nature portfolio

Peer Review File

Ventral tegmental area GABA neurons integrate positive and negative valence

Corresponding Author: Dr Benjamin Saunders

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 from Stelzner and colleagues, the authors investigate the activity of VTA GABAergic and dopaminergic neurons during reward, aversion, and conflict between the two. They use fiber photometry to measure gCaMP signals in dopaminergic or GABAergic neurons in the VTA They find that dopaminergic and GABAergic neurons show similar responses to rewarding cues and rewards, but that only GABAergic neurons show a robust response to aversive stimuli. Using a motivational conflict task in which rats face an escalating amplitude of footshock to retrieve a cued reward, they show that in both sets of neurons, the activity signal in response to the reward cue decreases with increasing cost. In dopaminergic neurons the gCaMP signal in response to the reward does not change with cost, but in GABAergic neurons, the activity in response to the reward increases as the cost increases. This is an important study that provides additional evidence in longstanding questions over the role of VTA dopaminergic and GABAergic neurons in reward and learning, and suggests that the two sets of neurons play different roles in these processes. The studies are well designed and controlled, and the data is of high quality. This is very nice work overall and I have only a few suggestions for improvement:
1. The dlx-cre results appear to be taken from an n of 2, this should be replicated to increase the sample size. Alternatively, I do think the key points of this paper stand without this data, so this figure could be removed.
2. While there is heterogeneity in dopaminergic neurons in the VTA, this is largely organized along spatial lines. The fiber tracks in this paper appear to all be located in the lateral portion of the VTA. The takeaway of this, for me, is not that the recordings are from a heterogenous population, but that they are likely from a relatively homogenous population that may differ significantly from the responses that would be found from dopaminergic neurons in the more medial portions of the VTA. Brief discussion of this is warranted.
3. I also think it is worth mentioning that the downstream effects from GABAergic activity are not necessarily local OR distal, but may encompass both-there's increasing evidence that projecting GABAergic neurons have local collaterals.

Reviewer #2

(Remarks to the Author)
The manuscript by Stelzner et al, investigates both VTA dopamine and VTA GABA neurons in reward and aversion following emerging data suggesting that these populations have a more nuanced role in this space. To do this they use a combination of pavlovian and conditioned stimulus task with fiber photometry targeting both populations of neurons. This is an elegant study that highlights and corroborates findings in the field suggesting that GABA neurons have the ability to encode reward and aversive stimuli. The findings here suggest that the GABA neurons engage to gate responses to reward and motivation together. This is a timely addition to the field and unique path of investigation.
Please see below for feedback and suggestions.

General Points:
Throughout the manuscript there are a lot of acronyms and labels missing that make interpretation for the reader more challenging which adds a level of unnecessary frustration.

In general, the brief description of the results (perhaps this is a word count limitation?), makes the interpretation of the data in the figures quite challenging.

Do the authors investigate potential sex differences?

Figure 1:
It would be helpful to include a description of what 'Early, Mid and Late' refer to in the figure legends or the results to help guide the reader. It might be helpful to include a timeline in the figure as an easy and informative reference guide for the reader as they go through the results.

What is the significance of the early, mid and late timeframes?

In Panel E, x axis label for what early, Mid and late refer to is missing.

In Panel G and H, the mid timepoint looks to be the same between CS+ and CS- which is quite different to what is observed in Panel O and P, dopamine vs GABA. Can the authors comment on what might be driving this for dopamine group.

Supp Fig 1: In panel A, the images are beautiful, but it would be helpful to have some indications as to what the author would like the reviewers to focus on. If it is simply viral expression in the region of interest, the VTA, a zoomed out image would also be helpful. If it is an example of probe placement with a track outlining, it would be good to highlight that.

Figure 2:
The additional of a timeline to show number of habituation days, testing paradigm, number of days between the first and last day would be helpful to guide the reader through the figure more informatively, rather than toggling through methods, results and figures to piece the information together.

The data in figure 2 is compelling though it would be nice to see how these neurons respond to additional aversive events rather than just freezing to really validate that these neurons are broadly 'strongly responsive to aversive events'. Foot shock represents a very strong aversive event. Would these responses look the same for a milder aversive event. Would the scale of responses look the same to a milder non-shock aversive event?

What do the authors make of the decrease in response from first to last in VTA GABA neurons? Is there some level of habituation/adaptation to the aversive event. If this shock was repeated at a future time point what would the response look like?

Figure 3:
Panel P: What do the authors make of the GABA response in the absence of a reward? Is this significant between rewarded vs unrewarded at each shock? This was difficult to discern from the figure legend description of stats analysis. What do the authors mean when they refer to phase in the figure legend?

Reviewer #3

(Remarks to the Author)
I have carefully reviewed the manuscript by Stelzner et al. examining the functional architecture of VTA microcircuitry in relation to appetitive and aversive conditioning. The study offers an incremental contribution to our understanding of VTA neuronal dynamics, though several concerns limit its suitability for publication in its current form.

The authors' investigation into the differential encoding patterns between dopaminergic and GABAergic VTA neurons represents a meticulous extension of the existing literature. Their confirmation that GABAergic neurons exhibit enhanced responsivity to aversive stimuli and uniquely encode valence information during feedback provides valuable empirical support for emerging computational models of midbrain function. However, the authors' framing of these findings as necessitating "an overall re-evaluation of VTA functional heterogeneity" appears disproportionate to the incremental nature of their contribution.
The manuscript positions itself as challenging prevailing theoretical frameworks. Yet, the results largely align with and amplify the established literature from the Uchida and Watabe-Uchida laboratories over the past decade and a half. The seminal works by Cohen et al. (2012) and Eshel et al. (2015) have already established the foundational architecture of VTA circuitry and delineated the distinctive contributions of DA and GABA neurons to appetitive and aversive learning paradigms. The present study does not fundamentally restructure this conceptual landscape but rather adds nuanced detail to an already richly developed model.
A critical limitation concerns the methodological approach. While fiber photometry offers certain advantages, its temporal and spatial resolution remains substantially inferior to single-unit electrophysiology. This methodological constraint potentially obscures the fine-grained heterogeneity in neuronal responses that has been previously documented through more temporally precise recording techniques. The bulk calcium signals captured in this study may homogenize important response diversity within neuronal subpopulations, potentially leading to oversimplified interpretations of circuit dynamics.
The manuscript would benefit from a more measured articulation of its contribution to the field. The suggestion that these findings necessitate a comprehensive reevaluation of VTA functional organization overstates their impact and may mislead readers of a broad-audience journal such as Nature Communications. A recalibration of language to acknowledge the

incremental, though valuable, nature of these results would enhance scientific accuracy.

Minor concerns:
- The abstract's reference to "recording genetically identified DA and GABA neurons" suggests single-cell recording precision, which mischaracterizes the bulk calcium imaging approach employed. This requires immediate correction to represent the methodological constraints accurately.

- Figure 1F presents averaged approach behavior but fails to capture the distribution of response latencies between tone onset and port entry. Including this distribution would provide crucial information about the temporal dynamics of conditioned responding.

Overall:
While the manuscript demonstrates technical competence and the findings are generally supported by the experimental design, the work does not achieve the level of conceptual innovation or methodological advancement required for publication in Nature Communications. The authors have conducted a careful investigation that builds incrementally upon well-established principles of VTA function, but the study would be more appropriately positioned as extending, rather than transforming, current models of midbrain dopamine circuitry.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have satisfactorily responded to my suggestion and I have no further concerns. The revised manuscript is greatly strengthened, and I think this is a nicely executed and really impactful manuscript.

Reviewer #2

(Remarks to the Author)
Thanks to the authors for going above and beyond what was recommended by the reviewers. I have no further comments other than I hope they are proud of the end product. Congratulations on a wonderful piece of work.

Reviewer #3

(Remarks to the Author)
I appreciate the careful consideration of all reviewers' concerns. I have nothing further to add and recommend publication as is. Thank you for your contribution to science.

We write in response to the review of our manuscript “Ventral tegmental area GABA neurons integrate positive and negative valence” for further consideration for publication in *Nature Communications*. There was strong interest in our initial manuscript and reviewers had excellent suggestions for improvement. We have substantially expanded and revised the manuscript along several lines:

1. Several new analyses that expand our initial dataset to offer new insights into the relationship between cue evoked activity in DA vs GABA neurons and behavior - including correlational analyses detailed movement analysis of behavior during the conflict task what show unique patterns of activity in GABA neurons that encode the initiation and termination of reward seeking under conflict.
2. New experimental data showing the effects of expectation violation on GABA neuron activity. These results show that both reward and shock omission engage error signals in GABA neurons. These results add depth to our central conclusions regarding the broader learning role for GABA neurons, and underscore the notion that they are encoding appetitive and aversive learning states.
3. New data from additional mdlx rats to increase the group size in line with reviewer comments and support the conclusion that different subpopulations of GABA neurons are engaged by reward and threat/aversive learning.
4. New data showing VTA GABA neurons are activated by bitter quinine drinking - these results suggest that these neurons are broadly engaged by aversive or unpleasant stimuli, beyond footshock.
5. A new functional optogenetic experiment wherein we demonstrate that brief inhibition of VTA GABA neurons disrupts reward seeking decisions under cost. These new data anchor the central conclusions of the paper, indicating that VTA GABA neurons are engaged to integrate reward and cost, and are functionally important in the generation of appropriate motivation to seek reward in the face of cost.
6. The paper has overall been expanded to a full format article, with 3 new main text figures and new supplemental figures, as well as additional text in every section. In particular related to reviewer comments, we have adjusted framing of the project to better fit in with the existing literature, and added comprehensive discussion of new issues, including those stemming from the new experimental results we show, and also questions of GABA neuron heterogeneity and other topics as suggested by reviewers.

The revised paper now offers deeper insight into our conclusions, and we hope the reviewers will agree the paper is much stronger. In particular, the revisions offer more insights into VTA GABA neuron encoding and function, supporting a framework for conceptualizing this population as key for integrating positive and negative aspects of learning to guide complex decision making. We think these results add significant new insight into several ongoing questions regarding neural encoding and control of motivation in the VTA and will be of great interest to the field. We also think the conflict-based tasks described here offer exciting opportunities for future study of learning and motivated behavioral states, including in disease models.

In light of our substantial revisions, we think this work is of broad interest, and well suited for publication in *Nature Communications*. Detailed responses to reviewer comments are below. Updated or new text in the manuscript file is denoted by **BLUE** coloring. We hope the reviewers agree that the paper is much improved in clarity, depth, and potential impact.

Reviewer #1:

In this manuscript from Stelzner and colleagues, the authors investigate the activity of VTA GABAergic and dopaminergic neurons during reward, aversion, and conflict between the two. They use fiber photometry to measure gCaMP signals in dopaminergic or GABAergic neurons in the VTA They find that dopaminergic and GABAergic neurons show similar responses to rewarding cues and rewards, but that only GABAergic neurons show a robust response to aversive stimuli. Using a motivational conflict task

in which rats face an escalating amplitude of footshock to retrieve a cued reward, they show that in both sets of neurons, the activity signal in response to the reward cue decreases with increasing cost. In dopaminergic neurons the gCaMP signal in response to the reward does not change with cost, but in GABAergic neurons, the activity in response to the reward increases as the cost increases. This is an important study that provides additional evidence in longstanding questions over the role of VTA dopaminergic and GABAergic neurons in reward and learning, and suggests that the two sets of neurons play different roles in these processes. The studies are well designed and controlled, and the data is of high quality. This is very nice work overall and I have only a few suggestions for improvement.

Response: We really appreciate these kind words about our studies, and we thank the reviewer for their great suggestions for improving the paper.

1. The dlx-cre results appear to be taken from an n of 2, this should be replicated to increase the sample size. Alternatively, I do think the key points of this paper stand without this data, so this figure could be removed.

Response: In the revised paper, we have added additional animals to this part of the data. While not a major focus of the paper, these data support the general conclusion that different subpopulations of GABA neurons show responsivity to positive and negative stimuli - and the rapid engagement of positive-going signals to reward indicates they do not act simply in opposition, but rather in parallel, to dopamine neurons. Certainly more work will be required in future studies to break down various sources of heterogeneity, but this part of the data suggests that a broad motivational salience encoding profile is likely a general property of VTA GABA neurons.

2. While there is heterogeneity in dopaminergic neurons in the VTA, this is largely organized along spatial lines. The fiber tracks in this paper appear to all be located in the lateral portion of the VTA. The takeaway of this, for me, is not that the recordings are from a heterogenous population, but that they are likely from a relatively homogenous population that may differ significantly from the responses that would be found from dopaminergic neurons in the more medial portions of the VTA. Brief discussion of this is warranted.

Response: This is an astute point. We targeted the “middle” of the VTA generally, and with the same coordinates for DA and GABA studies. Across all rats, the mean M/L placement was + .75mm, and there were some placements with more medial or lateral positions around that center (range + .5 to 1.1 mm from the midline). In revising the paper, we took a look at whether laterality of placement had any connection to variability in our recorded signals, and found that this was not the case - we did not see any correlation between M/L recording position and cue or outcome-related photometry signals, for either neuron type. We think this is interesting and have noted this detail in the revised paper. Regardless, the reviewer is correct that the recording position is likely important for VTA neural activity profiles, where subregional heterogeneity has been demonstrated in past studies.

3. I also think it is worth mentioning that the downstream effects from GABAergic activity are not necessarily local OR distal, but may encompass both-there’s increasing evidence that projecting GABAergic neurons have local collaterals.

Response: This is another great point, and we completely agree. As part of the overall expansion of the paper in revision, we have added more discussion, references, and context related to this point. We think that this anatomical complexity is likely directly related to the broader encoding profiles that we see for GABA, compared to dopamine. We think that our results ultimately support exciting future studies that can further disentangle the

relative contributions of carefully targeted subtypes, if indeed there is any significant distinction between “local” and “distal” populations.

Reviewer #2:

The manuscript by Stelzner et al, investigates both VTA dopamine and VTA GABA neurons in reward and aversion following emerging data suggesting that these populations have a more nuanced role in this space. To do this they use a combination of pavlovian and conditioned stimulus task with fiber photometry targeting both populations of neurons. This is an elegant study that highlights and corroborates findings in the field suggesting that GABA neurons have the ability to encode reward and aversive stimuli. The findings here suggest that the GABA neurons engage to gate responses to reward and motivation together. This is a timely addition to the field and unique path of investigation. Please see below for feedback and suggestions.

Response: We thank the reviewer for their kind words and enthusiasm about our studies. These are also great suggestions that we have incorporated into the revision to strengthen the paper.

General Points:
Throughout the manuscript there are a lot of acronyms and labels missing that make interpretation for the reader more challenging which adds a level of unnecessary frustration.

In general, the brief description of the results (perhaps this is a word count limitation?), makes the interpretation of the data in the figures quite challenging.

Response: We apologize for the lack of clarity in the manuscript. Overall, in revision the paper has been greatly expanded, and we made several edits in line with this set of comments. In terms of acronyms, without a specific example we were not sure which ones were a problem. We changed ‘PE’ to port entries, and ‘DA’ to dopamine throughout the paper, and have made other edits to clarify labels in the figures. We chose to keep GCaMP, GABA, and VTA as acronyms/abbreviations, as we felt those are very common shorthand in the field.

The results section has been reworked overall, to include all of the new data and analyses, and we have also moved the statistics from the figure captions to the main results text, to improve readability.

Do the authors investigate potential sex differences?

Response: In the original manuscript, we reported no consistent sex differences in behavior across all the tasks (Supplemental figure 2).

Figure 1:
It would be helpful to include a description of what ‘Early, Mid and Late’ refer to in the figure legends or the results to help guide the reader. It might be helpful to include a timeline in the figure as an easy and informative reference guide for the reader as they go through the results.

What is the significance of the early, mid and late timeframes?

In Panel E, x axis label for what early, Mid and late refer to is missing.

Response: Thank you for the suggestions. Overall we have made general edits to the figures and text for clarity and readability. The early mid and late periods in reward conditioning reflect beginning (day 1), middle (days 8

or 9) and end (days 13 or 15) stages of training for each task. We chose to visualize the data this way for simplicity, to illustrate the overall trend of changes in behavior/neural activity. This has been clarified in the revision.

In Panel G and H, the mid timepoint looks to be the same between CS+ and CS- which is quite different to what is observed in Panel O and P, dopamine vs GABA. Can the authors comment on what might be driving this for dopamine group.

Response: We think this is simply because the TH/dopamine group was slightly slower (though not significantly different) from the GAD group in the acquisition of simple reward seeking behavior. The behavioral data split by recording group are shown in Supplemental figure 2 - TH was qualitatively lower in CS+/- discrimination in the middle training phase. This is most likely just due to random cohort variability. Importantly, by late in training, TH and GAD groups were identical in behavior.

Supp Fig 1: In panel A, the images are beautiful, but it would be helpful to have some indications as to what the author would like the reviewers to focus on. If it is simply viral expression in the region of interest, the VTA, a zoomed out image would also be helpful. If it is an example of probe placement with a track outlining, it would be good to highlight that.

Response: Thank you for pointing this out. We have overall updated this set of panels to better illustrate the goal of showing the differential expression fo GCamp in TH+/- neurons across the dopamine and GABA targeted groups. We have included additional histology pictures, including closer zoom images with arrows to indicate neurons showing overlapping expression with/without TH.

Figure 2:
The addition of a timeline to show number of habituation days, testing paradigm, number of days between the first and last day would be helpful to guide the reader through the figure more informatively, rather than toggling through methods, results and figures to piece the information together.

Response: We thank the reviewer again for these great suggestions to improve clarity. We have added additional information regarding training/experimental timelines throughout, to figures, legends, and the main text and general updates along these lines.

The data in figure 2 is compelling though it would be nice to see how these neurons respond to additional aversive events rather than just freezing to really validate that these neurons are broadly 'strongly responsive to aversive events'. Foot shock represents a very strong aversive event. Would these responses look the same for a milder aversive event. Would the scale of responses look the same to a milder non-shock aversive event?

Response: This is an important question. We chose to focus on footshock as this is the standard for assessing aversive/threat/fear learning, and allows us to make the most direct comparisons with the existing literature. It also allows for a connection between the data in the simple aversive/fear conditioning task and the subsequent conflict paradigm. In our task, the physical barrier design, which is a key part of the framework for establishing an approach/avoidance conflict, is best conducted with a shock. But yes, it is important to know to what extent GABA neuron responses are engaged broadly by other types of negative stimuli. Previous studies suggest that there will be broad responsiveness to a variety of aversive events.

What exactly represents a "mild" aversive event to the animal is hard to really know, but we emphasize that we do use relatively mild shocks (.4mA for fear conditioning), at least in the context of the regular fear literature,

which can often include 1.0mA+ shock levels. The conflict task uses 0mA to.4mA - which is well below pain-inducing, and is likely a subthreshold level for directly inducing classic fear learning. So we would argue that the majority of our data reflect assessment of VTA signals in response to relatively mild to medium intensity aversive stimuli.

In the revised paper, we include new data showing that GABA neurons are activated when rats drink a moderate concentration of quinine. This would be considered a relatively mild unpleasant/aversive stimulus, and so this suggests that indeed there is likely a general engagement of VTA GABA neurons in response to an array of negatively valenced stimuli. We definitely think an important component of future studies will be to unpack this possible connection between stimulus intensity and modality further.

What do the authors make of the decrease in response from first to last in VTA GABA neurons? Is there some level of habituation/adaptation to the aversive event. If this shock was repeated at a future time point what would the response look like?

Response: This is an interesting observation. First, it's important to note there was not a statistically significant decrease in shock response on the last day of fear conditioning in the GABA neuron signals, although numerically the average response was slightly lower than the initial response. Primarily, we would therefore not really describe this as a decrease per se, but it is definitely interesting that this signal does seem to be adapting. We think the simplest explanation is shock predictability. Predicted aversive events may be encoded as less aversive, as uncertainty is a component of aversion. Because of the broader project goals to assess the integration of appetitive and aversive states in the conflict task, we did not focus on an exhaustive characterization of possible habituation to shock in the GABA signals.

To try to get at this a bit in our data, we plotted the peak shock signal trial by trial across shock presentations for the GABA neurons. Looking at the data this way, there is a very subtle overall downward trend in the later trials, but we don't think this is that particularly meaningful. Given everything else we show, we would suspect that signals would be similarly strong, or possibly stronger, if shock was given again at a future time point. This could reflect that it has become more surprising again, or that some kind of incubation/adaptation in the system happened. These would be interesting directions to follow up on in future studies.

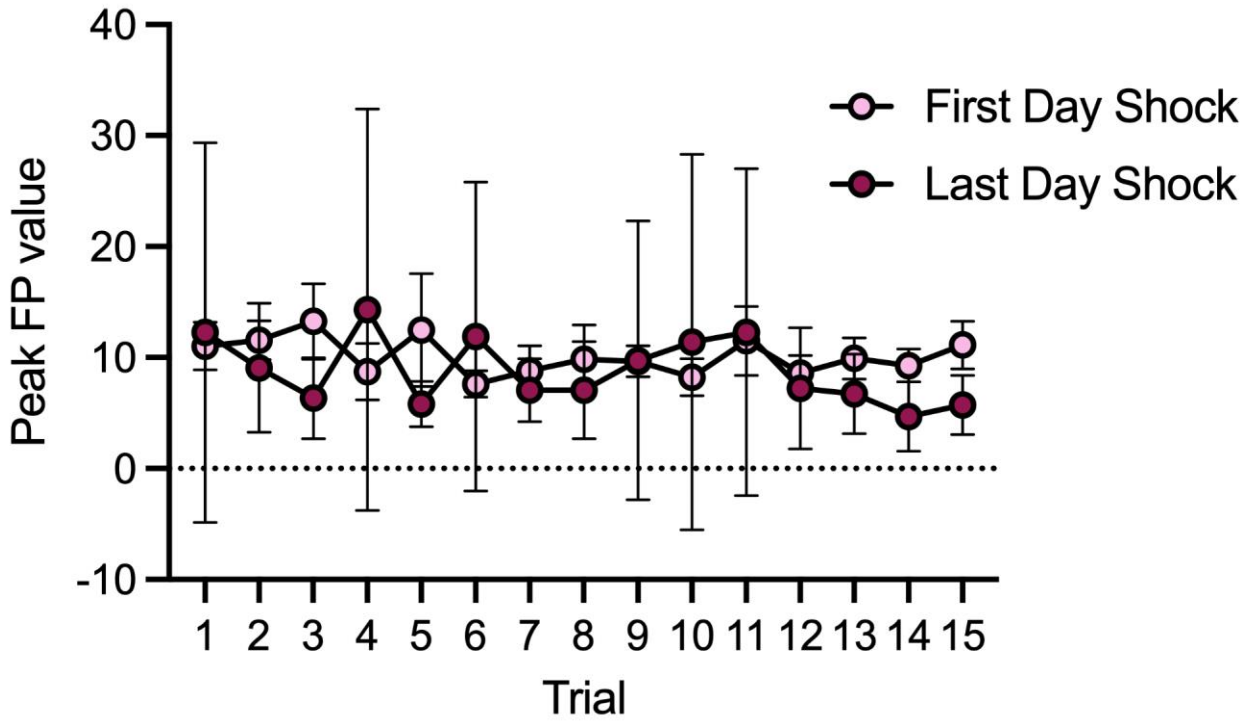

Figure 3:
Panel P: What do the authors make of the GABA response in the absence of a reward? Is this significant between rewarded vs unrewarded at each shock? This was difficult to discern from the figure legend description of stats analysis. What do the authors mean when they refer to phase in the figure legend?

Response: Another interesting observation. There is an overall main effect of the type of port entry (rewarded vs unrewarded) for the signal peak measure. The fact that the unrewarded port entry peak signal scales in parallel with rising cost we think may reflect a feedback or learning signal. Across our data, one theme is that GABA neurons seem to be more generally responsive to violations or surprises - in new data we include in the revision, we see that omission of expected reward or shock engages error signals in the GABA neurons. This fits with the notion that they possibly contribute to a broader learning space than dopamine neurons. In the conflict task, the GABA signal in response to unrewarded port entries may reflect that port entries that don't lead to reward are more costly, and therefore more important for driving updates to behavior - it makes sense in that line of reasoning that the responses reflect more of a transient peak (like a quick error/update) compared to the larger/longer signals in response to reward. A larger GABA signal then might signal that salient state. This interpretation fits when compared to the GABA signals in the reward conditioning task without conflict, where we don't see the same GABA response to unrewarded port entries - port entries in that context are less costly so GABA neuron "feedback" on unrewarded port entries is less important. Our new data, recording GABA neurons during omission of expected reward or shock, also speak to this point.

We cover this notion in the new discussion, including speculating that "Collectively this supports a framework where VTA GABA neurons are more reactive to new information - in this case, the presence or absence of reward - when threat or conflict is present."

In this figure legend, "phase" was meant to refer to the level of cost - we have changed this in the legend to say "shock level" to improve clarity.

—----------

Reviewer #3:

I have carefully reviewed the manuscript by Stelzner et al. examining the functional architecture of VTA microcircuitry in relation to appetitive and aversive conditioning. The study offers an incremental contribution to our understanding of VTA neuronal dynamics, though several concerns limit its suitability for publication in its current form.

The authors' investigation into the differential encoding patterns between dopaminergic and GABAergic VTA neurons represents a meticulous extension of the existing literature. Their confirmation that GABAergic neurons exhibit enhanced responsivity to aversive stimuli and uniquely encode valence information during feedback provides valuable empirical support for emerging computational models of midbrain function. However, the authors' framing of these findings as necessitating "an overall re-evaluation of VTA functional heterogeneity" appears disproportionate to the incremental nature of their contribution.

The manuscript positions itself as challenging prevailing theoretical frameworks. Yet, the results largely align with and amplify the established literature from the Uchida and Watabe-Uchida laboratories over the past decade and a half. The seminal works by Cohen et al. (2012) and Eshel et al. (2015) have already established the foundational architecture of VTA circuitry and delineated the distinctive contributions of DA and GABA neurons to appetitive and aversive learning paradigms. The present study does not fundamentally restructure this conceptual landscape but rather adds nuanced detail to an already richly developed model.

Response: We appreciate these comments and the chance to better explain our results in the context of past work. The data from the Harvard groups mentioned is essential/fundamental work that inspired a lot of ongoing investigation. Indeed, this past work was a direct inspiration for our studies, and we took care to heavily cite that work in this paper. In revision we have added even more discussion of this and other work that has been important for delineating current perspectives on VTA neural heterogeneity. We would pushback though on the suggestion that this earlier data demonstrates the same thing that we show here. The original Cohen and Eshel papers (and others) show that VTA GABA neurons can have excitation to rewards, but it is a very different pattern of activity and relationship to behavior compared to dopamine neurons. Their framework positions GABA neurons specifically within the reward prediction error context - GABA neurons support dopamine neurons to calculate RPEs, rather than directly encoding motivation or driving behavior. A major conclusion of those papers is that GABA neurons do not necessarily directly respond to rewards themselves, but have elevated activity at time of reward because of a ramping profile of activity preceding reward delivery. This was a very influential set of data that has sparked a lot of important work over the past decade. We generally find different patterns of activity in our studies, which anchor the conclusion that VTA GABA neurons can be active at the population level in parallel to dopamine neurons, and are engaged strongly by rewards themselves, rather than just in an expectation framework.

Our results expand this and other work to indicate a unique role for VTA GABA neurons in encoding and promoting reward seeking behavior under cost or motivational conflict. This is not a conclusion borne out of the earlier published data, as we show that VTA GABA neurons contribute their own parallel functions in motivation, being especially tuned to encoding and controlling multi-valent decision states. Our conclusions do not invalidate the past work, but we suggest it is supportive of the broadening framework for VTA neural heterogeneity, which

includes work cited here from other labs. In the revised paper, we include additional new data that further our conclusions and offer new insight. We also present a complete parallel dataset across paradigms in dopamine neuron recordings. In particular, the conflict paradigm results related to dopamine are also completely novel.

In line with our overall major revisions to the paper, we have tried to make these points of distinction clearer, to better get across how our data build on, and extend in important ways, the past literature. We have also added substantial new data to the manuscript that deepen our conclusions and add more functional insight to the role of VTA GABA neurons in motivation. These include, showing that VTA GABA neurons encode error signals for both reward and aversive learning states, and that VTA GABA neurons are necessary for decision making in the face of cost/conflict. Collectively, our results add a number of new insights to the field that we think are of broad interest.

Behaviorally, our conflict task also offers novel insight into cost-related decision making, and we think these studies give opportunities for new ways to interrogate classic learning systems.

A critical limitation concerns the methodological approach. While fiber photometry offers certain advantages, its temporal and spatial resolution remains substantially inferior to single-unit electrophysiology. This methodological constraint potentially obscures the fine-grained heterogeneity in neuronal responses that has been previously documented through more temporally precise recording techniques. The bulk calcium signals captured in this study may homogenize important response diversity within neuronal subpopulations, potentially leading to oversimplified interpretations of circuit dynamics.

Response: We agree that photometry is not the same as single-unit electrophysiology, and we apologize if the reviewer thinks we were trying to confuse readers to that point, as it was not our intention. There are many reasons to use a given technique, from conceptual to practical, and all offer different advantages/disadvantages. As a point of revision, we have overall expanded the paper to include a lot more context for the data, methods, and interpretation, including in the discussion section now mentioning that there are likely finer-level neural activity changes that an approach like photometry may miss, and we now more clearly emphasize that our results reflect population-level signals. So we agree with the reviewer on that point to a certain extent, but also would argue our approach - making use of genetically targeted calcium indicators to measure cell type specific population activity during behavior - is a well accepted approach and this kind of data are regularly published in the top neuroscience journals. Indeed, the Uchida groups cited above regularly publish papers in top journals that primarily rely on photometry data, including recordings of neurons in the VTA, to make conclusions about neural encoding.

Interestingly, the older ephys data cited above actually suggests relative uniformity in encoding profiles for a given VTA neural subtype. That is actually a main conclusion of one of the Eshel papers. There likely are actually a lot of unit-specific encoding profiles, if one were to record from all of them independently. But we would suggest that even though there may be more heterogeneity in neural signals at the unit by unit level, the broad conclusions of our paper do not depend on that outcome - every single GABA or dopamine neuron does not have to have the same activity profile for our conclusions to be true. There are also advantages to assessing a population level signal - one could argue it actually better reflects the cumulative output of a set of neurons, where heterogeneity of single unit responses may actually obscure an more prominent overall pattern. There are also other important methodological differences in our studies and some others, including the lack of head fixation that allows rats to freely move and engage in the learning environment.

The manuscript would benefit from a more measured articulation of its contribution to the field. The suggestion that these findings necessitate a comprehensive reevaluation of VTA functional organization

overstates their impact and may mislead readers of a broad-audience journal such as Nature Communications. A recalibration of language to acknowledge the incremental, though valuable, nature of these results would enhance scientific accuracy.

Response: We apologize for not more clearly getting across the novelty and importance of our studies to the reviewer. As stated, the manuscript has been greatly expanded on many fronts, including substantial new data and analyses. We have also revised with this specific line of critique in mind. We are careful to cite the past work that these studies build on, and suggest that our conclusions add important new insight into emerging updates to the way that the VTA functions. Our results support more interrogation of VTA cell types in more complex multi-valent decision contexts, which also need to be more thoroughly worked into conceptual frameworks of VTA functions. We disagree, however, with the notion that these studies are “incremental”, in the sense that they offer several novel insights into VTA GABA neuron function. Our studies, as is the case for any single paper, do not unilaterally call for a complete change in thinking - and we are careful to never state as such.

Minor concerns:

- The abstract's reference to "recording genetically identified DA and GABA neurons" suggests single-cell recording precision, which mischaracterizes the bulk calcium imaging approach employed. This requires immediate correction to represent the methodological constraints accurately.

Response: We have removed the “genetically identified” from this phrasing to avoid confusion. Although it is not uncommon to refer to the kind of approach we use - viral and transgenic mediated targeting of calcium sensors, as genetically specific.

- Figure 1F presents averaged approach behavior but fails to capture the distribution of response latencies between tone onset and port entry. Including this distribution would provide crucial information about the temporal dynamics of conditioned responding.

Response: This is a great suggestion, we have included port entry latency data in the revised manuscript.

Overall:

While the manuscript demonstrates technical competence and the findings are generally supported by the experimental design, the work does not achieve the level of conceptual innovation or methodological advancement required for publication in Nature Communications. The authors have conducted a careful investigation that builds incrementally upon well-established principles of VTA function, but the study would be more appropriately positioned as extending, rather than transforming, current models of midbrain dopamine circuitry.

Response: We appreciate the opportunity to better explain our results. While we disagree that this work is not sufficiently important enough to warrant publication in Nature Communications, we completely agree that these results build on an important set of existing ideas, as do all studies. We feel strongly that our conclusions regarding the unique contributions of VTA GABA neurons to conflict-related decision making is a novel and exciting set of results, which broaden the conceptual framework for thinking about VTA neural heterogeneity in learning and motivation. In revision, we have nearly doubled the data in the paper - and our new results - showing dynamic error signals in reward and threat learning for VTA GABA neurons, and a functional role for VTA GABA neurons in conflict-based reward seeking - add additional novel insights. Collectively these results add well beyond an incremental advance, and will set up a number of important investigations to further explore VTA control of learning and motivation. We appreciate the reviewer’s commitment to making sure results are not overstated. In revision we have carefully updated the paper to better reflect that, and we hope the reviewer sees these updates as better reflecting the state of the results.