nature portfolio

Peer Review File

Ascending and descending motor pathways converge in the centrolateral nucleus of the thalamus

Corresponding Author: Dr Martha Bickford

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 the manuscript by Naeem et al., the authors set out to characterize motor cortex (M1) and superior colliculus (SC) inputs to the centrolateral (CL) nucleus of the thalamus, especially characterizing the role of Pitx2 expressing neurons by virtue of Pitx2-Cre mice. They utilize viral tracing to show overlapping inputs from M1 and SC in CL, conventional BDA tracing with electron microscopy to characterize ultrastructural qualities of tectothalamic and corticothalamic synapses in CL, and finally employed optogenetic interrogation of these synaptic inputs in ex vivo slice recordings of CL with subsequent labeling of the recorded cells to find that nearly a third of CL cells recorded integrate inputs from M1 and SC, with populations that respond to each input alone. The authors frame this work in the context of corollary discharge, a form of motor efferents copy and discuss its relevance to motor planning and execution as CL integrates inputs from basal ganglia output, SC and motor cortex which are then relayed to striatum and back to motor cortex.

The work is technically sound. The methods are clear and carefully done. I particularly appreciated the dual opsin integration strategy employing the "occlusion protocol" as a control. The exclusion of GABAergic synapses in CL also demonstrates a deep understanding of the technical challenges of BDA tracer injections (e.g. retrograde transport to SNr, which then projects to CL). The number of animals and number of cells in all experiments is sufficient. The statistics seem appropriate in terms of reporting significance, though I would ask the authors to comment more on the relevance of the magnitude of differences between groups, not just whether they are significant or not (many of the differences found to be significant seem trivial in terms of the magnitude of difference).

My main concern with the manuscript is the novelty of the findings. While the paper provides rigorous details about the anatomical connections at the ultrastructural level, these connections have been known for decades so the novel contribution here seems modest. The ultrastructural data seems novel (though should check with some Yoland Smith work), but is not very informative about elucidating a differential role of M1 vs. SC inputs, though some minor findings are claimed. Likewise with the ephys data, though I see value in having this publication out there to be cited and say "yes, single cells in CL definitively integrate M1 and SC inputs", though there was little doubt of this.

I was also concerned with the references, and hoped to see some more modern citations on the CL nucleus, which could provide more context about its functional and behavioral relevance, especially in regard to its projections to striatum. Work by Brian Mathur, Yuri Saalman, Peter Magill and Yoland Smith to name a few.

Reviewer #2

(Remarks to the Author)
The manuscript presents a robust set of ex vivo experiments exploring functional convergence of ascending and descending motor commands in CL thalamus, utilising detailed anatomical characterisation of synaptic boutons with electron micrographs combined with cutting-edge dual colour optogenetics. However, there are some instances in which the claims made are not fully supported by the data, with a primary concern being discussion of concepts in the abstract that are present in the wider field, but are not explicitly addressed experimentally here, giving a potentially wrong impression about

the overall conclusions of the study.

Points for Authors' Consideration:

1. Corollary discharge is not explicitly investigated
The first four words in this manuscript are "Corollary discharge (CD) signals…", setting the tone for the rest of the manuscript's findings. While the investigation does indeed investigate a circuit ex vivo previously associated with CD in primates, there is no explicit in vivo experiment linking the authors' findings to CD in mice. Its emphasis in the abstract may give the impression the authors have directly investigated CD, which is not the case, and therefore may be a topic better suited for Discussion.

2. No explicit evidence that integrated signals in CL are sent to striatum
The abstract also asserts the striatum receives input from CL thalamus cells that receives integrated input from motor cortex (MCtx) and superior colliculus (SC). This is not supported by the data, as the evidence provided are histological images of projections in striatum from CL cells that receive SC input, not both SC and MCtx input. While it is very likely the convergent cells also send input to striatum, this has not been shown here, so should not be claimed in the abstract.

3. No experiment to suggest CL detects relative timing of cortical and subcortical motor commands
Abstract suggests the CL circuit may detect the relative timing cortical and subcortical movement commands, however there was again no experiment carried out to backup this claim. Figure 6 details functional convergence of MCtx and SC in CL at a single neuron level with dual colour optogenetics, but there was no variation in the timing of stimulation onset for either input.

4. Assignment of histological images to a common coordinate framework
Figure 1 would benefit from a robust assignment of the common coordinate framework (CCFv3) atlas on to histological slices. An atlas registration with software such as Aligning Big Brains and Atlases (ABBA) would give more confidence in the assignment of specific thalamic nuclei. This is also true for all figures in which patched cells are seemingly manually drawn onto a sketch of the CL (Figure 3, 4 and 6).

5. Pitx2::ChR2 in Figure – terminal stimulation may not be SC specific
Pitx2 expression is not exclusive to SC, so the axons stimulated in the Pitx2::ChR2 line may originate from other areas (MRN, Pons or hypothalamus). Authors could do a retrograde tracing experiment from CL to validate that most/all Pitx2 synaptic inputs in CL are from SC.

6. Cortical and subcortical synapses converge on a subset of CL neurons
Only a minority (28%) of CL cells responded to input from both SC and MCtx, which may require a rephrase throughout the manuscript from "convergence in CL single neurons" to "convergence onto a subset of CL single neurons".

7. No mention of Pitx2 cells in Figure 6
Much of the paper emphasises the importance of SCPitx2ON input to the CL; their synaptic boutons in EM micrographs are significantly larger than MCtx and other SC neurons (Fig. 2), and functionally they evoke a larger response amplitude per pixel density (Fig. 5). However, the actual experiment that interrogates convergence does not make use of a Pitx2-cre line; functional convergence in CL was only shown between SC and MCtx, not SCPitx2ON and MCtx. If SCPitx2ON cells are not also shown to converge functionally in CL with MCtx, why is their importance over other SC projections emphasised in figures prior?

8. Validation of anterograde transsynaptic tracing
Details about the transsynaptic anterograde tracing experiment would benefit from further clarification. It would be helpful to include information on viral titres and injection volumes, as well as a more thorough quantification of tracing efficiency, for example the number of labelled cells in CL. One possible approach could be to combine the anterograde transsynaptic Cre virus with a nuclear fluorescent Cre-dependent reporter in the CL to provide a more accurate estimate.

9. Description of Pitx2 targets
The Pitx2 projection patterns shown in Figure 1 appear consistent with those previously reported in Masullo et al., 2019. Unless the current work reveals substantially different targets, it may be sufficient to reference the earlier findings and frame the present data as a valuable confirmation and extension of that established projection map.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all of my concerns. I am particularly in agreement with the other reviewer that the authors do not explicitly investigate corollary discharge here, but rather look at a circuit known to be involved in corollary discharge, which they are now anatomically and physiologically characterizing. It seems they have made quite a few edits based on the other Reviewer's excellent points, and I believe the manuscript is suitable for publication.

Reviewer #2

(Remarks to the Author)
We feel that the authors have addressed all our points thoroughly. We congratulate them on their work and wish them the very best with their future efforts.

We thank the reviewers and editor for their insightful comments which have improved the presentation of our results. Below we address each point in red font.

1. Clarify why this study sheds a new light on M1 vs. SC inputs to the CL and relate this to previous publication (referee #1 refers to Yoland Smiths's work).

Please see our responses to Reviewer 1 regarding these points.

2. Provide an updated discussion that considers previous studies on projections to the striatum that are missing from the references. Please refer to the list of authors mentioned by referee #1.

Please see our response to Reviewer 1 regarding this point.

3. Clarify which concept mentioned in the abstract is or is not experimentally addressed in the study. For more details, see points 1-9 raised by referee #2. In some cases, additional experiments may be required (anterograde tracing – point 8).

Please see our responses to Reviewer 2 regarding these points.

For all graphs depicting a single point value (e.g., mean) with error bars, you must add individual data points or convert the graph to a boxplot or dot-plot to show data distribution.

This is the format of our graphs.

It's mandatory to provide access to the numerical source data for graphs and charts either through a repository or by providing the data in a Supplementary Data file (in excel format).

We have deposited excel files in Mendeley Data.

All blots/gels must be accompanied by size markers in every figure panel. Uncropped and unedited blot/gel images must be included as Supplementary Figure(s) in the Supplementary Information pdf.

NA

Please ensure that you have complied with the data deposition policies at the Nature Portfolio, please see here.

Confirmed

Please ensure that you have complied with our policies on research involving animals and humans, see here

Confirmed.

Please follow the ARRIVE guidelines for reporting animal experiments. Please fully complete an ARRIVE checklist including both the essential and recommended set of items (adding information to the manuscript where needed) and upload this with your revised manuscript.

Checklist completed.

Please also see our revision checklist for guidance on formatting the manuscript and complying with our policies. A comprehensive guide to our formatting requirements for final submissions is also available for your reference here.

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Please use the following link to submit your revised manuscript, point-by-point response to the referees' comments (which should be in a separate document to the cover letter) and any additional files:

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and superior colliculus (SC) inputs to the centrolateral (CL) nucleus of the thalamus, especially characterizing the role of Pitx2 expressing neurons by virtue of Pitx2-Cre mice. They utilize viral tracing to show overlapping inputs from M1 and SC in CL, conventional BDA tracing with electron microscopy to characterize ultrastructural qualities of tectothalamic and corticothalamic synapses in CL, and finally employed optogenetic interrogation of these synaptic inputs in ex vivo slice recordings of CL with subsequent labeling of the recorded cells to find that nearly a third of CL cells recorded integrate inputs from M1 and SC, with populations that respond to each input alone. The authors frame this work in the context of corollary discharge, a form of motor efferents copy and discuss its relevance to motor planning and execution as CL integrates inputs from basal ganglia output, SC and motor cortex which are then relayed to striatum and back to motor cortex.

The work is technically sound. The methods are clear and carefully done. I particularly appreciated the dual opsin integration strategy employing the "occlusion protocol" as a control. The exclusion of GABAergic synapses in CL also demonstrates a deep understanding of the technical challenges of BDA tracer injections (e.g. retrograde transport to SNr, which then projects to CL). The number of animals and number of cells in all experiments is sufficient. The statistics seem appropriate in terms of reporting significance, though I would ask the authors to comment more on the relevance of the magnitude of differences between groups, not just whether they are significant or not (many of the differences found to be significant seem trivial in terms of the magnitude of difference).
We thank Reviewer 1 for these positive comments regarding the rigor of our experimental techniques.

My main concern with the manuscript is the novelty of the findings. While the paper provides rigorous details about the anatomical connections at the ultrastructural level, these connections have been known for decades so the novel contribution here seems modest. The ultrastructural data seems novel (though should check with some Yoland Smith work), but is not very informative about elucidating a differential role of M1 vs. SC inputs, though some minor findings are claimed. Likewise with the ephys data, though I see value in having this publication out there to be cited and say "yes, single cells in CL definitively integrate M1 and SC inputs", though there was little doubt of this.

While the circuits studied may have been assumed based on previous studies, our studies provided several new results as described below.

1) **Ultrastructure of CL terminals that originate from motor cortex layer 5 neurons labeled using cre-dependent Apex2 expression in the RBP4-cre line.** Although

previous studies have speculated that large boutons in the CL nucleus originate from layer 5 cortical neurons, our study provides definitive evidence using viral tracing techniques.

2) **Ultrastructure of CL terminals that originate from superior colliculus premotor neurons labeled using cre-dependent Apex2 expression in the Pitx2-cre line.** Although previous anterograde tracing and degeneration studies examined superior colliculus projections to the centrolateral/mediodorsal thalamus, our study shows that the premotor neurons labeled in the Pitx2-cre line provide very large terminals that exceed the size of terminals originating from layer 5 of the motor cortex. We also compare this labeling to traditional BDA tracing from the superior colliculus, which as the reviewer appreciates is less specific.
3) **Synaptic properties of CL terminals that originate from superior colliculus premotor (Pitx2) neurons.** Our study provides the first description of the synaptic properties of SC terminals in the CL that originate from Pitx2 neurons.
4) **Synaptic properties of CL terminals that originate from layer 5 motor cortex neurons.** Our study provides the first description of the synaptic properties of layer 5 terminals in the CL that originate from the motor cortex.
5) **Convergent superior colliculus and layer 5 motor cortex innervation of single CL neurons using dual opsin optogenetics.** While superior colliculus and motor cortex terminals overlap in the CL neurons and may be assumed to innervate single neurons, it has also been suggested that terminals that originate from layer 5 cortical neurons provide a single source of "driver" inputs to individual thalamic neurons. We therefore tested whether 2 sources of "driver-like" inputs innervate single neurons.
6) **Tectorecipient CL neurons innervate the motor cortex and striatum.** While not a major part of the study, using transsynaptic labeling techniques we were able to specifically label the projections of CL neurons that receive input from the superior colliculus. This adds to the elegant studies of Yoland Smith examining corticostriatal and thalamostriatal circuits, addressed in more detail below.
7) **Detailed investigation of CL neuron morphology.** While previous studies have provided limited descriptions of CL neuron morphology, we provide a detailed analysis of CL dendritic arbors.

I was also concerned with the references and hoped to see some more modern citations on the CL nucleus, which could provide more context about its functional and behavioral relevance, especially in regard to its projections to striatum. Work by Brian Mathur, Yuri Saalman, Peter Magill and Yoland Smith to name a few.

We thank Reviewer 1 for the suggestion to discuss thalamostriatal circuits of the CL nucleus in more detail, adding recent citations. We have attempted to incorporate our

findings which focus on loops that include the superior colliculus into prevailing views. See intro lines 146-147 and discussion section lines 815-828.

The manuscript presents a robust set of ex vivo experiments exploring functional convergence of ascending and descending motor commands in CL thalamus, utilising detailed anatomical characterisation of synaptic boutons with electron micrographs combined with cutting-edge dual colour optogenetics. However, there are some instances in which the claims made are not fully supported by the data, with a primary concern being discussion of concepts in the abstract that are present in the wider field, but are not explicitly addressed experimentally here, giving a potentially wrong impression about the overall conclusions of the study.

Points for Authors' Consideration:

1. Corollary discharge is not explicitly investigated
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We have edited the abstract extensively (lines 40-50) to remove reference to corollary discharge (which was merely the inspiration for the study).

2. No explicit evidence that integrated signals in CL are sent to striatum
The abstract also asserts the striatum receives input from CL thalamus cells that receives integrated input from motor cortex (MCtx) and superior colliculus (SC). This is not supported by the data, as the evidence provided are histological images of projections in striatum from CL cells that receive SC input, not both SC and MCtx input. While it is very likely the convergent cells also send input to striatum, this has not been shown here, so should not be claimed in the abstract.

This is a good point, and we have edited that abstract appropriately.

3. No experiment to suggest CL detects relative timing of cortical and subcortical motor commands
Abstract suggests the CL circuit may detect the relative timing cortical and subcortical movement commands, however there was again no experiment carried out to backup this claim. Figure 6 details functional convergence of MCtx and SC in CL at a single neuron level with dual colour optogenetics, but there was no variation in the timing of stimulation onset for either input.

It is true that this was merely a suggestion and we have edited the abstract appropriately. The occlusion procedure can be used to detect convergence of two independently excited inputs onto single neurons but does not allow examination of the relative timing of their activation on neuron output.

4. Assignment of histological images to a common coordinate framework
Figure 1 would benefit from a robust assignment of the common coordinate framework (CCFv3) atlas on to histological slices. An atlas registration with software such as Aligning Big Brains and Atlases (ABBA) would give more confidence in the assignment of specific thalamic nuclei. This is also true for all figures in which patched cells are seemingly manually drawn onto a sketch of the CL (Figure 3, 4 and 6).

We have added a new supplementary figure (current **Figure S1**) which aligns the location of SC and motor cortex projections, recorded neurons, and tectorecipient neurons (labeled via transsynaptic tracing) with the Paxinos mouse atlas.

5. Pitx2::ChR2 in Figure – terminal stimulation may not be SC specific
Pitx2 expression is not exclusive to SC, so the axons stimulated in the Pitx2::ChR2 line may originate from other areas (MRN, Pons or hypothalamus). Authors could do a retrograde tracing experiment from CL to validate that most/all Pitx2 synaptic inputs in CL are from SC.

It is true that we used Pitx2-Ai32 mice for some of the experiments (n=18 responsive cells) but we also used SC injections in Pitx2-Cre mice (n= 36 responsive cells) to examine this projection. We have revised **Figure 5** to separate responses elicited in Pitx2-Ai32 versus virus injected animals (Pitx2-SC). See lines 598-723 of the results section.

6. Cortical and subcortical synapses converge on a subset of CL neurons
Only a minority (28%) of CL cells responded to input from both SC and MCtx, which may require a rephrase throughout the manuscript from “convergence in CL single neurons” to “convergence onto a subset of CL single neurons”.

We edited line 46 of the abstract and line 890 of the discussion.

7. No mention of Pitx2 cells in Figure 6
Much of the paper emphasises the importance of SCPitx2ON input to the CL; their synaptic boutons in EM micrographs are significantly larger than MCtx and other SC neurons (Fig. 2), and functionally they evoke a larger response amplitude per pixel density (Fig. 5). However, the actual experiment that interrogates convergence does not make use of a Pitx2-cre line; functional convergence in CL was only shown between SC and MCtx, not SCPitx2ON and MCtx. If SCPitx2ON cells are not also shown to converge functionally in CL with MCtx, why is their importance over other SC projections emphasised in figures prior?

We wanted to specifically examine the projections and synaptic properties of projections from premotor neurons of the SC to the CL. For the convergence studies, we wanted to specifically test the convergence with terminals that arise from layer 5 motor cortex neurons and therefore used the RPB4-cre line.

8. Validation of anterograde transsynaptic tracing
Details about the transsynaptic anterograde tracing experiment would benefit from further clarification. It would be helpful to include information on viral titres and injection volumes, as well as a more thorough quantification of tracing efficiency, for example the number of labelled cells in CL. One possible approach could be to combine the anterograde transsynaptic Cre virus with a nuclear fluorescent Cre-dependent reporter in the CL to provide a more accurate estimate.

We provided virus titers and volumes in the methods section. Since the Cre-dependent GFP injections in the CL labeled somata sufficiently, we have now quantified the number of cells labeled in the CL (see lines 444-446 of the results section), adding an image of labeled cells in a single optical section (**Figure S3C)** as well as their projections to the thalamic reticular nucleus (**Figure S3D**), and a plot of the counted cells (**Figure S1D)**.

9. Description of Pitx2 targets
The Pitx2 projection patterns shown in Figure 1 appear consistent with those previously reported in Masullo et al., 2019. Unless the current work reveals substantially different targets, it may be sufficient to reference the earlier findings and frame the present data as a valuable confirmation and extension of that established projection map.

We now emphasize that the Pitx2 tracing confirms that of Masullo et al., 2019. See lines 418-419 of the results.