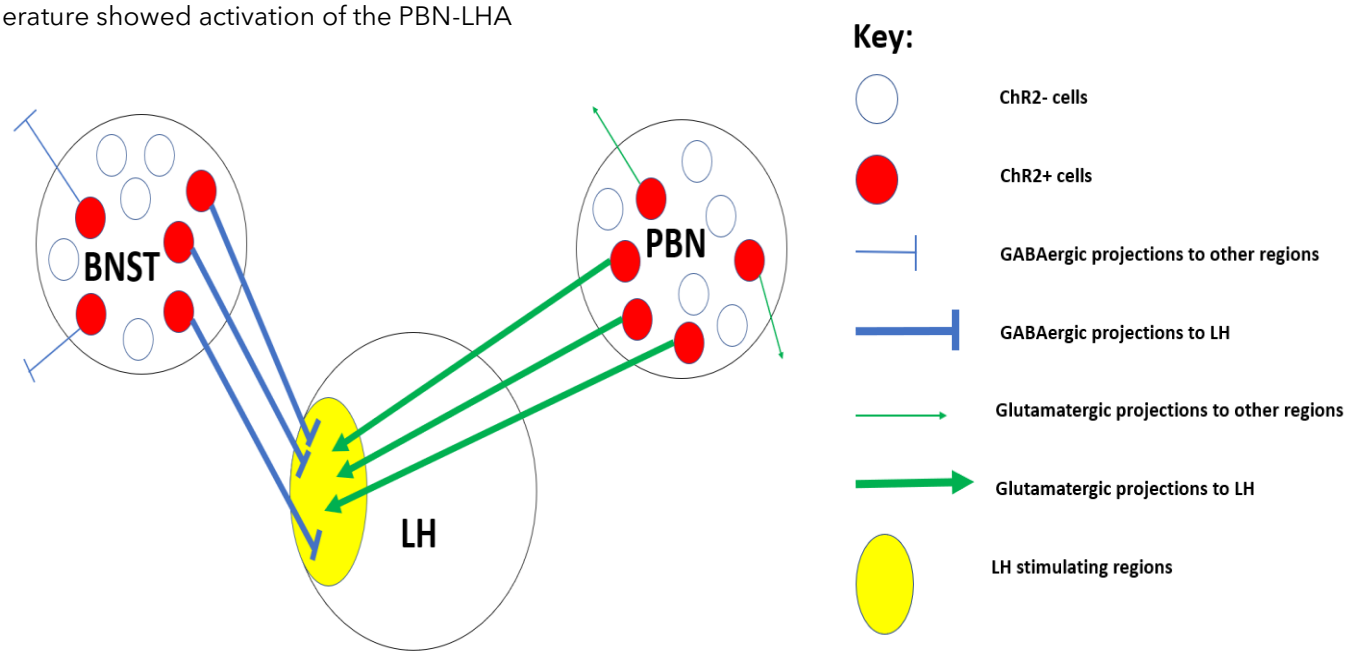

FIGURE 5: *Schematic of BNST and PBN cell projections to LHA. Figure 5 is a schematic of the cell input projections from the BNST and PBN inputs to the LHA. GABAergic inputs were from the BNST to the LH, while glutamatergic inputs were from the PBN to the LHA. The BNST, PBN, and LHA regions are each labeled. Stimulated cells were differentiated from non-stimulated cells, while projections towards the LHA were differentiated from projections towards other regions. A key was also included. Note: While the BNST and PBN does contain a mixture of both GABAergic and glutamatergic inputs, the BNST primarily contains GABAergic inputs and the PBN primarily contains glutamatergic inputs (viral expression was not restricted to solely GABAergic or glutamatergic inputs)*

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In most cases for both the BNST and PBN mice, increasing the frequency of stimulation caused an increase in licking behavior. There were also effects observed when changing the frequency, which was that increasing the stimulation frequency tended to cause an increase in sucrose licking. However, since many of the significant differences in feeding behavior increases revealed from post hoc tests occurred at a frequency of 20Hz but not as much with 40Hz, it is reasonable to believe that repeatedly increasing the frequency of light will not automatically increase feeding behaviors. This could be because 40 Hz stimulation is too high for the neurons to follow and could be detrimental for their health, which could ultimately decrease the rate of consummatory licking behavior. It is possible that licking cannot exceed the physical limits set by the central pattern generators.

It was also observed that the ChR2 mice tended to lick more than the YFP mice after stimulating the BNST-LHA pathway (the CHR2 mice did lick more for the PBN-LHA pathway, but the difference was not statistically significant). This could be due to the function of ChR2, which allows

stimulation of inputs to drive action potentials (Deisseroth & Hegemann, 2017). Furthermore, this can potentially imply that stimulation of the BNST-LHA pathway causes sucrose seeking. There tended to be more feeding when the mice were hungry (fasted) compared to when they were fed, which can imply that utilizing optogenetics to stimulate pathways to mimic neuronal activity in action may not be the only factor responsible for differences in feeding behaviors. This suggests that motivation (hunger) plays a role in the ability of BNST inputs to LHA to influence feeding. This idea can be relevant for human health and disease because it can show how feelings of hunger can cause people to eat more when they have food in front of them, rather than when they are already full. While obese mice were not focused on in this study, our findings support the hypothesis that fed mice were less sensitive to stimulation, while fasted mice were hypersensitive to stimulation.

One limitation of the study was that there were only two frequencies tested for the PBN mice (10 Hz and 20Hz). This could be limiting because it may not be as justified to predict a trend based upon the results. Testing more frequencies would allow for more reliable results for the PBN pathway mice. Another potential limiting factor is that since the surgeries were manually performed for both injecting the virus and implanting the optic fibers, it is possible that there could be potential variability in the data that is caused by variability in the location of the fibers and/or virus.

In summary, activation of the BNST-LHA pathway showed an increase in feeding, while activation of the PBN-LHA pathway did not have a significant effect on feeding behaviors. Future experiments will be needed to address the synaptic mechanisms behind the BNST-LHA and PBN-LHA pathways for why activation of these pathways results in certain effects. Also, further trials will be performed on the PBN-LHA pathway for more stimulation frequencies to see if new results are found. Additionally, future experiments can entail more satiety states, such as testing mice in a thirsty state. One reason for this would be to test whether the effects that we see are specific to hunger. Also, we can observe whether the mice respond differently to non-caloric rewards such as water.

Therefore, this could result in understanding whether the BNST-LHA or PBN-LHA pathways have functions that are specific to regulating food intake, or if they have more general functions related to all consummatory behaviors. The use of these mouse models will consequently help us develop a greater understanding of human neurobiology■

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