2020

Feeding Effects of GLP-1 Receptor Expressing Neurons in the Bed Nucleus of the Stria Terminalis

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FEEDING EFFECTS OF GLP-1 RECEPTOR EXPRESSING NEURONS IN THE BED NUCLEUS OF THE STRIA TERMINALIS

By

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A Thesis submitted to the
Department of Psychology
in partial fulfillment of the requirements for graduation with
Honors in the Major

Degree Awarded:
[Spring, 2021]

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Introduction

Glucagon-like peptide 1 (GLP-1) is an amino acid peptide hormone that can be found in the intestinal epithelial endocrine L-cells, as well as in a population of neurons in the brainstem. GLP-1 is released by the intestine in response to nutrients in the gastrointestinal tract, and acts as an incretin hormone, meaning that it leads to the secretion of insulin, as well as the inhibition of the release of glucagon. It also suppresses food intake and inhibits gastrointestinal motility, as part of the "ileal brake" mechanism (Holst 2007). This thesis focuses on brain GLP-1 and its receptors, which also play a role in the control of food intake. Because the active form of GLP-1 has an extremely short half-life, intestinal GLP-1 does not enter the brain in sufficient quantities to have effects there, and it is believed that the endogenous ligand for brain GLP-1 receptors (GLP-1R) is neuron-produced GLP-1 (Williams, 2009).

GLP-1 neurons are found in the caudal nucleus of the solitary tract (cNTS) and the medullary reticular formation. These neurons project to many other brain areas and release GLP-1 at those sites, where it binds to and activates the GLP-1R. In many of these nuclei, this leads to the suppression of food intake (Maniscalco & Rinaman 2018). GLP-1 neurons are activated by vagal afferent stimulation, which brings information about gastrointestinal satiety signals to the brain (Waise, Dranse & Lam, 2018). GLP-1 neurons seem to integrate different kinds of information, as these neurons are not only responding to gut signals, they are also activated by stress and they send information through their projections to other nuclei that have important roles in stress response, reward, and avoidance. The GLP-1R expressing neurons are spread throughout the forebrain, spinal cord and brainstem. The presence and feeding effects of GLP-1R in so many brain areas raises the question of whether all these GLP-1R populations do the same thing, and if

they do not, can we identify meaningful differences? To address this question, we will isolate and compare specific GLP-1R neuron pathways. This thesis will focus on one such pathway.

Our laboratory and others have shown that GLP-1R activity in the bed nucleus of the stria terminalis (BNST) can affect food intake and influence stress responses (Williams et al., 2018; Zheng et al., 2019). When GLP-1 was injected into this brain region in mice, it suppressed feeding, and injection of a GLP-1R antagonist increased food intake (Williams et al., 2018). The BNST is an integration center for limbic information, it is located in the basal forebrain and has a variety of neuron populations- one of them being GLP1-R neurons. This area contributes to feeding behavior and is also important for the stress response and the hypothalamic-pituitary-adrenal (HPA) axis (Lebow & Chen, 2016). In mice, GLP-1 directly inhibits about 60% of GLP-1R neurons in the BNST, and this finding was surprising because GLP-1R have been considered excitatory (Williams et al., 2018). This inhibition suggests a diversity of mechanisms for how GLP-1R neurons affect feeding. For these reasons, BNST GLP-1R neurons are a good target for us to investigate.

The BNST contains a variety of neuron populations and has many different efferent projections. In order to identify the roles each of these populations has in feeding, it is important to isolate a specific projection pathway. We focus here on the BNST GLP-1R neuron projection to the lateral hypothalamus (LH). The LH was chosen because it is known for its role in food control, and we have shown that GLP-1R neurons of the BNST send projections there (Williams et. al. 2018). Jennings and colleagues (2013) showed that inhibition of a BNST GABA projection to the LH suppresses feeding, and activation of this projection increases feeding. This is consistent with our finding that GLP-1 in the BNST both suppresses feeding and inhibits many BNST neurons.

This pathway can be targeted by using modern technology including genetically modified mice that express Cre recombinase in GLP-1R neurons. Cre recombinase is an enzyme that allows the control of site specific recombination in DNA, which means that it is able to turn genes 'on and off'. This allows us to use an adeno-associated virus (AAV) delivery approach that will insert DREADD only in GLP-1R neurons. There are excitatory and inhibitory versions of these receptors available, and this thesis used the excitatory kind. The drug clozapine-N-oxide (CNO) activates this receptor and causes the neuron in which it is expressed to have action potentials. Thus, we gain the ability to activate GLP-1R neurons (Roth, 2016). We used a retrograde virus injected into the LH, which travels retrogradely from there and infects the neurons that project to the LH, meaning that it infected the GLP-1R neurons in the BNST and induced DREADD expression there. Administration of CNO to the BNST then allows us to selectively activate this pathway. We injected CNO only into the BNST because there are many brain areas that express GLP-1R that also project to the LH, so other locations in the brain will have been infected by our AAV and express the DREADD. Isolating CNO to the BNST was meant to ensure that only the BNST to LH pathway is activated. Based on the findings that GLP-1R stimulation in the BNST both suppressed food intake and inhibited many BNST neurons (Williams et al., 2018), we hypothesized that activating these cells would have the opposite effect and increase food intake.

Methods

Subjects

Groups of GLP1R-Cre mice and C57Bl6 mice were age and weight matched (around 210 days old at time of surgery) and housed in a 12-12 light: dark cycle in standard mouse cages fitted with BioDAQ system (Research Diets). BioDAQ is a system that allows for continuous food

monitoring because the food hopper is on a sensitive load beam that constantly records the weight of the food. Mice had ad lib food and water except when otherwise noted. Ultimately the goal is to have a minimum of n=12 in each group, and at least 6-8 of each sex (based on power analysis done previously). However, because the study requires transgenic mice and the BioDAQ system has limited chambers, as well as COVID-19 pandemic limiting laboratory access, only one squad of 13 subjects was done.

Virus/Surgery

Mice had two weeks of habituation into the BioDAQ cage system before surgery, and were given about 10 days with continuous post-operative care to ensure recovery from surgery. Mice were sedated using 3-4% isoflurane (Zoetis) using an induction box and then using a nose cone during the stereotaxic surgery where the AAVs are injected in four locations (two injections delivered bilaterally) in the LH (-1.0mm & -0.5mm from bregma, +1.1mm & -1.1mm lateral to midline, and -5.2mm ventral to skull surface) using iontophoresis (Alternating pulse 7s for 5min, positive polarity, retention off, current within 4.8-5.0 μA). The viruses were either rgAAV-hSyn-DIO-hM3Dq-mcherry (DREADD) or rgAAV-hSyn-DIO-EGFP (GFP) (both AAVs from Addgene) GLP-1R-Cre and C57Bl6 mice were assigned to weight-matched groups, receiving either the DREADD or GFP virus. After the 4 virus injections, a bilateral cannula (Plastics One) was implanted into the BSNT (+0.75mm from bregma, +0.70mm & -0.70mm lateral to midline, and -2.5mm ventral to skull surface. Injectors (33G) extending 2.0mm below the end of the guide cannulas were used).

On surgery day, mice were given 10 mg/kg carprofen based on their body weight, and the day after surgery they were given 5mg/kg carprofen. They were monitored for 10 days, and

additional analgesic was given if needed. Cannula placement and viral expression was histologically validated at the end of the experiment (only partially completed; see Fig. 1 for representative placement), and only subjects with correct cannula placement will be included in the analysis. If sufficient numbers of incorrect cannula placement are found, they could be used as a control group.

Drugs

Mice were injected with CNO or vehicle. Doses of CNO (3 μM, 10μM, 30μM) were decided based on preliminary data from our laboratory that showed that these doses have no effect when injected into the ventricle. This is important so that we can be sure that any effect of CNO can be attributed to DREADD activation in the BNST, and not diffusion to other locations or non-specific effects.

Behavior/Experimental Design

To ensure peak DREADD or GFP expression, we started the experiment at least two weeks after surgery. Mice received at least one habituation training day where they all received vehicle (50% DMSO and 50% Saline) to get used to the injection procedure. On injection days, food was removed 3.5 h prior to injection. Vehicle or CNO was injected in a 200 nanoliter volume over 2-5 minutes within 1 hour before dark onset. A period of minimum 48-72h passed before the next treatment day. All subjects were supposed to receive vehicle and 3 doses of CNO in a counterbalanced order, but one treatment day was not done for some subjects due to COVID-19. Spontaneous chow intake data was collected through the BioDAQ system, and meal pattern

information including first meal size, average meal size, number of meals, hourly cumulative and non-cumulative intake was analyzed.

Statistical Analysis

For each variable collected, we would have performed a mixed-design analysis of variance. This would have had three factors: between subjects factors are genotype and DREADD vs. GFP, and the within-subjects factor is dose of CNO. $P < 0.05$ would have been taken as significant. Holm-Bonferroni tests would have been used to make planned pairwise comparisons between vehicle and CNO doses within each group. If we had completed the intended number of subjects, we would have presented data for males and females separately and could have statistically compared them to investigate possible sex differences.

Results

Due to the Coronavirus pandemic, only one cohort of subjects was run in this experiment, so we did not complete enough subjects in each group to reach a conclusion through statistical analysis. Combining male and female subjects, due to the absence of notable sex differences in our data so far, there was an average of n=3 per group, which is not enough subjects to statistically analyze and reach a conclusion. For this reason, although a large amount of data were collected, we only present an example of some of the food intake data here, cumulative chow intake 1 h into the dark phase (Fig. 2). Looking at the data, the indication is that there was slightly higher intake after 10μM CNO dose in GLP-1 DREADD mice. It is very difficult to identify any trends due to the variability across individual subjects within treatment conditions, so this could mean that there is no dose related trend. There are individual cases in which it appears that intake is higher after CNO in GLP-1R-Cre DREADD mice, but this is also the case in some animals that were wild

types, and in some GLP-1R-Cre mice that received the control AAV so they did not have the DREADD. Until we collect more data, we cannot make a conclusion.

The expected results of this experiment include that CNO will not have any effect in wild type C57Bl6 (non-Cre) DREAD animals, due to the fact that they did not have any DREADD expression because they have no Cre recombinase. We should also see no effects in the GLP-1R-Cre and C57Bl6 mice that received the GFP virus, again because they will not have any DREADD expression. Only the GLP-1R-Cre DREADD animals should exhibit effects of the CNO. If we do see an effect of CNO in one of the control groups, we would first need to show that the animals were not mixed up by looking at the reporter protein expression histologically. This could also happen if our CNO doses were too high, although our preliminary data suggest that these doses should not have effect in our control subjects. Effects at high CNO doses could be due to non-specific effects, for example if the CNO was binding to receptors it would not normally bind to. We do not expect this to be a problem in this experiment because we chose doses based on a preliminary study in which it was determined that there would not be any nonspecific effects at those specific doses.

We expected that CNO would activate the BNST GLP-1R neurons in the GLP-1R-Cre DREADD mice, and we hypothesized that this would increase food intake in these mice. Previous data suggests that BNST to LH inhibition increases intake (Jennings et al., 2013), and many GLP1-R neurons are inhibited by GLP-1 (Williams et al., 2018). If this were not the outcome, it could be because 40% of GLP-1R BNST neurons are not inhibited by GLP-1, and are excited instead (Williams et al., 2018). It is possible that the specific BNST neurons that we activated in this study are mostly the GLP-1R-activated type, or a mixed population. Another potential explanation for a lack of effect here is the possibility that repeated pressure injections into the BNST caused

sufficient damage to this brain area that many of the cells of interest were destroyed, thus affecting the results. Damage at the BNST injection site is visible in Figure 1.

Discussion

While we do not have enough data from this study to draw any conclusions, we have decided to change our methodological approach for future studies rather than continue this one. The original hypothesis was that activating the BNST GLP-1R neurons that project to the LH would increase feeding. We tried to address this hypothesis by injecting retrograde AAV into the LH. Because the AAVs are retrograde, it is transported from the axon terminals back to the cell bodies, and this means that any neuron that has axon terminals near the injection site will get infected regardless of where the cell bodies are. In the GLP-1R-Cre mice, this resulted in expression of either DREADD or GFP in any GLP-1R neurons that project to the area of that AAV injection, including GLP-1R neurons in the BNST, but also many other locations. GLP-1R neurons in the LH expressed the DREADD and mCherry reporter, as well, as seen in Figure 3. We implanted a bilateral cannula into the BNST, where CNO or vehicle was injected on treatment days. We intended the CNO injection to activate only the BNST GLP-1R-expressing neurons, and did not account for the possibility of DREADD-expressing axon terminals from GLP-1R neurons in other brain areas innervating the BNST. At the time we designed this study, we did not realize how interconnected these regions are. For example, GLP-1R neurons with cell bodies in LH project to the BNST (unpublished preliminary data, representative image shown in Figure 4). GLP-1R neurons in the lateral septum (LS) also project to the BNST as well as the LH (unpublished preliminary data), so it is possible that some LS neurons expressing DREADD in our experiment have axon terminals in the BNST. Therefore, when we injected CNO into the BNST, we not only

stimulated BNST cell bodies expressing the DREADD, we also stimulated LH neuron terminals and possibly also terminals from the LS and elsewhere. This means that the contribution of the BNST to LH pathway was not isolated as we intended, and we could not be sure that any results are due to the stimulation of only the BNST-LH pathway.

There is an additional concern regarding tissue damage due to the repeated injections and cannula placement in the BNST. This is a relatively small brain area so the multiple CNO injections as well as the presence of a bilateral cannula could have destroyed some of the neurons in the BNST, decreasing the number of GLP-1R neurons that could be activated by CNO injection. We hypothesized that stimulating the neurons there would increase food intake, but if a sufficient number of neurons are destroyed, it is possible that those that remain may not be enough to cause the effect we are looking for.

Before the COVID-19 pandemic, we began working on an alternative approach to address these problems. Different serotypes of AAVs have different effects in the nervous system. We used a retrograde AAV that was engineered to be robustly transported retrogradely, but there are other AAVs that are not transported retrogradely. We decided to inject one of those AAVs into the BNST, where neurons will be infected, and the GLP-1R-Cre neurons will express the DREADD or control GFP throughout their cell bodies and axons, including their axon terminals in the LH. Then we can implant the cannula in the LH, so the CNO activates the DREADD at those axon terminals coming from the BNST (e.g, Stachniak et al., 2014). We tested AAVs of serotypes 1 and 2, and found that AAV1 worked well for this. Others in the lab are now conducting this experiment. We do not have data to report right now since the experiment is ongoing, but the expectations and hypothesis remain the same: activating the BNST GLP-1R neuron terminals in the LH will increase food intake. In Spring 2020, as we were preparing to start over with this new approach,

the pandemic happened and all laboratory work stopped for a few months. I did not return to Tallahassee in Fall semester, and this why the new experiment is being done by other people.

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Figures

Figure 1: Representative image of a coronal brain section showing bilateral injection site in the dorsal BNST. LV = lateral ventricle; ac = anterior commissure; dotted lines surround the injection damage.

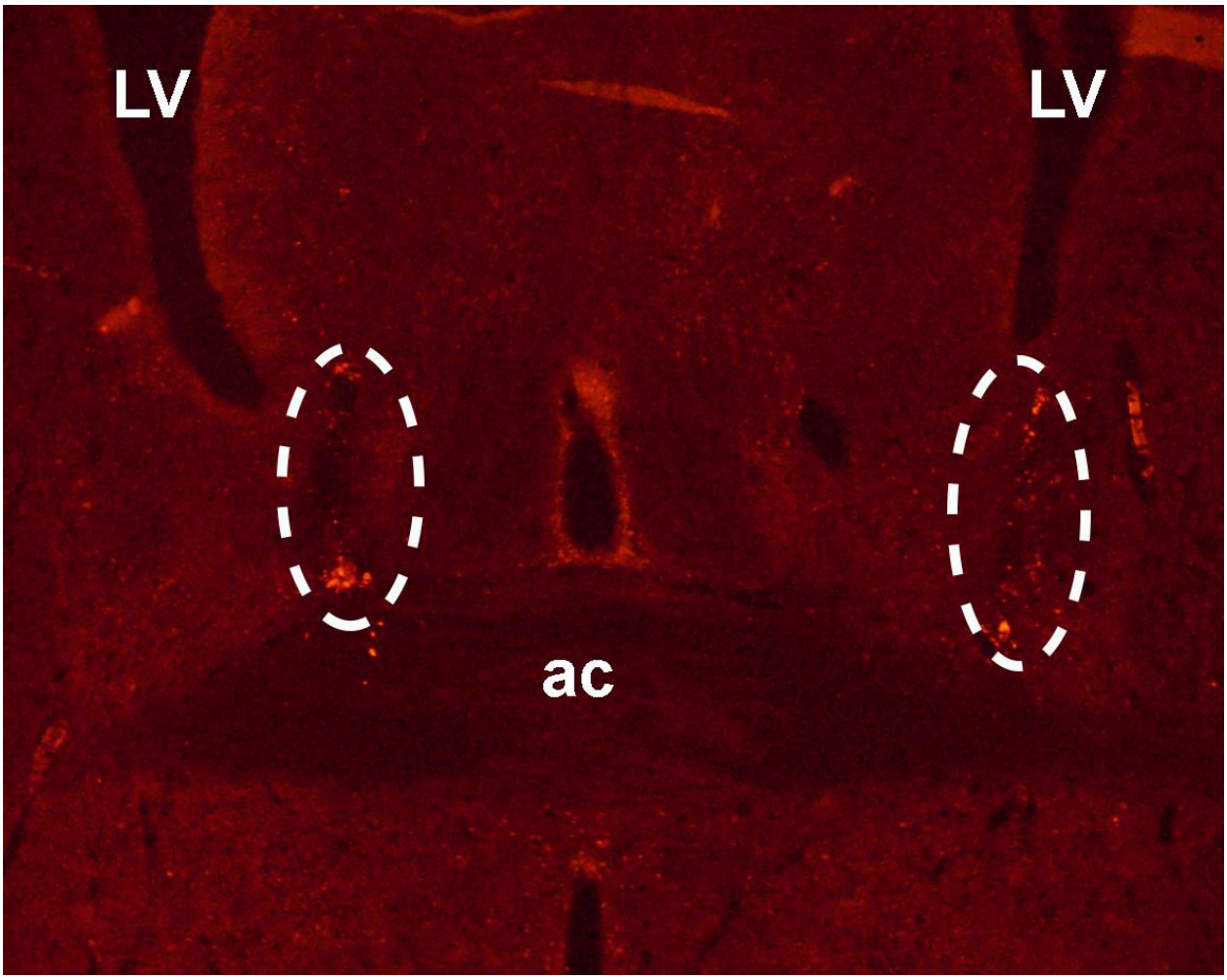

Figure 2: Cumulative 1-h chow intake, mean +/- SEM.

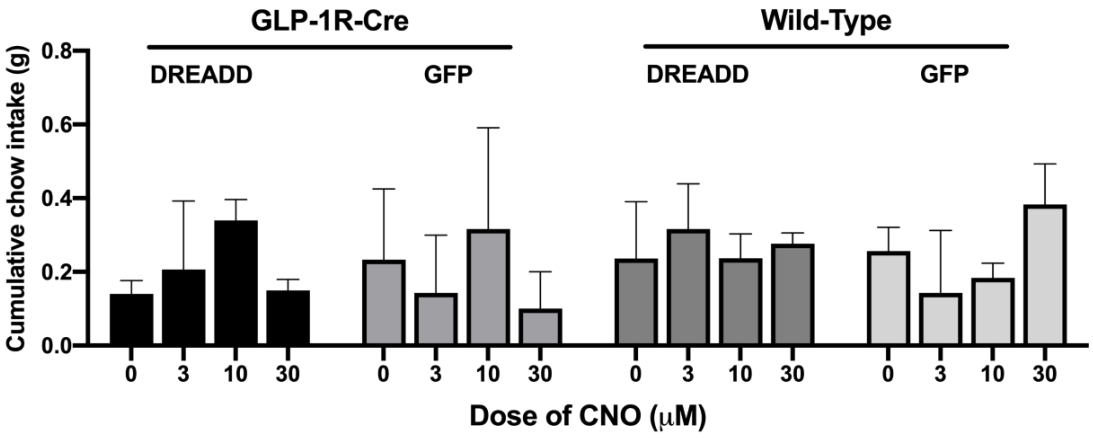

Figure 3: Representative image of a coronal section from a GLP-1R-Cre-DREADD mouse from this experiment, showing the LH. Red cells contain the DREADD-mCherry. f = fornix.

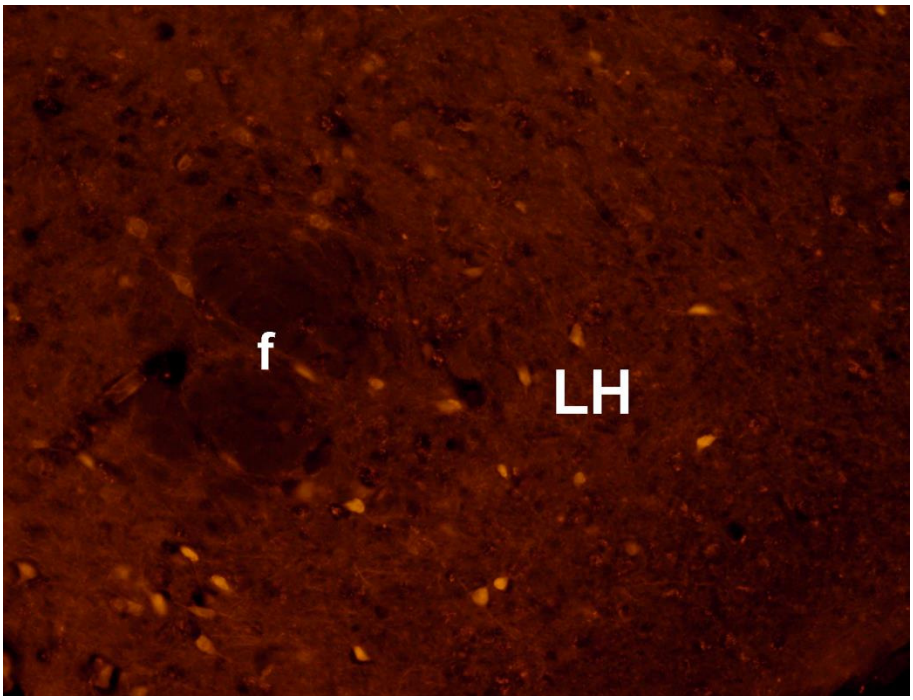

Figure 4: Representative image from a study in which GLP-1R-Cre mice were injected into the LH with an AAV for Cre-inducible expression of GFP that fills the cell including axonal projections. This image shows green GFP-containing axons of LH neurons innervating the BNST.