

Xue Han, Ph.D.

Peter Paul Career Development Professor

Associate Professor, Biomedical Engineering Department

Joint Professor, Experimental Therapeutics

Member, Photonics Center

Boston University

44 Cummington Street, Boston, MA, 02215

Phone: 617-358-6189  
Email: xuehan@bu.edu  
Web: http://www.bu.edu/hanlab

ATTN: EDITOR DI GIOVANNI

Dear Dr. Di Giovanni,

We thank you and the referees for their time and efforts on our manuscript. Both referees offered addressable, insightful and cogent criticisms of our manuscript, and we have addressed each in turn by adding two new figures, improving our graphical user interfaces, and adding detail in the manuscript where appropriate. Our responses to each remark are written underneath each comment, which are included below in italics.

**Referee #1 (Remarks to the Author):**  
*1. In their submitted manuscript, Romano and colleagues offer useful applications of a Teensy 3.2 board as a cheap, reliable and easy to build tool for behavioural research. The authors present here how this board can be used to implement a rotation encoder and a controller for CS-US learning association. Although the data presented serves the purpose, the manuscript could be somewhat improved.*

*2. Figure quality can be improved:  
Figure 2: connection schema are appreciated, but may in some cases be confusing. The figure could gain in clarity if the connections were color-coded, making them easier to follow (this might also help understand which of the connections in panel B are actually relevant).*

**Response:** We appreciate the reviewer’s insight, and agree that this figure could be improved by color-coding the connections in this figure. We done so and have also provided additional labels to all of the pins that are utilized by the prop shield as per their board schematics.

*3.* *Figure 3B: The temporal drift reported in the text might be efficiently represented using a smaller line thickness and inserting a magnification making the divergence more evident (e.g. of the first and the last 30us)*

**Response:** We appreciate the reviewer’s note, and agree that our visual display of temporal drift was insufficient. Further, we recognize that we were not sufficiently explicit in describing the contents of Figure 3B. The line described by the reviewer is the best fit of the data, and does not represent a theoretical recording with no temporal drift. We have included a better description of the data shown in Figure 3B, and have augmented this figure with 3 additional panels. 3Ci demonstrates the best-fit line of the measured data versus the theoretical time stamps in red superimposed on the theoretical, zero-drift line. 3Cii demonstrates the magnifications from the beginning and end of the recording session suggested by the reviewer.

*4. Figure 4: Regarding panel A, same observations raised for the panel B of the previous figure applies.*

**Response:** We appreciate the note, and have also included 3 subplots here to better demonstrate the time

delay.

*5. About the eye-blink paradigm, it is mentioned that the amplitude of the CS is set to 0 and then increased when needed; does this happen live via the custom GUI mentioned in the Methods? Once acquired, are the time-stamps in 0dB condition distinguishable from those produced in the 75dB? Moreover, once you plug an Arduino board, the script that is loaded on it starts automatically: does the researcher have a way of controlling start and end of session, pausing and restarting the paradigm?*

**Response:** We appreciate the note, and acknowledge that, in general, we insufficiently described the role of the GUI. We have clarified the role of the GUI in the *Methods* section. For both original implementations of the *Motion Tracking Experiment* and the *Trace Conditioning Eye Blink* experiments, when the Teensy 3.2 board is plugged in, it initializes a few options, for example setting various pins as input or output. It then waits for Serial input from the computer, which it receives when the user presses “Start” on a graphical user interface. This graphical user interface saves the Teensy-reported time stamps for each frame. For the *Motion Tracking Experiment*, it saves the velocities obtained by both ADNS-9800 sensors in both the x and y directions, and the amount of time that elapsed during that specific frame. For the *Trace Conditioning Eye Blink* *Experiments*, it reports for each frame the time elapsed in the experiment and in the current trial, the trial number, whether or not the LED was on in that particular frame, whether or not the sound was on in that particular frame, and whether or not the puff was active during that particular frame. Thus, the user is able to retrieve the time periods during which either the CS or the UCS are present.

In our original implementation, the timing of the CS and UCS, the durations of both of these, and the frequency of the CS are all hard-coded into the Teensy start-up script. We appreciate after reading your comment that it is more convenient, especially for a novice user, to have the ability to specify within the graphical user interface all of these parameters, so we have created a new graphical-user-interface and a minimally-modified accompanying Teensy library to allow the user to specify all of these features.

Originally, our graphical user interfaces only had the ability to control the start of an experiment and specify its length either explicitly (for the *Motion Tracking Experiment*) or by specifying the duration and number of trials (for the *Trace Conditioning Eye Blink* *Experiment*). We have now added a “Stop” feature to both the graphical user interface code and the Teensy code that allows a user to stop an experiment preemptively and then restart it without having to unplug the Teensy and restart MATLAB. This can function as a pre-emptive experimental termination, or as a pause, as the user is free to press the “Start” button at any time following usage of the “Stop”. The user does have to specify a new filename after pressing “Stop” and before pressing “Start” again, or the computer will by default assume the user wants to restart the paradigm completely.

**Referee #2 (Remarks to the Author):**

*3. Minor corrections:  
- page 2. The material reference for the Tindie sensors has a link, while other material have not  
- page 5. Typo: "for pre cise image capture"   
- page 5. " eye puff versus the sCMOS camera (Figure 4Bii)."  In this paragraph, Figure 4Biii and 4Biv should also be referred to in the text.  
- page 5. Technical details on the ADNS-9800 sensors would be more suitable for Methods:*

**Response:** We thank the author for these critiques and have implemented the changes suggested.

*‘Calcium event frequency generally scaled with motor output across population’  
  
‘the rise in PV population activity preceded MSNs by ~500 ms’.*

-**Response:** We thank the Referee for providing this feedback. We will edit the manuscript throughout to make these points more clear. We would like to emphasize that our claims are NOT based on single example results. Each major point was supported by an analysis of the entire population of MSNs and interneurons from every animal (and every recording session) that went in to these experiments. For example, Figures 1G and 1F reflect statistics run on the entire populations of identified MSNs (7518 neurons) and interneurons (47 cholinergic interneurons and 72 parvalbumin interneurons), across every instance of high and low states of motion (as described in the text). Thus, we derived the conclusion that “*Calcium event frequency generally scaled with motor output across population”. To increase clarity, we can revise this statement to “*event rates are higher when animals are engaged in vigorous locomotion versus low locomotion”.

The thank the referee’s second comment. with respect to the PV population is well taken, and although we did quantify and describe in the text “the average difference in the onset times of calcium events in PVs and MSNs around locomotion onset (523.1±304.3 ms, mean±SEM)”. We will revise the sentence to: *the rise in PV population activity preceded MSNs by* 523.1±304.3 ms (mean±SEM).  
  
*4. Line 86-87. ‘CHI population activity in contrast, reached max intensity several seconds after peak speed (Figure 2Di, ii)’. It is unclear whether the CHI population activity maximized at around 2s after peak speed. Furthermore, whether the ‘peak’ CHI activity is significantly different from the pre-peak period. Again, these qualitative descriptions are not convincing.*

**Response**: In the version of the manuscript, emphasis was placed upon findings consistent across all analyses in our imaging and optogenetics experiments, given the number of comparisons to be described and the limit on manuscript length. However, we recognize the need of these details, and will include analyses that more clearly capture these characteristics of each neuron class on a case by case basis. If length is a limit, we will include them in supplemental materials. We will include a longer duration time plot (showing the 16 second window) in supplemental materials, which clearly shows the increase in CHI population activity occurring on the down slope of the movement bout. This time-series data would make it easier to readers to visualize the specific time point the CHI population reaches its peak level of activity relative to this velocity curve and relative to the timing of the peak in the population of MSNs.

*5. Line 89. ‘Speed was not significantly different following PV and MSN activity (Figure 2E)’. In contrary to the author’s statement, the speed after MSN activity also shows a clear trend of decrease.*

**Response:** As we report in the legend for Figure 2, speed following MSN events was reduced relative to baseline by 2 seconds post. the reason for this is clear in the time-course of an average movement bout (included in revised manuscript of ~5 sec) shows that speed generally tapers off approximately 2s after peak velocity, and coincides with the period where the CHI population activity peaks. Speed declined more rapidly following CHI events (significantly reduced during 1-1.5 s post event), and to a greater extent than that following MSN events; an analysis which captures this population becoming active after MSNs and PVs, and just before the end of a movement bout will be included in a revised manuscript. Taken in the context of the remaining optogenetic and imaging data, we feel it appropriate to draw attention to the theme across across all levels of analysis: a major role for CHIs in terminating movement sequence within a specific time period. We will clarify this relationship in a revised manuscript with additional figures and more statistical characterization.

We appolagize for the confusion and misleading statement.

*6. Lines 92-95. The claims here should be clarified:  
- ‘PVs … encode the duration of a movement sequence. Can the authors morph several movement sequences and normalize them to average them and show that during movement sequences PV neurons’ activity is always elevated?*

**Response:** We think this is agreat idea, and we will add this in our revision. In deed, this is already captured by the analysesFigure 4 shows that PV activity is significantly, positively correlated with both instantaneous population MSN activity, and instantaneous velocity. Further the 16 second window plot referred to in response to comment 4 further demonstrates this feature.

*7. ‘MSN population activity more directly reflects the components of the movement bout’. Please clarify which components are being considered in this case.*

**Response:** We will clarify the ambiguity in a revised manuscript. We are referring to the tendency of MSN population to rise and fall with changes in the vigor of motor output, as shown in Figures 1E, Figure 2 A&B, Figure 4Ai, Aii, Bi, Bii, Ci, Cii, Di, and Dii.

*8. The authors haven’t shown convincing evidence for CHIs signaling the end of a movement bout, actually Fig. S9 suggests they do not.*

**Response:**  This statement is supported by three evidence. (1) movement velocity declines following CHI events (Fig. XXXX); (2) optogenetically stimulating CHIs (but not PVs) can cause the mice to stop moving. (3) CHIs play a causal role in synchronizing activity in the MSN population, and that such synchrony correlates with motion offset. Supplemental Figure 9 was included in response to the Referee’s previous request for more information on the possibility that different populations of CHIs contribute to different phases of movement. While we did find some evidence that this is a possibility, the optogenetic manipulations provide a clear and convincing demonstration for increased CHI population activity in terminating on-going movement. We agree that the parsing of CHIs for this new analysis is distracting to the population contributions to movement termination, and would be happy to remove the figure.   
  
*9. Line 105. ‘the same optogenetic stimulation of PVs during a movement bout introduced a transient change in movement trajectory characterized by repeated changes in direction over several seconds  
(Figure S7C; Video S4)’ It is important to report what is happening in more detail. It is not very illuminating to present this result just by bar graphs and an example video.*

-**Response**: This is another important point that we should have provided more detail on in the main text. As described in the Supplemental Materials and Methods, we noted that PV-stimulation appeared to increase the amount of side-to-side movement during locomotion bouts, and therefore specifically quantified X-axis zero crossings of the track ball during optical stimulation. We will provide these further details in a revised manuscript.  
  
*10. Figure 3E. The dependency on distance should be reported in a more detailed manner. Please report not just the two conditions (< 100 um versus >100 um) but more points in distance.*

**Response:** We have created and can include histograms illustrating the frequency of correlation values as a function of distance to enhance detail in a revision.  
  
*11. Figure 3G. The difference between the conditions is difficult to see with the 2D color plots. Plotting ‘Counts’ as a function of distance would be more illuminating*.

**Response:** This is a good idea, and we will do so in the revision.

*12. Line 139-140. ‘While the number of correlated MSN-interneuron pairs was reduced with distance for all cell pairs, the drop was most precipitous in MSN-PV pairs and least impacted in MSN-CHI pairs  
(Figure 3H)’. How this statement relates to the Figure 3H is unclear. Can the authors perform some statistics to support the statement?*

**Response:** We thank the Referee for raising this issue. We have histograms (as described above) that clearly illustrate this point, as well as a statistical quantification of the differences across interneuron-MSN pairs that we will readily include a revised manuscript.  
  
*13. Line 178. Remove ‘the’.*

**Response:** This will be revised.  
  
*14. The limitation of the present study should be acknowledged: (1)The inability to distinguish D1 and D2 MSNs, and (2) The overgeneralization of the findings in this particular area of the striatum.*

**Response:** We will include a more thorough discussion of the limitations and strengths of the current work in a revised manuscript including both of these points in the discussion.

**Referee #2 (Remarks to the Author):**  
  
*This resubmitted manuscript from Gritton and the colleagues added one major experiment which is using channelrhodopsin activation as gain-of-function to address PV and CHI impact on the locomotion  
(Figure 2F-H) and correlation to MSN activities (Figrue 5E); and one new analysis, which is the spatial relationship of MSN to PV and CHI (Figure3). These efforts strengthened the manuscript. However, I still  
have the following concerns, many of which are unaddressed issues from the previous submission.*  
  
**Major Issues:**

***1.*** *Novelty and the conceptual improvement from the existing work: calcium imaging of MSNs in the striatum has been carried out quite extensively recently. The strength of the current work is to imaging MSNs with PV or CHI simultaneously. In general, quite some previous in vitro work illustrated the mechanisms of how PVs and CHIs act through MSNs. Recent published work addressing PV function (e.g., Owen et al., 2018, Lee et al., 2017) in vivo, does decrease the novelty of this work, even though the authors try to argue in the discussions that current work is using a different behavioral paradigm (non-operant).*

**Response:** We believe and would like to reiterate that our study is the first and novel and highly significant in dissociation of the function of two types of striatal interneurons, cholinergic interneurons versus PV cell.

Since other calcium imaging studies of D1 and D2 MSNs, clearly shows that the *in vitro* manifestation of the interactions between interneurons and MSNs is not equivalent to the way these neural populations interact when striatal circuitry is being recruited in the service of behavior

***2.*** *The criticism that the results are mainly descriptive and have not formulated into clear theme or exactly how would the circuits work still stands. Do the authors still think that the PVs are feedforward inhibition? How does ChI activation fit in with the circuits that result in MSN activity? And what are the thoughts that how this increase in MSN activity then lead to suppression of speed/movement that fit in with existing literature? How do the authors explain the increase in locomotion when PV is activated? Overall inhibition of MSNs does not logically result in initiation of movement. Some in vitro confirmation of exactly what are the circuits proposed here would be very helpful, as well as the discussion about how this fits in with the bigger picture of the striatal circuits that are known. In the abstract, there are places that the wording is very strong although the evidence is weak, or indirect. For example, the key sentence “PV cells dictate which populations of MSNs are engaged during movement…..”. From the data presented, it does not show or define ‘which population’.*

**Response: We believe that these aspects raised here are excellent burning questions to striatal systems. We acknowledge that our data presented will not fully address these questions, but we will revise our discussion to specify how our data provide support different hypothesis.** .. (1) We think that PV cells provide feedforward inhibition, and our data supports this notion. computational modeling studies have formed the basis of the notion that PV interneuron-mediated feedforward inhibition can shut down active MSNs that are not important for the currently activated motor plan (e.g. Moyer et al., 2014); our data provide a *in vivo* demonstration of this process. (2) we think CHIs have complex effect on MSN activity…….

With respect to the specific comment that “o*verall inhibition of MSNs does not logically result in initiation of movement* “, we do not believe the activity of particular neurons can be addressed as ‘inhibitory” or “excitatory” components in a dynamical system, nor does our data suggest that PV activation results in general MSN inhibition. We have added new supplemental figures that we think addresses the dynamic nature of this inhibition specifically as it relates to PVs (Please also see response to Referee 3, comment 2). This includes a quantification of the populations of MSNs that recruited during optogenetic activation of PV cells, which suggests that not all MSNs are ubiquitously inhibited by their activation. Furthermore, reducing the effects of PV-stimulation to “MSN inhibition” ignores decades of data regarding lateral inhibition by MSNs onto MSNs, and the dis-inhibition that would result from PV control of such MSN-MSN interactions. As mentioned many times, heterogeneity exists in a behaving animal and reducing this preparation to slices removes the dynamic component, fundamentally changing the interpretation of such “mechanistic” data. The experimental tools to create the data the Referee is asking for simply do not exist, and as mentioned below in response to critique 5, to understand this mechanism is not a simple question that a single in vivo study is capable of uncovering. We are currently exploring ways to better reveal how systems are dynamically gated but this represents a massive, multi-lab and multi-year endeavor that extends beyond the scope of this or any other single paper.

***3.*** *Response heterogeneity within a given population (e.g, Figure 2E and S4 and others): It was brought up previously that the responses observed are heterogeneous within each population. For example, the  
same Figure 2E, I copied from the previous comments: ‘As stated in the manuscript, the responses are highly heterogeneous. Therefore, lumping all cells from one type together might bias the ones that just had bigger responses. For an extreme example, if all cells except one are silent, this one cell’s activity will dominant the outcome. The other end of the spectrum is that Chl neurons have equal numbers of cells that were positively (41%) and negatively (37%) regulated. So they are effectively different population of cells. It is hard to see how to fit things in when all the conclusions are based on the average of appositely responded neurons.’ Unless I missed something, instead of analyzing different response groups, the authors just removed those descriptions and get around the problem.*

**Response:** We did not further break down other cell type, because most PV and MSN <were negatively modulatedGiven that PV interneurons account for 2-3% of cells within the striatum, selecting the 15% of PV cells that are negatively modulated by movement for separate analysis is not be viable for any technique in neuroscience presently. We ed this in the revised manuscript, but we recognize that this may not have been obvious. We will this in a revision.

….”

***4.*** *A related issue is that it is really hard to interpret MSN results in the striatum if one lumps D1 and D2 neurons together. It is important to take D1/D2 identify into consideration both experimentally and data interpretation. Manipulation experiments: the added channelrhodopsin activation as gain-of-function does strengthen the manuscript. However, the loss-of-function experiments are also important. I am surprised this is not done here.*

**Response:** Again, we thank the Referee for drawing attention to this important point that we have acknowledged and expanded on significantly in the revised manuscript. Please also see response to Referee 3, comment 9 below. This specific point was brought up in the previous submission and we will reiterate our response here:

We acknowledge that a limitation of this study is that we cannot independently monitor more than two neuron types at once but to date no one has specifically addressed the influence of interneurons on MSNs in freely moving animals. However, at the time of submission, five recent studies: (Barbera et al., Neuron; Cui et al., Nature; Klaus et al., Neuron; Meng et al., Neuron; Parker et al., Nature) employing optical imaging to monitor activity in genetically defined D1 versus D2 MSN populations have revealed **entirely indistinguishable and overlapping activity-behavior relationships between D1 and D2 cells using GCaMP activity.**

Baring in mind that D1 and D2 populations cannot be differentiated from one another during normal behavior – though there is evidence these neuron populations are differentially impacted in a perfectly predictable way following dopamine depletion (e.g. Parker et al., 2018) – we have no reason to believe this distinction would change the conclusions based upon our analyses. While the similarity between D1 and D2 studies was mentioned in the opening of the discussion, we have now added a detailed characterization of this issue in the revised text to clarify why the MSN populations were treated as a combined population and the potential limitations that could arise from doing so.

Furthermore, we acknowledge that loss of function experiments with silencing could be useful, particularly as it relates to behavior, but since we can only track increases of activity with calcium sensors, we would not be able to relate loss of activity to an underlying calcium signal within the neural network. Given one of the strengths of this manuscript is our ability to relate behavior to neural activity, such optogenetic silencing experiments would not be fruitful in that regard. However, we have the tools and could add them if the editors deemed such experiments as necessary.

***5.*** *One technical point is also unaddressed from previous version.(1) Instead, the authors just removed the part related to cortical inputs. But the same criticism stands regarding the procedures used required removing the cortical tissue above the ROIs. I understand this is necessary for these experiments. But this should be at least mentioned in the discussion. (2) As mentioned by the authors previously different cortical inputs might differentially affects different cells or cell types. Such surgical procedure might work well in other brain regions, for example. hippocampus, but pose potential main caveat here. (3) Simply histology should be carried out at the end of experiments to see how much of the inputs to that piece of the striatum is compromised and to evalatue the impact. Along the above line and as brought up previously, it would be good to have optogenetic activation of motor inputs to the striatum and see how the GCaMP activity in the different subpopulations line up in onset time. In another word, is the difference in GCaMP onset timing between cell types dictated by the same input and does the local circuit, i.e., PV inhibiting MSN, ChI potentiating MSN, indeed line up relatively to the excitatory inputs from the motor cortex?*

**Response: (1)** As requested by the reviewer in the previous submission, we added this acknowledgement to the revised text in the Methods and Supplemental results but this point can be moved to the discussion in the next revision.

(2) Given that our results share many key similarities with other published striatal imaging studies (Cui et al., 2013; Klaus et al., 2017, Meng et al., 2018, Parker et al., 2018, Barbera et al, 2016, Owen et al., 2018; each employing cortical aspirations of various size), we believe that the findings are very relevant within this growing literature and reflect major as well as previously undescribed patterns of MSN-interneuron interactions. With regards to measurements of reduced cortical input to the striatum related to this study, we are not sure how that “measure” will change the interpretation of the results. The only possible comparison would be to imaging without cortical aspirations which is not currently possible. In general our results should be interpreted in comparison to other imaging studies where this approach is shared and all rely on common cortical aspirations to reveal the striatum.

Additionally, although we mentioned this point in the previous response, we did not observe any directional neglect (Figure S3D) in animals within our study, which might be expected with profound loss of input. Such hemi-specific neglect has been noted in experiments with lesions to striatal inputs limited to only one hemisphere (Whishaw et al., 1986).

(3) We have carefully considered the possibility of histology, but it is difficult to conclusively characterize…we acknowledge that by the end of the day, there will definitely be plasticity changes, but how would that impact our interpretation of the results? We can also cite the papers that examined PD motor cortex?

Finally, we also think it is extremely important to examine cortical inputs to the striatum, but it is beyond the scope of current study.

***6.*** *Cell numbers and cell identify: PV and CHI are very rare in numbers in the striatum. This brings up two issues: first, when the results are normalized to totally number, the small neuron number of PV and CHI  
might change the percentage significantly. Therefore, it is crucial to put cell numbers in prominent places. There are several places I can’t find exact cell numbers and there are places these numbers are relatively low;*

**Response:** We made a concerted effort to include within every figure and every analysis the total number of interneurons that were included if it deviated from the total number interneurons recorded in the study (i.e., representative animal figures). In instances where individual animals are used, these numbers are expected to be low as the number of interneurons recorded/session were 4.5 for PV’s and only 5.1 for CHI’s. Given that these populations represent between 1-5% of the total population, we believe the number of PVs and CHIs simultaneously recorded in a single subject is actually exceptionally large, and has never been achieved before. We will highlight this unique novelty in the revision.

***7.*** *More importantly, the cre lines used here need to be characterized by using antibody staining. Although PV line is widely used, but main characterization is done in the cortex. A simple control experiment would be to have the PV-Cre and ChI-Cre lines followed by the same viral co-injection scheme as noted above. Well characterized antibodies against PV and ChI can used and be visualized in IR-red using immunohistochemistry. A simple cell count experiment should provide the exact number of PV-positive or ChAT-positive (immune-positive) which have GCaMP6f expression but no tdTomato expression. With this experiment one can also appreciate how much false classified MSNs are incorporated into the dataset because All GCaMP-positive and tdTomato-negative cells were treated as MSNs for their analysis.*

**Response:** As mentioned below in response to reviewer 3, comment 1, the selectivity of each CRE line has been previously reported in the literature specifically in the striatum by Lee et al, and our previous publication Kondabolu et. al. (Lee et al., 2017; Kondabolu et al., 2016). We have now added this specific point in our revision.

***8.*** *(1) From the traces shown in Figure 1D, it is clear that the calcium peak is not corresponding to the action potential. As brought up before, GCaMP family of calcium indicators are slow in general compared to  
many actually calcium events, even with 6f depending on the questions. This poses limitations on temporal resolution. Again, depending on the questions, many applications might get by. Here, temporal profile is the key since many analyses rely solely on the time. (2) It is known that PV cells are fast spiking interneurons with short spike onset times. Therefore it is surprising that a slow rise time is found. One possible explanation is that the abundance of parvalbumin proteins which are calcium buffers, buffers the calcium far more compared to other cell types such as ChI and MSNs and therefore mask spike-activity related calcium fluctuations reported by GCaMP6f. Therefore the kinetics in GCaMP is more a byproduct of the cells biochemistry rather than it reporting the activity signature of the cell during motion. (3) I suggest showing the relation of the GCaMP signal in PV striatal cells and spiking behavior, preferably in vivo using optotaging as a way to confirm spike detection from PV cells.*

**Response: (1)** We absolutely agree with the limitation of the temporal resolution of Ca imaging. We acknowledged this and included a statement with citations that suggests the calcium signal likely reflects bursting within cell populations. (2) As PV cells within the striatum show bursts in excess of 60 Hz for sustained periods of time (Sharott et al., 2012), PV calcium signals likely reflect changes in the bursts of action potentials representing the highest periods of sustained cell activity, however as described in detail below, this information is not central to the prediction based upon, or interpretation of the results of our studies.

We believe that optotaging experiments will indeed confirm that GCaMP is not measuring single PV responses. It is interesting experiments, but we fell that this will take years to complete and the results will not affect our interpretation of our current results.

While the individual calcium event shape differs in PV cells, the analysis looking at their predictions related to movement onset and influences on MSN activity do not depend on the slow kinetics or duration of the signal. These analyses were constructed this way intentionally to overcome the temporal limitations of GCaMP. We also believe that the addition of the optogenetic manipulations is helpful in addressing this point. Both PV and CHI opto-stimulation showed rapid activation and unique influences on behavior, the timing and relationship of the responses on behavior were conserved, and most importantly, the results of the optogenetics experiments confirmed both our observations about interactions between interneurons and MSNs, as well as our predictions for how such interactions contribute to behavior. While determining the mechanisms underlying the calcium response in PV or CHI interneurons, as well as MSNs is important, we believe this is secondary to the functional role they have in influencing behavior that we reveal with calcium imaging.   
  
***9.*** *(1) Several issues with main figures: Figure 5Ei, MSNs Pr is no change. This is not consistent with Eiii, or the conclusion. (2) Also, there is no error area for blue traces (or the errors are so small that is not visible?). (3) Figure 2Ci, Chl traces do have a peak, it just had lower baseline. Why was that not significant? This will change the conclusion if the peak is considered significant; (4) Figure 5Ai, isn’t red trace has big AUC than the black, although black is above red within the 500ms window? This will bring different interpretation to the data.*

**Response:** With regards to Figure 5Ei and 5Eiii, PVs did not produce an overall increase in the MSN probability rate (blue trace; which does include error although the term is small given the sample size). Figure 5Eiii compares the probability of an MSN event co-occurring with a random calcium event from the population, co-occurring with a PV event, or co-occurring with a CHI event. In this case, when we look at such co-activity, PVs do not produce an increase in the probability of a cell being active. We have tried to make this point more clear in the revised submission.

The use of the term “significance” refers specifically to a statistically significant increase in activity over baseline in the windows analyzed as described in the first rebuttal letter.

The window size for Figure 2C (2 seconds) reflects that previously published for D1 and D2 populations by Barbera et al., 2016 (see their Figure 1F compared to Figure 2C in this manuscript). This window was selected **a priori**. Furthermore, our new analyses that expand the window of time in which we quantify changes in CHI population florescence clearly show the peak CHI population response just after peaks in velocity, as previously described and made more clear here.

With regards to Figure 5A, our analysis window was determined based on the rising phase of the calcium signal derived from the population (Figure S3Aii). Since we wanted to focus on how one cell event may alter the population fluorescence we only considered time points where the calcium signal in the triggering ROI was rising, which was 500ms. Importantly, the conclusions drawn from our original analysis of the data are maintained, and bolstered by the effect of optical stimulation of the different interneuron classes. We again thank the Referee for their helpful comments, and believe addressing them has enhanced the overall manuscript.

**Referee #3 (Remarks to the Author):**  
 *The authors utilize cell-type specific viral labeling and dual-color in vivo imaging to explore the activity of interneurons in dorsal striatum as they relate to MSN activity and running motion. To this effect, the authors claim the following major findings:  
  
1. PV interneuron activity precedes motor activity (and MSN activation)  
2. Activation of PVs increases motion  
3. Motion decreases following CHI activity  
4. Activation of CHIs decreases motion  
5. PV activity decreases MSN co-activity, CHI activity increases it  
6. MSN co-activity correlates negatively with motion  
  
The take home message is very clear, and this work is extremely timely, novel, and important.*

**Response:** We thank the Referee for their time and careful consideration of our work. We also want to acknowledge their generally positive review and their summary of the key findings which reflect the points that we also think are critically important. We further thank them for noting inconsistencies or points where our message was unclear. We have addressed each critique in the detailed responses below. **Major issues:**  
***1.*** *(1) PV interneuron activity precedes motor activity (and MSN activation) Fig 2Ci shows how PV activity precedes motion onset and MSN activity by ~500ms. The authors claim that Di shows CHI activity increasing  
shortly after peak velocity, but Dii does not reflect a significant effect. (2) Another important control is to demonstrate the specificity of the PV-Cre and confirm the PV expression using immunostaining of PV.*

**Response:** We agree and thank the Referee for pointing out the importance of this additional control and the use of qualitative statements with regards to unanalyzed points in time. In Fig 2Di our point was descriptive related to CHIs and we have removed descriptive statements within the revision that were not supported with quantitative statistical measures. Because periods of peak velocity can be maintained for tens of seconds, the exact timing of cholinergic activity can vary across movement bouts in a way that will not be well captured by the analysis performed in 2Dii. To more carefully address and fully characterize this point, we included a new time series analysis as a supplemental figure that identifies the timing of cholinergic peak activity within a movement bout. We thank the Referees’ for prompting this analysis as is better captures the timing of CHI activity in relation to movement. To re-iterate, this issue of timing is just one of 8 different results (including speed following CHI events, MSN-synchrony following CHI events, MSN-synchrony preceding movement offset, and a replication of each of these with optogenetic control of CHIs) all leading to the same conclusion regarding the influence of CHIs on MSN network activity and movement.

With regards to the specificity of our targeting with the PV line, two recent studies have previously used this same mouse line (e.g. Lee et al., 2017 and Owen et al., 2018). Within the Lee paper, in Supplemental Figure 1 they show that CRE mediated expression of a viral reporter was ~94% selective in relation to PV antibody staining. A similar comparison was reported in the striatum as it relates to the Chat-CRE line (Kondabolu, 2016). Given this quantification was previously done within the dorsal striatum where we were recording, we did not anticipate the necessity for antibody staining for the original submission. However, we will provide a similar quantification from our mice if reviewers and editors deem it necessary.

***2.*** *Experiments using Chrimson expression in PVs (Fig2F) showed that PV activation is sufficient to drive locomotion from rest. This observation is clear and robust. However, it is possible that the observation is an artefact of general disinhibition in the striatum. The authors go on to claim that there may be specific key populations of MSNs that are selectively disinhibited by this manipulation, but it is purely speculative and never demonstrated.*

**Response:** We agree that this statement about specific PV sub-populations was never demonstrated and that we did not rule out general disinhibition as a potential mechanism. However, general disinhibition would be expected to produce a general increase in activity in the MSN population following PV activation (both endogenous and optically triggered), and we did not observe such changes in any of our experiments. We felt this idea was the kind of speculation that might be included within the Discussion section, and as such we relegated it to a talking point in this part of the manuscript alone. However, given the Referee’s comments, we have since done new experiments involving network modeling during optogenetic activation that support the original statement of discrete populations of MSN being targeted through PV activation. These figures and quantitative analysis are now provided as supplemental figures in the revision.

***3.*** *Fig2E shows that speed decreases after CHI activity. This particular effect is clear, although the same figure is problematic for other reasons: (a) PV activity does not result in observed increased motion (which one would assume to be the case given the previous claim), and (b) it appears that speed decreases following MSN activity. The authors do not address these points and beyond making it difficult to interpret.*

**Response:** We agree that several of these points could be made easier to interpret with additional text or analysis and have made changes to the revised text and created new supplemental figures to address this confusion. Specifically with regard to points (a) and (b): The plot in 2E shows all locomotion following PV events. Similarly, in the optogenetic manipulation experiments, when all baseline speeds were considered (Figure 2G) this change in speed was not apparent. It was only when we limited our analysis to low movement periods (Figure 2H) that optogenetic stimulation of PVs was shown to promote movement. If we focus our analysis of endogenously generated events to these same behavioral conditions, we see the exact same pattern of increased movement following PV events as observed following optical stimulation during low movement time periods. This new plot will be included in a revised manuscript. For CHIs, neurons generally fire transiently when speed is high, and this manifests as a massive reduction in velocity following a CHI event across the population. As in the case of MSNs (point b), MSNs become active leading up to the peak in velocity within a given movement bout (Figure 2Di and Figure S3B). Thus a small reduction in velocity is expected several seconds after a MSN event given the natural rhythm and duration of a movement bout (~5 seconds) and MSN coding of it. However, we have now included new statistics and time series analysis that demonstrates the “slowing” that follows CHI events is profoundly different from that following MSN events (also see response to comment 4 below).

***4.*** *Fig2G shows that activation of CHIs results in overall decreases in speed. Once again, the authors do not address why PV activation only increases speed if the animal is at rest (2H) as 2G shows no positive  
modulation via PV activation. Furthermore, FigS3F shows that CHIs do not become active prior to motion offset, calling into question whether this effect is indeed physiological*.

**Response:** We again agree that several of these points could be made easier to interpret with additional text or analysis and have addressed this with new supplemental figures, analyses and additional discussion, as stated in response to comment (3). We speculate that during high movement, further activation in PV activity does not produce additional increases in velocity due to ceiling effects, which is supported by the absence of an effect on velocity when PVs are optically stimulated during periods of high locomotion. Thus the PV-triggered increase in speed, be it endogenously or optically triggered, is most apparent when focused on low-movement time periods. We have discussed this in more detail in the revised manuscript.

Based on the Referees concerns and as described above, we also looked more precisely at the timing of CHI activity to movement offset. We found that CHI activation follows peaks in velocity and just prior to movement termination, and further only CHIs had significant peaks in activity isolated to this time window.  
  
***5.*** *PV activity decreases MSN co-activity, CHI activity increases it Figures 3 and 5 focus on analyses of correlation between cells across cell types and motor states. A key element missing from these analyses  
is a true “baseline” measure of correlation/co-activity. Indeed, the first figure of the paper shows how MSN/PV/CHI activity are all very different from each other regarding their activity dynamics  
(frequency, AUC, etc.). Therefore, it is incorrect to directly compare “co-activity” between these distinct cell-type without providing a true baseline expectation of these measures. For example, shuffling dF/F values to yield “chance co-activation”. Another example of this error regards the comparison of co-activity/correlation during low vs. high speed (Fig 3) – higher speed naturally means more Ca2+ activity which means there is a greater chance of simultaneous activation of cells.*

**Response:** We thank the Referee for drawing attention to a point of confusion within this study. In the revised manuscript we have attempted to fully clarify this analysis both as it relates to this point and the point below (comment 6). Comparisons of co-activity were **always** **within the population of MSNs**, although centered at a randomly selected MSN event, or an interneuron event (red and green). Thus, these analyses include a baseline comparison, and would not be confounded by the different response characteristics of the individual cell types. We apologize for not presenting this finding in a more clear way. We have also added shuffled data comparisons when appropriate that reveal chance correlation values which is helpful in terms of interpreting the strength of the relationships that exist. It is also worth noting that in most cases, as described below, correlation analysis is derived from the entire session so the “motor activity” remains consistent in the analysis between specific interactions within those sessions. With regards to figures 3B and 3C, this addresses movement (B) or anatomical spacing (C) differences irrespective of cell type. We have included shuffled data in these revised plots for comparison.

For the other comparisons in Figure 3, the analysis is based on entire session data and we found no significant differences in movement event frequency between PV or CHI mice or between the MSN populations that came from PV or CHI mice. This suggests differences between these mice are driven by the cell-specific differences.   
  
***6.*** *Similar issues to the above section apply to these analyses, with the addition of the fact that Fig5Eii clearly shows that Chrimson activation of CHIs results in MSN activation. This is not observed with PV activation (Ei) – the authors do not address this, and it calls into question to exact nature of their optogenetic manipulation  
experiments.*

**Response:**  We agree the Referee that this result could be more clearly described. Also see response to Reviewer 2, comment 9. With regards to Figure 5Ei, PVs did not produce an overall increase in the MSN probability rate (blue trace). As the probability rate remains low under normal conditions, a reduction from this level is difficult to detect. As the reviewer mentioned in comment 5 above, it is important to consider changes from what might be expected with any other period of co-activity. Therefore, Figure 5Eiii compares the probability of an MSN event during PV optogenetic activation to that of a random calcium event from the population. Under these conditions, when we consider co-activity, PVs do not produce an increase in the probability of a cell being active, but rather produce a statistically significant reduction (which we interpret as evidence of these cells inhibiting a sub-set of MSNs). We have tried to make this point clear in the revised submission.  
  
***7.*** *Given that the PV-positive neurons, MSNs and CHIs have different Ca transient dynamics. It is important to calibrate the relationship between neuronal firing rate and the dynamics of Ca transient, i.e., number of APs, firing rate, vs Ca transient peak dF/F, rise time, decay time.*

**Response:**  The Referee makes an important point that exists within the literature with regard to what calcium activity actually represents and how faithful it is in reporting underlying action potentials. Based on reported firing rates from electrophysiology studies, calcium activity is most definitely not a proxy for individual action potentials although it does track spiking dynamics relatively well (Chen et al., 2013). While these points are interesting and relevant, we think a detailed analysis of the kinetics is beyond the scope of the manuscript. More importantly, the goal of these experiments was to use a technique that allowed us to form testable hypotheses about how neural populations interact, and how these interactions might contribute to behavior. Regardless of the relationship of calcium dynamics to spiking activity, we were able to uncover previously undescribed interactions between cell types, and then by causally manipulating the cells with optogenetics, show that these interactions do contribute to behavior. Characterization of the relationship of calcium kinetics to spiking activity might add another layer of detail, but it does not change the fact that the interactions exist and have a functional role.

**Other Comments:**  
 ***8.*** *Statistics: The authors utilize linear regression to assess the strength of the relationships between cell-types and motor activity. The conclusion is that PV cells, and not CHI cells, have significant predictive power regarding MSN activity and motor activity. It is unclear why the authors decided to solely use univariate regression and not include a multilinear approach, where each predictor variable can be more directly compared using regression coefficients (and additional factors such as redundancy can be addressed).*

**Response:** We thank the reviewer for suggesting this analysis. As we have no disagreement that a multilinear approach could be a better way to address this analysis, we will replicate the prediction analysis using multilinear modeling and present that data in parallel in a revised submission.

***9.*** *Another critique of this paper is how the authors group MSNs into a single cell type. It is perhaps misleading that a paper aiming to distinguish the cell-type-specific effects of distinct interneuron populations in the striatum does not respect the distinct cell-type-specific roles of the other 95% of cells present.*

*Specifically, there exist many papers (some using in vivo Ca2+ imaging) supporting the idea of distinct roles for D1 and D2 MSNs in controlling movement behavior (papers by Rui Costa, Xin Jin, and Mark Schnitzer etc). Throughout this entire study, the authors group these cell types together. At best, this results in a “diluted”, yet still significant relationship between MSNs and PV/CHI cells, resulting in the authors being not able to provide sufficient mechanistic evidence to support specific cell-type interactions. A more ideal approach would be to use two color GCaMP and RCaMP. Indeed, even in the regression analyses, the authors average the activity of many MSNs (which even they in FigS8 show have distinct motor encodings) and treat them as a single type (i.e. single response or predictor variable). It is clear that the authors are dismissive of the heterogeneity of MSN function.*

**Response:** We understand that this remains of great interest to the neuroscience community and we do not intend to be dismissive of the two different populations of MSN and have more fully embraced this discussion in the revised manuscript. Please also see response to Reviewer 2, comment 4.

Just as hypotheses concerning the roles of striatal interneurons have drawn from the results of studies employing different tasks, so have conclusions regarding the importance of activity within populations of D1- and D2-expressing MSNs during behavior. With respect to self-initiated locomotion and the studies noted by the Referee, particularly as it relates to calcium activity, five recent studies using GCAMP imaging to monitor activity dynamics selectively in D1 and D2 MSN populations **find that these populations** **are largely identical in their coding of movement onset and offset** (e.g. Barbera et al., 2016; Cui et al., 2013; Klaus et al., 2017, Meng et al., 2018, Parker et al., 2018), and only truly dissociate in the context of dopaminergic de-afferentation (Parker et al., 2018). Furthermore, while there does appear to be some topographic organization of MSNs in the dorsal striatum based upon the vector of movement the neurons code for as mentioned by the Referee (described in our manuscript figure S8, but also see Barbera et al., and Klaus et al.), **there does not appear to be differential segregation in clustering related to aspects of movement across the D1 and D2 populations.** Also, neither study found a difference in cluster strength, sparseness, or anatomical location between D1 and D2 neurons.

We entirely agree that the suggested experiment involving dual labeling may provide useful insight into a yet unidentified mechanism in how, or which, populations of neurons interneurons preferentially influence. Such an experiment would be an important follow-up to this work but we do not believe such a study should preclude publication of this work, which in light of recent striatal calcium imaging papers, draws attention to the **unique contributions of each interneuron class to MSN network activity and how they contribute to movement.**

***10.*** *Lastly, it appears that the authors mix up paired and unpaired nonparametric tests (or at least they are typos). One such example is line 1242.*

**Response:** Thank you for noting this error – this was indeed a typo. We have carefully reviewed the manuscript and corrected errors related to descriptions of statistical tests.