nature portfolio

Peer Review File

A Brain Reward Circuit Inhibited By Next-Generation Weight Loss Drugs in Mice

Corresponding Author: Professor Ali Güler

Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Note from the editor: Reviewer 2 raised issues in the third round of review. Reviewer #1 has overlapping expertise, and provided guidance on addressing these issues. The authors made these changes and it was signed off by reviewer #1".

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
The manuscript by Godschall et al. dissects the sites of action by which oral GLP1RAs regulate food intake, particularly hedonic feeding. The current orally available agonists are not active at the mouse GLP1 receptor so the studies employ a new mouse line with a humanized Glp1r S33W. They studied these knockin mice as well as animals in which the humanized receptor is introduced into discrete brain regions of GLP1r-cre mice using an AAV with a DIO- Glp1r S33W payload. They then analyzed the effects of the oral agents on a set of behaviors including consumption of chow in the dark phase as well as a highly palatable diet during the light phase, a time when animals consume the HFD but do not consume chow. They report that danuglipron reduces ingestion of a HFD activating inputs from preproglucagon (ie GLP1) expressing neurons in NTS to neurons expressing GLP1R in CEA. Finally, Godschall et al. report that GLP1RAs reduce dopamine responses in the NAc using a fluorescent reporter and (indirectly) link this effect to a projection from CEA to VTA. In aggregate the paper reports the novel and interesting findings that an oral GLPRA can specifically reduce hedonic feeding and this is a result of modulation of the neural targets of GCG neurons in NTS. However, several of the experiments that are reported are either flawed or insufficient and several additional experiments as well as significant revisions would be required for the paper to be suitable for publication.

Major Criticisms:

In Figure 1, the effects of orforglipron were recorded for 7-days. Studies over a longer time frame should be performed

Metabolic profiling was performed in both males and females in ext. Fig. 1), but the studies of the effects of GLP1RA administration in Figure 1 were limited to male mice. The authors need to add data for food intake in female mice.

In Fig. 2, the authors need to show that the neurons activated by the GLP1RAs express the GLP1R. Co-localization experiments between GLP1R and c-FOS labelling are necessary. In addition, the levels of c-FOS in control mice receiving vehicle is lacking. In addition, these images are of poor quality and the data, at least in the printed figures, do not justify the authors conclusion
The authors state that danuglipron preferentially activates NTS but not AP (see lines 147-153) raising the possibility that it will induce less nausea. The authors need to test this directly rather than simply referring to the distribution pattern of activated neurons. Studies using assays of nausea or conditioned taste aversion are necessary.

In Figure 3 the authors monitor the behavioral responses to the oral agents. However, these data are largely superfluous and omit the most relevant behaviors having to do with possible effects on nausea. These data do not bear on the key conclusions of the paper and appear to have been “thrown in”. In addition, the authors fail to observe significant effects on movement while at the same time the mice that received danuglipron or liraglutide are reported to spend significantly more

time resting? What is the explanation for this? What are GLP1RA effects on locomotion? What is the total distance traveled in the two groups? Furthermore, could changes in time spent in the shelter and grooming be a result of changes in anxiety as this has been demonstrated before in mouse and human studies? (Kornelius et al., 2024; Zheng et al., 2019)

In Figure 4U, synaptophysin tracing shows that NTS GCG neurons project to amygdala. However, data showing functional connections are lacking. Rabies tracing from CeA Glp1r neurons should be performed to determine whether these neurons receive direct inputs from NTS GCG. Pending this result, the authors need to provide functional evidence showing that activating NTS GCG terminals in CEA alters chow intake as well as hedonic feeding. In addition, the location of these terminals is unclear and the data in Ext. Fig 9 raise the possiblity that the terminals may be elsewhere in amygdala, see below.

In extended Figure 9 C–F, the authors state that eYFP-labeled hGLP1R-expressing neurons are in the CEA . However , the data appear to expression ventral to the CeA in STIA and MeA. More precise images should be provided with reference to a brain atlas showing precisely where these neurons are located. The based on the current images , it is unlcear whether these effects are specific to CeA vs. other amygdala nuclei.

In the electrophysiology experiments in Ext. Fig. 9, the authors do not state what the danuglipron was dissolved in and possible solvent effects on membrane deporarization were not tested. Furthermore, the depolarization that was reported did not induce any actions potentials or affect firing frequency of the neurons. These data are unimpressive.
In Fig. 5 the authors conclude that GLP1R expressing neurons in CEA regulate dopamine release in NAc . The authors need to use chemogenetics or neural ablation to show that inhibiting GLP1R expressing in CEA blunts the effects of GLPRAs to reduce hedonic feeding . It is relevant that GLP1R expressing neurons in NTS also project to NAc (Alhadeff et al., 2012) and that NAc expreses GLP1R and these neurons have also been shown before to regulate feeding (Mietlicki-Baase et al., 2014).

Minor Criticisms:
Figure 1H. The x-axis shows the starting point for GTT measurements a -240 min. This is not comparable to other GTT experiments in Figure 1.

In Figure 1G and U, Blood glucose appears to increase after danuglipron injection prior to the dextrose injection.

In Figure 1Z, the authors state that this experiment was performed in already obese mice but they do not state how long the mice had consumed a HFD before the experiment.

In Extended Data Fig. 8, it is not clear which hypothalamic areas the authors are referring to ie which specific areas correspond to the basomedial hypothalamus? The histologic sections seem to indicate that the injections of virus may also have spread into VMH and Arc see Panels C and D. The authors should provide posthoc images of whole slides of the injection areas with quantification of the total cells infected in each targeted brain area? If the virus has spread in the neighbouring areas, their conclusions may be inaccurate.

Referee #2

(Remarks to the Author)
This manuscript investigates the effects of next-generation, small-molecule GLP1RAs (danuglipron and orforglipron) on feeding behavior and neural circuits. Strengths include the timely interest in these classes of molecules and the development of genetically engineered mice expressing human-like GLP1Rs to study the effects of small-molecule GLP1RAs, similar to a rat model that was recently published (PMID: 39693407). Overall, the experiments seem well designed and executed but the real advance in our understanding of how these drugs is lacking, particularly with the growing number of publications exploring the mechanisms behind their efficacy. A few aspects that dampen my enthusiasm include a lack of pharmacokinetic comparisons/dose responses as well as limited oral administration (which is one of the major advantages of this class of molecules), limited long-term analysis of drug treatment, concerns over non-physiological artificial overexpression in the CeA, unconvincing evidence that the CeA pathway is specific to hedonic versus homeostatic food consumption, lack of testing the notion that less AP cFos activation translates into less aversion, and a circuitous leap to the canonical VTA to NAc dopamine reward system. Below I've listed some points I think would strengthen the manuscript:

1) It's unclear how the authors decided on the dose used throughout the experiments. It seems likely that altering the concentration may have far different effects (ie. cFos activation as well as appetite suppression)
2) Nothing really stands out with the cFos data that hasn't been reported with other GL1RAs. If the authors really want to make a claim that oral delivery leads to reduced AP cFos activation, then they should attempt to define what this means behaviorally. One would then hypothesize that these mice would demonstrate less aversion, although Figure 3 suggests behaviors are pretty similar. Also the representative images Fig 2j-k seem to show far less NTS expression via oral rote although this is not reported as significant. Of course, again these differences in cFos could merely come down to dose.
3) Figure 3 seems like a lot of work to show the drugs make the animals eat, drink, and move less while resting more which has been reported many times over using less flashy approaches.
4) There is a real concern with receptor overexpression. Examples in pharmacology have shown that ligands can act as antagonists at low receptor levels but switch to agonism when receptors are at higher-fold expression. This overexpression is likely non-physiological so it's difficult to interpret. Quantifying receptor levels would help clarify this.

5) The feeding experiments in Figure 4a-k are hard to take at face value given the variability of controls. For example vehicle injected mice tend to consume around 1 gram or more in most experiments run but the “key” experiment where the authors are claiming Dan does not inhibit SD consumed, the vehicle mice are super low making any change very difficult to detect. Therefore the conclusion that CeA is linked to hedonic feeding only is weakened…..
6) …..additionally, did the authors test whether opto activation of CeA Glp1r neurons suppressed chow. Given the numerous examples of different CeA populations that decrease food intake when activated, it seems highly likely this population would act similarly. It would be hard to imagine why the brain would be wired to specifically suppress “hedonic” food intake, especially when driven at supraphysiological levels of activation.
7) There’s a bit of confusion on the ephys experiments in Extended Data 9, are these recordings only performed in Cea of mice injected with the hGLP1R? What about in Glp1r-Cre mice just injected with DIO-YFP? It seems even without the humanized form, these Glp1r CeA neurons should still respond to the small-molecules in slice experiments, but may not in vivo.
8) Was there any validation of the AAV-DIO-mGlp1rS33W virus?
9) It’s unclear how many test animals were used for the photometry experiments, does each point in Fig 4n represent different animals or per injection. If the same animal was used repeatedly, those combined responses should be averaged in one data point. Also, all the mouse heat maps should be displayed as this allows the reader to see the raw data. The average number of events also seems to be variable during vehicle injections averaging around 12 in the hGLP1R mice but over 20 in the mGlp1r controls making interpretation cloudy. Could the authors look at area under the curve? What are the mice doing during the recordings and what happens to this activity during specific behaviors like eating?
10) The Glp1r knockout experiment is perplexing given the prior argument that liraglutide doesn’t reach/activate CeA Glp1r.
11) The jump to mesolimbic dopamine seems very sudden. Presumably these CeA Glp1r neurons send multiple projections to multiple sites, many of which have been documented. Is there a functional connection with the VTA? Are CeA Glp1r neurons inhibitory, when looking at scRNA data from the amygdala, what cluster(s) are they? Again, the evidence stated in lines 261-262 is weak.
12) Figure 5 is missing control non-Glp1r S33W mice.

Referee #3

(Remarks to the Author)
In this study, Godschall et al describe the development of mouse and viral vector tools to enable expression of humanized GLP-1R or mutated murine GLP-1R, responsive to oral small molecule GLP1RAs in clinical development, in mouse tissue. They characterize the behavioral, metabolic, and Fos activation profiles of danuglipron and orfoglipron in mice with S33WmGLP-1R. They go on to show that hGLP-1R expression in hypothalamus leads to agonist-induced suppression of ‘homeostatic feeding’, in brainstem leads to suppression of homeostatic and hedonic feeding, and in CeA leads to selective suppression of hedonic feeding. They perform functional studies to show that danuglipron or liraglutide activate CeA-GLP1R neurons, and that CeA-GLP1R neuron activation is sufficient and CeA-GLP1R signaling necessary to suppress HFD intake. Finally, they describe an NTS to CeA to VTA to NaC circuit by which GLP-1R agonist may suppress HFD intake.

The murine and viral tools developed are important and will likely be of broad use to the scientific community, and the dissection of different brain regions’ involvement in homeostatic vs hedonic food intake is interesting and adds to a growing literature dissecting mechanisms underlying GLP1RA-induced weight loss. A few controls are lacking and some important points glossed over, but with revision this study will be highly impactful.

Major points

- The fact that viral hGLP1R expression is an overexpression model should be discussed as a limitations. For example, it would be informative to see CeA-GLP1R fiber photometry data after lira injection in WT mice, or after Dan in S33W mice expected to have physiologic levels of receptor expression

- The distribution of fos-positive CeA neurons looks quite different after Dan vs Orfo vs Lira (fig 2). Presumably the effects of liraglutide on this region are indirect, and knowing the extent of overlap of Dan/Orfo vs Lira-induced fos expression in this region is ultimately going to be important in determining how small molecule vs peptide agonists work. An attempt at this using a Fos-trap or similar approach would be a good start. While this experiment is probably not absolutely essential for publication, this point needs to be discussed more clearly

- Looking at food intake following a fast (rather than just ad lib dark cycle food intake) may give a better sense of the role of GLP1R agonism on homeostatic food intake. Does not necessarily have to be done for all models but some data on this would be good

Minor points
- The authors somewhat overinterpret their findings in their proposed circuit diagram in Fig 5b. They do not actually show that NTS-GCG neurons synapse on CeA-GLP1R neurons, just that they project to CeA and that pharmacologic doses of GLP1R agonist activate CeA-GLP1R neurons. I’d add a question mark and address the remaining gaps.

- The authors state a couple of times that potentiation of insulin/suppression of glucagon release contribute to GLP-1RA-induced weight loss. This is incorrect – those responses to GLP-1RA are important for diabetes control but not weight loss so those phrases should be re-worded (lines 57 and 104)

- Commercially available GLP-1R antibodies are notoriously non-specific (eg see PMID: 23267050 and PMID: 23183176) so antibody validation of hGLP1R expression in extended figure 8 is not convincing without control staining. The authors show electrophysiologic validation of their virus in CeA in fig S9, and behavioral effects following viral injection, which mitigate this concern but would recommend showing antibody controls and/or mentioning this limitation for transparency.

- A few figure panels that are not noted as significantly different look fairly different (eg extended fig 1E, F – daytime EE in S33W mice looks almost higher than WT controls, extended fig 13B – sal vs lira look pretty different. A stats table or p values on the fig would be helpful.
Extended figure 11 shows an example raw photometry trace, and it would be good to see more raw data rather than just the highly processed heat maps. I understand that the team is analyzing events rather than sustained neural activity changes and so averages may not work, but at least showing sample control traces would be helpful

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)
Re-review of Nature 2024-12-26939B
Godschall et al. investigated the effects of oral GLP-1 receptor agonists (GLP-1RAs) in a humanized mouse model, focusing on food intake, body weight, and behavioral phenotypes. They also add a new component to the brain circuit through which these drugs exert their effects specifically an effect of actions on the amygdala to regulate reward pathways.
The authors have satisfactorily addressed all of my previous comments and in my estimation the paper is suitable for publication pending the authors addressing the following comments which are pertinent to the new experiments that were added.
What follows are some specific comments .
Figure 3 and 4
There is no change in cFOS activity in the DMH after administration of vehicle, danuglipron, liraglutide, or orforglipron (Fig. 3A). This is surprising because in Figure 4, danuglipron significantly suppresses chow intake in mice injected with AAV-DIO-hGLP1R in the DMH. The authors should address the basis for this apparent discrepancy.
Figure 2 and 3
The authors now demonstrate that orforglipron elicits less of a CTA than danuglipron which has a greater effect (Fig. 2, Extended Data Fig 3). However, orforglipron administration still results in significant cFOS activation in the area postrema (AP) to a similar extent as danuglipron (Fig. 3C). Given that both compounds activate the AP, previously shown by others to be a site at which nausea is induced, the authors should address why danuglipron has a greater effect. Specifically, they should determine whether different neuronal subpopulations within the AP are activated by these agents. Alternatively, this difference could reflect engagement of different downstream populations that in turn influence the response.
Figure 4
The rationale for focusing on the effects of danuglipronbut and not orforglipron on the central amygdala (CeA) is not clearly explained and should be clarified (Fig. 4).
Extended Data Fig. 11.
The authors note that Glp1r is expressed in the Vdr-expressing neuronal cluster within the CeA. However, MERFISH data from the Allen Brain Cell Atlas indicate that Glp1r is not limited to the Vdr population in amygdala. The authors should assess which other CeA neuronal subtypes express Glp1r.
In their response to Reviewer 2, the authors indicate that Glp1r-expressing neurons in the CeA partially overlap with Pnoc-positive neurons. However, previous work by Hardaway et al. (2019) demonstrated that chemogenetic inhibition of Pnoc+ neurons there suppresses palatable food intake. This effect contrasts with the decreased food intake in the current study following optogenetic stimulation of CeA Glp1r neurons. The authors should address the basis for these potentially contradictory findings.
Extended Data Fig. 12
The duration of recording in the analysis of membrane potential before and during danuglipron application is not clearly described. In addition, the recordings appear to have been performed in neurons from a single mouse, which is insufficient. Unless this is incorrect, recordings from CeA neurons from at least three animals per group should be included. Furthermore, the authors report an ~5 mV depolarization that is not durable. These data are thus unconvincing and additional experimental support for this response is necessary.
Extended Data Fig. 15
The authors should quantify the number of Glp1r neurons in CEA and the number of Glp1r neurons that are labeled after retrograde tracing from VTA using rabies.

Referee #2

(Remarks to the Author)
The authors should be commended for addressing the reviewer comments as the changes have significantly enhanced the conclusions of the manuscript, often times just by providing additional context and framing, but also through additional experiments and data analysis. My remaining comments are minor:

1) Figure 4m-n: it would be best to highlight all the cells showing co-expression with white arrows in all the panels as is depicted in the far right panel.

2) Figure 4x-y: it would have been good to see within-subject vehicle vs Lira comparison of both the AAV-GFP and AAV-Cre mice. It would also be insightful to see representative images of the injection sites particularly since the Cre virus seems to be fused to eGFP.

3) I am curious why the authors tested lira, instead of danu or orfo for Figure 4x-y as the effects of the former would be indirect while the latter as predicted by the other findings in this manuscript may act directly on CeA GLP1Rs?

4) Figure 5a and e: Add a panel showing representative image of injection site of the NTS and CeA, respectively.

Referee #3

(Remarks to the Author)
Overall, the authors have addressed my concerns with one exception and I think the work is suitable for publication and a contribution to the field across multiple dimensions.

The one issue that was not addressed is related to the distribution of fos-positive CeA neurons after Dan vs Orfo vs Lira. Seeing a similar FP response to these drugs in CeA GLP-1R expressing neurons does not rule out that it could be different sub-populations activated by the different drugs (through direct vs downstream circuit mechanisms), as FP is a bulk imaging approach. I do not think further experimental work is needed but did not see the discussion of this possibility in the revised text as noted in the reviewer response. I do think this needs to be discussed in a short sentence or two.

Referee #4

(Remarks to the Author)
Godschall et al. report the generation of a humanized GLPR mouse line and characterized their changes in behavior and neural activity after treatment with either danuglipron and orforglipron (and baselines). I am evidently a new reviewer added after a round of reviews. I have reviewed the manuscript and rebuttal, and I found the first reviewers asked excellent questions. However, in this round deeper phenotyping of behavior was added, which is my area of expertise. Therefore, I am going to specifically add comments on this aspect of the work (and not others).

Specific Major comments:

- How was the 2-hour filming window determined for home-cage monitoring?
- From the main text it appears only a single “neck/upper back” key point is used for keypoint estimation (this is then not called pose estimation) likely this is just for the locomotion, but they clarify in the main text (the method notes 9 keypoints for Keypoint-MoSeq, but then some nose heatmap processing? See minor below).
- Why do the authors use PCA/ how is it “significant”? Line 143 “This distinct profile led to its significant separation”. Provide statistical bootstrapping in the text.
- The major issue is the inappropriate use of Keypoint-MoSeq; the authors seems to use the “ ” configuration for pose estimation, and get the default 91 behavioral syllables, then go onto hand-curation, followed by nonlinear embedding (UMAP). This is a non-standard approach that would need significant validation. Why do they not follow the statistical approach from Wiltschko et al. 2020 Nature Neuroscience (see Figures 2 and 3), or even the Keypoint-MoSeq paper? For example, the authors could have used the pretrained SuperAnimal-TopViewMouse pose model that has more keypoints and leads to improved performance of MoSeq (see Weinreb et al. 2024 Nature Communications, Figure 5). Then they can take the full transition graph and perform statistics directly in this space (as in the 2020 paper) – UMAP and manual curation is not needed and not statistically robust. I strongly feel this analysis must be redone to be publishable.
-- Independently, why use UMAP? Is there something nonlinear in the behavior they needed to capture that PCA did not? How did they validate UMAP hyperparameters? Also measuring distances in UMAP global space is inappropriate – how did they determine the local space?
- The title must reflect this is in "humanized mice". The authors are surely aware of the media impact this work will have, and that is critical for appropriate communication.

Minor:

- Figure 1 has alignment issues (i.e., “y” is missed aligned) and could use more white space between panels for readability.
- Figure 2; the arrow is not straight under the term “OpenCV” and Keypoint-Moseq is mis-capitalized (MoSeq).
- Figure 3g, the stars cover the error bar
- Figure 4 should show raw calcium imaging data
- The Veh. in Figure 5m seems to be nearly symmetrical around retrial, why?
- Line 776; light cycle – was the light cycle inverted so you ran behavioral assays in the dark period?
- Line 797; it would be 90% train, 10% test (not validation, unless they finetuned parameters based on that 10% fraction); also to note, the mAP of 81 is actually very low given they trained on nearly 10K frames – this suggests the manual labeling was not high-quality.
- Line 801: “Representative heatmaps of the nose keypoint were generated by cropping to exclude cage walls and binning into 62x 27 spatial grids. Keypoint-MoSeq54,55 was used to infer behavioral syllables from 80 hours of keypoint data.” Does

this mean ONLY the nose was used? What is the relevance of the heatmap generation step?
- Line 841 links to an org repo on GitHub (https://github.com/UVACircMetNeuLab), there is no source code I can review available.
- Overall, the figures has many small alignment issues.

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)

Referee #2

(Remarks to the Author)
Since I have already addressed the authors commitment to revising the work on a couple of occasions, I just want to highlight a few things, particularly as reviews have become more transparent.

• The authors conclude dan and orfo are not aversive because they don't significantly change CTA (sacch preference), however it is certainly trending in that direction. They then perform an elaborate behavioral syllable monitoring analysis to "better" answer this. However, in my honest opinion, a more robust way of testing this question is a) increasing the power (n=6 looked like it was tested) and b) using another conditioned stimulus such as Ensure that would yield a much higher preference ratio over water.
• The manuscript leans very heavily on this idea that AP = nausea/malaise and NTS = satiety when it comes to these drugs but it is far more probable that both areas play roles in mediating both effects and there is likely much crosstalk between these two structures.
• It's not clear why the authors observe oral dan reduces AP Fos activation compared to injections…might be worth mentioning why.
• The major finding to me is Figure 4 so I just want to point out a couple points that I'd want to be absolutely certain on. Firstly, it is unclear if each animal was just run once with vehicle vs drug treatment. If it was me, I would want to ensure that the effects I were seeing were entirely reproducible (maybe they did and I in fact confident which negates my point). It's also clear that some of these experiments involved n=6 which again would be worrisome for me unless the same 6 mice were run for vehicle vs drug across 3 different trials and then averaged. While within-subject analyses were run, if the effects are robust enough, statistical tests could be run against mCherry control cohorts but it a) doesn't look like this was done for the BMH and b) these controls are missing from the other regions. This would control for instances when the vehicle controls from one experiment look vastly different than other (i.e. Fig 4c vs 4f).
• It's impossible to tell how specific the DIO-hGLP1R virus is to the region of interest as images are zoomed in to a point where outside expression cannot be assessed (except in Ext Fig 12 using the antibody which suggests the virus is hitting outside the intended targets). Secondly, beyond region specificity, there is no verification that this virus is actually only expressed in Cre-expressing GLP1R neurons.
• For ephys experiments, how was it determined that every YFP recorded cell also expressed the hGLP1R? It's probable that many cells express one or the other. Obviously the authors are relying on the YFP to visualize the cells but it is unclear how it was determined these had the hGLP1R.
• The fact that expressing hGLP1R in all four areas of interest lead to dan-mediated food intake suppression raises the question that if you put these hGLP1R anywhere in the brain, even places where endogenous GLP1R isn't normally expressed, would the authors make similar observations.
• I'm having a very difficult time coming to terms with the notion that optoactivation of GLP1R CeA neurons ONLY perturbs HFD consumption but not SD. What happens to HFD intake after a fast? The same can be said about the NTS GCG inputs to the CeA and GLP1R CeA to VTA terminals. Dopamine is not just rewarding for HFD but all food so my biggest struggle is determining why this dichotomy exists, particularly when it comes to appetite suppression effects. In simplest terms I don't know how an artificial activation of a brain pathway would reduce food intake of one food substrate but not another. Do the authors propose similar results would be observed with any "palatable" or "hedonic" food?
• Just want to make sure I'm reading this right, deletion of GLP1R in the CeA (Fig 4x-y) ONLY effects HFD intake after Lira injection. Why would that be? Certainly, lira is not getting anywhere near these GLP1R expressing CeA neurons. It is possible lira activates upstream pathways which then engage these cells but it's not shown that GLP1R CeA neurons are indeed activated by the drug. It's also not confirmed that GLP1R was successfully deleted from these cells.

Referee #4

(Remarks to the Author)
I thank the authors for the clarifications and edits to the behavioral aspects of the manuscript. I believe it is now clear for a reader to understand the pipeline. I would urge the authors to release the full code and dataset with the paper, vs. the demos now provided.

Version 4:

Reviewer comments:

Referee #1

(Remarks to the Author)
I am satisfied with the authors Responses to Reviewer #2 and my comments

We thank all three reviewers for their constructive critiques, which have led to substantial improvements in our manuscript. We have addressed every concern raised with extensive new experiments and analyses, including chronic dosing studies, comprehensive behavioral profiling, functional circuit validation, and enhanced anatomical characterization. These additions transform our initial findings into a comprehensive mechanistic dissection of how small-molecule GLP1R agonists modulate feeding behavior and reward processing. Please find our detailed, point-by-point responses below.

Referee #1 (Remarks to the Author):

The manuscript by Godschall et al. dissects the sites of action by which oral GLP1RAs regulate food intake, particularly hedonic feeding. The current orally available agonists are not active at the mouse GLP1 receptor so the studies employ a new mouse line with a humanized Glp1r S33W. They studied these knockin mice as well as animals in which the humanized receptor is introduced into discrete brain regions of GLP1r-cre mice using an AAV with a DIO- Glp1r S33W payload. They then analyzed the effects of the oral agents on a set of behaviors including consumption of chow in the dark phase as well as a highly palatable diet during the light phase, a time when animals consume the HFD but do not consume chow. They report that danuglipron reduces ingestion of a HFD activating inputs from preproglucagon (ie GLP1) expressing neurons in NTS to neurons expressing GLP1R in CEA. Finally, Godschall et al. report that GLP1RAs reduce dopamine responses in the NAc using a fluorescent reporter and (indirectly) link this effect to a projection from CEA to VTA. In aggregate the paper reports the novel and interesting findings that an oral GLPRA can specifically reduce hedonic feeding and this is a result of modulation of the neural targets of GCG neurons in NTS. However, several of the experiments that are reported are either flawed or insufficient and several additional experiments as well as significant revisions would be required for the paper to be suitable for publication.

Major Criticisms:

In Figure 1, the effects of orforglipron were recorded for 7-days. Studies over a longer time frame should be performed.

We have extended our chronic dosing regimen to 21 days of daily orforglipron injections, which reveals durable weight loss and aligns with protocols used in recent preclinical studies (3–5). This longer treatment period confirms sustained efficacy (Fig. 1z).

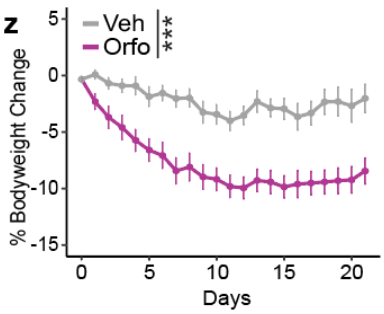


Figure 1 (in revised manuscript). **z**, 21-day weight changes of mice on chronic HFD treated with orforglipron or vehicle daily at ZT6 (*n* = 10 per injection, paired t-test, ****P*<0.001).

Metabolic profiling was performed in both males and females in ext. Fig. 1), but the studies of the effects of GLP1RA administration in Figure 1 were limited to male mice. The authors need to add data for food intake in female mice.

All acute feeding experiments in Figure 1 (panels b–y) include both male and female mice; only the 21-day chronic weight-loss study (Figure 1z) was restricted to males due to well-documented sex differences in diet-induced obesity on this genetic background (6,7) Female C57BL/6J mice consistently fail to gain the ≥20% body weight required for meaningful weight-loss studies. We have clarified this in the Methods.

In Fig. 2, the authors need to show that the neurons activated by the GLP1RAs express the GLP1R. Co-localization experiments between GLP1R and c-FOS labelling are necessary. In addition, the levels of c-FOS in control mice receiving vehicle is lacking. In addition, these images are of poor quality and the data, at least in the printed figures, do not justify the authors conclusion.

We have added high-resolution Fos images from vehicle-treated WT and Glp1r^{S33W} mice, demonstrating comparable baseline Fos levels across all control groups (Figure 3). Although prior studies have shown that GLP1RAs activate regions with known Glp1r expression (4,8,9,10), direct Fos/Glp1r co-staining of native proteins has proven technically challenging in the field. Indirect approaches used elsewhere (e.g. labeling of Glp1r-positive neurons in Glp1r-Cre mice using cre-dependent reporters (11)) are not feasible in our model, because Glp1r^{S33W} mice require homozygosity for functionality and the Glp1r-IRES-Cre allele expresses the wild-type mGlp1r. To demonstrate that small-molecule GLP1RAs selectively activate hGLP1R but not mGlp1r, we therefore used a viral strategy: we unilaterally injected AAV-DIO-hGLP1R into one CeA and AAV-DIO-mGlp1r into the contralateral CeA of Glp1r-Cre;tdTomato mice (Fig. 4l). Following danuglipron administration, Fos induction in tdTomato-positive neurons was significantly higher on the hGLP1R-expressing side than on the mGlp1r side (Fig. 4m-o), providing definitive evidence that small-molecule GLP1RAs selectively activate hGlp1r-positive neurons in the CeA. Additionally, all representative micrographs have been replaced with higher-resolution, atlas-annotated images that accurately reflect our quantification (Fig. 3).

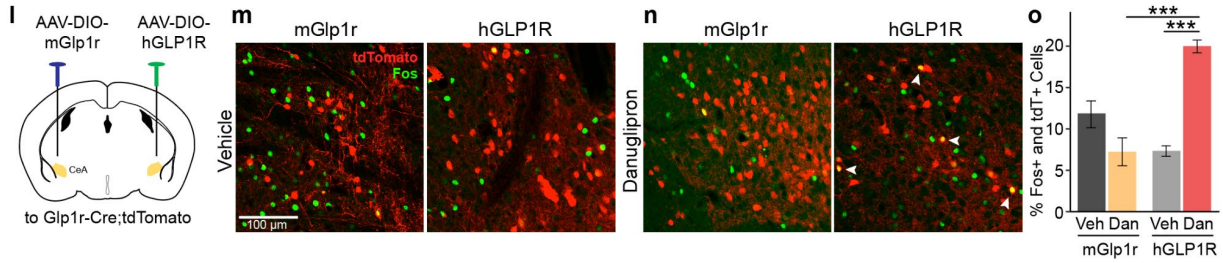
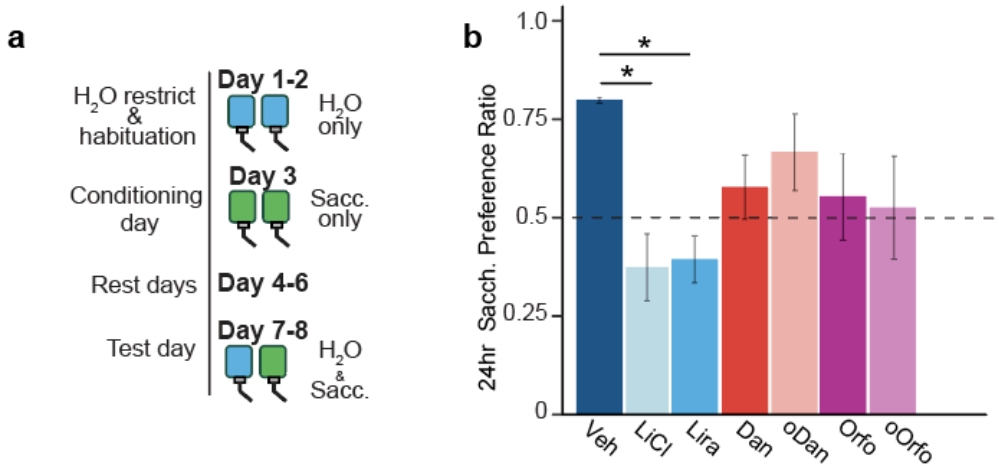


Figure 4 (in revised manuscript). Danuglipron directly activates hGLP1R-expressing cells in the CeA. l, Schematic of AAV-DIO-mGlp1r (left) and AAV-DIO-hGLP1R (right) injections in the CeA of Glp1r-Cre;tdTomato mice. **m,n**, Representative images of the CeA of (**m**) vehicle or (**n**) danuglipron injected mice with unilateral mGlp1r (left) and hGLP1R (right) infection (red = tdTomato; green = Fos; colocalization = white arrows). Scale bars = 100 µm. **o**, Quantification of the percent of Fos+ cells co-express tdTomato in the CeA of mGlp1r and hGLP1R infected mice (n = 3-4 per condition, two-way ANOVA with Tukey's HSD correction, **P<0.01; ***P<0.001).

The authors state that danuglipron preferentially activates NTS but not AP (see lines 147-153) raising the possibility that it will induce less nausea. The authors need to test this directly rather than simply referring to the distribution pattern of activated neurons. Studies using assays of nausea or conditioned taste aversion are necessary.

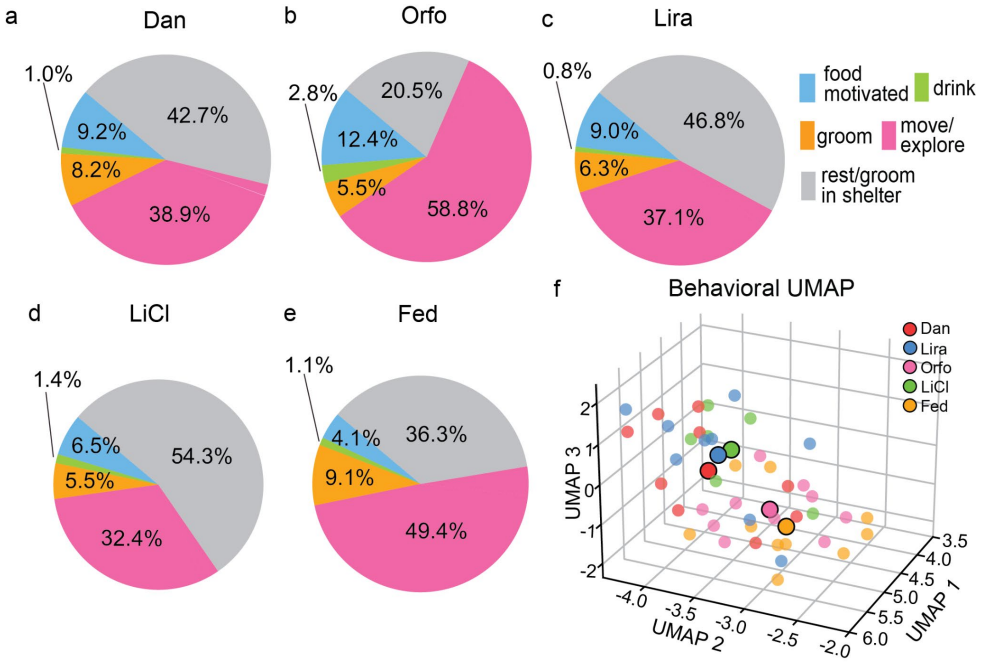
We have added comprehensive conditioned taste avoidance (CTA) experiments to address this important point and nausea associated with GLP1RAs. We performed CTA assays, which confirmed that liraglutide induces strong avoidance comparable to lithium chloride (12), but failed to detect more subtle effects of small-molecule GLP1RAs (Extended Data Figure 3a,b). To capture these nuanced behaviors, we developed high-resolution home-cage video tracking with machine-learning behavioral profiling across five treatment groups—LiCl (nausea control), pre-fed (satiation control), liraglutide, danuglipron, and orforglipron (Figure 2). This new approach revealed that liraglutide and danuglipron cluster with LiCl in behavioral space, exhibiting nausea-like signatures, while orforglipron maintains a distinct profile. These results demonstrate that our video-based methodology captures nuanced aversive behaviors that traditional assays miss and directly correlates with each drug's differential AP versus NTS activation pattern.



Extended Data Fig. 3 (in revised manuscript). Liraglutide but not small molecules GLP1RAs induced conditioned taste avoidance (CTA) **a**, Pipeline for CTA protocol. **b**, 24-hour saccharin preference after injections of vehicle (Veh), lithium chloride (LiCl), liraglutide (Lira), danuglipron (Dan), oral Dan (oDan), orforglipron (Orfo) or oral Orfo (oOrfo) (*n* = 6-8 per injection, one-way ANOVA with Tukey's HSD correction, **P*<0.05; ***P*<0.01).

In Figure 3 the authors monitor the behavioral responses to the oral agents. However, these data are largely superfluous and omit the most relevant behaviors having to do with possible effects on nausea. These data do not bear on the key conclusions of the paper and appear to have been "thrown in".

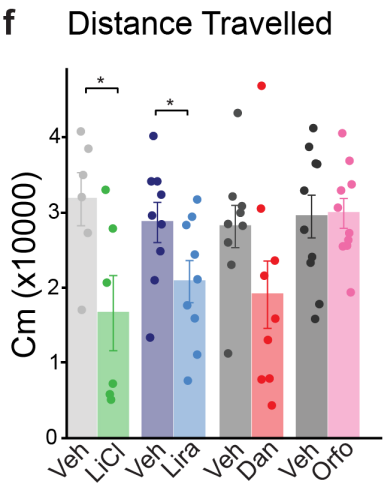
We have substantially expanded this behavioral analysis based on your feedback. The revised Figure 2 now includes two critical control groups—LiCl (nausea control) and pre-fed mice (satiation control)—alongside our original GLP1RA treatments. This comprehensive comparison reveals that different GLP1RAs produce fundamentally different subjective experiences. Liraglutide and danuglipron cluster with LiCl in PCA/UMAP space of behavioral analysis, while orforglipron forms a distinct profile that differs from both nausea and satiation states (Reviewer Response Figure 1; Fig. 2s-t). These behavioral patterns directly validate our Fos data, where low NTS/AP ratios for liraglutide and danuglipron correspond to nausea-like behaviors, while orforglipron's high ratio correlates with minimal behavioral disruption.



Reviewer Response Figure 1 (Adapted from Figure 2 in the revised manuscript). Liraglutide and danuglipron induce behavioral phenotypes closer to nausea-like state than pre-fed state. **a-e,** Proportion of time spent performing each of the 5 behavioral categories during the 2-hour recording (ZT 12-14). (*n* = 9 Dan, *n* = 10 Orfo, *n* = 9 Lira, *n* = 6 LiCl, *n* = 10 Fed). **f**, UMAP visualization of the full behavioral dataset, including normalized transitions, bout metrics (average, maximum length, and total count), time spent in each behavioral category, food and water sensor data, distance traveled, and velocity. PCA was used for preprocessing, retaining 16 components that explain 95% of the variance. Opaque circles represent condition-wise mean embeddings; smaller points indicate individual mice.

In addition, the authors fail to observe significant effects on movement while at the same time the mice that received danuglipron or liraglutide are reported to spend significantly more time resting? What is the explanation for this? What are GLP1RA effects on locomotion? What is the total distance traveled in the two groups?

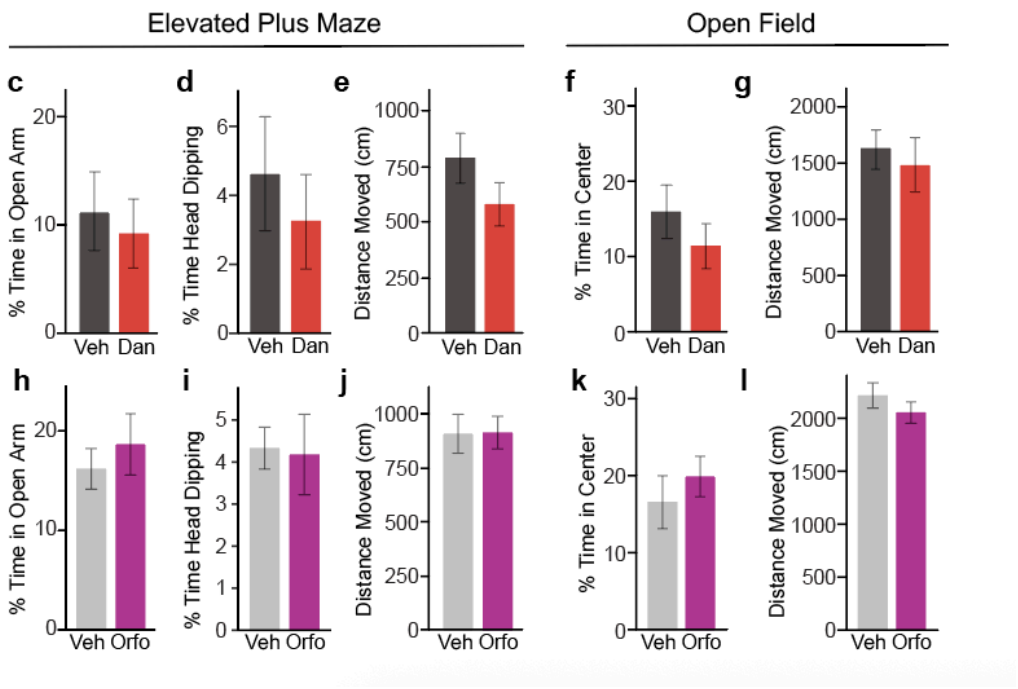
We have added quantitative locomotion analysis to clarify this apparent contradiction. Using SLEAP neck-keypoint tracking, we measured total distance traveled and found that mice treated with liraglutide or LiCl showed significant reductions compared to vehicle controls, while danuglipron showed a similar trend and orforglipron had no effect (Extended Data Fig. 7f). These locomotion data are consistent with the increased resting and reduced exploration we observed in Figure 2l-m.



Extended Data Fig. 7 (in revised manuscript). LiCl and liraglutide reduce distance traveled. **f,** Locomotion in a home cage during the dark cycle (ZT 12-14) of mice after injections of lithium chloride (LiCl), danuglipron (Dan), liraglutide (Lira), orforglipron (Orfo), or paired vehicle control (Veh). Total distance traveled in centimeters using the neck/upper back keypoint from SLEAP tracking results (n = 6 Veh/LiCl, n = 9 Veh/Lira, n = 9 Veh/Dan, n = 10 Veh/Orfo, paired t-test, $^*P < 0.05$).

Furthermore, could changes in time spent in the shelter and grooming be a result of changes in anxiety as this has been demonstrated before in mouse and human studies? (Kornelius et al., 2024; Zheng et al., 2019)

We have added comprehensive anxiety testing to rule out this alternative explanation. Using both elevated plus maze and open field tests, we found that neither danuglipron nor orforglipron altered anxiety-related behaviors compared to vehicle controls (Extended Data Figure 3c-l). These results confirm that the increased shelter-resting and grooming we observe does not reflect anxiety responses.



Extended Data Fig. 3 (in revised manuscript). Danuglipron and orforglipron do not induce anxiety-like behaviors. **c-l**, Elevated plus maze and open field anxiety tests after injections of (**c-g**) vehicle (Veh) or danuglipron (Dan) and (**h-l**) vehicle (Veh) or orforglipron (Orfo). Glp1r^{S33W} mice were injected with Veh/Dan at ZT 11.5 or Veh/Orfo at ZT 8.5 and tested at ZT 12.5. For elevated plus maze, the percentage of time spent in open arms, percentage of time spent head dipping, and distance traveled were measured. For the open field test, the percentage of time spent in the center (defined as 4/9 of center area) and distance traveled were measured (*n* = 8 per injection, paired t-test).

In Figure 4U, synaptophysin tracing shows that NTS GCG neurons project to amygdala. However, data showing functional connections are lacking. Rabies tracing from CeA Glp1r neurons should be performed to determine whether these neurons receive direct inputs from NTS GCG. Pending this result, the authors need to provide functional evidence showing that activating NTS GCG terminals in CEA alters chow intake as well as hedonic feeding.

We have functionally validated the NTS^{Gcg}→CeA projection using optogenetic experiments. In Gcg-Cre mice, we expressed Cre-dependent ChrimsonR in NTS^{Gcg} neurons and stimulated their axon terminals in the CeA during feeding sessions. Light stimulation selectively reduced HFD intake by 62% compared to control while having no effect on standard-diet consumption (Figure 5b–d). These data demonstrate that NTS^{Gcg} terminal activation in the CeA is sufficient to engage downstream circuitry and specifically suppress hedonic feeding, providing direct functional evidence for this pathway's role in reward-driven intake.

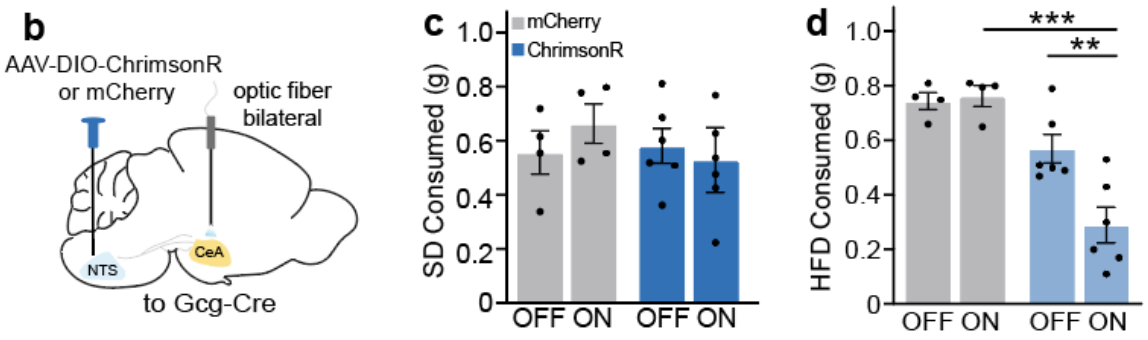


Figure 5 (in revised manuscript). NTS^{Gcg} axon stimulation in the CeA inhibits HFD intake. **b**, Schematic of AAV-DIO-ChrimsonR or AAV-DIO-mCherry injection into the NTS of Gcg-Cre mice with bilateral fiber-optic implants in the CeA. **c,d**, 1-hour consumption of (**c**) standard diet (SD) or (**d**) high-fat diet (HFD) in mCherry (control) versus ChrimsonR-expressing mice under light ON versus OFF conditions (n = 4–6 per group, two-way ANOVA with Tukey's HSD, *P < 0.05).

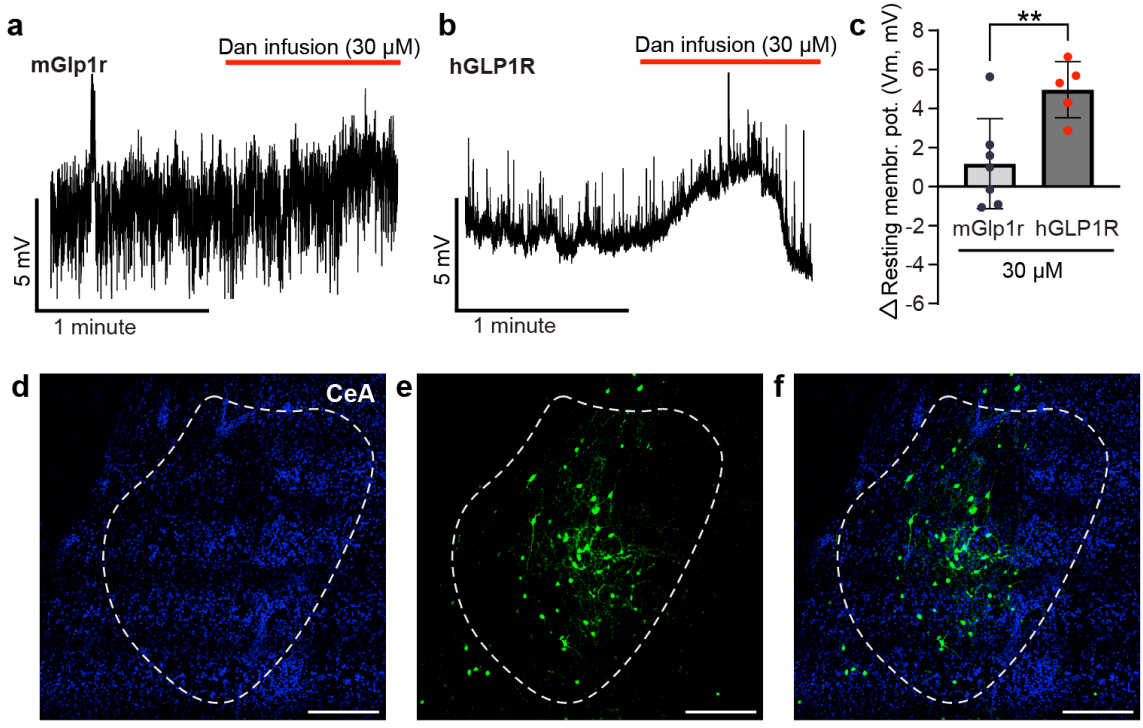
In addition, the location of these terminals is unclear and the data in Ext. Fig 9 raise the possibility that the terminals may be elsewhere in amygdala, see below. In extended Figure 9 C–F, the authors state that eYFP-labeled hGLP1R-expressing neurons are in the CEA . However , the data appear to expression ventral to the CeA in STIA and MeA. More precise images should be provided with reference to a brain atlas showing precisely where these neurons are located. The based on the current images , it is unclear whether these effects are specific to CeA vs. other amygdala nuclei.

We have conducted detailed histological validation to ensure CeA-specific targeting across all our experiments. For the electrophysiology recordings shown in Extended Data Figure 12 (previous Extended Data Figure 9), we intentionally targeted a broader amygdala region to maximize recording yield, as the primary goal was to demonstrate functional hGLP1R expression and danuglipron responsiveness. We have clarified this broader targeting in the revised Methods section. For all experiments requiring strict CeA-specific manipulation—including tracing, behavioral assays, optogenetics, and knockout studies—we used atlas-defined boundaries and included only mice with virus expression confined to the CeA (Extended Data Figures 9, 10, 13, 14). To address the concern about NTS^{Gcg} terminal localization, we have added high-resolution images showing that synaptophysin terminals are present within the atlas-defined CeA boundaries (Fig. 5a). Our histological validation criteria are detailed in the Methods, and any mice with off-target expression were excluded from analysis. This approach ensures that our behavioral and circuit findings accurately reflect CeA^{Glp1r} neuron function.

In the electrophysiology experiments in Ext. Fig. 9, the authors do not state what the danuglipron was dissolved in and possible solvent effects on membrane depolarization were not tested. Furthermore, the depolarization that was reported did not induce any actions potentials or affect firing frequency of the neurons. These data are unimpressive.

We have added detailed solvent information and appropriate controls to address these concerns. Danuglipron was dissolved in ethanol then diluted in vehicle (1N NaOH, 2% Tween 80, 5% PEG

400, 5% dextrose) and further diluted in ACSF to yield final concentrations of 30 µM danuglipron with 0.056% ethanol, 0.011% Tween 80, and 0.028% PEG 400. As a critical control, we recorded from CeA neurons in Glp1r-Cre mice injected with AAV-DIO-mGlp1r + eYFP and found no depolarization response to identical danuglipron treatment, ruling out solvent effects (Extended Data Fig. 12a-c). The modest but statistically significant depolarization we observed is consistent with Gs-coupled receptor activation and cAMP-mediated effects reported for other GPCRs. This membrane potential shift primes neurons for increased excitability without necessarily triggering action potentials in slice conditions(13–16). Importantly, we validated these slice findings with robust in vivo responses: danuglipron drove significant Fos induction specifically in hGLP1R-expressing neurons (Fig. 4l–o) and evoked strong calcium transients in fiber photometry recordings (Fig. 4q–t). Together, these complementary approaches demonstrate that our viral hGLP1R construct produces functional receptor expression that responds appropriately to danuglipron both in vitro and in vivo.



Extended Data Fig. 12 (in revised manuscript). Danuglipron depolarizes the resting membrane potential in hGLP1R-expressing amygdala neurons. Representative traces showing the effect of danuglipron perfusion (30 µM) on the resting membrane potential of **a,** mGlp1r-expressing **b,** hGLP1R-expressing amygdala neurons. **c,** Average depolarization induced by danuglipron in mGlp1r- and hGLP1R-expressing amygdala neurons (*n* = 5-7 cells, Unpaired t-test, ***P*<0.01). **d-f,** Localization of eYFP-labeled hGLP1R-expressing neurons in the amygdala. Scale bars = 100 µm.

In Fig. 5 the authors conclude that GLP1R expressing neurons in CEA regulate dopamine release in NAc. The authors need to use chemogenetics or neural ablation to show that inhibiting GLP1R expressing in CEA blunts the effects of GLPRAs to reduce hedonic feeding. It is relevant that GLP1R expressing neurons in NTS also project to NAc (Alhadeff et al., 2012) and that NAc expresses GLP1R and these neurons have also been shown before to regulate feeding (Mietlicki-Baase et al., 2014).

We have conducted both loss-of-function and gain-of-function experiments to demonstrate that CeA^{Glp1r} neurons are necessary for GLP1RA-mediated suppression of hedonic feeding. To test necessity, we performed CeA-specific genetic knockout of Glp1r by injecting AAV-Cre into the CeA of $Glp1r^{flox/flox}$ mice (versus AAV-GFP controls). CeA^{Glp1r} deletion significantly impaired liraglutide's ability to suppress HFD intake while leaving standard diet responses intact (Figure 4x,y), demonstrating that CeA^{Glp1r} signaling is required for hedonic feeding suppression.

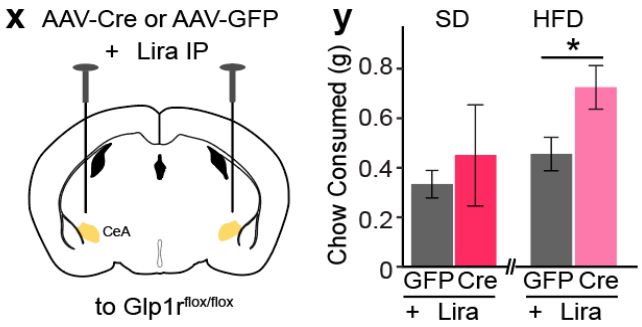


Figure 4 (in revised manuscript). Feeding effects of Glp1r knockout in the CeA. **x**, Schematic of AAV-Cre to conditionally knockout Glp1r, or AAV-GFP control injection to the CeA of Glp1rflox/flox mice. **y,** SD (left) and HFD (right) consumption 4 hours after injection of liraglutide in AAV-Cre or AAV-GFP expressing mice. (n = 6, Welch's t-test, *P<0.05).

To validate the CeA^{Glp1r} → VTA pathway, we performed terminal stimulation experiments by expressing Cre-dependent ChrimsonR in CeA^{Glp1r} neurons and stimulating their axon terminals in the VTA. Optogenetic activation reduced HFD intake by 48.8% compared to controls while having no effect on standard diet consumption (Fig. 5f-h), confirming that CeA^{Glp1r} projections to the VTA are sufficient to suppress hedonic feeding. We acknowledge that multiple GLP1R-expressing circuits likely contribute to feeding regulation, including the NTS→NAc and local NAc circuits cited by the reviewer. Our findings establish CeA^{Glp1r} neurons as an additional critical node that specifically regulates reward-driven feeding through the mesolimbic system.

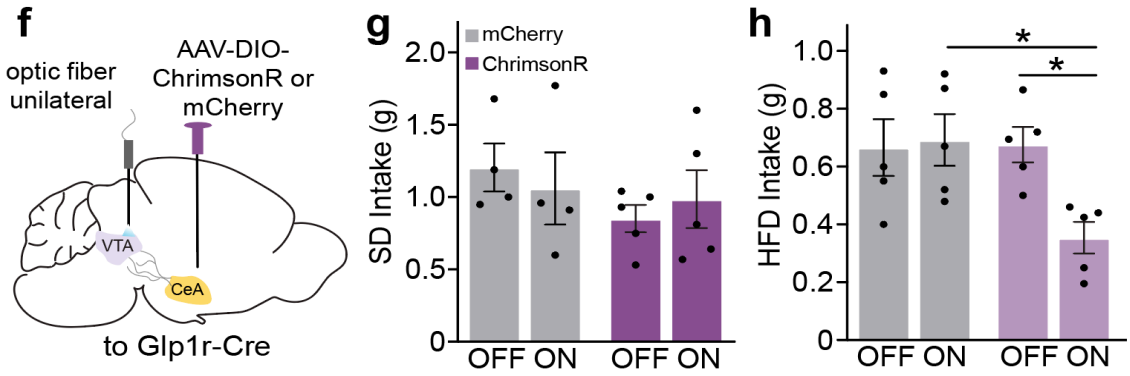


Figure 5 (in revised manuscript). CeA^{Glp1R} axon stimulation in the VTA inhibits HFD intake. **f**, Schematic of AAV-DIO-ChrimsonR or AAV-DIO-mCherry injection into the CeA of Glp1r-Cre mice with a unilateral fiber optic implant in the VTA. **g,h**, 1-hour consumption of (**g**) SD or (**h**) HFD in mCherry versus ChrimsonR-expressing mice under light ON versus OFF conditions (*n* = 4- 5, two-way ANOVA with Tukey's HSD, *P < 0.05).

Minor Criticisms:

Figure 1H. The x-axis shows the starting point for GTT measurements at -240 min. This is not comparable to other GTT experiments in Figure 1.

We have clarified the timing in the Methods and figure legend. Orforglipron is administered at –240 min to accommodate its slower pharmacodynamics, consistent with published protocols (5,17). Time 0 remains the dextrose injection across all GTT panels, ensuring direct comparability of glucose responses between drugs.

In Figure 1G and U, Blood glucose appears to increase after danuglipron injection prior to the dextrose injection.

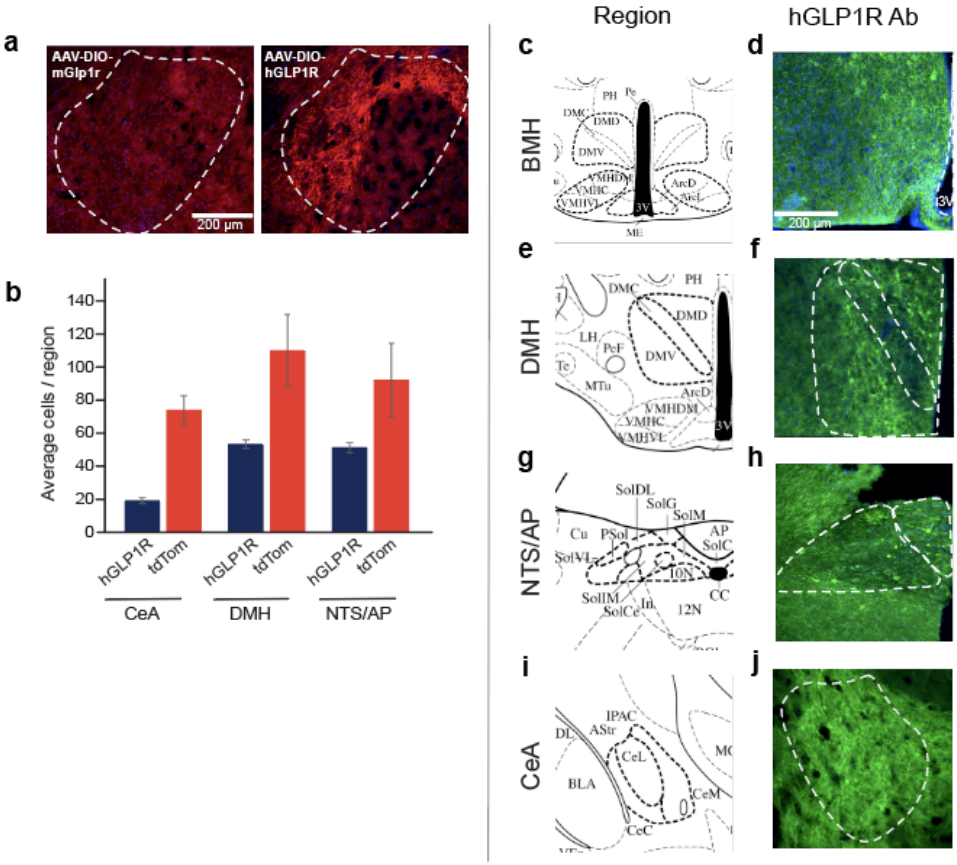
We appreciate the reviewer's observation. The modest increase in blood glucose observed after danuglipron injection, prior to the dextrose challenge, is likely attributable to a transient stress response from the injection and blood draw procedures. Although the mice are acclimated to handling, these procedures can still induce a brief elevation in glucose levels that stabilizes by time 0 (the dextrose injection). Moreover, both Glp1r^{S33W} (responsive) and WT (non-responsive) mice exhibited a similar upward trend at this time point, further supporting the notion that this effect reflects a response in this cohort rather than a direct pharmacologic action of danuglipron.

In Figure 1Z, the authors state that this experiment was performed in already obese mice but they do not state how long the mice had consumed a HFD before the experiment.

This information is detailed in the Methods section. Mice were maintained on a high-fat diet for at least 8 weeks prior to treatment, with inclusion criteria requiring ≥20% body weight gain from baseline.

In Extended Data Fig. 8, it is not clear which hypothalamic areas the authors are referring to ie which specific areas correspond to the basomedial hypothalamus? The histologic sections seem to indicate that the injections of virus may also have spread into VMH and Arc see Panels C and D. The authors should provide posthoc images of whole slides of the injection areas with quantification of the total cells infected in each targeted brain area? If the virus has spread in the neighbouring areas, their conclusions may be inaccurate.

In the revised manuscript's Methods section we are more explicit in our definition of the basomedial hypothalamus (BMH) which includes the arcuate nucleus, median eminence, dorsomedial hypothalamus, and ventromedial hypothalamus. Total cells infected per area are quantified in the updated figures (Extended Data Fig. 9b).



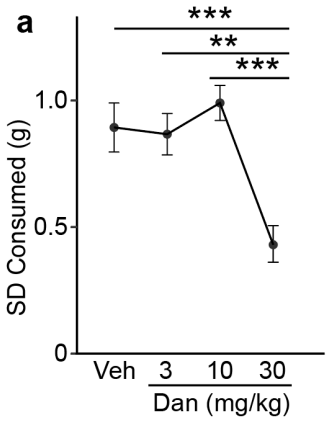
Extended Data Fig. 9 (in revised manuscript). Validation of the AAV-hSyn-DIO-hGLP1R virus. a, Validation of human GLP1R (hGLP1R; red) expression in the CeA of Glp1r-Cre mice injected with AAV-DIO-hGLP1R (right). hGLP1R protein expression is absent in Glp1r-Cre mice injected with AAV-DIO-mGlp1r in the CeA (left). Scale bar = 200 µm. **b**, Quantification of hGLP1R+ cells and average number of Glp1r+ cells (measured in Glp1r-Cre;tdTomato mice) in the CeA, DMH, or NTS/AP regions. **c-j,** Fluorescent microscopy images of the brain regions stereotaxically injected with AAV-DIO-hGLP1R. Images from Allen Brain Atlas, with regions of interest in bold and hGLP1R antibody staining (green) in the (**c,d**) basomedial hypothalamus (BMH), (**e,f**) DMH, (**g,h**) NTS/AP, or (**i,j**) CeA. Scale bars = 200 µm.

Referee #2 (Remarks to the Author):

This manuscript investigates the effects of next-generation, small-molecule GLP1RAs (danuglipron and orforglipron) on feeding behavior and neural circuits. Strengths include the timely interest in these classes of molecules and the development of genetically engineered mice expressing human-like GLP1Rs to study the effects of small-molecule GLP1RAs, similar to a rat model that was recently published (PMID: 39693407). Overall, the experiments seem well designed and executed but the real advance in our understanding of how these drugs is lacking, particularly with the growing number of publications exploring the mechanisms behind their efficacy. A few aspects that dampen my enthusiasm include a lack of pharmacokinetic comparisons/dose responses as well as limited oral administration (which is one of the major advantages of this class of molecules), limited long-term analysis of drug treatment, concerns over non-physiological artificial overexpression in the CeA, unconvincing evidence that the CeA pathway is specific to hedonic versus homeostatic food consumption, lack of testing the notion that less AP cFos activation translates into less aversion, and a circuitous leap to the canonical VTA to NAc dopamine reward system. Below I've listed some points I think would strengthen the manuscript:

1) It's unclear how the authors decided on the dose used throughout the experiments. It seems likely that altering the concentration may have far different effects (ie. cFos activation as well as appetite suppression)

We conducted systematic dose optimization for each compound to ensure robust and comparable effects. For danuglipron, we performed dose-response studies (3, 10, 30 mg/kg) and selected the minimum dose producing maximal feeding suppression (Extended Data Fig. 2a). For orforglipron and liraglutide, we chose doses that matched danuglipron's anorectic magnitude, enabling direct comparisons across compounds. These doses align with established protocols in the literature(5,17,18) and have been described in our Methods section.



Extended Data Fig. 2 (in revised manuscript). **a**, 2-hour SD intake (ZT 12 - ZT 14) after vehicle (Veh) or escalating doses of danuglipron (3mg/kg, 10 mg/kg, or 30 mg/kg) (n = 8-15 per group, one-way ANOVA with Tukey's HSD, $^{**}P<0.01$; $^{***}P<0.001$).

2) Nothing really stands out with the cFos data that hasn't been reported with other GL1RAs. If the authors really want to make a claim that oral delivery leads to reduced AP cFos activation, then they should attempt to define what this means behaviorally. One would then hypothesize that these mice would demonstrate less aversion, although Figure 3 suggests behaviors are pretty similar.

We have expanded our Fos analysis to reveal distinct activation patterns between oral and IP delivery routes. While the brain regions activated by GLP1RAs have been described, we show for the first time that oral danuglipron produces significantly lower AP activation compared to IP delivery, and that orforglipron consistently exhibits higher NTS/AP ratios than other compounds (Fig. 3j-n). To determine the behavioral significance of these patterns, we developed comprehensive nausea assessment combining CTA with high-resolution behavioral profiling (Figure 2). This integrated approach demonstrates that NTS/AP activation ratios predict behavioral tolerability—orforglipron's favorable ratio correlates with reduced nausea-like behaviors compared to danuglipron and liraglutide.

Also the representative images Fig 2j-k seem to show far less NTS expression via oral route although this is not reported as significant. Of course, again these differences in cFos could merely come down to dose.

We replaced all images with high-resolution versions and added Fos quantification to Figure 3. These revisions show that orforglipron produces a higher NTS/AP activation ratio than danuglipron or liraglutide, and that oral danuglipron yields lower AP Fos compared to IP delivery. Despite these regional Fos differences, glucose lowering and feeding suppression are equivalent across treatment type or administration route (Fig. 1). In other words, similar behavioral and physiological outcomes paired with distinct activation patterns indicate that NTS/AP ratios reflect bioavailability differences rather than inconsistent dosing.

3) Figure 3 seems like a lot of work to show the drugs make the animals eat, drink, and move less while resting more which has been reported many times over using less flashy approaches.

This point is well taken, which motivated us to expand this analysis significantly. We added two critical control groups—LiCl (nausea control) and pre-fed mice (satiation control)—and developed comprehensive machine-learning behavioral profiling to address a fundamental question: do the small-molecule GLP1RAs suppress feeding by reducing hunger, or by inducing subtle malaise? This expanded analysis led to observations that represent a significant new contribution to the field. We discovered that danuglipron similar to liraglutide produce behavioral signatures closely resembling LiCl-induced nausea, while orforglipron maintains a profile distinct from both nausea and satiation states (Fig. 2s-t). For the first time, we can separate satiety from malaise-like effects and demonstrate that different GLP1RAs produce fundamentally different subjective experiences. These behavioral patterns directly correlate with our neural activation data—drugs with high

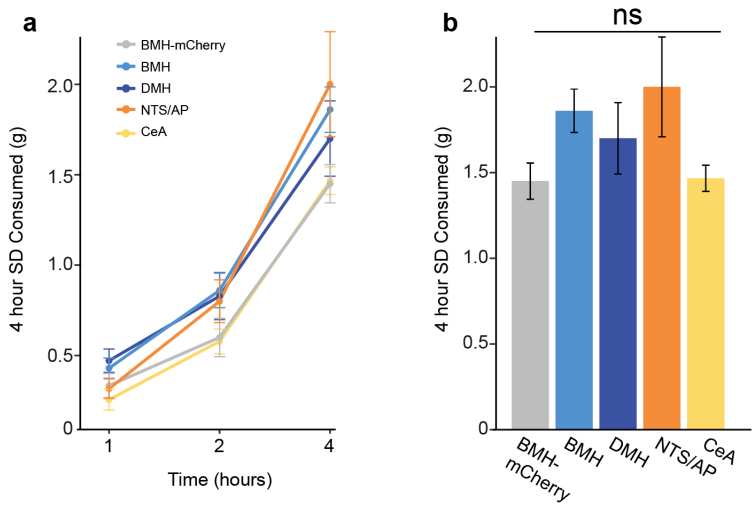
AP/NTS ratios produce nausea-like behaviors, while orforglipron's favorable ratio corresponds to minimal behavioral disruption (Fig. 2i-r, Extended Data Fig. 7, Extended Data Fig. 8). This integrated approach provides insights into drug tolerability and mechanism that are invisible to conventional behavioral assays.

4) There is a real concern with receptor overexpression. Examples in pharmacology have shown that ligands can act as antagonists at low receptor levels but switch to agonism when receptors are at higher-fold expression. This overexpression is likely non-physiological so it's difficult to interpret. Quantifying receptor levels would help clarify this.

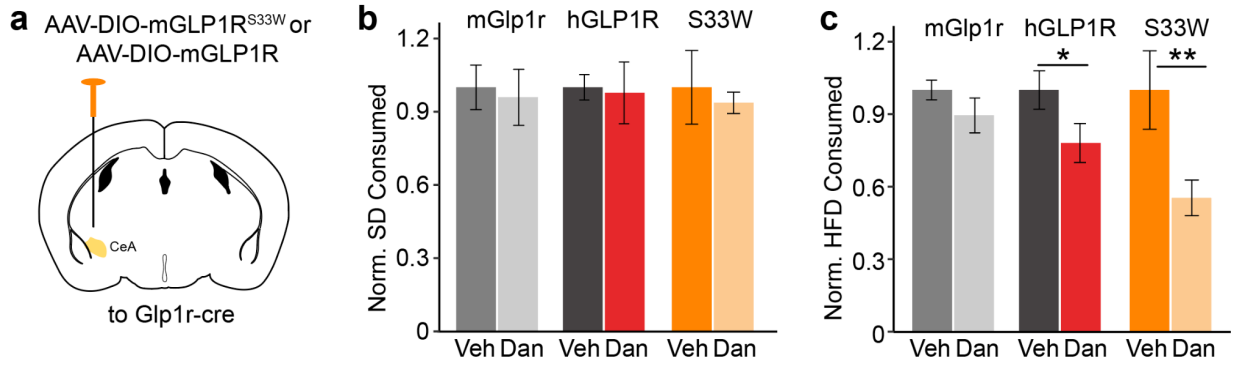
We have conducted extensive validation to ensure our viral approach recapitulates native GLP1R signaling rather than producing artifacts from receptor overexpression. We first quantified hGLP1R-positive cells versus native Glp1r-Cre;tdTomato neurons in each targeted region (CeA, DMH, NTS/AP) and consistently found fewer virally transduced cells than endogenous Glp1r-Cre populations (Extended Data Fig. 9b), ruling out gross over-infection. We then confirmed that these hGLP1R-expressing neurons respond to agonist as expected for a Gs-coupled receptor, but not mGlp1r controls, across three independent readouts: (1) Electrophysiology – only hGLP1R slices depolarize upon danuglipron (Extended Data Fig. 12c); (2) Fos histology – only hGLP1R-expressing CeA neurons show robust Fos induction after danuglipron (Fig. 4l–o); and (3) In vivo fiber photometry – only hGLP1R mice exhibit calcium transients to danuglipron (Fig. 4q–t, Extended Data Fig. 13e–g). Together, these complementary validations confirm that our viral strategy recapitulates native GLP1R signaling in target neurons, rather than producing artifactual effects from receptor overexpression.

5) The feeding experiments in Figure 4a-k are hard to take at face value given the variability of controls. For example vehicle injected mice tend to consume around 1 gram or more in most experiments run but the "key" experiment where the authors are claiming Dan does not inhibit SD consumed, the vehicle mice are super low making any change very difficult to detect. Therefore the conclusion that CeA is linked to hedonic feeding only is weakened…..

We have validated the consistency of our feeding paradigms across all brain-region experiments. All studies used a paired design where each mouse was tested under both vehicle and drug conditions. We compared baseline vehicle SD intake across all brain-region groups and found no significant differences, confirming consistent experimental conditions (Reviewer Response Figure 2). To further validate CeA specificity for hedonic feeding, we tested two additional viral constructs: AAV-DIO-mGlp1r (negative control) and AAV-DIO-mGlp1r^{S33W} (renders mGlp1r responsive to small-molecule GLP1RAs; Extended Data Fig. 10). When targeted to the CeA, both hGLP1R- and mGlp1r^{S33W}-expressing mice showed robust HFD suppression with danuglipron but no change in SD intake. These data demonstrate that CeA activation by danuglipron consistently and selectively suppresses hedonic feeding without affecting homeostatic intake, supporting our conclusion that CeA^{Glp1r} neurons specifically regulate reward-driven eating.



Reviewer Response Figure 2. Baseline standard diet (SD) consumption is equivalent across experiments. Comparison across SD fed mice injected with AAV-DIO-mCherry (in the BMH) or AAV-DIO-hGLP1R in the basomedial hypothalamus (BMH), dorsomedial hypothalamus (DMH), nucleus of the solitary tract/ area postrema (NTS/AP) or the central amygdala (CeA). **a**, SD intake over 4 hours of vehicle injected animals with different brain targets. **b**, SD intake after 4 hours of vehicle injected mice expressing mCherry or hGLP1R in the BMH, DMH, NTS/AP, or CeA (*n* = 6-10 mice per group, One-way ANOVA with Tukey's HSD, ns = no significance).



Extended Data Fig. 10 (in revised manuscript). AAV-DIO-mGLP1RS33W-HA targeted to the CeA of Glp1r-Cre mice recapitulates effects of AAV-DIO-hGLP1R. a, Schematic of Glp1r-Cre mice injected with a Cre-dependent AAV carrying full length mouse Glp1r (mGlp1r) or AAV carrying full length mouse GLP1R with the S33W mutation at position 33 (mGLP1RS33W) in the CeA. b,c, Normalized 4-hour (b) SD and (c) HFD consumption post-injection of vehicle or danuglipron in mGlp1r, full length human GLP1R (hGLP1R) or mGlp1r-S33W (S33W) expressing mice (n = 4-9 per group, two-way ANOVA with Bonferroni correction, *P<0.05; **P<0.01). Data are represented as means ± SEM. *P<0.05; **P<0.01; ***P<0.001.

6) …..additionally, did the authors test whether opto activation of CeA Glp1r neurons suppressed chow. Given the numerous examples of different CeA populations that decrease food intake when activated, it seems highly likely this population would act

similarly. It would be hard to imagine why the brain would be wired to specifically suppress "hedonic" food intake, especially when driven at supraphysiological levels of activation.

We have conducted comprehensive optogenetic validation to test CeA^{Glp1r} neuron specificity for hedonic versus homeostatic feeding. We expressed ChR2 in CeAGlp1r neurons and tested stimulation across multiple frequencies (0, 5, 10, and 20 Hz) during both homeostatic feeding (standard diet after overnight fasting) and hedonic feeding (HFD during inactive phase). Optogenetic activation robustly suppressed HFD intake in a frequency-dependent manner but had minimal impact on standard diet consumption (Fig. 4u–w), confirming that these neurons preferentially regulate reward-driven feeding. This selectivity is supported by emerging evidence that distinct CeA subpopulations specifically modulate hedonic consumption (19–23). Our single-nucleus RNA-seq analysis demonstrates that Glp1r expression overlaps partially with other reward-regulating populations (e.g., Pnoc-positive neurons) in the CeA (Reviewer Response Figure 3), supporting the existence of a dedicated subpopulation that specifically influences hedonic feeding responses.

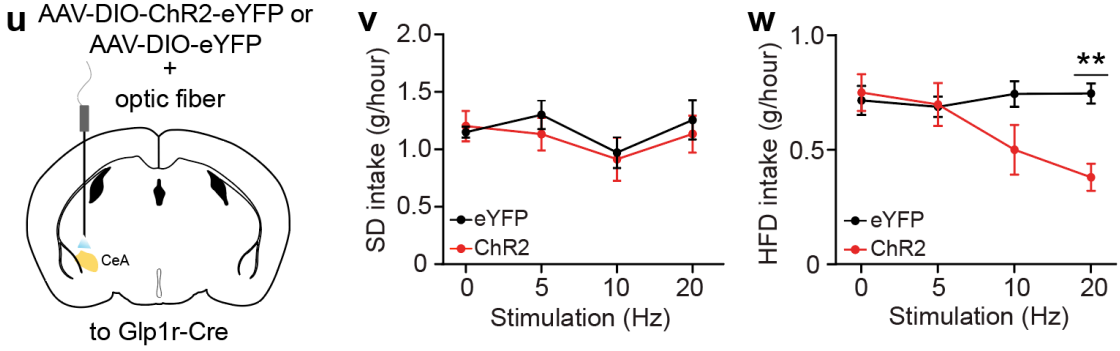
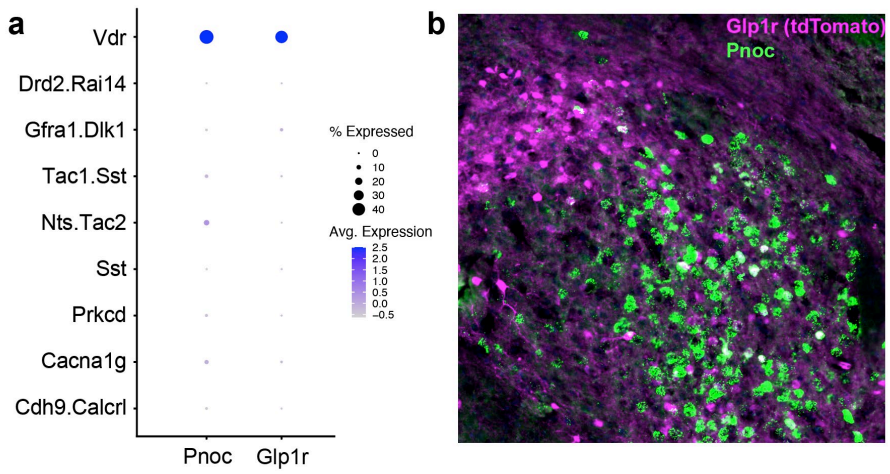


Figure 4 (in revised manuscript). Stimulation of CeA Glp1r neurons selectively inhibits HFD intake. u, Schematic of AAV-DIO-eYFP or AAV-DIO-ChR2-eYFP injection with a fiber-optic implant in the CeA of Glp1r-Cre mice. **v,w,** 1-hour SD (**v**) and 1-hour HFD (**w**) consumption of eYFP (control) or ChR2-expressing mice with different frequencies of light stimulation on different days (n = 5 per group, two-way ANOVA with Bonferroni correction, *P<0.05).



Revision Response Figure 3. Central amygdala scRNA-seq (24) and RNA FISH co-expression of hedonic-related markers. **a**, Dotplot showing Glp1r and Pnoc are expressed in the Vdr cluster. **b**, Colocalization of Pnoc (green; RNA) and Glp1r (magenta; tdTomato protein) with some but not total overlap between cell types in the CeA.

7) There's a bit of confusion on the ephys experiments in Extended Data 9, are these recordings only performed in Cea of mice injected with the hGLP1R? What about in Glp1r-Cre mice just injected with DIO-YFP? It seems even without the humanized form, these Glp1r CeA neurons should still respond to the small-molecules in slice experiments, but may not in vivo.

We have clarified the electrophysiology experimental design and controls. Recordings were made exclusively from amygdala neurons expressing either hGLP1R or mGlp1r as controls. Since danuglipron selectively activates the human receptor, we recorded from Glp1r-Cre mice injected with AAV-DIO-mGlp1r + eYFP as negative controls. As expected, these mGlp1r-expressing neurons showed no change in resting membrane potential when exposed to 30 μM danuglipron, while hGLP1R-expressing neurons showed significant depolarization (Extended Data Fig. 12a-c). This confirms that the depolarization reflects human-receptor-specific activation rather than off-target effects of native mGlp1r or solvent artifacts.

8) Was there any validation of the AAV-DIO-mGlp1r S33W virus?

We have validated the AAV-DIO-mGlp1r-S33W virus through both immunohistochemical and functional approaches. HA-tag immunostaining confirms correct expression and localization of mGlp1r-S33W in the CeA (Extended Data Figure 10d). Functionally, Cre-dependent mGlp1r-S33W expression in the CeA reproduced the danuglipron sensitivity seen with hGLP1R: danuglipron suppressed HFD intake in mGlp1r-S33W-expressing mice but not in wild-type mGlp1r controls (Extended Data Figure 10b,c). This behavioral rescue demonstrates that the S33W mutation confers the expected human receptor-like pharmacology in vivo, confirming the virus functionality.

9) It's unclear how many test animals were used for the photometry experiments, does each point in Fig 4n represent different animals or per injection. If the same animal was used repeatedly, those combined responses should be averaged in one data point. Also, all the mouse heat maps should be displayed as this allows the reader to see the raw data. The average number of events also seems to be variable during vehicle injections averaging around 12 in the hGLP1R mice but over 20 in the mGlp1r controls making interpretation cloudy. Could the authors look at area under the curve? What are the mice doing during the recordings and what happens to this activity during specific behaviors like eating?

We have clarified the photometry experimental design and enhanced data presentation to address these concerns. Each data point represents one mouse's paired recording (vehicle vs. drug) from six hGLP1R mice and six mGlp1r controls. To normalize across sessions, we calculated z-scores using each mouse's own vehicle recording as baseline: $z(t) = [\Delta F/F(t) - \mu(\text{vehicle})] / \sigma(\text{vehicle})$. This paired normalization removes baseline differences and enables direct comparison across mice and conditions. After normalization, the mean number of significant calcium events during vehicle sessions was similar between groups (17.8 vs. 20 events), confirming that drug effects reflect true receptor activation rather than baseline variability.

We have added a comprehensive data presentation including heatmaps for each individual mouse (Fig. 4q,r; Extended Data Fig. 13e) and area under the curve (AUC) analyses (Fig. 4s; Extended Data Fig. 13g), which validate our event-based findings. All recordings were performed in freely-behaving mice in their home cages without food or water to avoid confounding behavioral effects, following 2-hour habituation sessions over 2-3 days to minimize stress artifacts.

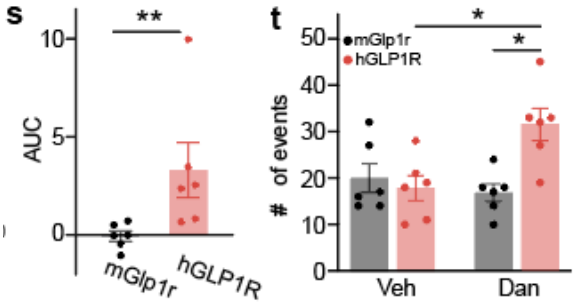


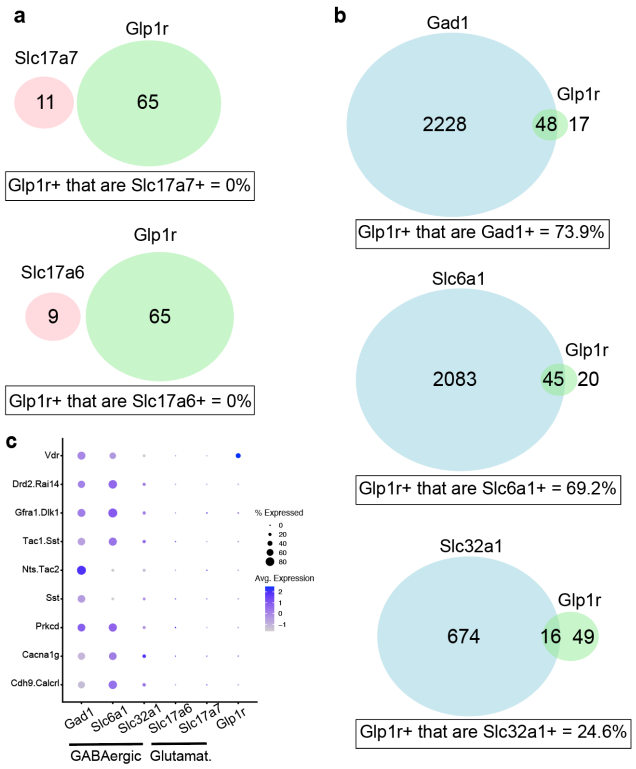
Figure 4 (in revised manuscript). Calcium levels in the CeA increase after danuglipron in mice expressing hGLP1R but not mGlp1r confirming danuglipron selectively activates the human receptor. **s,** Area under the curve (AUC) quantification of calcium recordings for mice expressing mGlp1r or hGLP1R after danuglipron injection (n = 6 mice per condition, unpaired t-test, $^{**}P<0.01$). **t**, Number of significant calcium events averaged per condition during 1-hour recording session following vehicle or danuglipron in mGlp1r or hGLP1R expressing mice (n = 6 mice per injection, two-way ANOVA with Bonferroni correction, $^{*}P<0.05$).

10) The Glp1r knockout experiment is perplexing given the prior argument that liraglutide doesn't reach/activate CeA Glp1r.

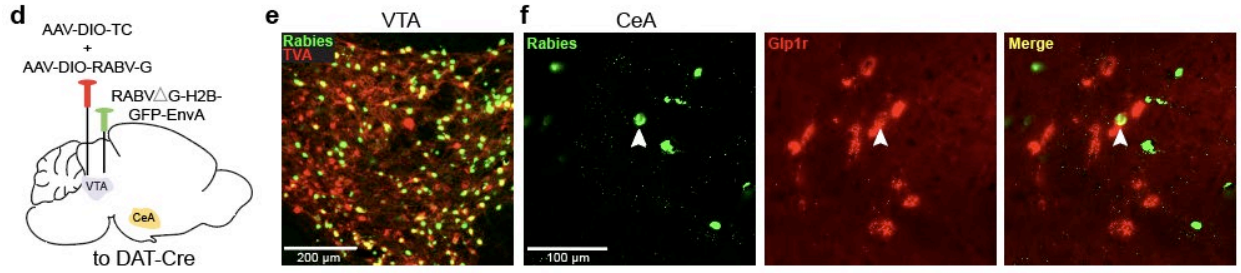
While peptide GLP1R agonists like liraglutide may not penetrate deep brain regions as efficiently as small molecules, multiple Fos studies (4,10) show robust CeA activation after systemic delivery. This possibly reflects indirect engagement of CeA^{Glp1r} neurons via upstream inputs (e.g., vagal, brainstem circuits, or circumventricular organs) rather than direct receptor binding. Moreover, some have proposed that low-level CeA occupancy by peripherally administered peptides cannot be ruled out, especially under chronic dosing conditions near cerebrospinal fluid (23,25). Our CeA-specific Glp1r knockout reveals that intact CeA^{Glp1r} signaling is required for liraglutide's full suppression of HFD intake (Figure 4x,y). Even if liraglutide acts on CeA neurons only via indirect pathways, removing CeA Glp1r abolishes the downstream anorectic effect, demonstrating that CeA^{Glp1r} neurons form a critical node in the reward-driven feeding circuit regardless of the engagement mechanism.

11) The jump to mesolimbic dopamine seems very sudden. Presumably these CeA Glp1r neurons send multiple projections to multiple sites, many of which have been documented. Is there a functional connection with the VTA? Are CeA Glp1r neurons inhibitory, when looking at scRNA data from the amygdala, what cluster(s) are they? Again, the evidence stated in lines 261-262 is weak.

We have revised the manuscript to provide a clearer transition into the mesolimbic dopamine system before presenting our new data. Based on studies by Alhadeff and colleagues(26), the VTA receives both direct and indirect GLP1-mediated input from multiple brain regions, making it a critical convergence point. We conducted cell-type specific, monosynaptic rabies tracing from VTA dopamine neurons and confirmed direct connectivity between CeA^{Glp1r} neurons and VTA dopamine neurons (Extended Data Fig. 15d-f). Additionally, we performed CeA^{Glp1r} fiber stimulation in the VTA and found that optogenetic activation reduced HFD intake by 48.8% while having no effect on standard diet consumption (Fig. 5f-h), demonstrating functional connectivity. Our single-nucleus RNA-seq analysis using published data confirms that CeA^{Glp1r} neurons are predominantly GABAergic (27,28) (Extended Data Fig. 11), consistent with direct inhibition of VTA dopamine neurons. Together, these experiments provide robust evidence for a functional CeA→VTA→NAc circuit that regulates hedonic feeding.



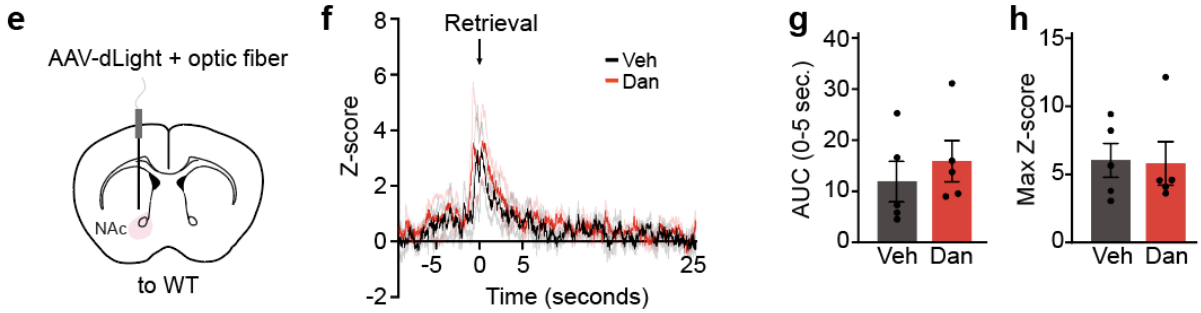
Extended Data Fig. 11 (in revised manuscript). Central amygdala transcriptomic analyses reveal Glp1r neurons are GABAergic (24). **a-b**, Coexpression analysis of Glp1r mRNA and glutamatergic markers (Slc17a7 and Slc17a6) and Glp1r and GABAergic markers (Gad1, Slc6a1, and Slc32a1). **c**, Dotplot showing Glp1r is expressed in the Vdr cluster that contains GABAergic markers.



Extended Figure 15 (in revised manuscript). Tracing from CeA to VTA reveals connections between CeA^{Glp1r} and VTA^{DAT} neurons. d, Illustration of cell-type specific, monosynaptic retrograde rabies tracing approach targeting VTA dopamine neurons. **e,** Representative image showing the VTA injection site in Dat-Cre mice (scale bar = 200 µm). **f**, Left: GFP+ input neurons identified in the CeA. Middle: In situ hybridization for Glp1r mRNA in the CeA. Right: merged image demonstrating colocalization (white arrow) of Glp1r mRNA with rabies-derived GFP in the CeA (scale bar = 100 µm).

12) Figure 5 is missing control non-Glp1r S33W mice.

We have now added WT (non-Glp1r^{S33W}) mice expressing dLight in the nucleus accumbens as controls. In these animals, we repeated the dopamine recordings under both GLP1RA and vehicle conditions. As expected, GLP1RA administration did not alter NAc dopamine transients in WT mice, confirming that the reductions observed in Glp1r^{S33W} mice require the humanized receptor. These control data are presented in Extended Data Figure 16e–h.



Extended Data Fig. 16 (in revised manuscript). e, Schematic of genetically-encoded dopamine sensor, AAV-dLight1.3b, injection and fiber optic implant in the NAc of WT mice. **f**, Averaged Z-score traces showing dopamine release in the NAc in response to HFD following administration of vehicle or danuglipron in WT mice. Traces are aligned to food retrieval time (t = 0) and averaged across five food trials per mouse. **g,h**, Quantified (**g**) area under the curve (AUC) for Z-scores and (**h**) maximum fluorescence Z-scores within the food retrieval window (*n* = 5 per injection, paired t-test).

Referee #3 (Remarks to the Author):

In this study, Godschall et al describe the development of mouse and viral vector tools to enable expression of humanized GLP-1R or mutated murine GLP-1R, responsive to oral small molecule GLP1RAs in clinical development, in mouse tissue. They characterize the behavioral, metabolic, and Fos activation profiles of danuglipron and orfoglipron in mice with S33WmGLP-1R. They go on to show that hGLP-1R expression in hypothalamus leads to agonist-induced suppression of 'homeostatic feeding', in brainstem leads to suppression of homeostatic and hedonic feeding, and in CeA leads to selective suppression of hedonic feeding. They perform functional studies to show that danuglipron or liraglutide activate CeA-GLP1R neurons, and that CeA-GLP1R neuron activation is sufficient and CeA-GLP1R signaling necessary to suppress HFD intake. Finally, they describe an NTS to CeA to VTA to NaC circuit by which GLP-1R agonists may suppress HFD intake.

The murine and viral tools developed are important and will likely be of broad use to the scientific community, and the dissection of different brain regions' involvement in homeostatic vs hedonic food intake is interesting and adds to a growing literature dissecting mechanisms underlying GLP1RA-induced weight loss. A few controls are lacking and some important points glossed over, but with revision this study will be highly impactful.

Major points

- The fact that viral hGLP1R expression is an overexpression model should be discussed as a limitation. For example, it would be informative to see CeA-GLP1R fiber photometry data after lira injection in WT mice, or after Dan in S33W mice expected to have physiologic levels of receptor expression.

We acknowledge that viral hGLP1R expression has inherent limitations and have conducted extensive validation to address this concern. We quantified hGLP1R-positive cells versus native Glp1r-Cre;tdTomato neurons in each targeted region (CeA, DMH, NTS/AP) and consistently found fewer virally transduced cells than endogenous Glp1r-expressing populations (Extended Data Fig. 9b), confirming our viral approach does not result in over-infection. However, performing fiber photometry calcium imaging experiments in Glp1r expressing neurons with danuglipron in $Glp1r^{S33W}$ mice is not feasible because the Glp1r-Cre allele is an IRES knock-in that retains the wild-type mGlp1r sequence; generating animals with both Cre expression and S33W mutation would compromise the homozygous S33W functionality required for small-molecule responsiveness. We have incorporated these experimental considerations in the revised manuscript to provide transparency about our methodological approach.

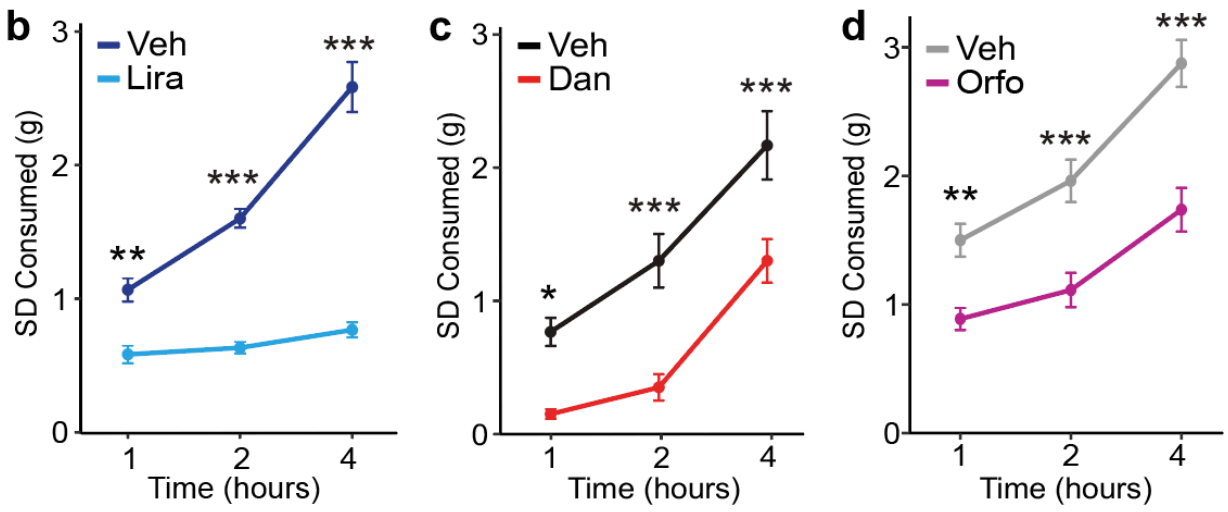
- The distribution of fos-positive CeA neurons looks quite different after Dan vs Orfo vs Lira (fig 2). Presumably the effects of liraglutide on this region are indirect, and knowing the extent of overlap of Dan/Orfo vs Lira-induced fos expression in this region is ultimately going to be important in determining how small molecule vs peptide agonists work. An attempt at this using a Fos-trap or similar approach would be a good start. While this experiment is probably not absolutely essential for publication, this point needs to be discussed more clearly

Although we can't definitively state that Fos expression patterns differ across treatment conditions (Figure 3), potential differences after liraglutide versus small-molecule GLP-1RAs might reflect variations in direct versus indirect routes of action or pharmacokinetics. To address this, we used fiber photometry in mice expressing hGLP-1R exclusively in the CeA^{Glp1r} neurons and recorded calcium responses to liraglutide, danuglipron, and vehicle (Figure 4 p-t and Extended Data Fig. 13 e-g). Remarkably, both liraglutide and danuglipron triggered similar calcium transient event rates in CeA^{Glp1r} neurons, indicating that peptide and small-molecule agonists converge on these cells in the CeA with comparable strength. We've added these photometry data and a discussion about the potential differential engagement of this circuit by peptide versus small-molecule GLP1RAs.

- Looking at food intake following a fast (rather than just ad lib dark cycle food intake) may give a better sense of the role of GLP1R agonism on homeostatic food intake. Does not necessarily have to be done for all models but some data on this would be good

We have added overnight-fast refeeding assays to better assess homeostatic feeding responses. We measured standard diet intake at 1, 2, and 4 hours post-fast following administration of liraglutide, danuglipron, and orforglipron in $Glp1r^{S33W}$ mice (Extended Data Fig. 2b-d). These

experiments confirm that all three GLP1RAs suppress this “high-drive” homeostatic feeding, supporting our previous findings from ad libitum dark-cycle feeding and providing a more comprehensive assessment of how GLP1R agonism modulates different feeding states.



Extended Data Fig. 2 (in revised manuscript). **b-d**, Post-fast refeeding of SD intake 1, 2, and 4 hours post-treatment of (**b**) Lira (n = 6) or (**c**) Dan (n = 6) (**d**) Orfo (n = 8) and vehicle controls in $Glp1r^{S33W}$ mice (two-way ANOVA with Bonferroni correction, *P<0.05; **P<0.01; ***P<0.001).

Minor points:

- The authors somewhat over interpret their findings in their proposed circuit diagram in Fig 5b. They do not actually show that NTS-GCG neurons synapse on CeA-GLP1R neurons, just that they project to CeA and that pharmacologic doses of GLP1R agonist activate CeA-GLP1R neurons. I’d add a question mark and address the remaining gaps.

We have addressed this concern by providing functional validation of the NTS→CeA connection. We expressed Cre-dependent ChrimsonR in NTS^{Gcg} neurons and optically stimulated their terminals in the CeA during feeding sessions. This stimulation selectively reduced HFD intake compared to controls while having no effect on standard-diet consumption (Figure 5b–d), demonstrating functional connectivity that complements our anatomical tracing. To avoid over-interpretation of remaining synaptic details, we removed the speculative circuit diagram from the manuscript.

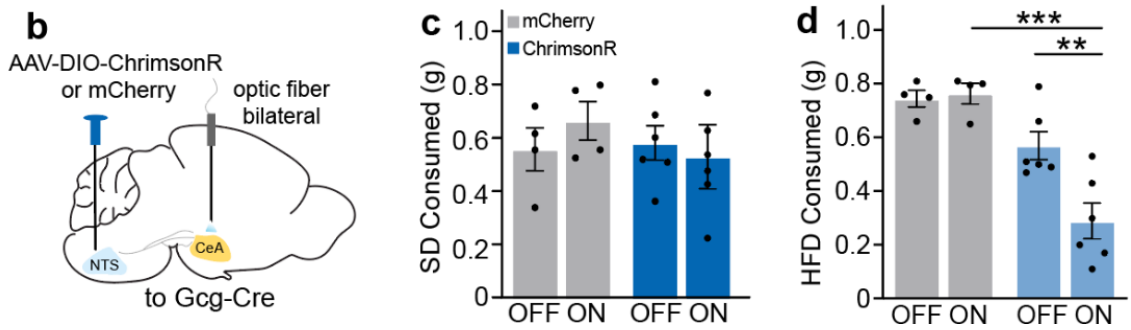


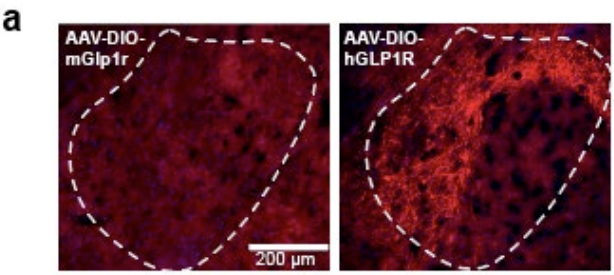
Figure 5 (in revised manuscript). b, Schematic of AAV-DIO-ChrimsonR or AAV-DIO-mCherry injection into the NTS of Gcg-Cre mice with bilateral fiber-optic implants in the CeA. **c,d**, 1-hour consumption of (**c**) standard diet (SD) or (**d**) high-fat diet (HFD) in mCherry (control) versus ChrimsonR-expressing mice under light ON versus OFF conditions (n = 4–6 per group, two-way ANOVA with Tukey's HSD, $^*P < 0.05$).

- The authors state a couple of times that potentiation of insulin/suppression of glucagon release contribute to GLP-1RA-induced weight loss. This is incorrect – those responses to GLP-1RA are important for diabetes control but not weight loss so those phrases should be re-worded (lines 57 and 104)

We have corrected these statements throughout the manuscript.

- Commercially available GLP-1R antibodies are notoriously non-specific (eg see PMID: 23267050 and PMID: 23183176) so antibody validation of hGLP1R expression in extended figure 8 is not convincing without control staining. The authors show electrophysiologic validation of their virus in CeA in fig S9, and behavioral effects following viral injection, which mitigate this concern but would recommend showing antibody controls and/or mentioning this limitation for transparency.

We recognize the known specificity issues with commercial GLP-1R antibodies and have addressed this concern with appropriate controls. We performed parallel immunolabeling in Glp1r-Cre mice injected with AAV-DIO-mGlp1r (wild-type mouse receptor) and observed no detectable signal under identical conditions (Extended Data Fig. 9a), demonstrating that our anti-hGLP1R antibody does not cross-react with mGlp1r. This negative control, combined with our functional validation through electrophysiology, fiber photometry, and behavioral responses, provides confidence in the specificity of our hGLP1R detection.



Extended Data Fig. 9 (in revised manuscript). hGLP1R antibody selectively recognizes virally expressed human GLP1R. **a**, Validation of human GLP1R (hGLP1R; red) expression in the CeA of Glp1r-Cre mice injected with AAV-DIO-hGLP1R (right). hGLP1R protein expression is absent in Glp1r-Cre mice injected with AAV-DIO-mGlp1r in the CeA (left). Scale bar = 200 µm.

- A few figure panels that are not noted as significantly different look fairly different (eg extended fig 1E, F – daytime EE in S33W mice looks almost higher than WT controls, extended fig 13B – sal vs lira look pretty different. A stats table or p values on the fig would be helpful. Extended figure 11 shows an example raw photometry trace, and it would be good to see more raw data rather than just the highly processed heat maps. I understand that the team is analyzing events rather than sustained neural activity changes and so averages may not work, but at least showing sample control traces would be helpful

We have provided comprehensive statistical details in Supplementary Table 1 for all experiments in the manuscript, including exact p-values and statistical tests used. For the photometry experiments, we have enhanced data presentation by including individual mouse heatmaps showing the complete 60-minute recording sessions (Fig. 4q,r; Extended Data Fig. 13e) alongside our event-based analyses. While raw traces spanning the full hour are difficult to visualize clearly when compressed into figure panels, the individual heatmaps provide complete transparency of the data for each animal. We have also added area under the curve (AUC) analyses (Fig. 4s; Extended Data Fig. 13g) that complement our event-based findings and demonstrate the robustness of our calcium signal changes. This comprehensive data presentation ensures both statistical rigor and full transparency of our photometry findings.

References

1. Daily Pill May Work as Well as Ozempic for Weight Loss and Blood Sugar - The New York Times [Internet]. [cited 2025 Jun 9]. Available from: https://www.nytimes.com/2025/04/17/health/pill-glp-1-eli-lilly.html
2. How a New Weight-Loss Pill Could Transform Health | TIME [Internet]. [cited 2025 Jun 9]. Available from: https://time.com/7278429/weight-loss-obesity-pill-eli-lilly/
3. Huang K-P, Acosta AA, Ghidewon MY, McKnight AD, Almeida MS, Nyema NT, et al. Dissociable hindbrain GLP1R circuits for satiety and aversion. Nature. 2024 Aug;632(8025):585–93.
4. Gabery S, Salinas CG, Paulsen SJ, Ahnfelt-Rønne J, Alanentalo T, Baquero AF, et al. Semaglutide lowers body weight in rodents via distributed neural pathways. JCI Insight. 2020 Mar 26;5(6).
5. Sloop KW, Cox AL, Wainscott DB, White A, Droz BA, Stutsman C, et al. The pharmacological basis for nonpeptide agonism of the GLP-1 receptor by orforglipron. Sci Transl Med. 2024 Dec 18;16(778):eadp5765.
6. Oraha J, Enriquez RF, Herzog H, Lee NJ. Sex-specific changes in metabolism during the transition from chow to high-fat diet feeding are abolished in response to dieting in C57BL/6J mice. Int J Obes (Lond). 2022 Oct;46(10):1749–58.
7. Gelineau RR, Arruda NL, Hicks JA, Monteiro De Pina I, Hatzidis A, Seggio JA. The behavioral and physiological effects of high-fat diet and alcohol consumption: Sex differences in C57BL6/J mice. Brain Behav. 2017 Jun;7(6):e00708.
8. Adams JM, Pei H, Sandoval DA, Seeley RJ, Chang RB, Liberles SD, et al. Liraglutide Modulates Appetite and Body Weight Through Glucagon-Like Peptide 1 Receptor-Expressing Glutamatergic Neurons. Diabetes. 2018 Aug;67(8):1538–48.
9. Yacawych WT, Wang Y, Zhou G, Hassan S, Kernodle S, Sass F, et al. A single dorsal vagal complex circuit mediates the aversive and anorectic responses to GLP1R agonists. BioRxiv. 2025 Jan 24;
10. Salinas CBG, Lu TT-H, Gabery S, Marstal K, Alanentalo T, Mercer AJ, et al. Integrated brain atlas for unbiased mapping of nervous system effects following liraglutide treatment. Sci Rep. 2018 Jul 9;8(1):10310.
11. Huang Z, Liu L, Zhang J, Conde K, Phansalkar J, Li Z, et al. Glucose-sensing glucagon-like peptide-1 receptor neurons in the dorsomedial hypothalamus regulate glucose metabolism. Sci Adv. 2022 Jun 10;8(23):eabn5345.
12. Sisley S, Gutierrez-Aguilar R, Scott M, D'Alessio DA, Sandoval DA, Seeley RJ. Neuronal GLP1R mediates liraglutide's anorectic but not glucose-lowering effect. The Journal of Clinical Investigation. 2014 Jun 2;
13. Cong Z, Zhao F, Li Y, Luo G, Mai Y, Chen X, et al. Molecular features of the ligand-free GLP-1R, GCGR and GIPR in complex with Gs proteins. Cell Discov. 2024 Feb 13;10(1):18.

14. Harikumar KG, Wootten D, Pinon DI, Koole C, Ball AM, Furness SGB, et al. Glucagon-like peptide-1 receptor dimerization differentially regulates agonist signaling but does not affect small molecule allostery. Proc Natl Acad Sci USA. 2012 Nov 6;109(45):18607–12.
15. Alcacer C, Andreoli L, Sebastianutto I, Jakobsson J, Fieblinger T, Cenci MA. Chemogenetic stimulation of striatal projection neurons modulates responses to Parkinson's disease therapy. The Journal of Clinical Investigation. 2017 Feb 1;
16. Nakajima K, Cui Z, Li C, Meister J, Cui Y, Fu O, et al. Gs-coupled GPCR signalling in AgRP neurons triggers sustained increase in food intake. Nat Commun. 2016 Jan 8;7:10268.
17. Kawai T, Sun B, Yoshino H, Feng D, Suzuki Y, Fukazawa M, et al. Structural basis for GLP-1 receptor activation by LY3502970, an orally active nonpeptide agonist. Proc Natl Acad Sci USA. 2020 Nov 24;117(47):29959–67.
18. Saxena AR, Gorman DN, Esquejo RM, Bergman A, Chidsey K, Buckeridge C, et al. Danuglipron (PF-06882961) in type 2 diabetes: a randomized, placebo-controlled, multiple ascending-dose phase 1 trial. Nat Med. 2021 Jun 14;27(6):1079–87.
19. Chen Z, Chen G, Zhong J, Jiang S, Lai S, Xu H, et al. A circuit from lateral septum neurotensin neurons to tuberal nucleus controls hedonic feeding. Mol Psychiatry. 2022 Dec;27(12):4843–60.
20. Liu H, Bean JC, Li Y, Yu M, Ginnard OZ, Conde KM, et al. Distinct basal forebrain-originated neural circuits promote homoeostatic feeding and suppress hedonic feeding in male mice. Nat Metab. 2024 Sep;6(9):1775–90.
21. Hardaway JA, Halladay LR, Mazzone CM, Pati D, Bloodgood DW, Kim M, et al. Central Amygdala Prepronociceptin-Expressing Neurons Mediate Palatable Food Consumption and Reward. Neuron. 2019 Jun 5;102(5):1037-1052.e7.
22. Minère M, Wilhelms H, Kuzmanovic B, Lundh S, Fusca D, Claßen A, et al. Thalamic opioids from POMC satiety neurons switch on sugar appetite. Science. 2025 Jan 2;387(6735):750–8.
23. Duran M, Willis JR, Dalvi N, Fokakis Z, Virkus SA, Hardaway JA. Integration of Glucagon-Like Peptide 1 Receptor Actions Through the Central Amygdala. Endocrinology. 2025 Feb 5;166(3).
24. Peters C, He S, Fermani F, Lim H, Ding W, Mayer C, et al. Transcriptomics reveals amygdala neuron regulation by fasting and ghrelin thereby promoting feeding. Sci Adv. 2023 May 24;9(21):eadf6521.
25. Chen H, Liu S, Gao S, Shi H, Yan Y, Xu Y, et al. Pharmacovigilance analysis of neurological adverse events associated with GLP-1 receptor agonists based on the FDA Adverse Event Reporting System. Sci Rep. 2025 May 24;15(1):18063.
26. Alhadeff AL, Rupprecht LE, Hayes MR. GLP-1 neurons in the nucleus of the solitary tract project directly to the ventral tegmental area and nucleus accumbens to control for food intake. Endocrinology. 2012 Feb;153(2):647–58.

27. Graham DL, Durai HH, Trammell TS, Noble BL, Mortlock DP, Galli A, et al. A novel mouse model of glucagon-like peptide-1 receptor expression: A look at the brain. J Comp Neurol. 2020 Oct;528(14):2445–70.

28. Zeng N, Cutts EJ, Lopez CB, Kaur S, Duran M, Virkus SA, et al. Anatomical and Functional Characterization of Central Amygdala Glucagon-Like Peptide 1 Receptor Expressing Neurons. Front Behav Neurosci. 2021 Dec 24;15:724030.

We thank the reviewers for their thoughtful and constructive feedback. We have carefully addressed all comments below and made the corresponding revisions in the main text (changes marked in red). In our view, these suggestions have substantially strengthened the manuscript, and we appreciate the reviewers' insightful critiques and recommendations.

Referees' comments:

Referee #1 (Remarks to the Author):

Re-review of Nature 2024-12-26939B
Godschall et al. investigated the effects of oral GLP-1 receptor agonists (GLP-1RAs) in a humanized mouse model, focusing on food intake, body weight, and behavioral phenotypes. They also add a new component to the brain circuit through which these drugs exert their effects specifically an effect of actions on the amygdala to regulate reward pathways.
The authors have satisfactorily addressed all of my previous comments and in my estimation the paper is suitable for publication pending the authors addressing the following comments which are pertinent to the new experiments that were added.
What follows are some specific comments .

Figure 3 and 4
There is no change in cFOS activity in the DMH after administration of vehicle, danuglipron, liraglutide, or orforglipron (Fig. 3A). This is surprising because in Figure 4, danuglipron significantly suppresses chow intake in mice injected with AAV-DIO-hGLP1R in the DMH. The authors should address the basis for this apparent discrepancy.

The absence of Fos induction in the DMH after GLP1RA administration is consistent with prior reports showing that these drugs do not reliably induce immediate early gene expression in this region[1–4]. Moreover, our finding that small-molecule GLP1RAs reduce chow intake in mice with AAV-mediated hGLP1R expression in the DMH aligns with studies showing that selective activation of DMH-GLP1R neurons suppresses feeding[4]. Thus, while GLP1RAs do not elicit a bulk Fos response, they still engage DMH-GLP1R neurons to influence feeding. We have clarified this point in the revised manuscript.

Figure 2 and 3
The authors now demonstrate that orforglipron elicits less of a CTA than danuglipron which has a greater effect (Fig. 2, Extended Data Fig 3). However, orforglipron administration still results in significant cFOS activation in the area postrema (AP) to a similar extent as danuglipron (Fig. 3C). Given that both compounds activate the AP, previously shown by others to be a site at which nausea is induced, the authors should address why danuglipron has a greater effect. Specifically, they should determine whether different neuronal subpopulations within the AP are activated by these agents. Alternatively, this difference could reflect engagement of different downstream populations that in turn influence the response.

The different nausea-like effects of danuglipron and orforglipron likely stem from their pharmacological properties. Danuglipron is a fast-acting GLP1R agonist that potently stimulates cAMP and also recruits β-arrestin, with a short half-life of ~6 hours, whereas orforglipron is slower-acting, more cAMP-selective, and has a longer half-life of ~36 hours[5–7]. These features are expected to shape how each engages brainstem circuits controlling feeding and aversion. Indeed, we found that orforglipron produced a significantly higher NTS/AP Fos ratio, reflecting a satiety-dominant signal[8], whereas danuglipron and liraglutide showed lower ratios, consistent with a more nausea-like profile (Fig. 3j). We have clarified this reasoning in the revised manuscript to highlight how pharmacological differences may account for the distinct behavioral effects.

Figure 4
The rationale for focusing on the effects of danuglipron but and not orforglipron on the central amygdala (CeA) is not clearly explained and should be clarified (Fig. 4).

Our rationale for focusing on danuglipron in the nucleus-specific experiments of Figure 4, including the CeA, was its pharmacokinetic profile. Danuglipron has faster onset and shorter duration of action[5–7], which allowed us to align drug exposure with feeding assays and the time course of fiber photometry recordings, enabling more precise interpretation of region-specific GLP1R responses. We have clarified this rationale in the revised manuscript.

Extended Data Fig. 11.
The authors note that Glp1r is expressed in the Vdr-expressing neuronal cluster within the CeA. However, MERFISH data from the Allen Brain Cell Atlas indicate that Glp1r is not limited to the Vdr population in amygdala. The authors should assess which other CeA neuronal subtypes express Glp1r.

In their response to Reviewer 2, the authors indicate that Glp1r-expressing neurons in the CeA partially overlap with Pnoc-positive neurons. However, previous work by Hardaway et al. (2019) demonstrated that chemogenetic inhibition of Pnoc+ neurons there suppresses palatable food intake. This effect contrasts with the decreased food intake in the current study following optogenetic stimulation of CeA Glp1r neurons. The authors should address the basis for these potentially contradictory findings.

Although our findings may initially seem to contrast with Hardaway et al. (2019), several distinctions help reconcile the results. Hardaway et al. used chemogenetic inhibition of Pnoc+ neurons with a Gi-coupled receptor, whereas we employed optogenetic stimulation of CeA^{Glp1r} neurons, which differ in both target population and cellular consequences. Consistent with the Allen Brain Cell Atlas[9,10], our data show that Glp1r expression in the CeA is not confined to a single subtype; Glp1r neurons only partially overlap with Pnoc+ neurons (~30%), with most being Pnoc-negative. Furthermore, in Extended Data Fig. 15 we now show additional CeA populations that express Glp1r beyond Vdr and Pnoc, albeit at lower proportions. Together,

these results underscore the cellular heterogeneity of CeA circuits in feeding regulation. We have added the new data and revised the text.

Extended Data Fig. 12
The duration of recording in the analysis of membrane potential before and during danuglipron application is not clearly described. In addition, the recordings appear to have been performed in neurons from a single mouse, which is insufficient. Unless this is incorrect, recordings from CeA neurons from at least three animals per group should be included. Furthermore, the authors report an ~5 mV depolarization that is not durable. These data are thus unconvincing and additional experimental support for this response is necessary.

We expanded the electrophysiological dataset and clarified the Methods. The updated figure (Extended Data Fig. 16) includes recordings from CeA neurons obtained from seven independent mice per group (mGlp1r: 13 cells from 7 mice; hGLP1R: 10 cells from 7 mice). In addition to the groupwise comparison, we now include within-cell paired analyses (Extended Data Fig. 16g-i) showing that danuglipron depolarizes the resting membrane potential and reduces the time to spike-threshold in response to a current injection ramp in hGLP1R expressing neurons, indicating increased excitability.

Extended Data Fig. 15
The authors should quantify the number of Glp1r neurons in CEA and the number of Glp1r neurons that are labeled after retrograde tracing from VTA using rabies.

In the revised manuscript, we quantified the percentage of *Glp1r* RNA–expressing CeA cells infected with rabies via the VTA DA neurons. We found that 10.9% ± 1.7% of neurons are positive for both rabies and Glp1r mRNA expression (Extended Data Fig. 20h-j). We note, however, that this percentage likely represents an under-estimate due to well-known technical limitations of monosynaptic rabies tracing. First, rabies spread is inherently inefficient[11] and can be affected by the type and titer of helper AAV and rabies[12]. Given the specific helper AAV and rabies vectors used in our study, we expect that our rabies experiment labeled fewer than a third of the *Glp1r*+ CeA neurons synapsing on VTA neurons[12]. Second, rabies infection is known to alter gene expression, downregulating hundreds of genes, including many canonical cell-type markers[13–15]. Loss of *Glp1r* expression following rabies infection could therefore result in “false negatives,” artificially lowering the apparent proportion of rabies+/*Glp1r*+ CeA cells (out of the ~500 Glp1r+ neurons/mm^2 in the CeA). Finally, rabies spread is insufficient to infer functional relationships between the pre- and post-synaptic cells[16], meaning that even a relatively low degree of co-localization between rabies and *Glp1r* RNA should not be interpreted as evidence of a functionally unimportant circuit. We use monosynaptic rabies mapping here to generate a hypothesis about connectivity from *Glp1r*+ CeA neurons to VTA neurons, which we then test functionally using other methods (Figures 5e-h).

Referee #2 (Remarks to the Author):

The authors should be commended for addressing the reviewer comments as the changes have significantly enhanced the conclusions of the manuscript, often times just by providing additional context and framing, but also through additional experiments and data analysis. My remaining comments are minor:
1) Figure 4m-n: it would be best to highlight all the cells showing co-expression with white arrows in all the panels as is depicted in the far right panel.

We have updated the figure to highlight all the cells showing co-expression in all the panels as suggested.

2) Figure 4x-y: it would have been good to see within-subject vehicle vs Lira comparison of both the AAV-GFP and AAV-Cre mice. It would also be insightful to see representative images of the injection sites particularly since the Cre virus seems to be fused to eGFP.

These data are now provided in Extended Data Fig. 19, which includes within-subject vehicle versus liraglutide comparisons for both AAV-GFP and AAV-Cre mice, along with representative images of the injection sites.

3) I am curious why the authors tested lira, instead of danu or orfo for Figure 4x-y as the effects of the former would be indirect while the latter as predicted by the other findings in this manuscript may act directly on CeA GLP1Rs?

For this necessity experiment in Figure 4x-y, the only available tool for genetic deletion is the wild-type $Glp1r^{flox/flox}$ line. This precludes the use of small-molecule agonists, since cells outside the CeA that continue to express wild-type *mGlp1r* remain unresponsive to small molecules. We therefore used liraglutide to test the necessity of CeA *Glp1r* in reward-based feeding. Although some of its effects on CeA neurons are likely indirect, liraglutide induces CeA activation (Figure 3d,h,i and Extended Data Fig.21h-j), and our data show that CeA^{Glp1r} expression is necessary, at least in part, for GLP1RA-dependent suppression of reward-driven feeding (Figure 4y). Together with the sufficiency experiments in Figure 4f, where hGLP1R expression in CeA neurons enabled small-molecule–dependent inhibition of high-fat diet intake, these results demonstrate that CeA Glp1r signaling is both necessary and sufficient for GLP1RA-mediated suppression of hedonic feeding. We have clarified this rationale in the revised manuscript.

4) Figure 5a and e: Add a panel showing representative image of injection site of the NTS and CeA, respectively.

Representative images of the NTS and CeA injection sites are now included in Extended Data Fig. 20b-d.

Referee #3 (Remarks to the Author):

Overall, the authors have addressed my concerns with one exception and I think the work is suitable for publication and a contribution to the field across multiple dimensions.

The one issue that was not addressed is related to the distribution of fos-positive CeA neurons after Dan vs Orfo vs Lira. Seeing a similar FP response to these drugs in CeA GLP-1R expressing neurons does not rule out that it could be different sub-populations activated by the different drugs (through direct vs downstream circuit mechanisms), as FP is a bulk imaging approach. I do not think further experimental work is needed but did not see the discussion of this possibility in the revised text as noted in the reviewer response. I do think this needs to be discussed in a short sentence or two..

We agree that, as fiber photometry measures bulk calcium signals, our results reflect average activity across CeA^{Glp1r} neurons and do not exclude the possibility that different subpopulations may be differentially engaged by danuglipron, orforglipron, and liraglutide through direct or circuit-mediated mechanisms. To address this, we have added the following clarification to the Results: "*Because fiber photometry measures population-level activity, we cannot rule out that different subsets of CeA Glp1r neurons are differentially recruited by individual GLP1RAs, with similar bulk responses arising from overlapping but non-identical populations.*"

Referee #4 (Remarks to the Author):

Godschall et al. report the generation of a humanized GLPR mouse line and characterized their changes in behavior and neural activity after treatment with either danuglipron and orforglipron (and baselines). I am evidently a new reviewer added after a round of reviews. I have reviewed the manuscript and rebuttal, and I found the first reviewers asked excellent questions. However, in this round deeper phenotyping of behavior was added, which is my area of expertise. Therefore, I am going to specifically add comments on this aspect of the work (and not others).

Specific Major comments:

- How was the 2-hour filming window determined for home-cage monitoring?

The 2-hour filming window for home-cage monitoring was chosen to capture the peak behavioral effects of the drugs. Some drugs, such as danuglipron (~6-hour half-life) and LiCl, have relatively short half-lives or rapid impacts on behavior, so injections were timed based on these pharmacokinetics and the results shown in Figure 1. All behavioral assays were conducted at the same ZT time, and paired controls accounted for differences in injection timing across drugs. We clarified this point in the methods section.

- From the main text it appears only a single "neck/upper back" key point is used for keypoint estimation (this is then not called pose estimation) likely this is just for the locomotion, but they

clarify in the main text (the method notes 9 keypoints for Keypoint-MoSeq, but then some nose heatmap processing? See minor below).

The reviewer is correct that a single keypoint was used to calculate total distance traveled (Extended Data Figure 10f) and to generate representative heatmaps (Figure 2b and Extended Data Figure 4c). However, for Keypoint-MoSeq analysis, we used the full set of nine tracked keypoints (Extended Data Figure 4b). We have updated the main text and Methods section to clarify that all nine keypoints were used for behavioral syllable inference with Keypoint-MoSeq.

- Why do the authors use PCA/ how is it “significant”? Line 143 “This distinct profile led to its significant separation”. Provide statistical bootstrapping in the text.

We used PCA as a dimensionality reduction approach to visualize overall behavioral structure and summarize group-level trends in two dimensions. This analysis was intended for exploratory visualization rather than statistical inference, to illustrate how behavioral features contribute to apparent group separation. We have revised the text to reflect this.

- The major issue is the inappropriate use of Keypoint-MoSeq; the authors seems to use the “” configuration for pose estimation, and get the default 91 behavioral syllables, then go onto hand-curation, followed by nonlinear embedding (UMAP).

We apologize for causing this confusion. To clarify, we did not use a fixed or default number of syllables. We ran the automatic kappa scan in Keypoint-MoSeq to identify values of kappa that produced syllable durations consistent with prior mouse work (e.g., Weinreb et al. 2024). We then fit models using the three closest kappa values and selected the model that yielded syllables most consistent with recognizable behavioral motifs. We recognize that we did not make this procedure clear in the original Methods section, and we updated the text accordingly.

This is a non-standard approach that would need significant validation. Why do they not follow the statistical approach from Wiltschko et al. 2020 Nature Neuroscience (see Figures 2 and 3), or even the Keypoint-MoSeq paper? For example, the authors could have used the pretrained SuperAnimal-TopViewMouse pose model that has more keypoints and leads to improved performance of MoSeq (see Weinreb et al. 2024 Nature Communications, Figure 5). Then they can take the full transition graph and perform statistics directly in this space (as in the 2020 paper) – UMAP and manual curation is not needed and not statistically robust. I strongly feel this analysis must be redone to be publishable.

Thank you for this thoughtful and detailed comment. We acknowledge that our approach differs from the statistical pipeline described in Wiltschko et al. (2020) and the original Keypoint-MoSeq paper. To ensure the robustness of our findings, we first analyzed the syllable output directly, without manual curation, and observed a consistent trend: orforglipron had a smaller impact on

behavior compared to LiCl, danuglipron, or liraglutide, relative to their respective paired controls. To more closely align with the cited methodologies, in the revised manuscript, we have incorporated syllable-level analysis, including syllable frequency and transition structure, as shown in Figure 2d-f and Extended Data Figures 7-9.

Manual curation was subsequently applied to refine the analysis, particularly to distinguish food-motivated behaviors from superficially similar actions (e.g., head probing or sniffing away from the food hopper) using spatial context. Grouping syllables by behavioral similarity allowed for clearer biological interpretation. For example, while individual movement-related syllables may increase or decrease under a given drug, curated categories provide a more interpretable summary of overall locomotor changes. Manual merging of similar syllables is part of the Keypoint-MoSeq analysis pipeline and follows the general procedures outlined in their documentation (https://keypoint-moseq.readthedocs.io/en/latest/advanced.html#merging-similar-syllables).

Regarding the suggestion to use the SuperAnimal-TopViewMouse model from Weinreb et al. (2024), we appreciate this suggestion but note that most of their figures use 8 top-view keypoints, which is comparable to our 9-keypoint setup. Their enhanced performance in Figure 5 stems from additional keypoints obtained via multi-angle 3D tracking, which are not reliably visible in 2D top-down recordings. Due to the physical constraints of our current PhenoTyper system, we were unable to capture multi-angle videos and thus could not implement their 3D tracking approach.

Finally, we appreciate the reviewer's point regarding UMAP. We have removed this analysis from the revised manuscript. We have also updated both the main text and Methods section to clearly describe the uncurated syllable-level analysis, the rationale for manual refinement, and how these steps complement each other in our behavioral interpretation.

-- Independently, why use UMAP? Is there something nonlinear in the behavior they needed to capture that PCA did not? How did they validate UMAP hyperparameters? Also measuring distances in UMAP global space is inappropriate – how did they determine the local space?
As mentioned above, we've removed UMAP as suggested by the reviewer which has not altered any of our conclusions.

- The title must reflect this is in "humanized mice". The authors are surely aware of the media impact this work will have, and that is critical for appropriate communication.

We have modified the title as suggested by the reviewer.

Summary of Revisions: We clarified the use of 1 keypoint for heatmap visualization and distance traveled, versus 9 keypoints for Keypoint-MoSeq behavioral analysis. We refined our PCA descriptions, detailed the Keypoint-MoSeq workflow including kappa selection, and added

the pre-curation syllable analysis to the manuscript. UMAP has been removed, and the Methods and Results sections have been updated for clarity and reproducibility.

Minor:

- Figure 1 has alignment issues (i.e., “y” is missed aligned) and could use more white space between panels for readability.
- Figure 2; the arrow is not straight under the term “OpenCV” and Keypoint-Moseq is mis-capitalized (MoSeq).
- Figure 3g, the stars cover the error bar

We have corrected the alignment issues throughout the figures to the best of our ability, adjusted the spacing between panels for readability, fixed the mis-capitalization and the arrow placement in Figure 2, and corrected the positioning of the stars in Figure 3g.

- Figure 4 should show raw calcium imaging data

As detailed in the Methods, calcium recordings were analyzed using z-score normalization with the vehicle condition as the baseline to isolate drug-specific changes while minimizing inter-animal variability. In response to the reviewer’s suggestion, we have now included representative data showing the full raw recordings, ΔF/F traces, and ΔF/F heatmaps (Extended Data Fig.17b-g), which illustrate how the raw signals relate to the normalized visualizations. For the heatmaps in Figure 4, we applied min–max (0–1) normalization relative to each mouse’s vehicle session strictly for visualization, allowing temporal patterns to be compared across trials without being dominated by amplitude differences. All quantitative analyses, including event detection and AUC, were performed on the z-scored ΔF/F traces. This workflow is consistent with established practice in the fiber photometry field, where raw ΔF/F data are shown for context and normalized signals are used for group-level comparison and visualization[17–21].

- The Veh. in Figure 5m seems to be nearly symmetrical around retrial, why?

The symmetry of the vehicle trace in Figure 5m reflects the typical pattern of NAc dopamine activity around reward retrieval. Dopamine ramps during approach, peaks at contact, and rapidly declines afterward, producing a near-symmetric average. This response is consistent across panels (Fig. 5j, m, q, t) and with prior dopamine sensor fiberphotometry studies in NAc for reward-related dopamine dynamics[22]. In these experiments, both danuglipron and orforglipron reduced dopamine levels during retrieval of rewarding food.

- Line 776; light cycle – was the light cycle inverted so you ran behavioral assays in the dark period?

We clarify in the revised manuscript that experiments conducted during ZT 0-12 occurred in the light phase and those during ZT 12-24 in the dark phase of a standard 12:12 light-dark cycle without light exposure. All behavioral recordings in PhenoTypers were carried out in light-tight boxes equipped with infrared cameras, which allowed monitoring during the dark phase without light exposure and ensured preservation of the animals' circadian rhythms.

- Line 797; it would be 90% train, 10% test (not validation, unless they finetuned parameters based on that 10% fraction);
-also to note, the mAP of 81 is actually very low given they trained on nearly 10K frames – this suggests the manual labeling was not high-quality.

Our dataset consists of 11,847 labeled frames across 19 videos, split 80/10/10 into training, validation, and test sets. The test set was drawn from an independent video to assess generalization. Metrics in the revised manuscript are reported from this test set, and the Methods have been updated to clearly describe the dataset size and split.

Regarding the mAP of 0.81, we appreciate the reviewer's attention to tracking quality. Several factors inform our interpretation of this metric. First, mAP is a stringent, distance-based threshold metric that can underestimate performance in complex environments where even small pixel offsets are penalized despite being biologically negligible for behavioral classification. Second, our enriched home-cage setup—with bedding, shelter, wheel, and dynamic lighting—creates occlusions and postural variations that challenge any tracking algorithm. Pereira et al. (2022) reported mAP < 0.60 in similar home-cage environments (albeit during tracking of two mice), suggesting our 0.81 represents reasonable performance for this context.

Most importantly, the biological validity of our tracking is supported by multiple quality controls: (1) Keypoint-MoSeq's built-in outlier filtering interpolated erroneous keypoints to improve modeling accuracy; (2) behavioral syllables identified by the model are visually interpretable and correspond to recognizable mouse behaviors (Extended Data Fig. 6); (3) sensor-based measures (food hopper entries, wheel rotations, water licks) independently corroborate the tracking-derived behavioral patterns (Extended Data Fig. 10l-n); and (4) drug effects detected in our behavioral analysis (Fig. 2d-f, Extended Data Fig. 8) align with established GLP1RA phenotypes. We therefore conclude that tracking quality was sufficient for the downstream biological conclusions, though we acknowledge that higher mAP values would be ideal and represent an area for future technical improvement.

- Line 801: "Representative heatmaps of the nose keypoint were generated by cropping to exclude cage walls and binning into 62x 27 spatial grids. Keypoint-MoSeq54,55 was used to infer behavioral syllables from 80 hours of keypoint data." Does this mean ONLY the nose was used? What is the relevance of the heatmap generation step?

We clarified in the Methods that the heatmaps were generated using only the nose coordinates to visualize time spent at different cage locations (Figure 2b, Extended Data Figure 4c). All nine tracked keypoints were used for Keypoint-MoSeq model fitting to infer behavioral syllables.

- Line 841 links to an org repo on GitHub (https://github.com/UVACircMetNeuLab), there is no source code I can review available.
We apologize for the earlier oversight. Our GitHub repository is now public and can be accessed at: UVACircMetNeuLab/glp1r-reward-circuit. We have updated this link in the Methods.

- Overall, the figures has many small alignment issues.
We have fixed the alignment errors to the best of our abilities.

References

1. Salinas, C. B. G. *et al.* Integrated brain atlas for unbiased mapping of nervous system effects following liraglutide treatment. *Sci. Rep.* **8**, 10310 (2018).
2. Gabery, S. *et al.* Semaglutide lowers body weight in rodents via distributed neural pathways. *JCI Insight* **5**, (2020).
3. Hansen, H. H. *et al.* Whole-brain activation signatures of weight-lowering drugs. *Mol. Metab.* **47**, 101171 (2021).
4. Kim, K. S. *et al.* GLP-1 increases preingestive satiation via hypothalamic circuits in mice and humans. *Science* **385**, 438–446 (2024).
5. Saxena, A. R. *et al.* Danuglipron (PF-06882961) in type 2 diabetes: a randomized, placebo-controlled, multiple ascending-dose phase 1 trial. *Nat. Med.* **27**, 1079–1087 (2021).
6. Griffith, D. A. *et al.* A Small-Molecule Oral Agonist of the Human Glucagon-like Peptide-1 Receptor. *J. Med. Chem.* **65**, 8208–8226 (2022).
7. Zhang, X. *et al.* Differential GLP-1R Binding and Activation by Peptide and Non-peptide Agonists. *Mol. Cell* **80**, 485-500.e7 (2020).

8. Huang, K.-P. *et al.* Dissociable hindbrain GLP1R circuits for satiety and aversion. *Nature* **632**, 585–593 (2024).
9. Zhang, M. *et al.* Molecularly defined and spatially resolved cell atlas of the whole mouse brain. *Nature* **624**, 343–354 (2023).
10. Zhuang, X., Jung, W. & Zhang, M. A molecularly defined and spatially resolved cell atlas of the whole mouse brain. *Brain Image Library* (2023) doi:10.35077/act-bag.
11. Wickersham, I. R. *et al.* Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. *Neuron* **53**, 639–647 (2007).
12. Patiño, M. *et al.* Postsynaptic cell type and synaptic distance do not determine efficiency of monosynaptic rabies virus spread measured at synaptic resolution. *eLife* **12**, (2023).
13. Huang, K. W. & Sabatini, B. L. Single-Cell Analysis of Neuroinflammatory Responses Following Intracranial Injection of G-Deleted Rabies Viruses. *Front. Cell. Neurosci.* **14**, 65 (2020).
14. Patiño, M. *et al.* Single-cell transcriptomic classification of rabies-infected cortical neurons. *Proc Natl Acad Sci USA* **119**, e2203677119 (2022).
15. Webster, A. N. *et al.* Molecular connectomics reveals a glucagon-like peptide 1-sensitive neural circuit for satiety. *Nat. Metab.* **6**, 2354–2373 (2024).
16. Rogers, A. & Beier, K. T. Can transsynaptic viral strategies be used to reveal functional aspects of neural circuitry? *J. Neurosci. Methods* **348**, 109005 (2021).
17. Kaur, S. *et al.* Lateral parabrachial FoxP2 neurons regulate respiratory responses to hypercapnia. *Nat. Commun.* **15**, 4475 (2024).
18. Xiao, Q. *et al.* A new GABAergic somatostatin projection from the BNST onto accumbal parvalbumin neurons controls anxiety. *Mol. Psychiatry* **26**, 4719–4741 (2021).
19. Cassidy, R. M. *et al.* A lateral hypothalamus to basal forebrain neurocircuit promotes feeding by suppressing responses to anxiogenic environmental cues. *Sci. Adv.* **5**, eaav1640 (2019).

20. Cai, J. *et al.* An excitatory projection from the basal forebrain to the ventral tegmental area that underlies anorexia-like phenotypes. *Neuron* **112**, 458-472.e6 (2024).
21. Luchsinger, J. R. *et al.* Delineation of an insula-BNST circuit engaged by struggling behavior that regulates avoidance in mice. *Nat. Commun.* **12**, 3561 (2021).
22. Lodder, B. *et al.* Absolute measurement of fast and slow neuronal signals with fluorescence lifetime photometry at high temporal resolution. *Neuron* (2025) doi:10.1016/j.neuron.2025.08.013.

REVIEWER #2
Since I have already addressed the authors commitment to revising the work on a couple of occasions, I just want to highlight a few things, particularly as reviews have become more transparent.

• The authors conclude dan and orfo are not aversive because they don't significantly change CTA (sacch preference), however it is certainly trending in that direction. They then perform an elaborate behavioral syllable monitoring analysis to "better" answer this. However, in my honest opinion, a more robust way of testing this question is a) increasing the power (n=6 looked like it was tested) and b) using another conditioned stimulus such as Ensure that would yield a much higher preference ratio over water.

Reviewer #1 suggests increasing the N number for this experiment or using another conditioned stimulus test (Ensure).

We appreciate the reviewer's suggestion to strengthen the conditioned taste avoidance (CTA) analysis. Accordingly, we have increased the sample sizes across all intraperitoneal treatment groups. The updated group sizes are: vehicle (n = 9), LiCl (n = 7), liraglutide (n = 8), danuglipron (n = 9), and orforglipron (n = 10) (Extended Data Fig. 3b). With the expanded cohort, our conclusions are unchanged. Only LiCl and liraglutide produced statistically significant reductions in saccharin preference relative to vehicle (both $P < 0.001$, one-way ANOVA with Tukey's HSD correction), whereas danuglipron and orforglipron did not differ significantly from vehicle. Moreover, saccharin preference in LiCl-treated mice was significantly lower than danuglipron- or orforglipron-treated mice ($P = 0.02$ for both comparisons), indicating that any apparent "trend" toward aversion with the small-molecule GLP1RAs in the original dataset does not reflect a robust aversive effect.

• The manuscript leans very heavily on this idea that AP = nausea/malaise and NTS = satiety when it comes to these drugs but it is far more probable that both areas play roles in mediating both effects and there is likely much crosstalk between these two structures.

Reviewer #1 suggests a toning down and explicit acknowledgement of this potential model.

We appreciate the reviewer's point that the functions of the area postrema (AP) and nucleus tractus solitarius (NTS) are not strictly dissociable and that both regions likely contribute to both satiety and aversive signaling, with substantial crosstalk between them. In the revised manuscript, we have toned down our interpretation and now explicitly acknowledge that AP and NTS operate as part of a distributed hindbrain network rather than as isolated "nausea" versus "satiety" centers. Specifically, in the section describing the NTS/AP Fos ratio (Fig. 3j), we clarify that this metric is intended to capture a relative bias in regional recruitment, not a categorical separation of function. We now state that the elevated NTS/AP Fos ratio observed with orforglipron reflects a shift toward NTS-dominant engagement within this shared network, consistent with the distinct behavioral profile we observe, while recognizing that both nuclei likely participate in both appetitive and aversive processes.

Updated text (Line 174-182): Although AP activation is often associated with nausea and malaise and NTS activation with satiety-related signaling, these functions are not mutually exclusive and both nuclei likely contribute to multiple aspects of ingestive behavior[9,30]. Within this framework, we reasoned that differences in the relative recruitment of AP and NTS might help explain the distinct behavioral profiles elicited by different GLP1RAs[14,31]. To test this, we calculated the NTS/AP Fos ratio for each treatment. Orforglipron produced a significantly higher NTS/AP ratio, reflecting a bias toward NTS-dominant engagement, whereas danuglipron and liraglutide yielded lower ratios that align with a more AP-skewed, nausea-like activation profile in keeping with their distinct pharmacokinetic and signaling properties (Fig. 3j)[16–18].

• It's not clear why the authors observe oral dan reduces AP Fos activation compared to injections…might be worth mentioning why.

Reviewer #1 recommends that you should address this.

The reduced AP Fos activation after oral danuglipron relative to intraperitoneal injection likely reflects route-dependent pharmacokinetics and differences in systemic exposure. Oral dosing typically produces slower absorption and lower peak plasma concentrations than parenteral routes at equivalent doses (Al Shoyaib et al., 2019; Griffith et al., 2022). These differences in the rate and magnitude of circulating drug exposure may reduce the blood-borne signals that recruit AP neurons, which are known to be particularly sensitive to rapidly rising circulating factors due to their relatively permeable blood–brain barrier. We now explicitly note this interpretation in the Results.

Updated text (Line 187-191): This likely reflects the fact that oral dosing produces slower absorption and lower peak exposure, which may reduce recruitment of AP neurons that are sensitive to circulating factors. Preferential recruitment of NTS over AP may therefore help limit nausea and malaise, side effects often linked to AP engagement, while preserving NTS-linked therapeutic benefits[9].

• The major finding to me is Figure 4 so I just want to point out a couple points that I'd want to be absolutely certain on. Firstly, it is unclear if each animal was just run once with vehicle vs drug treatment. If it was me, I would want to ensure that the effects I were seeing were entirely reproducible (maybe they did and I in fact confident which negates my point). It's also clear that some of these experiments involved n=6 which again would be worrisome for me unless the same 6 mice were run for vehicle vs drug across 3 different trials and then averaged. While within-subject analyses were run, if the effects are robust enough, statistical tests could be run against mCherry control cohorts but it a) doesn't look like this was done for the BMH and b) these controls are missing from the other regions. This would control for instances when the vehicle controls from one experiment look vastly different than other (i.e. Fig 4c vs 4f).

Reviewer #1 recommends better clarification of how their experiments were performed.

All feeding experiments in Figure 4 used a within-subject paired design. Within each viral cohort, the same mice received vehicle or danuglipron on separate, counterbalanced test days. The reported n values therefore correspond to paired vehicle and drug datasets for each animal, and the plotted intake values reflect one test session per condition per mouse, averaged across animals. This design produced robust, consistent within subject effects on SD and HFD intake that were detectable within the reported cohorts. We have updated the Methods section to clarify the experimental design.

We applied this paired within-subject strategy across multiple Glp1r-expressing brain regions to identify sites where receptor activation is sufficient to alter SD or HFD intake. The CeA emerged as the region with the most interesting dissociation, with selective suppression of HFD intake, prompting focused follow-up experiments in this region. In the CeA we used three viral constructs in parallel: hGLP1R and mGlp1r^{S33W} as danuglipron sensitive receptors, and wild type mGlp1r as a receptor level control that is matched for expression but insensitive to danuglipron (Figure 4 and Extended Data Fig. 13). Danuglipron suppressed HFD intake only when a danuglipron sensitive receptor was expressed (hGLP1R or mGlp1r^{S33W}), consistent with a direct and region specific Glp1r mechanism. Together with the electrophysiological (Extended Data Fig. 16), optogenetic (Figure 4 u-w), and conditional Glp1r deletion (Figure 4 x-y and Extended Data Fig. 19), these experiments support the robustness and CeA specificity of the Figure 4 findings.

• It's impossible to tell how specific the DIO-hGLP1R viruhats is to the region of interest as images are zoomed in to a point where outside expression cannot be assessed (except in Ext Fig 12 using the antibody which suggests the virus is hitting outside the intended targets). Secondly, beyond region specificity, there is no verification that this virus is actually only expressed in Cre-expressing GLP1R neurons.

Reviewer #1 suggests that you should clarify this in a revised paper.

GLP1R, like many GPCRs, is challenging to detect immunohistochemically, so we relied on genetic tools to constrain and validate receptor expression. The Glp1r-Cre line used in our studies has been extensively validated for faithful labeling of endogenous Glp1r neurons (Zeng et al., 2021). To assess antibody specificity, we injected AAV-DIO-mGlp1r into Glp1r-Cre mice as a negative control and observed no detectable hGLP1R signal (Extended Data Fig. 12a), indicating that the antibody does not label tissue in the absence of hGLP1R expression. To confirm that DIO-hGLP1R expression is restricted to Cre expressing Glp1r neurons, we co-injected AAV-DIO-hGLP1R with a widely used Cre dependent reporter virus (AAV-DIO-GCaMP7s; Extended Data Fig. 17a). In this setting, hGLP1R staining co-localized with Cre dependent fluorescence, demonstrating that hGLP1R is expressed within the intended Glp1r positive population. The apparent spread in Extended Data Fig. 12 largely reflects axonal and fiber labeling rather than off target somatic infection, because membrane bound receptor

staining follows processes that extend beyond the injection site. In these experiments, clearly labeled somata were confined to the targeted sites and their immediate vicinity.

• For ephys experiments, how was it determined that every YFP recorded cell also expressed the hGLP1R? It's probable that many cells express one or the other. Obviously the authors are relying on the YFP to visualize the cells but it is unclear how it was determined these had the hGLP1R.

Reviewer #1 suggests that you should clarify this in a revised paper.

In all CeA recordings, AAV-DIO-mGlp1r or AAV-DIO-hGLP1R was co-injected at a 1:1 ratio with AAV-DIO-eYFP into Glp1r-Cre mice; therefore, YFP fluorescence identified Cre-positive neurons that were competent to express the receptor. This approach is consistent with prior electrophysiological studies using fluorescent reporters to target neurons expressing exogenous receptors without fluorescent tags (Y. Cai et al., 2021). We have confirmed viral co-expression of Cre dependent receptor with a Cre dependent fluorescent reporter in a parallel dataset where we co-injected AAV-DIO-hGLP1R and AAV-DIO-GCaMP7s (Extended Data Fig. 17a) and demonstrated efficient co-transduction under these conditions. Most importantly, electrophysiological recordings were performed without bias and restricted to YFP positive neurons in both conditions, allowing a direct comparison between mGlp1r (danuglipron insensitive) and hGLP1R (danuglipron sensitive) cohorts. We now clarify this in the Methods text.

• The fact that expressing hGLP1R in all four areas of interest lead to dan-mediated food intake suppression raises the question that if you put these hGLP1R anywhere in the brain, even places where endogenous GLP1R isn't normally expressed, would the authors make similar observations.

Reviewer #1 suggests that you should add a comment on this in a revised paper.

In all rescue experiments, hGLP1R was expressed using a Cre-dependent DIO construct in Glp1r-Cre mice, so expression was confined to neurons that endogenously express Glp1r within each targeted region rather than arbitrarily throughout the brain. Thus, these experiments restore small molecule sensitivity specifically to native Glp1r populations in anatomically defined feeding circuits. Importantly, the outcomes are not uniform across sites: BMH/DMH, NTS/AP, and CeA manipulations produce distinct patterns of SD versus HFD suppression and differ in magnitude, which is inconsistent with a generic, location-independent effect of hGLP1R expression on food intake. Instead, the data support a model in which danuglipron acts through a distributed set of endogenous GLP1R expressing nodes that differentially regulate homeostatic and hedonic feeding. We now clarify these design features in the revised text.

Updated text (Line 193-197): To distinguish direct from indirect effects of small molecule GLP1RAs, we used Glp1r-Cre mice and a Cre-dependent AAV expressing full length human GLP1R (AAV-hSyn-DIO-hGLP1R) (**Fig. 4a**). In this configuration, hGLP1R expression is

restricted to Cre-positive, endogenously *Glp1r* expressing neurons within each targeted region (**Extended Data Fig. 12a,b**), allowing direct assessment of region specific GLP1R activation.

• I'm having a very difficult time coming to terms with the notion that optoactivation of GLP1R CeA neurons ONLY perturbs HFD consumption but not SD. What happens to HFD intake after a fast? The same can be said about the NTS GCG inputs to the CeA and GLP1R CeA to VTA terminals. Dopamine is not just rewarding for HFD but all food so my biggest struggle is determining why this dichotomy exists, particularly when it comes to appetite suppression effects. In simplest terms I don't know how an artificial activation of a brain pathway would reduce food intake of one food substrate but not another. Do the authors propose similar results would be observed with any "palatable" or "hedonic" food?

Reviewer #1 suggests that you should add a comment on this in a revised paper.

Dopamine contributes to the motivational value of both SD and HFD, and we do not interpret CeA^{Glp1r} circuit activation as imposing a hard boundary between nutrient types. Instead, the apparent selectivity for HFD reflects motivational context rather than nutrient specific coding. In our assays, HFD is consumed during the rest phase in *ad libitum* fed mice, when homeostatic drive is low and intake is strongly hedonic, whereas SD intake is measured after an overnight fast under high homeostatic drive. Under these conditions, CeA^{Glp1r} neurons and their upstream NTS^{Gcg} inputs or CeA^{Glp1r} to VTA outputs are positioned to gate hedonic feeding, so optogenetic activation preferentially suppresses HFD consumption while leaving strongly homeostatic SD intake largely intact. This framework is consistent with prior work showing that CeA ensembles encode the valence of consumed food and bidirectionally modulate intake through positive and negative valence signals, rather than generating a single hunger or satiety state (H. Cai et al., 2014; Canovas et al., 2025; Douglass et al., 2017; Schiff et al., 2018; Stern et al., 2021). Our interpretation therefore predicts that CeA^{Glp1r} activation would preferentially constrain hedonic feeding across other highly palatable foods as well.

Importantly, selective effects on palatable foods are not unique to our study and there are numerous elegant studies demonstrating hedonic and homeostatic feeding can be differentially modulated (Chen et al., 2022; Hardaway et al., 2019; Minère et al., 2025) . For instance, chemogenetic inhibition of CeA^{Pnoc} neurons has previously been shown to reduce HFD intake without altering chow intake (Hardaway et al., 2019). Taken together, our data continue to support the broad role for the CeA in encoding the valence of consumed food, rather than promoting a global state of hunger or satiety.

Updated text (Line 331-336): Specifically, CeA^{Glp1r} neurons are well-positioned to receive endogenous and exogenous GLP-1 input and modulate VTA activity, thereby linking metabolic signals to dopamine-dependent reward circuits. Consistent with this role, our experiments show that activating CeA^{Glp1r} neurons preferentially suppresses palatable food consumption, supporting a broad role for the CeA in encoding food valence rather than a global hunger or satiety state.

• Just want to make sure I'm reading this right, deletion of GLP1R in the CeA (Fig 4x-y) ONLY effects HFD intake after Lira injection. Why would that be? Certainly, lira is not getting anywhere near these GLP1R expressing CeA neurons. It is possible lira activates upstream pathways which then engage these cells but it's not shown that GLP1R CeA neurons are indeed activated by the drug. It's also not confirmed that GLP1R was successfully deleted from these cells.

Reviewer #1 suggests that you should comment on the potential limitations of the experiments raised by the reviewer.

Deletion of Glp1r in the CeA selectively reduced liraglutide-induced suppression of HFD intake without affecting SD intake (Fig. 4y, Extended Data Fig. 19a-f). Since systemic liraglutide is unlikely to reach CeA^{Glp1r} neurons at high concentrations, these effects support a model in which CeA^{Glp1r} neurons function as a required downstream node through which upstream GLP1R pathways influence hedonic feeding via activation of Glp1r receptors in the CeA, likely through NTS^{Gcg} inputs as supported by our functional connectivity data (Fig. 5a-d), rather than as a primary site of drug action. CeA fiber photometry and Fos mapping after peripheral liraglutide or other peptide-based GLP1R agonist administration show robust activation of CeA neurons (Extended Data Fig. 17h-j and Figure 3d) (Duran et al., 2025; Falk et al., 2023; Gabery et al., 2020; Hansen et al., 2021; Salinas et al., 2018; Trevaskis et al., 2017), consistent with this framework. We now state this model explicitly in the revised text.

Because available GLP1R antibodies do not reliably detect endogenous mouse receptors, we cannot directly visualize receptor loss at the single cell level after Cre mediated recombination. Instead, as in prior work using this validated Glp1r flox allele with virally induced gene ablation, (Ding et al., 2025; Kim et al., 2024; Yacawych et al., 2025), we rely on robust local Cre expression together with the selective loss of liraglutide's effect on HFD intake in CeA Cre injected mice compared with GFP controls as convergent evidence that CeA GLP1R signaling is effectively disrupted.

Updated text (Line 275-278): Since systemic liraglutide is unlikely to reach CeA Glp1r neurons at high concentrations, CeA fiber photometry (**Extended Data Fig. 17h–j**) and Fos data (**Fig. 3d,h**)[7,25,28,29] support a model in which liraglutide acts via upstream GLP1R pathways yet requires Glp1r in the CeA for full suppression of HFD intake.

Referee #4 (Remarks to the Author):

I thank the authors for the clarifications and edits to the behavioral aspects of the manuscript. I believe it is now clear for a reader to understand the pipeline. I would urge the authors to release the full code and dataset with the paper, vs. the demos now provided.

We have added the syllable-level analysis and the full MoSeq syllable dataset to our GitHub repository for the paper. Running the entire pipeline from raw data also requires access to the underlying behavioral videos and SLEAP tracking files, which are too large to host on GitHub.

We will provide these raw datasets to interested investigators upon request as stated in the Data Availability section.

Bibliography

Al Shoyaib, A., Archie, S. R., & Karamyan, V. T. (2019). Intraperitoneal route of drug administration: should it be used in experimental animal studies? *Pharmaceutical Research*, *37*(1), 12. https://doi.org/10.1007/s11095-019-2745-x

Cai, H., Haubensak, W., Anthony, T. E., & Anderson, D. J. (2014). Central amygdala PKC-δ(+) neurons mediate the influence of multiple anorexigenic signals. *Nature Neuroscience*, *17*(9), 1240–1248. https://doi.org/10.1038/nn.3767

Cai, Y., Nielsen, B. E., Boxer, E. E., Aoto, J., & Ford, C. P. (2021). Loss of nigral excitation of cholinergic interneurons contributes to parkinsonian motor impairments. *Neuron*, *109*(7), 1137-1149.e5. https://doi.org/10.1016/j.neuron.2021.01.028

Canovas, J. A., Wang, L., Mohamed, A. A. M., Abbott, L. F., & Zuker, C. S. (2025). A brain center that controls consummatory responses. *Cell*, *188*(24), 6907-6922.e17. https://doi.org/10.1016/j.cell.2025.08.021

Chen, Z., Chen, G., Zhong, J., Jiang, S., Lai, S., Xu, H., Deng, X., Li, F., Lu, S., Zhou, K., Li, C., Liu, Z., Zhang, X., & Zhu, Y. (2022). A circuit from lateral septum neurotensin neurons to tuberal nucleus controls hedonic feeding. *Molecular Psychiatry*, *27*(12), 4843–4860. https://doi.org/10.1038/s41380-022-01742-0

Ding, H., Wang, M., Zhang, J., Wan, C., Huang, Z., Liu, L., & Liu, J. (2025). Exendin-4 induced retching-like behavior mediated by postsynaptic effect via AMPA receptors in the area postrema of mice. *American Journal of Physiology. Endocrinology and Metabolism*, *329*(2), E254–E265. https://doi.org/10.1152/ajpendo.00174.2025

Douglass, A. M., Kucukdereli, H., Ponserre, M., Markovic, M., Gründemann, J., Strobel, C., Alcala Morales, P. L., Conzelmann, K.-K., Lüthi, A., & Klein, R. (2017). Central amygdala circuits modulate food consumption through a positive-valence mechanism. *Nature Neuroscience*, *20*(10), 1384–1394. https://doi.org/10.1038/nn.4623

Duran, M., Willis, J. R., Dalvi, N., Fokakis, Z., Virkus, S. A., & Hardaway, J. A. (2025). Integration of Glucagon-Like Peptide 1 Receptor Actions Through the Central Amygdala. *Endocrinology*, *166*(3). https://doi.org/10.1210/endocr/bqaf019

Falk, S., Petersen, J., Svendsen, C., Romero-Leguizamón, C. R., Jørgensen, S. H., Krauth, N., Ludwig, M. Q., Lundø, K., Roostalu, U., Skovbjerg, G., Nielsen, D. A. G., Ejdrup, A. L., Pers, T. H., Dmytriyeva, O., Hecksher-Sørensen, J., Gether, U., Kohlmeier, K. A., & Clemmensen, C. (2023). GLP-1 and nicotine combination therapy engages hypothalamic and mesolimbic pathways to reverse obesity. *Cell Reports*, *42*(5), 112466. https://doi.org/10.1016/j.celrep.2023.112466

Gabery, S., Salinas, C. G., Paulsen, S. J., Ahnfelt-Rønne, J., Alanentalo, T., Baquero, A. F., Buckley, S. T., Farkas, E., Fekete, C., Frederiksen, K. S., Helms, H. C. C., Jeppesen, J. F., John, L. M., Pyke, C., Nøhr, J., Lu, T. T., Polex-Wolf, J., Prevot, V., Raun, K., … Hogendorf, W. F. J. (2020). Semaglutide lowers body weight in rodents via distributed neural pathways. *Journal of Clinical Investigation Insight*, *5*(6). https://doi.org/10.1172/jci.insight.133429

Griffith, D. A., Edmonds, D. J., Fortin, J.-P., Kalgutkar, A. S., Kuzmiski, J. B., Loria, P. M., Saxena, A. R., Bagley, S. W., Buckeridge, C., Curto, J. M., Derksen, D. R., Dias, J. M., Griffor, M. C., Han, S., Jackson, V. M., Landis, M. S., Lettiere, D., Limberakis, C., Liu, Y., … Tess, D. A. (2022). A Small-Molecule Oral Agonist of the Human Glucagon-like Peptide-1 Receptor. *Journal of Medicinal Chemistry*, *65*(12), 8208–8226. https://doi.org/10.1021/acs.jmedchem.1c01856

Hansen, H. H., Perens, J., Roostalu, U., Skytte, J. L., Salinas, C. G., Barkholt, P., Thorbek, D. D., Rigbolt, K. T. G., Vrang, N., Jelsing, J., & Hecksher-Sørensen, J. (2021). Whole-brain activation signatures of weight-lowering drugs. *Molecular Metabolism*, *47*, 101171. https://doi.org/10.1016/j.molmet.2021.101171

Hardaway, J. A., Halladay, L. R., Mazzone, C. M., Pati, D., Bloodgood, D. W., Kim, M., Jensen, J., DiBerto, J. F., Boyt, K. M., Shiddapur, A., Erfani, A., Hon, O. J., Neira, S., Stanhope, C. M., Sugam, J. A., Saddoris, M. P., Tipton, G., McElligott, Z., Jhou, T. C., … Kash, T. L. (2019). Central Amygdala Prepronociceptin-Expressing Neurons Mediate Palatable Food Consumption and Reward. *Neuron*, *102*(5), 1037-1052.e7. https://doi.org/10.1016/j.neuron.2019.03.037

Kim, K. S., Park, J. S., Hwang, E., Park, M. J., Shin, H. Y., Lee, Y. H., Kim, K. M., Gautron, L., Godschall, E., Portillo, B., Grose, K., Jung, S.-H., Baek, S. L., Yun, Y. H., Lee, D., Kim, E., Ajwani, J., Yoo, S. H., Güler, A. D., … Choi, H. J. (2024). GLP-1 increases preingestive satiation via hypothalamic circuits in mice and humans. *Science*, *385*(6707), 438–446. https://doi.org/10.1126/science.adj2537

Minère, M., Wilhelms, H., Kuzmanovic, B., Lundh, S., Fusca, D., Claßen, A., Shtiglitz, S., Prilutski, Y., Talpir, I., Tian, L., Kieffer, B., Davis, J., Kloppenburg, P., Tittgemeyer, M., Livneh, Y., & Fenselau, H. (2025). Thalamic opioids from POMC satiety neurons switch on sugar appetite. *Science*, *387*(6735), 750–758. https://doi.org/10.1126/science.adp1510

Salinas, C. B. G., Lu, T. T.-H., Gabery, S., Marstal, K., Alanentalo, T., Mercer, A. J., Cornea, A., Conradsen, K., Hecksher-Sørensen, J., Dahl, A. B., Knudsen, L. B., & Secher, A. (2018). Integrated brain atlas for unbiased mapping of nervous system effects following liraglutide treatment. *Scientific Reports*, *8*(1), 10310. https://doi.org/10.1038/s41598-018-28496-6

Schiff, H. C., Bouhuis, A. L., Yu, K., Penzo, M. A., Li, H., He, M., & Li, B. (2018). An Insula-Central Amygdala Circuit for Guiding Tastant-Reinforced Choice Behavior. *The Journal of Neuroscience*, *38*(6), 1418–1429. https://doi.org/10.1523/JNEUROSCI.1773-17.2017

Stern, S. A., Azevedo, E. P., Pomeranz, L. E., Doerig, K. R., Ivan, V. J., & Friedman, J. M. (2021). Top-down control of conditioned overconsumption is mediated by insular cortex Nos1 neurons. *Cell Metabolism*, *33*(7), 1418-1432.e6. https://doi.org/10.1016/j.cmet.2021.03.001

Trevaskis, J. L., Sacramento, C. B., Jouihan, H., Ali, S., Le Lay, J., Oldham, S., Bhagroo, N., Boland, B. B., Cann, J., Chang, Y., O'Day, T., Howard, V., Reers, C., Winzell, M. S., Smith, D. M., Feigh, M., Barkholt, P., Schreiter, K., Austen, M., … Sharma, A. (2017). Neurturin and a GLP-1 Analogue Act Synergistically to Alleviate Diabetes in Zucker Diabetic Fatty Rats. *Diabetes*, *66*(7), 2007–2018. https://doi.org/10.2337/db16-0916

Yacawych, W. T., Wang, Y., Zhou, G., Hassan, S., Kernodle, S., Sass, F., DeVaux, M., Wu, I., Rupp, A., Tomlinson, A. J., Lin, Z., Secher, A., Raun, K., Pers, T., Seeley, R. J., Myers, M., & Qiu, W. (2025). A single dorsal vagal complex circuit mediates the aversive and anorectic responses to GLP1R agonists. *BioRxiv*.

Zeng, N., Cutts, E. J., Lopez, C. B., Kaur, S., Duran, M., Virkus, S. A., & Hardaway, J. A. (2021). Anatomical and Functional Characterization of Central Amygdala Glucagon-Like Peptide 1 Receptor Expressing Neurons. *Frontiers in Behavioral Neuroscience*, *15*, 724030. https://doi.org/10.3389/fnbeh.2021.724030

Thank you to Reviewer 1 for their thoughtful consideration of our manuscript, and for carefully engaging with Reviewer 2’s comments as well as their own. Their guidance helped us substantially improve the clarity and strength of the manuscript. We are grateful for their time and constructive input throughout the review process.

The manuscript by Godschall et al. dissects the sites of action by which oral GLP1RAs regulate food intake, particularly hedonic feeding. The current orally available agonists are not active at the mouse GLP1 receptor so the studies employ a new mouse line with a humanized Glp1r S33W. They studied these knockin mice as well as animals in which the humanized receptor is introduced into discrete brain regions of GLP1r-cre mice using an AAV with a DIO- Glp1r S33W payload. They then analyzed the effects of the oral agents on a set of behaviors including consumption of chow in the dark phase as well as a highly palatable diet during the light phase, a time when animals consume the HFD but do not consume chow. They report that danuglipron reduces ingestion of a HFD activating inputs from preproglucagon (ie GLP1) expressing neurons in NTS to neurons expressing GLP1R in CEA. Finally, Godschall et al. report that GLP1RAs reduce dopamine responses in the NAc using a fluorescent reporter and (indirectly) link this effect to a projection from CEA to VTA. In aggregate the paper reports the novel and interesting findings that an oral GLPRA can specifically reduce hedonic feeding and this is a result of modulation of the neural targets of GCG neurons in NTS. However, several of the experiments that are reported are either flawed or insufficient and several additional experiments as well as significant revisions would be required for the paper to be suitable for publication.

Major Criticisms:

In Figure 1, the effects of orforglipron were recorded for 7-days. Studies over a longer time frame should be performed

Metabolic profiling was performed in both males and females in ext. Fig. 1), but the studies of the effects of GLP1RA administration in Figure 1 were limited to male mice. The authors need to add data for food intake in female mice.

In Fig. 2, the authors need to show that the neurons activated by the GLP1RAs express the GLP1R. Co-localization experiments between GLP1R and c-FOS labelling are necessary. In addition, the levels of c-FOS in control mice receiving vehicle is lacking. In addition, these images are of poor quality and the data, at least in the printed figures, do not justify the authors conclusion

The authors state that danuglipron preferentially activates NTS but not AP (see lines 147-153) raising the possibility that it will induce less nausea. The authors need to test this directly rather than simply referring to the distribution pattern of activated neurons. Studies using assays of nausea or conditioned taste aversion are necessary.

In Figure 3 the authors monitor the behavioral responses to the oral agents. However, these data are largely superfluous and omit the most relevant behaviors having to do with possible effects on nausea. These data do not bear on the key conclusions of the paper and appear to have been “thrown in”. In addition, the authors fail to observe significant effects on movement while at the same time the mice that received danuglipron or liraglutide are reported to spend significantly more time resting? What is the explanation for this? What are GLP1RA effects on locomotion? What is the total distance traveled in the two groups? Furthermore, could changes in time spent in the shelter and grooming be a result of changes in anxiety as this has been demonstrated before in mouse and human studies? (Kornelius et al., 2024; Zheng et al., 2019)

In Figure 4U, synaptophysin tracing shows that NTS GCG neurons project to amygdala. However, data showing functional connections are lacking. Rabies tracing from CeA Glp1r neurons should be performed to determine whether these neurons receive direct inputs from NTS GCG. Pending this result, the authors need to provide functional evidence showing that activating NTS GCG terminals in CEA alters chow intake as well as hedonic feeding. In addition, the location of these terminals is unclear and the data in Ext. Fig 9 raise the possiblity that the terminals may be elsewhere in amygdala, see below.

In extended Figure 9 C–F, the authors state that eYFP-labeled hGLP1R-expressing neurons are in the CEA . However , the data appear to expression ventral to the CeA in STIA and MeA. More precise

images should be provided with reference to a brain atlas showing precisely where these neurons are located. The based on the current images , it is unlcear whether these effects are specific to CeA vs. other amygdala nuclei.

In the electrophysiology experiments in Ext. Fig. 9**,** the authors do not state what the danuglipron was dissolved in and possible solvent effects on membrane deporarization were not tested. Furthermore, the depolarization that was reported did not induce any actions potentials or affect firing frequency of the neurons. These data are unimpressive.

In Fig. 5 the authors conclude that GLP1R expressing neurons in CEA regulate dopamine release in NAc . The authors need to use chemogenetics or neural ablation to show that inhibiting GLP1R expressing in CEA blunts the effects of GLPRAs to reduce hedonic feeding . It is relevant that GLP1R expressing neurons in NTS also project to NAc (Alhadeff et al., 2012) and that NAc expreses GLP1R and these neurons have also been shown before to regulate feeding (Mietlicki-Baase et al., 2014).

Minor Criticisms:

Figure 1H. The x-axis shows the starting point for GTT measurements a -240 min. This is not comparable to other GTT experiments in Figure 1.

In Figure 1G and U, Blood glucose appears to increase after danuglipron injection prior to the dextrose injection.

In Figure 1Z**,** the authors state that this experiment was performed in already obese mice but they do not state how long the mice had consumed a HFD before the experiment.

In Extended Data Fig. 8, it is not clear which hypothalamic areas the authors are referring to ie which specific areas correspond to the basomedial hypothalamus? The histologic sections seem to indicate that the injections of virus may also have spread into VMH and Arc see Panels C and D. The authors should provide posthoc images of whole slides of the injection areas with quantification of the total cells infected in each targeted brain area? If the virus has spread in the neighbouring areas, their conclusions may be inaccurate.

Re-review of Nature 2024-12-26939B

Godschall et al. investigated the effects of oral GLP-1 receptor agonists (GLP-1RAs) in a humanized mouse model, focusing on food intake, body weight, and behavioral phenotypes. They also add a new component to the brain circuit through which these drugs exert their effects specifically an effect of actions on the amygdala to regulate reward pathways.

The authors have satisfactorily addressed all of my previous comments and in my estimation the paper is suitable for publication pending the authors addressing the following comments which are pertinent to the new experiments that were added.

What follows are some specific comments .

Figure 3 and 4

There is no change in cFOS activity in the DMH after administration of vehicle, danuglipron, liraglutide, or orforglipron (Fig. 3A). This is surprising because in Figure 4, danuglipron significantly suppresses chow intake in mice injected with AAV-DIO-hGLP1R in the DMH. The authors should address the basis for this apparent discrepancy.

Figure 2 and 3

The authors now demonstrate that orforglipron elicits less of a CTA than danuglipron which has a greater effect (Fig. 2). However, orforglipron administration still results in significant cFOS activation in the area postrema (AP) to a similar extent as danuglipron (Fig. 3C). Given that both compounds activate the AP, previously shown by others to be a site at which nausea is induced, the authors should address why danuglipron has a greater effect. Specifically they should determine whether different neuronal subpopulations within the AP are activated by these agents. Alternatively this difference could reflect engagement of different downstream populations that in turn influence the response.

Figure 4

The rationale for focusing on the effects of danuglipronbut and not orforglipron on the central amygdala (CeA) is not clearly explained and should be clarified (Fig. 4).

Extended Data Fig. 11.

The authors note that *Glp1r* is expressed in the Vdr-expressing neuronal cluster within the CeA. However, MERFISH data from the Allen Brain Cell Atlas indicate that *Glp1r* is not limited to the Vdr population in amygdala. The authors should assess which other CeA neuronal subtypes express Glp1r.

In their response to Reviewer 2, the authors indicate that *Glp1r*-expressing neurons in the CeA partially overlap with *Pnoc*-positive neurons. However, previous work by Hardaway et al. (2019) demonstrated that chemogenetic inhibition of *Pnoc*+ neurons there suppresses palatable food

intake. This effect contrasts with the decreased food intake in the current study following optogenetic stimulation of CeA *Glp1r* neurons. The authors should address the basis for these potentially contradictory findings.

Extended Data Fig. 12

The duration of recording in the analysis of membrane potential before and during danuglipron application is not clearly described. In addition, the recordings appear to have been performed in neurons from a single mouse, which is insufficient. Unless this is incorrect, recordings from CeA neurons from at least three animals per group should be included. Furthermore, the authors report an ~5 mV depolarization that is not durable. These data are thus unconvincing and additional experimental support for this response is necessary.

Extended Data Fig. 15

The authors should quantify the number of Glp1r neurons in CEA and the number of Glp1r neurons that are labeled after retrograde tracing from VTA using rabies.