

Brain-wide input-output analysis of tuberal nucleus somatostatin neurons reveals hierarchical circuits for orchestrating feeding behavior

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript by Senol et al, the authors perform a series of experiments to determine the anatomical connectivity of a population of neurons in the tuberal nucleus that are positive for the marker somatostatin. They show a wide range of retrogradely labelled inputs, and axonal projections across the brain. They then carry out input:output mapping using cTRIO, and show some qualitative differences in the input to tuberal neurons that have different afferent projections. This is a nice dataset and the experiments are in general performed well. However, overall I think the advance given by this study – investigating one specific cell type in one brain region at an anatomical level, with only limited insight into the functional consequences of this connectivity, is more suitable for a more specialized journal. In addition, for the data presented, a number of extra control experiments are required, as well as more robust statistics and more in depth methodological detail.

I have outlined some specific comments below.

1. A number of key controls are missing in the rabies experiments, especially for the more complex flp and cre experiments. For the simple tracing experiments in figure 1, the authors need to show results with no G protein expression, and also with no TVA expression to show that rabies labelling is specific and properly pseudotyped. For the cTRIO these controls are very important as this technique is very leaky (see Kohl et al Nature 2018, Extended data figure 5). The authors must show that the same experiments without either cre or flp (and TVA and G protein) result in different distributions of labelling. This is especially important considering the majority of labelling is local (which is most affected by leaky tracing).
2. For the axon tracing experiments, the text and figures refer to 'individual boutons' but how these were quantified is not described. It would be extremely challenging to measure individual boutons across the entire brain, and if this is what the authors are doing there would need to be robust control data and evidence that this was achievable across mice in a quantitative way. If (which I think might be the case) the authors are instead integrating fluorescence in each brain region, then this mitigates this issue somewhat, but the language needs to be amended and the methods need to be much clearer on what was actually done.
3. the means of registration to the allen atlas, and subsequent cell and bouton detection is very opaque – with a simple reference to 'magellanmapper'. There needs to be specific walkthrough of how the images were registered, what settings and what parameters were used, and how cell and bouton classifiers were trained (if they were used). This is because there are multiple options and settings in the megellanmapper code that would make large differences to the data. As an illustration, even after spending a large amount of time on the github for this package it is still unclear to me exactly what is done in this analysis pipeline.
4. For a large dataset with many regions and comparisons, it is essential to have proper statistical measures. Due to the relatively low n (as low as 3 mice), this is presumably not possible (and so hasn't been done). In figure 3 multiple comparisons are made without correction, and in figure 4 when corrections for multiple comparisons are made all but certain accumbens input conditions do not pass the authors significance threshold. It is unclear how to interpret these results is given this.

5. The main comment I have is that this study is quite preliminary, and would really need some more in-depth insight. The authors include a final figure aiming to address this but unfortunately I found it underwhelming. The goal of this figure is to exemplify the idea that the subtle differences in input : output connectivity reflects functional differences in the circuit that underlie behavioral function. However, this figure only investigates one pathway. For example, showing that there is cFos staining in VTA neurons projecting to TN is fine, but it doesn't answer the question the authors are posing. Moreover, there is no comparison - would it not be more appropriate to test all the different combinations of input and output? And with and without HFD or fasting? Second, the authors show that artificially activating one of the projection pathways increases locomotion – but again there is no comparison. Again, an increase in locomotion is fine, but what does this mean in terms of feeding behavior? Do the other pathways have other unique roles? What are they? Consumption? Approach? Grooming? On top of this, this experiment does not inform the question of the connectivity – the authors would need to show that differences in unique input to TN also cause distinct behavioral phenotypes and logically map these on to the consequences of output activation. Unfortunately I do not think this issue can be addressed easily with some quick revision experiments.

Reviewer #2

(Remarks to the Author)

This manuscript by Senol et al. describes the newly developed VITALISTIC, a whole brain imaging and analysis method using tissue clearing and light-sheet imaging. They utilized this technique to determine the input-output circuitry of TNSST neurons, orexigenic neurons found in ref6, and compared them to AgRP neural circuits on a whole-brain basis. This reviewer found the text to be well-written, the experiments to be well-designed, and the majority of the results to be clear and significant. During the reading of the manuscript, however, the reviewer found that some documentation should be more elaborated. cTRIO's experimental design was distinct from the original procedure (ref21). This reviewer advises the following adjustments to the article in order to prepare it for publication.

#1 Line 186-

Here is an analysis of the neural circuits of AgRP provided, however, it would be beneficial to include more information about the experimental system. For example, information on where and which viral tools were introduced is lacking .

#2 Line 204-205

"while the connection between LHA and TNSST neurons survived TTX treatment." Which data supports this result?

#3 Line 206-218

The manuscript compares the inputs of AgRP and TNSST neurons here. However, it could benefit from more organized descriptions. For example, more detailed and comprehensive documentation from data mining on histamine, serotonin, dopamine, and acetylcholine would be beneficial. The authors should also explain why they only focused on dopamine and acetylcholine receptor expression in the ISH experiment. Additionally, it would be more appropriate to compare receptor expressions in AgRP neurons using data obtained from the same experimental system rather than relying on literature.

#4 Line 220-

The cTRIO used in this manuscript is different from the original method (ref21). While this reviewer is convinced that the authors' method may work in principle, discussions regarding the background of the modification and the pros and cons of the modified method should be elaborated to justify their choice.

#5 Line 259-

This part suggests an indirect relationship between the DA nuclei (VTA), TNSST, and PAG, but it does not provide direct evidence for a specific circuit of DA (VTA) -> TNSST -> PAG, as the experiments did not target the limited perturbation of this circuit. So, the conclusions that the authors draw from this part should be cautious and take this limitation in the experimental system into account.

#6

Several references are missing or inappropriately cited. Specifically, Menegas et al. eLife (2015) (<https://doi.org/10.7554/eLife.10032>) should be cited. It appears that the CUBIC methods used are from the protocols of Tainaka et al. Cel Reports (2018) (10.1016/j.celrep.2018.07.056) and Matsumoto et al. Nature Protocols (2019) (<https://doi.org/10.1038/s41596-019-0240-9>).

#7

The starting nuclei targeted by the VITALISTIC analysis should be indicated in the Circos maps in Fig 1d, Fig. 2c, Fig. 3a.

#8

Fig1e

To evaluate the location of the AONs where Chr2 was introduced, it would be helpful to brighten the DAPI image more. The location of the patch image should be specified. In the electrophysiology figures, explanation for PTX/CNQX/APV should be added to the legend.

#9

Fig3c

This graph should include a legend.

#10

Fig4g

This figure is too abstract and contains little information, this reviewer recommends to either make it more detailed or remove it.

#11

Fig5h

Similar to Fig 1e, the location of the ChR2 introduction cannot be evaluated. Please use the brightened DAPI image with indications of the region in the figure.

#12

FigS2

In this figure, the Fig panels and legend do not correspond and should be rearranged.

Reviewer #3

(Remarks to the Author)

Senol and colleagues describe an elegant study mapping input-output of TN SST neurons, which reveals hierarchical circuits for orchestrating feeding behavior. In this work the investigators employed the cutting-edge VITALISTIC method to unbiasedly acquire whole-brain connections of SST neurons and made a very nice and comprehensive analysis and interpretation of the anatomical results. Interestingly, they found that SST neurons receive inputs from and send output to many cortical and subcortical neurons, which they further confirmed using the optogenetics-assist circuitry mapping approach. Thus, this work is not only an anatomical study, but also an in-depth analysis of functional relations and mechanisms. Moreover, the investigators studied projection-specific input tracing of SST neurons and revealed that the input-output configuration matches specific functional need. These results provide an important insight in understanding the roles of SST neurons in the regulation of feeding behavior. Lastly, the investigators revealed the disparity of input between AgRP neurons and SST neurons and found that SST neurons received relative more inputs from extra-hypothalamus and neuromodulatory centers, which is interesting to the reviewer. Because these results suggest that different population of orexigenic neurons in the hypothalamus may receive distinct top-down control and thus play distinct roles in different contexts. Overall, this work is meticulous, and well controlled and mechanistic studies are pursued in depth.

Minors:

1) Line 201-203, what does 'responded to LED' mean? Is it an optogenetic experiment? If yes, which brain area did the author inject into AAV? Please clarify these details to avoid confusion.

2) Thickness of scale bars in figures are not always consistent, e.g., Fig 1b, 4f.

3) The CTB signals in Fig. 5f, are not clear enough. It is better to use a higher quality image.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done an enormous amount of extra work to address the comments.

First, the authors have now included controls for their rabies which is good, and confirms their methods is effective. They have also added more methods for the pipeline in the rebuttal, but cited plagiarism worries for not including it in the main manuscript. I think this should be added in full to the methods – I don't think plagiarism is an issue here – it is important to have the methods of exactly what parameters were used in the current study, and not create a reference history spiral.

However, as far as I can tell (as there is a lot of extra data) the majority of the extra experiments done, while very large in number, in many cases don't actually address the points made in the original review, which is quite confusing.

First, the authors have added in a huge amount of single neuron tracing data - which was seemingly done by another lab for a different project that will be published separately in a different journal. I am not sure if this is appropriate, but will defer to the editors for this. Scientifically, in these experiments the authors show that there is not a clean, parallel output connectivity as previously reported, but that this connectivity can be clustered into 5 clusters based on projections to PAG, pons, raphe, pallidum, septum and pvt. This is very nice (if only discussed extremely briefly!), and consistent with a number of other single neuron tracing studies to come out recently. If included, I would suggest really spelling out what this beautiful data actually show more clearly. As right now its not very clear.

The issue is that after this there is now no internal consistency, as the authors next go on to compare the input to 4 different output projections – to PVN, BST, CeA and PAG. This is internally very inconsistent? The authors need to explain why this was done, and why these 4 were chosen? Does the new single neuron data not make the second half a bit invalid? Based on the single neuron tracing, it would not be that surprising that the correlations between inputs of these arbitrary projections

were similar, as they are likely the same population of neurons? Maybe reworking the tracing data to compare PAG to 'others' might be more beneficial? Or at least explicitly explaining why this disconnect is now apparent.

Second, the response to my comments asking for validation that there might be functional consequences of the different input patterns found is confusing. The reason this was pointed out is because there should be a comparison between different inputs to TN:SST neurons. This is because this is the only way to assign a function to specific connectivity - otherwise you have no idea if your manipulation is specific to the input, or simply activating or inhibiting the overall circuit - for example see Britt et al 2012 Neuron. Similar to the output tracing above, the authors have provided a huge number of extra experiments, but explicitly avoided testing this key concept. Instead they provide evidence via multiple different means that activating 1 input into TN:SST neurons (VTA input) has an effect on 1 TN:SST output (to PAG). They have not done the key test, which is to test that a different input (potentially acting on a different output) does not have the same effect. As the authors point out - they previously found that the projections to e.g. BNST provide a different phenotype (eating). If their hypothesis is correct (and dopamine specifically innervates TN:SST neurons that project to PAG), then activating dopamine input should only affect the TN:SST-PAG pathway and have little effect on the TN:SST-BNST pathway. Additionally, this should also manifest as having no effect on eating, but only locomotion in their behavioural experiments. I feel a bit bad raising this, but it was very explicitly laid out in the original review, and despite a huge amount of experiments, these new data do not address this issue.

Finally, with the new behaviour data, the authors interpret the increased locomotion as increased approaches to food bouts, but at least in the example, the control mouse is sitting next to the food port most of the session - and so overall the effect of the led seems to be to make the animal spend less time overall near the food port. This is not apparent from the authors discussion (and in fact it is hinted that it may even be the opposite). Some quantitative and more detailed behavioural analysis is needed here to understand what is actually going on. This is not helped by the very low n of these experiments. It will also be helped by the point above - showing how the phenotype differs from a different input.

Minor point:

In many places in the text the figures look like they have been lifted directly out of a visualisation program, with labels that are not understandable or legible and with very small fonts - it would be good to make it clear what labels are and what the data actually shows. At present it is extremely hard to understand the logic of the data and what the conclusions are from the figures.

Reviewer #2

(Remarks to the Author)

Concerns raised by this reviewer have been addressed properly.

Reviewer #3

(Remarks to the Author)

All my concerns have been thoroughly addressed, and the revised manuscript has been significantly improved. Therefore, I strongly recommend it for publication.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I thank the authors for their detailed response - and appreciate the time taken to address my comments. The authors have now addressed my comments. I look forward to future work from the lab addressing some of the remaining questions!

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Reviewer #1 (Remarks to the Author):

In this manuscript by Senol et al, the authors perform a series of experiments to determine the anatomical connectivity of a population of neurons in the tuberal nucleus that are positive for the marker somatostatin. They show a wide range of retrogradely labelled inputs, and axonal projections across the brain. They then carry out input:output mapping using cTRIO, and show some qualitative differences in the input to tuberal neurons that have different afferent projections. This is a nice dataset and the experiments are in general performed well. However, overall I think the advance given by this study – investigating one specific cell type in one brain region at an anatomical level, with only limited insight into the functional consequences of this connectivity, is more suitable for a more specialized journal. In addition, for the data presented, a number of extra control experiments are required, as well as more robust statistics and more in depth methodological detail.

We appreciate the critical comments of this reviewer and would like to thank for the comments that motivated us to substantially improve our manuscript by providing additional functional assays. We performed additional experiments showing that stimulating the dopaminergic nerve terminals in TN, similar to stimulating the ^{TN}SST nerve terminals in PAG, promoted food approaching/locomotion. Such effect is blocked by inhibiting PAG-projecting ^{TN}SST neurons (new Figure 6), revealing key functional insights based on our quantitative anatomical mapping. Moreover, we also provided single cell projectome of 186 individually traced tuberal nucleus SST neurons (new Figure 3), which provided unprecedented details of the input-output configuration of this important feeding regulation neurons.

On the other hand, we want to highlight that the value of our manuscript is not simply providing a set of mapping results for a specific cell type in hypothalamus. The importance of our manuscript is to reveal the rich extra-hypothalamic input to an important feeding regulation neuron, and that seemingly functionally similar hypothalamic orexigenic neurons receive very different inputs. The fact that deep hypothalamic orexigenic neurons receive a lot of cortical and other extra-hypothalamic inputs suggests the important, yet still under-appreciated, value of top-down modulation of feeding behavior. I'm sure that some connections revealed in our work may inspire new ideas studying feeding regulation circuit mechanisms. The unbiased anatomical mapping, combining rabies virus tracing and whole-brain mapping techniques, could provide important functional insights guiding experimental design. The value of such quantitative brain-wide mapping is still not well appreciated, nor widely applied, for studying the function of hypothalamic feeding regulation circuits; and our work will set a starting point for more comprehensive understanding of hypothalamic feeding circuits.

I have outlined some specific comments below.

1. A number of key controls are missing in the rabies experiments, especially for the more complex flp and cre experiments. For the simple tracing experiments in figure 1, the authors need to show results with no G protein expression, and also with no TVA expression to show that rabies labelling is specific and properly pseudotyped. For the cTRIO these controls are very important as this technique is very leaky (see Kohl et al Nature 2018, Extended data figure 5). The authors must show that the same experiments without either cre or flp (and TVA and G protein) result in different

distributions of labelling. This is especially important considering the majority of labelling is local (which is most affected by leaky tracing).

We do agree that the original cTRIO method is leaky, as correctly pointed out by the reviewer. As you can tell from the Extended Figure 1 of Schwarz et al. 2015 Nature paper and the Extended Figure 5 of Kohl et al. 2018 Nature paper, the leakiness of cTRIO method is most likely due to non-specific recombination of CAV-FLEX^{loxP}-Flp virus, resulting in flp-expression without the presence of Cre, therefore a lot of leaky expression of TVA and subsequent infection by rabies virus. Without the CAV-FLEX^{loxP}-Flp virus, the leakiness is much restricted (Extended Figure 1b of Schwarz et al., 2015 Nature paper and Extended Figure 1a of Kohl et al. 2018 Nature paper). As suggested by the reviewer, we performed the following control experiments: 1) Inject retrograde AAV-cre in PVH, AAV-DIO-G and AAV-FLEX^{FRT}-TVA in TN of wild type B6 mice, followed by injecting rabies virus in TN; and 2) inject retrograde AAV-cre in PVH, AAV-DIO-G in TN of SST-flp mice without AAV-FLEX^{FRT}-TVA virus, followed by injecting rabies virus in TN. In both cases, we found near absence of leaky infection of rabies virus (Suppl Fig. 7). This set of data suggests improvement of our strategy over original cTRIO method with much enhanced specificity.

2. For the axon tracing experiments, the text and figures refer to ‘individual boutons’ but how these were quantified is not described. It would be extremely challenging to measure individual boutons across the entire brain, and if this is what the authors are doing there would need to be robust control data and evidence that this was achievable across mice in a quantitative way. If (which I think might be the case) the authors are instead integrating fluorescence in each brain region, then this mitigates this issue somewhat, but the language needs to be amended and the methods need to be much clearer on what was actually done.

We apologise for the ambiguous text. In this manuscript, we actually used the number of boutons to make plots. We completely agree with this reviewer that it's extremely challenging to measure individual boutons across the entire brain. We actually manually validated over 50 thousand identified boutons but unfortunately couldn't conclude that the quantification is consistently above 90% accurate across all randomly picked regions. While on the other hand, input mapping by identifying cell bodies is much more robust and accurate. We also tried to use the integrated fluorescent signal in each brain region, but found this to be also very noisy, because different brain regions have different level of background signal. Therefore, despite our huge efforts and time put into imaging, quantifying and validating presynaptic mapping (especially for revision), we decided to remove this part of results. Fortunately, our colleagues at Shanghai Institute of Neuroscience are doing a comprehensive and heroic projectome mapping of hypothalamic peptidergic neurons. We had a discussion and started a collaboration to analyze the single cell projectome of tuberal nucleus somatostatin neurons. Through a heroic effort, they managed to provide single cell projectome of 186 ^{TN}SST neurons, based on analyzing axon length of sparsely labelled ^{TN}SST neurons. This effort provided the most comprehensive output mapping of ^{TN}SST neurons with great detail, and we included this set of results as our new Figure 3 of output mapping (and also new Suppl Fig 5 and 6). The major body of that work was recently accepted at Nature Neuroscience, and the detailed results of ^{TN}SST neurons are exclusively included in our manuscript.

We need to point out that the fMOST based output mapping is based on measuring axon length in each brain region, which does have its limitations. For example, it's unclear whether the axon length is proportional to actual presynaptic bouton number and could over-estimate the 'weight' of output in regions closer to cell body (i.e. within tuberal nucleus or in nearby lateral hypothalamus). Notably, our colleagues spent a huge amount of efforts in their Nature Neuroscience paper to show that actually the axon length correlates well with presynaptic bouton number in most of the brain regions. We therefore also mentioned that axon length 'shows high correlation with synaptic terminal numbers', and will cite the Nature Neuroscience paper if our paper could be published. We also discussed about the potential limitation and caution in interpretation of this output mapping in the manuscript. This part of work consumed a lot of time and we hope that such efforts could convince the reviewer that our work would provide important value for better understanding of ^{TN}SST neurons.

3. the means of registration to the allen atlas, and subsequent cell and bouton detection is very opaque – with a simple reference to 'magellanmapper'. There needs to be specific walkthrough of how the images were registered, what settings and what parameters were used, and how cell and bouton classifiers were trained (if they were used). This is because there are multiple options and settings in the megellanmapper code that would make large differences to the data. As an illustration, even after spending a large amount of time on the github for this package it is still unclear to me exactly what is done in this analysis pipeline.

We are sorry for not making the methods clearer, mainly because the 'magellanmapper' has been published. We indeed changed settings for each brain and manually validated over 500 cells for each brain to make sure that the program can detect over 90% of cells identified by human eyes in randomly picked brain regions.

The details of using this program is copied below from eLife paper:

We detected nuclei in cleared mouse brains using a 3D Laplacian of Gaussian blob detection technique throughout each whole brain. To perform detections in large images several hundred gigabytes (GB) to over a terabyte (TB) in size, we subdivided the image into many smaller chunks to reduce RAM requirements and maximize parallel processing. We loaded images through the Numpy library's memory mapped method (load with the mmap_mode option) to load only the necessary parts of the image on-the-fly, allowing us to load small images a chunk at a time without reading the entire volumetric image into memory. We divided the image shape into overlapping chunks to ensure that nuclei at borders would not be missed, with overlap size of approximately the nucleus diameter. After determining the offset and shape of each chunk, we set the image array as a class attribute, initiated multiprocessing (the multiprocessing.Pool in the standard Python library), and accessed each chunk as a view in a separate process via class methods to avoid duplicating arrays in memory. Thus, we could control total memory usage by the size of chunks and the number of CPU (central processing unit) cores available for separate processes.

3D cell detection poses a number of challenges including adapting to local variation such as staining inhomogeneity, background variation, and autofluorescence, in addition to overlapping cells in dense tissue (Shuvaev et al., 2017). To address these issues, we analyzed images in a local manner by further subdividing each chunk for preprocessing based on its

immediate surroundings. We split each chunk into sub-chunks using the same approach as above but ran each sub-chunk serially within each CPU process. In each sub-chunk, we first clipped intensity values at the 5th and 98.5th percentiles (percentile in Numpy) to remove extreme outliers, rescaled the intensities from 0 to 1, and further saturated signal by clipping the rescaled intensities at the 50th percentile. We next enhanced edges by using unsharp masking with a Gaussian sigma of 8 (filters.gaussian in scikit-image) to identify sharp details as the difference between an image and its blurred version (which we amplified by a factor of 0.3) and adding back those details to the original image. We mildly eroded the resulting signal with an erosion filter using an octahedron structuring element of size 1 (morphology.erosion with morphology.octahedron in scikit-image) to separate out blobs.

To detect blobs, we implemented the 3D Laplacian of Gaussian blob detector from the scikit-image library (feature.blob_log) as a multi-scale interest point operator (Lindeberg, 1993). We set the minimum and maximum sigma based on the microscopy resolution, with 10 intermediate values, detection threshold of 0.1, and overlap fraction threshold of 0.55 below which duplicated blobs are eliminated. Initially we missed many nuclei positioned above one another in the z-direction, likely because the anisotropy necessitated by the relatively thick lightsheet at 5x in our setup limited resolution in the z-direction. To improve detection along the z-axis, we interpolated the images in this direction to near isotropy before detection (transform.resize in scikit-image). The blob detector had a tendency to cluster detections in the bottom and topmost z-planes in each ROI from nuclei visible within the ROI but whose centroids are outside. To avoid this clustering and minimize duplication with adjacent ROIs, we cropped nuclei from these planes on the assumption that they would be captured better in the adjacent, overlapping ROIs.

Overlapping chunks minimized missing nuclei at edges but also necessitated pruning blobs duplicately detected in adjacent chunks. Pruning involves checking for duplicates within all potentially overlapping regions. Since the overlapping portions of the regularly spaced chunks collectively form a grid pattern throughout the full volumetric image, we could limit our search to these grid planes along each axis. After completing detection on the whole image, we first pooled all detected blobs into a single array. Along a given axis of the full image, we determined the boundaries for each overlapping region and all of its blobs. Within each overlapping region, we found all blobs close to another blob by taking the absolute value of the difference between all blobs with one another and finding blobs within a given tolerance in all dimensions. The tolerance was titrated so that the ratio of final blobs in overlapping regions to the next adjacent regions of same volume was about 1:1. For each close pair of blobs found, we replaced both blobs with a new blob that took the mean of their coordinates. To minimize memory usage, we checked smaller groups of blobs against one another until completing all comparisons. We checked overlapping regions simultaneously in multiprocessing along a given axis for efficiency, re-pooled all blobs, and pruned along the next axis to account for blobs that may have been duplicately detected in overlapping chunks along multiple axes, at grid intersections.

Parameters for preprocessing, detections, and pruning steps were optimized through a Grid Search approach, a type of hyperparameter tuning, to check combinations of parameters systematically. To evaluate the accuracy of each set of parameters, two students in our lab generated truth sets of nuclei locations and radii using our serial 2D nuclei annotation tool, taking ROIs of size 42×42×32 pixels (x, y, z) from representative ROIs of all major brain structures (n = 15 forebrain, eight midbrain, and seven hindbrain; n = 2766 nuclei). They

separately generated additional truth sets at a slightly lower magnification (4x), size 60×60×14 pixels (n = 40 ROIs, 1116 nuclei) to increase representation. After detecting nuclei on these images with a given set of parameters, matches between detections and ground truth was determined using the Hungarian algorithm, a combinatorial optimization method to determine optimal assignments between two sets (Kuhn, 1955), as implemented in `optimize.linear_sum_assignment` in Scipy. After scaling nuclei coordinates for isotropy, we found the Euclidean distances between detected and truth nuclei points through `distance.cdist`, which serves as the cost matrix input to `optimize.linear_sum_assignment` to find optimal pairings between points based on closest distance. We took correctly identified detections, or true positives (TP), as pairings within a given tolerance distance. Unpaired detections or those in pairs exceeding this threshold were considered false positives (FP), and the same for ground truth were false negatives (FN). Since a match for a given nucleus within the ROI may lie outside of it and thus go unseen, we first searched for pairings only within an inner sub-ROI, followed by a secondary search for pairings between only unmatched inner sub-ROI objects and the rest of the ROI (Ho et al., 2017). This approach reduced the total number of nuclei available (n = 1118 nuclei) but avoided missed border matches. As measures of performance of our detection compared with ground truth, we used the following standard equations:

(6) Sensitivity(Recall)= $\frac{TP}{TP+FN}$

(7) PositivePredictiveValue(PPV,orPrecision)= $\frac{TP}{TP+FP}$

Image downsampling and registration

To assign nuclei to the proper brain label, we employed automated label propagation by registering the E18.5 atlas to each of our imaged mouse brains using SimpleElastix (Marstal et al., 2016), a toolkit that combines the programmatic access of SimpleITK (Lowekamp et al., 2013) to the Insight Segmentation and Registration Toolkit (ITK) (Yoo et al., 2002) with the Elastix (Klein et al., 2010; Shamonin et al., 2014) image registration framework. Elastix has been recently validated as a computationally efficient and accurate tool for registration of mouse brains cleared by CUBIC (Nazib et al., 2018).

As stitched images are typically several hundreds of GBs per file, image downsampling was necessary to reduce memory utilization during image registration. To reduce file size efficiently in both time and memory usage, we employed the same chunking strategy as used during nuclei detection except with larger, non-overlapping units to resize multiple sections of the image simultaneously. We also reduced memory required for the output array by saving directly to disk with a memory-mapped array (`lib.format.open_memmap` in Numpy). We matched the target final size to the E18.5 atlas, which would be registered to each downsampled image.

Registration involved rigid followed by non-rigid alignment. For rigid registration, we employed a translation (translation parameter map in SimpleElastix) with default settings except increasing to 2048 iterations (MaximumNumberOfIterations setting) followed by an affine (affine parameter map) with 1024 iterations, applied with an `ElastixImageFilter`, to shift, resize, and shear the atlas microscopy image to the same space as that of the sample brain. For non-rigid alignment, we employed a b-spline strategy (bspline parameter map) guided by the `AdvancedNormalizedCorrelation` similarity metric (Nazib et al., 2018; Hammelrath et al., 2016) with grid spacing of size 60, measured in voxels rather than physical units (`FinalGridSpacingInVoxels` setting in place of `FinalGridSpacingInPhysicalUnits`), over 512 iterations. The `TransformixImageFilter` in SimpleElastix allowed us to apply the identical registration transformation to the atlas labels image, except that we set the final b-

spline interpolation order (FinalBSplineInterpolationOrder) to 0 to avoid interpolating any new values, preserving the labels' specific set of integer values. We applied this identical transformation to both the mirrored and edge-refined atlas labels.

To evaluate the level of alignment from registration, we measured the similarity between each registered atlas histology and its corresponding sample image using a Dice Similarity Coefficient (DSC) (Dice, 1945) as implemented by the GetDiceCoefficient function in SimpleElastix/SimpleITK, given by the equation (Tustison and Gee, 2009):

$$(8) \text{ DiceSimilarityCoefficient(DSC)} = 2|S \cap T| / |S| + |T|$$

where S and T are two different sets of voxels. We took the foreground of each atlas and sample microscopy image to be its mean threshold (filters.threshold_mean function in scikit-image) and input them to a LabelOverlapMeasuresImageFilter to take the DSC."

Since it's published, we cited the literature and didn't repeat all these details in our manuscript to avoid plagiarism report.

4. For a large dataset with many regions and comparisons, it is essential to have proper statistical measures. Due to the relatively low n (as low as 3 mice), this is presumably not possible (and so hasn't been done). In figure 3 multiple comparisons are made without correction, and in figure 4 when corrections for multiple comparisons are made all but certain accumbens input conditions do not pass the authors significance threshold. It is unclear how to interpret these results is given this.

We appreciate the reviewer's comments regarding sample size and multi-comparison correction. For better statistics, we performed more tracing experiments and enhanced the power of our analysis. Specifically, for Figure 2 (previously Figure 3), we increased sample size of AgRP group from 3 to 7, and performed multi-comparison (unpaired t test with Welch correction and Holm-Sidak correction) for all plots. We are now more confident to conclude that, among major neuromodulatory inputs, THSST neurons receive more inputs from PPN and VTA (Figure 2c). Likewise, all the statistics in Figure 2b are also corrected for multi-comparison. For major input regions comparison, THSST neurons in general receive more extra-hypothalamic inputs from the ipsilateral side, but AgRP neurons receive much more hypothalamic inputs. Regarding the comparisons in Fig 4e, we want to discuss with the reviewer our rationale of the analysis. We actually first looked for differences in VTA and SN regions because we want to know if dopaminergic inputs have some differential innervation pattern, given its important role in motivation and reward signalling. Therefore, the differences in SN and VTA could be considered hypothesis-driven and thus not applicable for multi-comparison correction. We agree with the reviewer that proper statistics is important and agree that our sample size is small. Our intention is to use such brain-wide mapping system as an exploratory tool for discovering potential interesting differences for future work. For example, if some brain region shows difference, even without more rigorous multi-comparison correction, we can still perform a new set of experiments to specifically look for changes in this brain region (i.e. accumbens) as a post hoc validation. We want to bring it to the reviewer's attention that a rigorous multi-comparison correction for hundreds of comparisons will for sure dramatically reduce Type I error, but with the notable risk of increasing Type II error, which is a topic of discussion among

statisticians. Whole brain mapping is not as easy as, or as high-throughput as, single cell sequencing in which the sample size (number of cells) could be large enough for more stringent statistics. Our intention is therefore to introduce this pipeline as an exploratory tool so that some potential interesting differences could be further validated using a different set of experiments.

5. The main comment I have is that this study is quite preliminary, and would really need some more in-depth insight. The authors include a final figure aiming to address this but unfortunately I found it underwhelming. The goal of this figure is to exemplify the idea that the subtle differences in input : output connectivity reflects functional differences in the circuit that underlie behavioral function. However, this figure only investigates one pathway. For example, showing that there is cFos staining in VTA neurons projecting to TN is fine, but it doesn't answer the question the authors are posing. Moreover, there is no comparison - would it not be more appropriate to test all the different combinations of input and output? And with and without HFD or fasting? Second, the authors show that artificially activating one of the projection pathways increases locomotion – but again there is no comparison. Again, an increase in locomotion is fine, but what does this mean in terms of feeding behavior? Do the other pathways have other unique roles? What are they? Consumption? Approach? Grooming? On top of this, this experiment does not inform the question of the connectivity – the authors would need to show that differences in unique input to TN also cause distinct behavioral phenotypes and logically map these on to the consequences of output activation. Unfortunately I do not think this issue can be addressed easily with some quick revision experiments.

Thanks this reviewer for raising this concern, which is indeed helpful for us to improve the significance of our manuscript. We also feel that revealing functional differences based on quantitative neural circuit mapping will greatly improve the significance of our work. We previously showed that activating ^{TN}SST terminals in PVN and BNST increased food consumption but stimulating terminals in PAG and CeA did not. In this manuscript, we reported that stimulating terminals in PAG, although didn't increase food consumption, increased locomotion. We agree that simply report increase in locomotion is not informative enough. Therefore, we did dive deeper into the VTA-TN-PAG circuit and found that this circuit is specific for food approaching behavior. None of the terminal stimulation caused grooming behavior. Based on our anatomical mapping, we examined whether stimulating the terminals of VTA dopaminergic neurons in TN also enhance locomotion or food approaching behavior. We imported TH-cre mice and bred it with SST-cre mice, for taking advantage of the fact that TN region doesn't have obvious TH-positive neurons and VTA region doesn't have obvious SST-positive neurons. We first expressed ChR2 in VTA dopaminergic neurons, retro AAV in PAG to express mCherry in PAG-projecting ^{TN}SST neurons, and implanted optic fiber in TN for stimulating dopaminergic terminals in TN. Consistent with the effect of stimulating ^{TN}SST terminals in PAG, we found that such optogenetic stimulation of dopaminergic nerve terminals in TN significantly enhanced locomotion (Figure 6e). We further analysed animal behavior by DeepLabCut and found that such enhanced locomotion indeed resulted in more food approaching events, reflected by more back-and-forth traveling between other locations in the chamber and food port (Figure 6g). To confirm that such effect is due to the activation of PAG-projecting ^{TN}SST neurons, we injected retrograde transporting AAV-DIO-GtACR1 in PAG to express GtACR1 in

PAG-projecting ^{TN}SST neurons. Taking advantage of the spectrum of ChR2 and GtACR1, we can use different light for stimulating dopaminergic terminals in TN while inhibiting PAG-projecting ^{TN}SST neurons. Notably, such dual-optogenetic manipulation abolished the effect of stimulating dopaminergic terminals in TN (Figure 6m-p), further supporting the hypothesis that VTA dopaminergic neurons stimulate ^{TN}SST neurons that project to PAG to enhance food approaching behavior. We hope to convince this reviewer that we examined the functional significance by logically manipulating neural circuits according to our quantitative mapping. To further confirm the connection between VTA dopaminergic neurons and PAG-projecting ^{TN}SST neurons, we injected AAV-DIO-ChR2 in VTA and retrograde transporting AAV-DIO-mCherry in PAG, and recorded the activity of red-labelled PAG-projecting ^{TN}SST neurons in response to optogenetic stimulation of dopaminergic terminals. We found that optogenetic stimulation increased the firing frequency of ^{TN}SST neurons (with a delay), consistent with the neuromodulatory role of these dopaminergic input to ^{TN}SST neurons (Fig. 6a-d).

In summary, with much more effort put into revising this manuscript, we did show that differences in anatomical mapping can guide rational design of experiments for confirming the functional specificity of neural circuits.

Reviewer #2 (Remarks to the Author):

This manuscript by Senol et al. describes the newly developed VITALISTIC, a whole brain imaging and analysis method using tissue clearing and light-sheet imaging. They utilized this technique to determine the input-output circuitry of ^{TN}SST neurons, orexigenic neurons found in ref6, and compared them to AgRP neural circuits on a whole-brain basis. This reviewer found the text to be well-written, the experiments to be well-designed, and the majority of the results to be clear and significant. During the reading of the manuscript, however, the reviewer found that some documentation should be more elaborated. cTRIO's experimental design was distinct from the original procedure (ref21). This reviewer advises the following adjustments to the article in order to prepare it for publication.

We appreciate the comments of this reviewer. We want to highlight that we now included additional circuit specific functional experiments (Figure 6) and complete projectome of 186 ^{TN}SST neurons (now Figure 3), which greatly enhanced the manuscript.

#1 Line 186-

Here is an analysis of the neural circuits of AgRP provided, however, it would be beneficial to include more information about the experimental system. For example, information on where and which viral tools were introduced is lacking .

Similar as mapping the inputs to ^{TN}SST neurons, we injected AAV-DIO-TVA-G-mCherry in the arcuate of AgRP-cre mice, followed by injecting deltaG-rabies virus in the arcuate. It's now indicated in the text.

#2 Line 204-205

"while the connection between LHA and TNSST neurons survived TTX treatment."
Which data supports this result?

We apologize for the negligence here. We want to clarify that the TTX-insensitive response was noted for a few cells but not systematically analysed. For avoiding any confusion, we decided to delete this sentence. We also updated the figure legends of Suppl Fig. 2.

#3 Line 206-218

The manuscript compares the inputs of AgRP and TNSST neurons here. However, it could benefit from more organized descriptions. For example, more detailed and comprehensive documentation from data mining on histamine, serotonin, dopamine, and acetylcholine would be beneficial. The authors should also explain why they only focused on dopamine and acetylcholine receptor expression in the ISH experiment. Additionally, it would be more appropriate to compare receptor expressions in AgRP neurons using data obtained from the same experimental system rather than relying on literature.

We agree with this reviewer that a more organized description will benefit the manuscript. We actually performed more input mapping of AgRP neurons and now updated the quantification of Fig. 2c with a more rigorous multi-comparison correction. We noted that only the inputs from PPN and VTA showed a significant higher percentage for ^{TNSST} neurons, after multi-comparison correction. We focused on dopamine and acetylcholine receptor expression because of the more significant higher input numbers from the dopaminergic center VTA and cholinergic center PPN (Fig. 2c). We also agree that comparing receptor expression will be better if using the same experimental system. Therefore, we performed additional FISH for dopaminergic and cholinergic receptors in AgRP neurons (Fig 2e and g), which further supported the idea that AgRP neurons tend to receive less neuromodulator inputs than ^{TNSST} neurons.

#4 Line 220-

The cTRIO used in this manuscript is different from the original method (ref21). While this reviewer is convinced that the authors' method may work in principle, discussions regarding the background of the modification and the pros and cons of the modified method should be elaborated to justify their choice.

We'd like to thank the reviewer for this comment and please refer to our reply to reviewer1's first comment. Briefly, with more control experiments (suppl Fig. 7), we showed that our method is less leaky than cTRIO method that relied on the potentially leaky cre-dependent CAV-flex-Flp virus.

#5 Line 259-

This part suggests an indirect relationship between the DA nuclei (VTA), TNSST, and PAG, but it does not provide direct evidence for a specific circuit of DA (VTA) -> TNSST -> PAG, as the experiments did not target the limited perturbation of this circuit. So, the conclusions that the authors draw from this part should be cautious and take this limitation in the experimental system into account.

Thanks to the reviewer for this comment, which is related with Reviewer1's 5th comment. Briefly, with additional circuit specific manipulation, we showed that optogenetic stimulation of VTA dopaminergic terminals in TN promoted food approaching behavior, and such effect is abolished by optogenetically inhibiting PAG-projecting ^{TN}SST neurons (Fig 6). We also performed additional electrophysiology experiments to show that optogenetic stimulation of dopaminergic terminals resulted in enhanced excitability of ^{TN}SST neurons (Fig. 6).

#6

Several references are missing or inappropriately cited. Specifically, Menegas et al. eLife (2015) (<https://doi.org/10.7554/eLife.10032>) should be cited. It appears that the CUBIC methods used are from the protocols of Tainaka et al. Cel Reports (2018) (10.1016/j.celrep.2018.07.056) and Matsumoto et al. Nature Protocols (2019) (<https://doi.org/10.1038/s41596-019-0240-9>).

Thank this reviewer for the suggestion, and we now included suggested literature.

#7

The starting nuclei targeted by the VITALISTIC analysis should be indicated in the Circos maps in Fig 1d, Fig. 2c, Fig. 3a.

Now indicated with pointers to make it easier to notice.

#8

Fig1e

To evaluate the location of the AONs where ChR2 was introduced, it would be helpful to brighten the DAPI image more. The location of the patch image should be specified. In the electrophysiology figures, explanation for PTX/CNQX/APV should be added to the legend.

Thanks and modified accordingly.

#9

Fig3c

This graph should include a legend.

Thanks and modified accordingly.

#10

Fig4g

This figure is too abstract and contains little information, this reviewer recommends to either make it more detailed or remove it.

Thanks and modified accordingly.

#11

Fig5h

Similar to Fig 1e, the location of the ChR2 introduction cannot be evaluated. Please use the brightened DAPI image with indications of the region in the figure.

Thanks and modified accordingly with a new image showing clear landmark of 3V and edge of hypothalamus.

#12

FigS2

In this figure, the Fig panels and legend do not correspond and should be rearranged.

Thanks and modified accordingly.

Reviewer #3 (Remarks to the Author):

Senol and colleagues describe an elegant study mapping input-output of TN SST neurons, which reveals hierarchical circuits for orchestrating feeding behavior. In this work the investigators employed the cutting-edge VITALISTIC method to unbiasedly acquire whole-brain connections of SST neurons and made a very nice and comprehensive analysis and interpretation of the anatomical results. Interestingly, they found that SST neurons receive inputs from and send output to many cortical and subcortical neurons, which they further confirmed using the optogenetics-assist circuitry mapping approach. Thus, this work is not only an anatomical study, but also an in-depth analysis of functional relations and mechanisms. Moreover, the investigators studied projection-specific input tracing of SST neurons and revealed that the input-output configuration matches specific functional need. These results provide an important insight in understanding the roles of SST neurons in the regulation of feeding behavior. Lastly, the investigators revealed the disparity of input between AgRP neurons and SST neurons and found that SST neurons received relative more inputs from extra-hypothalamus and neuromodulatory centers, which is interesting to the reviewer. Because these results suggest that different population of orexigenic neurons in the hypothalamus may receive distinct top-down control and thus play distinct roles in different contexts. Overall, this work is meticulous, and well controlled and mechanistic studies are pursued in depth.

Thanks this reviewer for the comments.

Minors:

1) Line 201-203, what does 'responded to LED' mean? Is it an optogenetic experiment? If yes, which brain area did the author inject into AAV? Please clarify these details to avoid confusion.

thanks reviewer for pointing this out. We now included more details in Figure S6. We forgot to mention that it's similar as Figure S2a, using AgRP: Ai14 mice to label AgRP neurons, and injected AAV-hSyn-ChR2-EYFP in lateral hypothalamus. The texts are also updated.

2) Thickness of scale bars in figures are not always consistent, e.g., Fig 1b, 4f.

Thanks and modified accordingly.

3) The CTB signals in Fig. 5f, are not clear enough. It is better to use a higher quality image.

Thanks and modified accordingly.

Reviewer #1 (Remarks to the Author):

Reply: I sincerely appreciate the reviewer's critical comments, which have been invaluable in helping us improve our manuscript. I acknowledge that some miscommunication and unintended confusion may have occurred, as rightly pointed out by the reviewer (please see our detailed reply below). With the inclusion of additional results and discussion (text highlighted in green for the second round revision), we hope the reviewer can agree that we have made every effort to address his/her suggestions and enhance the manuscript. We believe the revised version has sufficient merit for consideration for publication in *Nature Communications*.

Once again, I want to express my gratitude to the reviewer for raising critical issues needed to be addressed. Your thoughtful comments, aimed at improving the manuscript, are deeply appreciated.

The authors have done an enormous amount of extra work to address the comments.

First, the authors have now included controls for their rabies which is good, and confirms their methods is effective. They have also added more methods for the pipeline in the rebuttal, but cited plagiarism worries for not including it in the main manuscript. I think this should be added in full to the methods – I don't think plagiarism is an issue here – it is important to have the methods of exactly what parameters were used in the current study, and not create a reference history spiral.

Reply: thanks for confirming our efforts and we have modified methods accordingly.

However, as far as I can tell (as there is a lot of extra data) the majority of the extra experiments done, while very large in number, in many cases don't actually address the points made in the original review, which is quite confusing.

First, the authors have added in a huge amount of single neuron tracing data - which was seemingly done by another lab for a different project that will be published separately in a different journal. I am not sure if this is appropriate, but will defer to the editors for this. Scientifically, in these experiments the authors show that there is not a clean, parallel output connectivity as previously reported, but that this connectivity can be clustered into 5 clusters based on projections to PAG, pons, raphe, pallidum, septum and pvt. This is very nice (if only discussed extremely briefly!), and consistent with a number of other single neuron tracing studies to come out recently. If included, I would suggest really spelling out what this beautiful data actually show more clearly. As right now it's not very clear.

Reply: We have had extensive communication with the last corresponding author of the other manuscript, Dr. Xiaohong Xu, who agreed to be a co-corresponding author of our manuscript. As I said in the previous rebuttal letter, this part of data/analysis

will NOT be published in their other manuscript and single cell projectome of TU was not their initial plan, so I hope to assure this reviewer that there will be no editorial concerns. In our previous report (PMID 29976824), we concluded that 'TN^{SST} neurons showed a one-to-many projection pattern', with relatively few overlap between the ones projecting to PAG and BNST or CeA, which is actually supported by this new single cell projectome result. We did not want to leave the reviewer with a false impression that we reported a 'clean, parallel output connectivity' in our previous paper. I agree that this set of data should be discussed more, so we included more discussion. However, I also want to highlight that each method has its limitations so the data should be interpreted with caution. As we mentioned in the manuscript, this single cell tracing data is based on neurite GFP signal in each brain region, not the amount of synaptic puncta, so the presence of signal in a specific region does not strictly mean synaptic output, even though there is a correlation between the two. We now highlighted the advance brought by such single cell projectome mapping in discussion, as well as mentioned that caution should be taken in interpreting this data.

The issue is that after this there is now no internal consistency, as the authors next go on to compare the input to 4 different output projections – to PVN, BST, CeA and PAG. This is internally very inconsistent? The authors need to explain why this was done, and why these 4 were chosen? Does the new single neuron data not make the second half a bit invalid? Based on the single neuron tracing, it would not be that surprising that the correlations between inputs of these arbitrary projections were similar, as they are likely the same population of neurons? Maybe reworking the tracing data to compare PAG to 'others' might be more beneficial? Or at least explicitly explaining why this disconnect is now apparent.

Reply: We chose these four regions based on our previous report (PMID 29976824), which was mentioned as the reason for conducting subsequent experiments. We have to admit that if we knew the single cell projectome results, we will choose a different set of targets. That said, we do not agree that single neuron data made the second half invalid. First of all, the limited overlap between BNST and PAG-projecting TN^{SST} neurons is supported by the single neuron data; so that at least these two population of neurons are unlikely to be the same population of neurons. When looking at Figure 4e, the biggest difference is usually between PAG and BNST groups. Second, the single neuron projectome results are biased toward posterior part of TN, with relatively less sampling in more anterior part, which may have different projection pattern. We now stated more explicitly why we choose the four targets. We also mentioned the potential bias of the single neuron data, and we sincerely hope that this reviewer can agree that it's not necessary to do more single neuron tracing data to have a more comprehensive covering of the whole TN. We all know that no data is perfect and it's important to honestly point out the limitation, and show our best effort within a certain timeline.

Second, the response to my comments asking for validation that there might be functional consequences of the different input patterns found is confusing. The reason this was pointed out is because there should be a comparison between different inputs to TN:SST neurons. This is because this is the only way to assign a function to specific connectivity - otherwise you have no idea if your manipulation is specific to the input, or simply activating or inhibiting the overall circuit – for example see Britt et al 2012 Neuron. Similar to the output tracing above, the authors have provided a huge number of extra experiments, but explicitly avoided testing this key concept. Instead they provide evidence via multiple different means that activating 1 input into TN:SST neurons (VTA input) has an effect on 1 TN:SST output (to PAG). They have not done the key test, which is to test that a different input (potentially acting on a different output) does not have the same effect. As the authors point out – they previously found that the projections to e.g. BNST provide a different phenotype (eating). If their hypothesis is correct (and dopamine specifically innervates TN:SST neurons that project to PAG), then activating dopamine input should only affect the TN:SST-PAG pathway and have little effect on the TN:SST-BNST pathway. Additionally, this should also manifest as having no effect on eating, but only locomotion in their behavioural experiments. I feel a bit bad raising this, but it was very explicitly laid out in the original review, and despite a huge amount of experiments, these new data do not address this issue.

Reply: I want to confirm with this reviewer that we fully agree with this reviewer's suggestion and did try to test the idea of 'assign a function to specific connectivity'. We apologise that we didn't make it clearer that activating dopaminergic inputs in TN did not change food consumption, which is now included as Suppl Fig8b. We did find that stimulating ^{TN}SST terminals in BNST did not increase locomotion many years ago (although not reported), which is now also included as Suppl Fig8a. I think we explicitly showed that activating VTA-TN-PAG pathway enhanced locomotion without changing food consumption. On the other hand, we did try to find a specific input for stimulating TN-BNST pathway, but had no luck. First of all, we didn't see a clear potential excitatory input preferentially targeting BNST-projecting ^{TN}SST neurons. Second, as we showed before (PMID 34168339), stimulating a single excitatory input to ^{TN}SST neurons seemed to have no effect on food consumption. We also tried stimulating other inputs, such as AON, but also found no change in food consumption (not shown in current manuscript). Based on current results, it seems that multiple excitatory inputs need to be simultaneously active to provide sufficient drive for food consumption (and may need concurrent neuromodulatory inputs for amplifying such drive), and we're testing this hypothesis in a different project. Although we tried (unsuccessfully) to find a specific input for showing specific food consumption output (TN-BNST), we have to say that nature is sometimes more complicated than we would have expected and no model is perfect. I hope this reviewer can agree that we addressed at least one hypothesis, which is that VTA-TN-PAG circuit promotes locomotion without impacting food consumption.

Finally, with the new behaviour data, the authors interpret the increased locomotion as increased approaches to food bouts, but at least in the example, the control mouse is sitting next to the food port most of the session – and so overall the effect of the led seems to be to make the animal spend less time overall near the food port. This is not apparent from the authors discussion (and in fact it is hinted that it may even be the opposite). Some quantitative and more detailed behavioural analysis is needed here to understand what is actually going on. This is not helped by the very low n of these experiments. It will also be helped by the point above – showing how the phenotype differs from a different input.

Reply: thanks the reviewer for raising this concern. I think this is a very good point and we didn't pay attention to. We went through the results and found that it just happened to be the mice that liked to stay near the food port. Different mice have different preferences. We now included another pair of mice which liked to stay in another corner. Regarding the activity of TN-PAG pathway, I think it needs to be precisely controlled for enabling food approaching behavior and needs to be inhibited when animals actually start to consume food. We indeed previously showed that the bulk activity of ^{TN}SST neurons ramps up when approaching food but starts to decrease when consuming food. How exactly the activity of dopaminergic terminals in TN region coordinate with other excitatory inputs together to fine-tune the activity of ^{TN}SST neurons will be a very interesting project that we hope to explore in greater detail using in vivo imaging and in vivo recording techniques. I hope the reviewer can agree that further investigation is beyond the scope of this manuscript.

Minor point:

In many places in the text the figures look like they have been lifted directly out of a visualisation program, with labels that are not understandable or legible and with very small fonts – it would be good to make it clear what labels are and what the data actually shows. At present it is extremely hard to understand the logic of the data and what the conclusions are from the figures.

Reply: We feel sorry for these mistakes and now tried to make the fonts bigger in some plots (i.e. Fig 1g, Fig 3e). I'm not sure if the reviewer wanted us to make the fonts in Fig 1d, 2a and 3c bigger; I'm afraid it's quite challenging given the huge amount of texts. These texts are for all the brain regions analysed. We now made it clearer in figure legend. If the reviewer has further confusion regarding any other figure/plots, we'll be happy to further modify.

Reviewer #1 (Remarks to the Author):

Reply: I sincerely appreciate the reviewer's critical comments, which have been invaluable in helping us improve our manuscript. I acknowledge that some miscommunication and unintended confusion may have occurred, as rightly pointed out by the reviewer (please see our detailed reply below). With the inclusion of additional results and discussion (text highlighted in green for the second round revision), we hope the reviewer can agree that we have made every effort to address his/her suggestions and enhance the manuscript. We believe the revised version has sufficient merit for consideration for publication in *Nature Communications*.

Once again, I want to express my gratitude to the reviewer for raising critical issues needed to be addressed. Your thoughtful comments, aimed at improving the manuscript, are deeply appreciated.

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First, the authors have now included controls for their rabies which is good, and confirms their methods is effective. They have also added more methods for the pipeline in the rebuttal, but cited plagiarism worries for not including it in the main manuscript. I think this should be added in full to the methods – I don't think plagiarism is an issue here – it is important to have the methods of exactly what parameters were used in the current study, and not create a reference history spiral.

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Reply: We have had extensive communication with the last corresponding author of the other manuscript, Dr. Xiaohong Xu, who agreed to be a co-corresponding author of our manuscript. As I said in the previous rebuttal letter, their other manuscript

focused on AgRP, POMC, orexin and other hypothalamic neurons, but not SST neurons. Indeed, the whole manuscript does not have a single place mentioning 'tuberal nucleus' and there is no duplicated report of the same data, so I hope to assure this reviewer that there will be no editorial concerns. We also sent the editor their accepted manuscript for them to evaluate.

In our previous report (PMID 29976824), we concluded that 'TN SST neurons showed a one-to-many projection pattern', with relatively few overlap between the ones projecting to PAG and BNST or CeA, which is actually supported by this new single cell projectome result. We did not want to leave the reviewer with a false impression that we reported a 'clean, parallel output connectivity' in our previous paper. I agree that this set of data should be discussed more, so we included more discussion. However, I also want to highlight that each method has its limitations so the data should be interpreted with caution. As we mentioned in the manuscript, this single cell tracing data is based on neurite GFP signal in each brain region, not the amount of synaptic puncta, so the presence of signal in a specific region does not strictly mean synaptic output, even though there is a correlation between the two. We now highlighted the advance brought by such single cell projectome mapping in discussion, as well as mentioned that caution should be taken in interpreting this data.

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Reply: We chose these four regions based on our previous report (PMID 29976824), which was mentioned as the reason for conducting subsequent experiments. We have to admit that if we knew the single cell projectome results, we will choose a different set of targets. That said, we do not agree that single neuron data made the second half invalid. First of all, the limited overlap between BNST and PAG-projecting ^{TN}SST neurons is supported by the single neuron data; so that at least these two population of neurons are unlikely to be the same population of neurons. When looking at Figure 4e, the biggest difference is usually between PAG and BNST groups. Second, the single neuron projectome results are biased toward middle part of TN, with relatively less sampling in more anterior and posterior parts, which may have different projection pattern. We now stated more explicitly why we choose the four targets. We also mentioned the potential bias of the single neuron data, and we sincerely hope that this reviewer can agree that it's not necessary to do more single

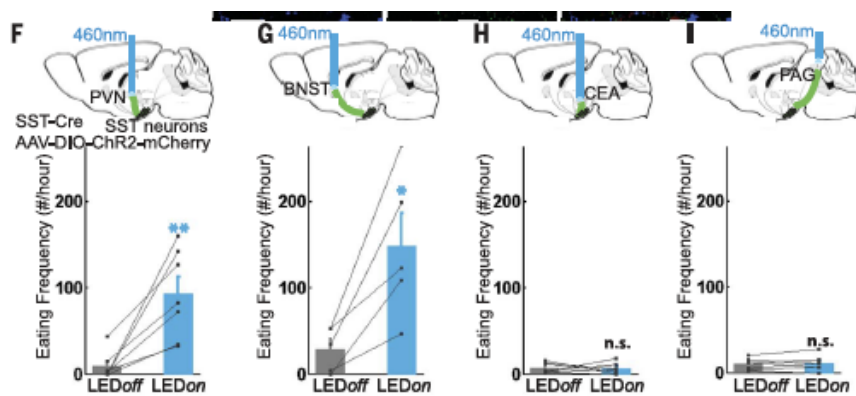
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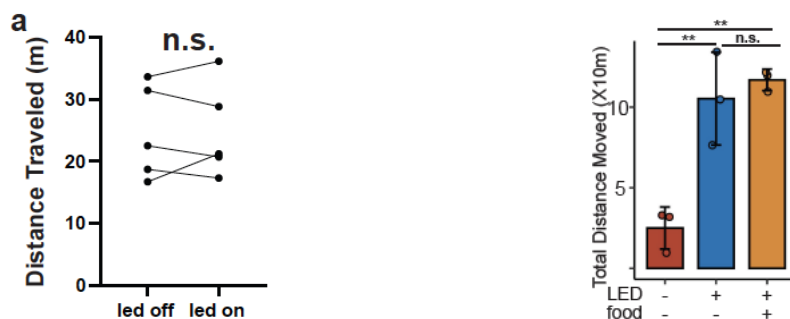
Reply: I want to confirm with this reviewer that we fully agree with this reviewer's suggestion and did try to test the idea of 'assign a function to specific connectivity'. We apologise that we didn't make it clearer that activating dopaminergic inputs in TN did not change food consumption, which is now included as Suppl Fig8b. We did find that stimulating ^{TN}SST terminals in BNST did not increase locomotion many years ago (although not reported), which is now also included as Suppl Fig8a. I think we explicitly showed that activating VTA-TN-PAG pathway enhanced locomotion without changing food consumption.

To make the comparison easier, I included the key findings in this rebuttal letter to show that activating BNST output enhanced eating without impacting locomotion, while activating PAG output enhanced locomotion without impacting eating. We also showed that activating dopaminergic input enhanced locomotion without impacting eating.

This figure is copied from our previous publication (Luo et al., Science, 2018, 361, 76-81) Figure 3 to show that activating output in BNST promoted food consumption but stimulating output in PAG did not:



We further showed in this manuscript (Suppl Figure 8a, below left) that activating BNST output did not enhance locomotion (analysis of previous unpublished data), but activating PAG output did (Figure 5i, below right)



On the other hand, we did try to find a specific input for stimulating TN-BNST pathway, but had no luck. First of all, we didn't see a clear potential excitatory input preferentially targeting BNST-projecting ^{TN}SST neurons. Second, as we showed before (PMID 34168339), stimulating a single excitatory input to ^{TN}SST neurons seemed to have no effect on food consumption. We also tried stimulating other inputs, such as AON, but also found no change in food consumption (not shown in current manuscript). Based on current results, it seems that multiple excitatory inputs need to be simultaneously active to provide sufficient drive for food consumption (and may need concurrent neuromodulatory inputs for amplifying such drive), and we're testing this hypothesis in a different project. Although we tried (unsuccessfully) to find a specific input for specific food consumption output (TN-BNST), we have to say that nature is sometimes more complicated than we would have expected and no model is perfect. I hope this reviewer can agree that we addressed at least one hypothesis, which is that VTA-TN-PAG circuit promotes locomotion without impacting food consumption.

Finally, with the new behaviour data, the authors interpret the increased locomotion as increased approaches to food bouts, but at least in the example, the control mouse is sitting next to the food port most of the session – and so overall the effect of the led seems to be to make the animal spend less time overall near the food port. This is not apparent from the authors discussion (and in fact it is hinted that it may

even be the opposite). Some quantitative and more detailed behavioural analysis is needed here to understand what is actually going on. This is not helped by the very low n of these experiments. It will also be helped by the point above – showing how the phenotype differs from a different input.

Reply: thanks the reviewer for raising this concern. I think this is a very good point and we didn't pay attention to. We went through the results and found that it just happened to be the mice that liked to stay near the food port. Different mice have different preferences. We now included another pair of mice which liked to stay in another corner. We also performed a new set of experiments to further enhance the n number. How exactly the activity of dopaminergic terminals in TN region coordinate with other excitatory inputs together to fine-tune the activity of ^{TN}SST neurons will be a very interesting project that we hope to explore in greater detail using in vivo imaging and in vivo recording techniques.

Minor point:

In many places in the text the figures look like they have been lifted directly out of a visualisation program, with labels that are not understandable or legible and with very small fonts – it would be good to make it clear what labels are and what the data actually shows. At present it is extremely hard to understand the logic of the data and what the conclusions are from the figures.

Reply: We feel sorry for these mistakes and now tried to make the fonts bigger in some plots (i.e. Fig 1g, Fig 3e). I'm not sure if the reviewer wanted us to make the fonts in Fig 1d, 2a and 3c bigger; I'm afraid it's quite challenging given the huge amount of texts. These texts are for all the brain regions analysed. We now made it clearer in figure legend. If the reviewer has further confusion regarding any other figure/plots, we'll be happy to further modify.