Role of raphe GABAergic neurons in leptin-regulated food intake: effect of L-allylglycine on GAD67 expression

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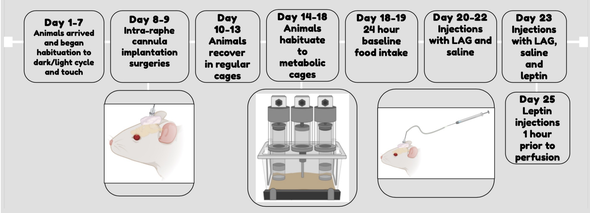
Introduction

The brain is a complex organ that is responsible for controlling many aspects of our life such as memory, emotion, motor skills, breathing, and hunger. Of these functions, the regulation of hunger is an important pathway to understand, as it is essential for life. Within the brain, there are multiple neural pathways that play an integral role in the basis of food intake, and it is imperative to understand how each piece fits together. Through previous studies, it has been demonstrated that leptin has direct control on food intake. Along with this notion, studies have also shown that many neurons that respond to leptin are GABAergic; however, it is unknown what specific role gamma-aminobutyric acid (GABA) may have. GABA is an amino acid that acts as a primary inhibitory neurotransmitter for nerve cells located in the brain. The synthesis of GABA takes place in the cytoplasm of the presynaptic neuron and results from the decarboxylation of glutamate due to the activity of the enzyme glutamic acid decarboxylase (GAD). In order to study the role of GABA in the regulation of food intake by leptin, we decreased the levels of GABA using L-allylglycine (LAG), a compound that inhibits GAD activity. As a result of decreased GAD activity, GABA synthesis is blocked, which leads to lower levels of the neurotransmitter. The purpose of this experiment is to evaluate the importance of GABA in leptin's ability to reduce food intake in the dorsal raphe nucleus (DRN) by reducing the amount of available GABA in the DRN prior to leptin administration.

Methods



Habituation and Baseline: Eighteen adult, male Sprague-Dawley rats were habituated individually and given access to food and water. The animals were weighed and handled before noon every day.

Days 8-9: Intra-raphe cannula implantation procedures were performed using stereotactic surgery techniques. A small incision was made on the top of the skull & strategic holes were drilled to insert the cannula. Once finished, cement was used to keep the cannula in place.

Days 10-18: After intra-raphe cannula implantation surgeries, the rats were returned to their regular cages for recovery. Three days after surgery, rats were then relocated to metabolic cages in order to begin habituation to the new environment.

Days 18-19: Food intake was automatically collected by the metabolic cages for 24 hours starting at the onset of the dark cycle to serve as the baseline food intake.

Days 20-23: Injections were completed with eight animals at a given time. L-allylglycine (LAG) was injected once per day for 4 days. 20 µg of LAG was given to half of the rats and vehicle was given to the other half. On the fourth day of injections, 5 µg of leptin was administered into the raphe of all rats prior to the dark cycle.

Methods

Euthanasia (Day 25): One hour prior to sacrifice, injections of 2 µL of leptin or vehicle were administered into the DRN. Animals were perfused transcardially using 0.1M PBS and 4% paraformaldehyde. Brains were removed and post fixed for 24 hours in 4% paraformaldehyde at 4°C, then cryoprotected in 30% sucrose for several days at 4°C.

Tissue Preparation: Brains were removed from 4ºC and frozen using 2-methylbutane and stored in -80ºC. Each brain was then sectioned at 20 µm using a cryostat and immediately mounted onto slides.

RNAscope: RNAscope procedure was performed on tissue that contained the raphe nuclei. On day 1, slides were washed in 200mL 1X PBS, incubated at 60ºC for 30 minutes, fixed in 4ºC PFA for 10 minutes, dehydrated using increasing concentrations of EtOH, underwent target retrieval, and were left to dry overnight. On day 2, the tissue was washed then incubated in protease III for 30 minutes 40°C, washed with distilled water, then hybridized with probes for vesicular GABA transporter (VGAT), GAD, and the leptin receptor (lepR.). Probes were then amplified and labeled with opal dyes.

Imaging: Mounted slides were coverslipped and imaged using Leica SP8 confocal microscope.

Results

Figure 1

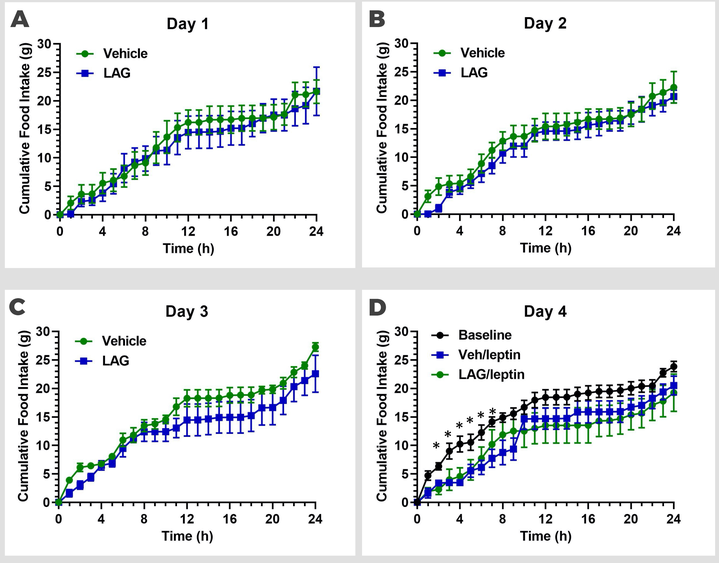


Figure 1. Effect of LAG and leptin treatments on food intake when administered to the DRN. Food intake data automatically collected by the metabolic cages were collapsed into 1-hour bins over the 24-hour period, with T=0 as the onset of the dark cycle. (A-B) On days 1-2 of LAG treatment, there is not a noticeable difference in food intake levels between LAG and vehicle treated groups. (C) On day 3 of LAG treatment, the graph shows greater separation between the two groups as the animals in the LAG group had a decline in food intake. (D) On day 4, leptin injections were also given to both groups just prior to the dark cycle, which results in a reduced food intake for around 8 hours after administration compared to baseline food intake. However, the effect of leptin was similar between LAG and vehicle treated animals. Statistics were performed using a one-way ANOVA.

Figure 2

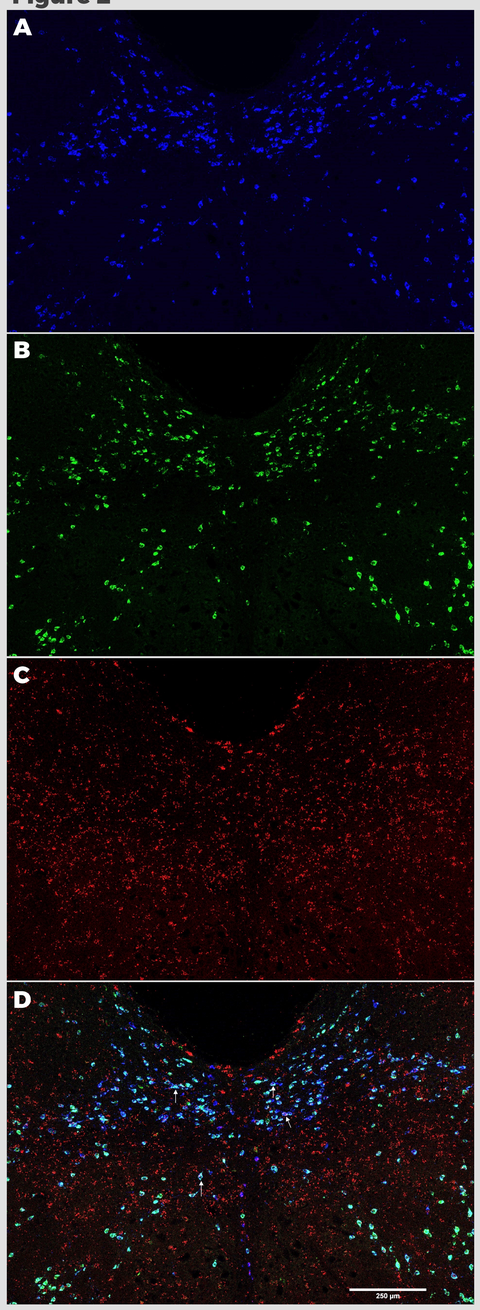


Figure 2. RNAScope of the DRN showing GABAergic neurons sensitive to leptin. Oligo probes for (A) vesicular GABA transporter (VGAT), (B) GAD67, and (C) leptin receptor mRNA are present in the raphe. (D) The merged image shows colocalization between the markers for GABAergic neurons and the leptin receptor mRNA (White arrows), suggesting that GABA may play a role in leptin's function in the raphe.

Conclusions

- Leptin successfully decreased food intake levels in both experimental groups.
- The presence of GABA is lowered as a result of LAG injections.
- GABAergic neurons that express leptin receptors are present in the raphe nuclei.
- Behavioral data (food intake) has not displayed a definitive answer on whether GABA is involved in this immediate pathway.
- RNAscope images show colocalization of vesicular GABA transporter (VGAT), leptin receptors, and GAD67 mRNA, suggesting a role for GABA in leptin's functions in the DRN.

Future Directions

- Use 4 different treatment groups (vehicle-vehicle, vehicle-leptin, LAG-vehicle, LAG-leptin)
- Increase the number of rats used in study (n-size)
- Alter the dosage of LAG injections
- Conduct the experiment over a longer time frame
- Use micropumps to inject LAG slowly over time rather than in 1 injection
- Quantify the expression of leptin receptor and GAD67 in the experimental groups

References

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Acknowledgements

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