nature portfolio

Peer Review File

Dopaminergic signaling to ventral striatum neurons initiates sniffing behavior

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Johnson et al. highlight the fundamental nature of sniffing behavior, emphasizing its association with olfaction, affective states, and physiological rhythms. It outlines the intricate neural mechanisms underlying sniffing, mainly focusing on the role of the mesolimbic dopamine (DA) system in underlying this behavior. By projecting into regions like the ventral striatum, known for its integration of cortical and limbic circuitry, DA modulates behaviors such as reinforcement, motivation, and – as shown by the authors - sniffing. This paper delves into the mesolimbic DA system's involvement in initiating sniffing behavior, shedding light on its crucial role in this ubiquitous aspect of animal behavior. We were very enthusiastic about reviewing this paper. However, we would like to raise some major and minor points that might be reevaluated by the authors.

Major:

1) Characterizing the causal relationship between DA release and sniffing: Potentially, the most important conclusion of the paper would be proving that “sniffing is initiated by dopamine’s actions upon ventral striatum neurons.” However, there are numerous problems with this conclusion. The cautious language used by the authors suggests that they are aware of these issues, but they should address them more directly:

1A) Under physiological conditions, is striatal DA released before or after the initiation of sniffing? This lacking piece of information is crucial for interpreting other findings. Even faced with synchronization issues, the authors could address this question in a couple of ways, for example: a) show cross-correlation of inhalations vs. DA events, corrected for average lag expected for both signals b) perform this or similar analysis on the calcium data from D1&D2 cells – because the data comes from head-fixed recordings, synchronization shouldn’t be an issue there c) perform extra experiments, for example record electrophysiological activity from striatum.

1B) Are the effects of DA release on sniffing direct (i.e., motor) or mediated through arousal? The data provided by the authors is very consistent with the findings of Li et al. (2023), who demonstrated that the effects of stimulating the DA system on facial movements are relatively slow (an order of magnitude slower than the effects of stimulating the motor cortex). This raises the question of the striatal neurons could indeed evoke sniffing through some well-defined pathway (“even if just bisynaptically”, as suggested by the authors), or whether the observed sniffing is a by-product of an overall increase of global arousal. The authors suggest that only the minor effects observed in the YFP group can be caused by the latter mechanism. If they are correct, the latency of the effect in the YFP group could be expected to be higher than in the ChR2 group – yet this comparison is not shown in Figure 4Gi! (The same comment applies to the whole panel 4H – the legend for YFP is present, but the data is not shown.) It should also be noted that the time course of the stimulation-evoked sniffing, shown in Figure 5D, seems to suggest that the dopaminergic modulation of breathing is too slow to play a role in the initiation of responses to odors (which can be as fast as ~100msec).

1C) Is it possible that striatal DA regulates breathing and not only sniffing rate? The authors should demonstrate if DA levels are also correlated with baseline breathing rate. This applies especially to fiber photometry and pharmacological (blocking D1/D2/D3 receptors) experiments.

To sum up, the authors should address the issues 1A-1C and then, citing the relevant literature, propose what is the most plausible causal relationship between dopamine and sniffing. To summarize a few possibilities: a) DA ---> motor circuits --->

sniffing/ breathing? B) DA ---> arousal ---> breathing? C) sniffing initiation ---> DA ---> further invigoration of sniffing?

2) Explaining discrepancies with the studies of the tail of the striatum. A previous study (Mengas et al., 2018, 10.7554/eLife.21886) demonstrated that only the tail of striatum – but not ventral striatum – is activated in animals exposed to novel odors (which sniff vigorously). This is a clear discrepancy with the results presented here. Why the authors did not record DA from the tail of striatum? This discrepancy should be addressed.

3) Description of sniffing types:
- The phrase “vigor of sniffing” is not entirely clear. Do the authors mean frequency, or does the rate of frequency increase? Maybe it’s better to replace the phrase with a more literal “frequency of sniffing”? Providing a more detailed classification of sniffing types could enhance the clarity and interpretation of the results.

Minor:
Impact of early olfactory regions with neuromodulation: The presence of dopaminergic interneurons in the olfactory bulb and norepinephrine (NE) input from the locus coeruleus to the olfactory bulb suggests potential interactions with the mesolimbic DA and NE system. The changes in the variability of the sniff frequency to a novel odor might already be explained at the olfactory bulb level. At least some information in the discussion would be beneficial for the reader.

Response of dopaminergic neurons to novel stimuli vs. reward: Previous papers mention that some dopamine neurons do not always respond to novel stimuli but consistently respond to reward. These observations might be related to functional subtypes of dopaminergic neurons. It's essential to discuss whether a subset of dopaminergic neurons mediates the observed effects on sniffing behavior.

Accounting for lag in sniffing analysis: The paper subtracts a fixed lag time from the onset of detected respiratory cycles to adjust for the delay between nasal inhalation and plethysmograph detection. However, considering the variability in peak detection during fast sniffing bouts, a single fixed lag (28ms) may not accurately represent the timing error. Further analysis could explore methods to account for variable lag times, especially during rapid sniffing episodes.

Classification of sniffing dynamics: The classification of sniffing dynamics based on different stimuli reveals distinct respiratory patterns. However, questions arise regarding the methods used for comparison and sampling. Even though the number of observations for each class is quite similar (almost all of the classes are around 70), some resampling by keeping the same distribution properties (same sample size) might be beneficial (e.g. SMOTE). In addition, part of the classification analysis concerning buzz stimuli is missing from the results. If authors do not need that comparison, they can consider removing it.

Experiment regarding co-expression of glutamate: The rationale for the VGlutT2fl/fl x TH-Cre experiment is unclear before the discussion. (It’s not explained that the strategy excludes the cells that co-express glutamate.)

DA tonic/phasic release: The statement “This suggests that DA release into the ventral striatum is more so to initiate and possibly invigorate sniffing than to maintain its tonic display.” - would be much more understandable if the authors would mention the fact that DA neurons typically fire in short bursts.

Laser power: In the methods section, the laser power is not mentioned. This is a really important detail given the effects observed in the YFP group.

Reconsideration of the phrase: The phrase “anterograde AAV expressing” is misleading in many cases, as the authors use local (somatic) expression.

Color scale: The heatmaps on Fig. 5 require adjusting scale – currently, they are just blue and provide no information.

Reviewer #2

(Remarks to the Author)
The authors carefully measured the relationship between dopamine release into the ventral striatum and sniffing in mice. They found that dopamine release was strongly correlated with sniffing and proceeded to show that dopamine terminal activation could drive sniffing. They also recorded from D1 and D2 striatal neurons, showing that their activity was also strongly correlated with sniffing - and that antagonists reduced sniffing.

This work is well done and will be of interest to a broad audience of neuroscientists. However, I think that the introduction and discussion of the results focuses too heavily on sniffing itself. As the authors note, "rodents, dogs, and even semi-aquatic vertebrates increase their sniffing frequency while foraging for food". Notably, sniffing is not a very important way that humans direct their attention, learn how to solve new problems, investigate new situations, or shape their social interactions. Instead, humans rely more on their visual system and auditory system (language).

But, since mice are the most important model organism for understanding the mechanisms underlying these processes (learning, memory, attention, social interaction) and learning about diseases that affect these processes - we must have a strong understanding of what sensory information the mouse uses, how the mouse directs its' attention and learns, and how the mouse selects actions.

Because olfaction is so crucial for mice when learning, performing social interactions, or doing any task - it is crucial that we understand the systems governing the intake of olfactory information. In this way, the authors have made a very nice contribution to the field. I believe that the discussion should focus more heavily on this aspect - and also discuss how their results could be analogous to dopamine guiding attention in other sensory domains in humans (which do not rely as heavily on olfactory cues).

Experimentally, I believe that the core claims being made by the authors are well supported by the data. However, I would suggest avoiding over-interpreting effects with marginal significance if they are not critical to the main conclusions. For example, I would suggest removing the * asterisk in figure 3I indicating a significant difference between response to the buzz between TuS and NAcSh since the p value is only 0.01 and there are multiple comparisons being made with a relatively low sample size. Whether or not there is a real difference between these two regions matters very little to the overall conclusions that are being drawn related to these regions' relation to sniffing. The effect size and p values for the statistical tests needed to support the core claims in the paper are convincing.

Two key measurements for this study are the measurement of sniffing and the photometry measurement. In figure 1, there is a nice quantification of the number of labeled puncta in each region of each animal. This gives confidence that the photometry measurement is consistent across mice. However, figure 2 shows mostly example traces of the sniff detection quality. The traces from the example mouse show an excellent correlation between intranasal cannula pressure recording and plethysmograph pressure recording. I think this figure would benefit from a quantification of the correlation between these measures across a few mice to demonstrate consistency of the measurement. This could be data from a separate set of mice, since it is only meant to show the reliability of the measurement.

In terms of methodology, the authors use standard methods for the field. They do note in their discussion that the ChR2 paradigm likely causes "DA release onto postsynaptic neurons in supraphysiological manners". However, this is a well known and understood aspect of this type of experiment. The authors do a good job of limiting their interpretation to saying that stimulation of these DA terminals is sufficient to initiate sniffing. It would be very interesting to include any data related to titrating the light delivery in these experiments, or any data showing the range in naturally evoked DA signal in different experimental conditions if available. But I don't think these sorts of experiments are essential to add if the data doesn't already exist.

The methods section of the paper provides enough detail that the experiments could be precisely repeated, and I have no concerns or suggestions for that section.

Reviewer #3

(Remarks to the Author)
The authors performed a set of experiments designed to test the role of ventral striatal dopamine in sniffing responses to novel odors. By performing fiber photometry in conjunction with whole body plethysmography, they show that novel odors promote both sniffing and dopamine release in the olfactory/tubular striatum (TuS) and nucleus accumbens shell regions (NAcS), which could be recapitulated by optogenetic stimulation of ventral tegmental area (VTA) dopaminergic neuron terminals in these loci. Novel olfactory stimuli also increased the activity of both D1 and D2 striatal medium spiny neurons (MSNs) that receive dopamine input, and pharmacological blockade of these receptors in the NAcS or TuS attenuates different aspects of odor-induced sniffing. The authors conclude that odor-evoked dopamine release acting through D1 and D2 MSNs is a critical neurobiological mechanism mediating sniffing. While the manuscript is generally well written and methodologically sound, there are a few significant weaknesses that could be addressed in a revision.

First, the authors don’t specifically state what odorants were used as ‘novel stimuli’ in the body of the manuscript (although some information is in the methods). This is problematic because dopamine may encode the stimulus valence (e.g. aversive vs. appetitive) independent of sniffing. Providing these details will be critical for full interpretation of the results.

Second, there are inconsistencies in the methods used. For example, the authors perform photometry in freely moving mice in some experiments but head-fixed mice in others, which is partly to reduce any confounds caused by motion artifact. This is an appropriate rationale for head fixation, however no dead sensor or fluorophore control was used in freely moving mice, making it impossible to tell if data collected in these experiments were influenced by animal movement. Analysis of raw data from the isosbestic photometry channel would probably alleviate this concern, but it is my belief that at least some subset of the experiments should be performed with inert sensor controls. A second example of inconsistent methodology is the use of Dat-Cre mice in some experiments but Th-Cre mice in others. There are well-described differences in the populations of

neurons targeted between the two lines, so why change the genetic strategy for targeting dopaminergic neurons between experiments?

Third, the authors make the point that dopamine release is critical for the initiation of sniffing but not its duration (Figure 4). A more thorough testing of this hypothesis – in my opinion – would involve optogenetic inhibition of dopaminergic terminals precisely at the onset of a spontaneous sniff bout to see if it delays sniffing onset, reduces total bout length, or blocks the bout altogether. While the D1 and D2 receptor antagonist experiments provide some evidence that ventral striatal dopamine is necessary for sniffing, the temporal kinetics of the manipulation is quite long and affects both between tonic and phasic modes of dopamine release.

Additionally, I have included a more comprehensive list of comments and concerns below that the authors may want to consider if they revise their manuscript:

In the introduction, the discussion of spinal circuits that orchestrate sniffing is relatively superficial. It may be helpful to provide more detail if the authors feel that background knowledge of respiratory circuitry is required to contextualize the experimental question explored in the manuscript.

Similarly, the stated rationale for studying the role of the ventral striatum is not as clear or strong as it could be. I would recommend revising the final paragraph of the introduction to make it more compelling.

In the Results, it is stated that it is “well known especially in rats that the ventral striatum receives dense input from mesolimbic dopamine neurons…” However, dopaminergic projection targets, axonal arborization, and monosynaptic inputs have been thoroughly studied in mice (e.g. Beier et al., Cell, 2015). Likewise, one of the citations used to support this statement (30) was a study performed in C57Bl/6J mice. Although the tracing data in Figure 1 is well done and supports the use of the recording sites chosen, it is likely confirmatory and not necessarily novel. It would be helpful to the reader if the authors acknowledged similar previous work and stated if the current findings deviate from those studies in a meaningful way.

In Figure 2Bi and 2Biii, the authors state that their approach can distinguish between basal breathing and high frequency sniffing bouts. They then provide quantitative details establishing their criteria for identifying a ‘sniff bout’. Their approach seems sound based on provided references; however, I am concerned that only relying on plethysmography data to identify sniff bouts does not capture other motor-related dopamine signals (e.g. head rotation or changes in body position) or provide a visual ‘sanity check’ to confirm that purposeful odor sampling is occurring (instead of anxiety-related increases in respiratory rate). While not necessary for revision, it might be helpful to video record a subset of animals to capture more information about the behavior being studied (e.g. with pose estimation algorithms). This would both validate the approach and provide a more feature-rich dataset that could be correlated with the GRAB-DA data.

In Figure 3, it is not clear from the text or the caption which odorants were used as a ‘novel’ odor. Were all three odorants listed in the Methods used? Or just one? If multiple, in what order were they delivered? Why were these specific compounds chosen? These are critical details, as dopamine responses could represent the valence of the sensory stimulus, their novelty, etc. Furthermore, at the end of the paragraph associated with Figure 3, the authors state that “these results indicate that sniffing corresponds with increases in DA release in two specific sub-regions of the ventral striatum, and that the level of DA correlates to the vigor of sniffing.” I still find these correlations hard to interpret, as the experiments presented do not really explore whether the dopamine response to odor could reflect sensory stimulus novelty (i.e. is dopaminergic habituation a function of sensory prediction error encoding?) or its hedonic value (i.e. does sniffing-related dopamine release only occur when the odor is appetitive? Or aversive?).

This is a minor point, but the authors refer to injection of an ‘anterograde AAV’. To me, this means they will use an AAV that will transduce downstream neurons when injected into an upstream, synaptically connected site (e.g. the work of Zhang and others using AAV1). Since the authors are performing terminal activation with an AAV5 ChR2 viral construct, I’d probably delete the word ‘anterograde’ to avoid confusion.

Significant changes in sniffing and dopamine release was observed in YFP control mice during optogenetic stimulation in Figure 4. While this could be due changes in arousal state secondary to laser light leakage from the fiber ferrule, light stimuli that are barely perceptible to humans can evoke dopamine release in the ventral striatum (see Gonzalez et al., eLife, 2023). If dopamine release in the ventral striatum drives sniffing, then that could explain the response seen in YFP controls. Additionally, what was the laser power used for optogenetic stimulation (I didn’t see it in the Methods)? This is relevant given the possibility of heating artifacts. Overall, additional discussion about the results in 4D and E is probably warranted.

In Supplementary Figure 3, why was the Th-Cre mouse line used instead of the Dat-Cre mouse line? Given the controversy surrounding the use of the Th-Cre mouse to study VTA dopamine neurons (see Lammel et al., Neuron, 2016), this seems like a strange choice of mouse line. While it likely does not change the results of the experiment, the inconsistency is worth noting and, perhaps, justifying.

Regarding the experiments in Figure 4H, the authors state that the results of their optogenetic excitation experiments “indicate that DA release into the ventral striatum initiates sniffing, but that it does not persistently driving a sniff bout.” I’m of the opinion that you can’t fully substantiate this claim without optogenetically inhibiting dopamine release precisely at the onset of a spontaneous sniff bout to show that transiently reducing dopamine release causally suppresses sniffing behavior.

In Figure 6, why was head-fixation necessary to limit motion artifacts for photometry experiments, whereas it wasn't for Figures 2 and 3? This begs the question: if motion artifacts were truly a concern, why didn't the authors use a dead sensor control for GRAB-DA experiments? Or a GFP control for the GCaMP experiments? These concerns may be addressable by analysis of the raw isosbestic signal during freely moving behavior. However, repeating at least a subset of the photometry with an inert sensor control would probably strengthen the robustness of the approach.

Also in Figure 6, which odorants were used as the 'novel odors'? Without knowing this, it's impossible to fully interpret changes in the dopaminergic response to these odors, especially if those odors are pleasurable, aversive, or neutral.

Reviewer #4

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
We appreciate the authors' thorough revisions. The new experiments and analyses have greatly improved the manuscript, especially in clarifying the relationship between sniffing, dopamine, and neuron activity. The added figures and updated text are beneficial. However, we have a few minor suggestions for further enhancing the single-unit data presentation. Specifically, we have some comments regarding analyses shown on the following panels:
Figure panel 7B:
- It is unclear if the data comes from 'Odor' or 'Spontaneous' periods.
- We suggest removing the spike waveforms from the plot, especially because they come from a previously published data set.
- Instead, we propose to show some standard peri-event time histograms (PSTH). The accompanying analysis clearly shows that most spikes are emitted after the initiation of sniffing (which is expected). Adding PSTH would allow the readers to understand the dynamics of neuronal activity better.
Figure panel 7C:
- Please present this panel before 7B. Now, the order of panels does not correspond to the main text, which is very confusing (breathing rate is discussed in the text first, but in the figure, it is shown only after some spike analysis).
Figure panel 7D:
- The main text says: 'Among these, they were more likely to fire within a short respiratory cycle during the inter-trial interval when the background respiratory rate is slower (Fig 7D, mixed ANOVA, $F(3, 76)=31.48$, $p<0.0001$).' This sentence is confusing for several reasons: a) Did the authors mean faster, not slower? b) Does the 'inter-trial interval' refer simply to the 'Spontaneous' periods? If yes, we would suggest sticking to the same language in the main text and figures for clarity.
- The fact that the distribution of spiking relative to breathing frequency is completely flat during 'Odor' periods is very interesting. The authors explain this observation simply as an effect of insufficient trials with low-frequency breathing (which, by the way, could probably be tested by plotting scatter plots with individual trials). However, an alternative explanation - supported by the results of inhibition of the DA terminals - would be that odor-evoked sniffing is controlled by slightly different mechanisms than the spontaneous one. Do the authors find such an interpretation plausible?
Figure panel 7E:
- In the 'Odor' condition, there seems to be a trend for increasing breathing frequency already during the first spikes. From our experience, normalizing breathing frequency by simply subtracting it from each trial's baseline is a great way to improve the power of statistical tests (otherwise, there is much variability coming from differences between animals). Would the results remain the same after such a normalization?

Reviewer #2

(Remarks to the Author)
The authors have done a good job of addressing the comments raised by the reviewers, and I think the paper is now ready for publication.

Reviewer #3

(Remarks to the Author)
The authors have sufficiently addressed my concerns, and I recommend publication of the paper at this time. Nice job!

Reviewer #4

(Remarks to the Author)

Response to reviewers

We thank the reviewers for their time and consideration of this manuscript and we appreciate their constructive feedback. We have incorporated all of their suggestions as outlined below and as indicated throughout our revised text in red font. This included performing additional fiber photometry experiments to address concerns regarding possible artifacts in the sniff-evoked $GRAB_{DA}$ signal, bilateral optical inhibition of dopamine (DA) terminals in the NAc and TuS, and also extensive analyses of existing and new data sets to better describe the relationship between sniffing, DA, and ventral striatum neuron activity. In addition to revisions to some figure panels, there are 6 new figures included in this revision (new Figs 6 & 7, and new Supplementary Figs 2, 3, 4, and 7). Due to this we note the figure numbering is different from in the original submission.

The reviewer comments helped to strengthen this manuscript. Again, we thank the reviewers for their time in providing thoughtful comments.

Reviewer #1

Johnson et al. highlight the fundamental nature of sniffing behavior, emphasizing its association with olfaction, affective states, and physiological rhythms. It outlines the intricate neural mechanisms underlying sniffing, mainly focusing on the role of the mesolimbic dopamine (DA) system in underlying this behavior. By projecting into regions like the ventral striatum, known for its integration of cortical and limbic circuitry, DA modulates behaviors such as reinforcement, motivation, and – as shown by the authors - sniffing. This paper delves into the mesolimbic DA system's involvement in initiating sniffing behavior, shedding light on its crucial role in this ubiquitous aspect of animal behavior. We were very enthusiastic about reviewing this paper. However, we would like to raise some major and minor points that might be reevaluated by the authors.

We thank this reviewer for their enthusiasm and thoughtful critiques. We have addressed their points as outlined below.

Major:

1) Characterizing the causal relationship between DA release and sniffing: Potentially, the most important conclusion of the paper would be proving that “sniffing is initiated by dopamine’s actions upon ventral striatum neurons.” However, there are numerous problems with this conclusion. The cautious language used by the authors suggests that they are aware of these issues, but they should address them more directly:

While the optogenetic stimulation results in **Figures 4 & 5** do indeed support that conclusion noted above since a key finding is that optical stimulation of DAergic terminals evokes sniffing, we entirely agree that the following questions are important and have addressed them in several ways described below.

1A) Under physiological conditions, is striatal DA released before or after the initiation of sniffing? This lacking piece of information is crucial for interpreting other findings. Even faced with synchronization issues, the authors could address this question in a couple of ways, for example: a) show cross-correlation of inhalations vs. DA events, corrected for average lag expected for both signals b) perform this or similar analysis on the calcium data from D1&D2 cells – because the data

comes from head-fixed recordings, synchronization shouldn't be an issue there c) perform extra experiments, for example record electrophysiological activity from striatum.

We have included several additional analyses to examine this:

First, we neglected to provide a long time-scale example of sniffing behavior in relation to DA levels while an animal spontaneously transitions in and out of sniffing, as well as when delivered odors and the buzz stimulus. We now provide this as new **Supplementary Fig 2** and mention this in Results and Discussion. We believe this will help readers see the relationship between DA levels and sniffing. One point we make in this new Figure (which was already evident in our correlation scatter plots (e.g., **Fig 2Fii and Gii**) is that not always will DA detectably rise when animals engage in sniffing. While in most cases DA does rise with sniffing, and indeed these two are positively correlated in most of our analyses as in the original submission, we think it will be useful for readers to recognize it is not a 1:1 predictive relationship and we now Discuss why this may be (see second to last paragraph on Page 30-31).

Next, using a single-unit data set wherein we also recorded intranasal respiration in awake mice (Gadziola et al 2015, JNeurosci), we were able to better capture temporal dynamics between sniffing and ventral striatum neural activity (though without knowing cell type in ventral striatum as we did in Fig 8). As shown in new **Figure 7**, we found that spiking of neurons coincides with sniffing (either a single fast respiratory cycle or a bout of sniffing) to the point that when a spike is detected, that is associated with an escalation in the frequency of the next breath (**Fig 7Ei**). This suggests that spiking in the ventral striatum may be a component of an 'detection circuit' which through known pathways between the ventral striatum and VTA, could feedback on the VTA to influence subsequent DA release upon the ventral striatum, therefore initiating vigorous sniffing. We discuss this in our revised discussion and also note important future experiments which need to be done to pinpoint the major components of the circuitry which certainly spans beyond the TuS, NAc, and VTA (e.g., frontal cortex, motor cortex, reticular system subregions, *etc*). Please see our response to this reviewer's point 1D below.

1B) Are the effects of DA release on sniffing direct (i.e., motor) or mediated through arousal? The data provided by the authors is very consistent with the findings of Li et al. (2023), who demonstrated that the effects of stimulating the DA system on facial movements are relatively slow (an order of magnitude slower than the effects of stimulating the motor cortex). This raises the question of the striatal neurons could indeed evoke sniffing through some well-defined pathway ("even if just bisynaptically", as suggested by the authors), or whether the observed sniffing is a by-product of an overall increase of global arousal. The authors suggest that only the minor effects observed in the YFP group can be caused by the latter mechanism. If they are correct, the latency of the effect in the YFP group could be expected to be higher than in the ChR2 group – yet this comparison is not shown in Figure 4Gi! (The same comment applies to the whole panel 4H – the legend for YFP is present, but the data is not shown.)

We now include additional analyses of optically-evoked sniffing latencies for our EYFP controls (see lines 289-293) and have updated **Figure 4Gi** accordingly. We found that sniffing upon opto-stimulation of VTA DAT terminals occurred on average ~260-280ms (across ventral striatum subregions) following stimulation onset, yet with some mice showing latencies as low as ~200ms. EYFP control mice show latencies on average ~339ms. Our reported latency of 260-280ms in ChR2-treated mice is more rapid than that reported by Li and colleagues wherein the latency to facial motor movements was ~400ms. These results further support that the minor effects observed in our EYFP

controls is a byproduct of global arousal, though also see the interesting alternative explanation regarding optically/visually-evoked DA release as raised by Reviewer 3 which we also incorporate into our revision.

Please note that in **Figure 4Gi**, less data contributed to the EYFP group since only control animals that reached 6Hz on >40% of trials (as quantified in **Figure 4Giv**) were included to give a more accurate comparison of latency. The excluded mice simply did not display a comparable number of sniffing bouts as the DA-evoked sniffing groups. We include note of this in our figure legend. We have also included the EYFP data for the panel insets in **Figure 4H**.

It should also be noted that the time course of the stimulation-evoked sniffing, shown in Figure 5D, seems to suggest that the dopaminergic modulation of breathing is too slow to play a role in the initiation of responses to odors (which can be as fast as ~100msec).

We note this in our revised Discussion (second paragraph).

1C) Is it possible that striatal DA regulates breathing and not only sniffing rate? The authors should demonstrate if DA levels are also correlated with baseline breathing rate. This applies especially to fiber photometry and pharmacological (blocking D1/D2/D3 receptors) experiments.

DAergic antagonism, like that used in some main-stay anti-psychotics, certainly can drive a sedative state and that should impact respiration by reducing its frequency among possibly other manners. We have included new analyses to address these questions. In new **Supplementary Figure 3B**, we analyzed whether pharmacologically antagonizing D1, D2, and D3 receptors influences the distributions of baseline respiratory rates (*i.e.,* those breaths far removed from any stimulus deliveries) compared to the within-subject vehicle treatment days. From this we found that there is a slight reduction in the occurrence of higher frequency breaths when D1 and D2 receptors are antagonized, which allowed for more low frequency breaths (~4Hz) to occur especially in the D2 antagonized group. The effect of D2 antagonism was significant only in the NAcSh (Wilcoxon signed-rank test W=-279, p=0.012) which we now report in our results.

As shown in **Supplementary Figure 3A**, we also analyzed the $GRAB_{DA}$ signals relative to baseline breaths (slow respiration between 2-4Hz), and found no change in $\Delta F/F$ averaged across baseline breaths. In **Figure 2Ei** we also now include an example trace of $GRAB_{DA}$ during baseline breathing and lack of coupling with respiration is evident – and especially no single transient during that phase compares in the amplitude to that during sniffing (**Fig 2Eii**).

1D) To sum up, the authors should address the issues 1A-1C and then, citing the relevant literature, propose what is the most plausible causal relationship between dopamine and sniffing. To summarize a few possibilities: a) DA ---> motor circuits ---> sniffing/ breathing? B) DA ---> arousal ---> breathing? C) sniffing initiation ---> DA ---> further invigoration of sniffing?

We have more elaborated upon the plausible causal relationship in our revised discussion (see second to last paragraph). Also, considering that future literature will build upon this work to show additional circuits whereby DA can initiate sniffing, we have modified our primary conclusion that “sniffing IS initiated by dopamine’s actions on ventral striatum neurons” to read “sniffing CAN BE initiated by…” to leave open possibilities for descending DA and other pathways to play roles in

sniffing without presenting too rigid of statement to the field.

2) Explaining discrepancies with the studies of the tail of the striatum. A previous study (Mengas et al., 2018, 10.7554/eLife.21886) demonstrated that only the tail of striatum – but not ventral striatum – is activated in animals exposed to novel odors (which sniff vigorously). This is a clear discrepancy with the results presented here. Why the authors did not record DA from the tail of striatum? This discrepancy should be addressed.

This is an important point. The ventral striatum DA recordings performed by Menegas and colleagues in their interesting manuscript were largely acquired from the NAcCore, which as we also report, do not display elevations in $GRAB_{DA}$ upon sniffing. Our findings are therefore similar to what was reported by Menegas and colleagues.

We now incorporate in the discussion (line 588 - 596): "*Previous work from Menegas and colleagues reported that DA is only released in the tail of the striatum upon novel odor presentation (which likely would entail sniffing), with negligible changes in DA levels seen in the ventral striatum[32]. It is interesting to note that in their study, ventral striatum recordings were largely acquired from the NAcC, wherein we similarly did not see increases in DA levels. Furthermore, the results of Menegas and colleagues even suggest subtle reductions in DA levels, which agrees with our current findings from the NAcC. We expand upon this work by investigating DA dynamics across all three ventral striatal regions which include the NAcC, NAcSh, and TuS while animals sniff.*"

3) Description of sniffing types:
- The phrase "vigor of sniffing" is not entirely clear. Do the authors mean frequency, or does the rate of frequency increase? Maybe it's better to replace the phrase with a more literal "frequency of sniffing"? Providing a more detailed classification of sniffing types could enhance the clarity and interpretation of the results.

We agree that a clearer explanation will enhance the clarity and interpretation of our results to readers. We have changed all mentions of "vigor of sniffing" and "sniffing vigor" to "frequency of sniffing" or "sniffing frequency" as suggested.

Minor:

4. Impact of early olfactory regions with neuromodulation: The presence of dopaminergic interneurons in the olfactory bulb and norepinephrine (NE) input from the locus coeruleus to the olfactory bulb suggests potential interactions with the mesolimbic DA and NE system. The changes in the variability of the sniff frequency to a novel odor might already be explained at the olfactory bulb level. At least some information in the discussion would be beneficial for the reader.

We now discuss that interesting point in our revised Discussion, see second paragraph (Page 26).

5. Response of dopaminergic neurons to novel stimuli vs. reward: Previous papers mention that some dopamine neurons do not always respond to novel stimuli but consistently respond to reward. These observations might be related to functional subtypes of dopaminergic neurons. It's essential to discuss whether a subset of dopaminergic neurons mediates the observed effects on sniffing behavior.

We now discuss that, "*Furthermore, different DA neuron populations in the VTA display heterogenous responses to novelty vs. reward[32,81,92,93], which might stem from functional subtypes of DAergic neurons. It will be important to investigate the differing VTA neuron populations which may underly these subregion-specific differences in DA dynamics in the ventral striatum during sniffing"* in lines 609-613.

6. Accounting for lag in sniffing analysis: The paper subtracts a fixed lag time from the onset of detected respiratory cycles to adjust for the delay between nasal inhalation and plethysmograph detection. However, considering the variability in peak detection during fast sniffing bouts, a single fixed lag (28ms) may not accurately represent the timing error. Further analysis could explore methods to account for variable lag times, especially during rapid sniffing episodes.

First, we want to highlight that as requested by reviewer 2 (See comment #3), we have bolstered these analyses by collecting data from an additional 2 mice and have updated **Figure 2Biii** to include these data. Directly to this reviewer's point here, we fully agree and appreciate this being mentioned. We found that during periods where mice rapidly transitioned in and out of sniffing, the plethysmograph-intranasal lag was on average 28-36ms (across-mouse range). During resting or baseline breathing (2-4Hz in a mouse), the lag increases to 53-100ms (across mouse range). We have incorporated this longer lag in our newly included analyses of DA levels relative to resting respiration (as requested by this Reviewer, see comment 1C). Since our identification of the onset of sniffing bouts for the photometry analyses utilized the first short duration respiratory cycle (the first 'fast sniff'), we want to note that this would be the first respiration that was encountered with the shorter lag so that does not impact how the data should be calculated from how it was done in our original submission. Notably, in order to not misrepresent a shorter than real latency from sniffing onset to DA flux, we have selected to keep the lag constant as 28ms and not use the longer lag as a constant (36ms).

7. Classification of sniffing dynamics: The classification of sniffing dynamics based on different stimuli reveals distinct respiratory patterns. However, questions arise regarding the methods used for comparison and sampling. Even though the number of observations for each class is quite similar (almost all of the classes are around 70), some resampling by keeping the same distribution properties (same sample size) might be beneficial (e.g. SMOTE). In addition, part of the classification analysis concerning buzz stimuli is missing from the results. If authors do not need that comparison, they can consider removing it.

We have addressed this in two ways and updated **Figure 5** accordingly as well as our results section (lines 361-373). First, we performed SMOTE to 'bootstrap' the groups to have equal numbers of samples and found similarly large classification of opto-evoked sniffing compared to all other types of sniffing. Second, we reran the classifier with incrementally reduced samples (60, 40) in all sniff-type groups and found that, even with as little as 40 samples per group, the classifier discriminated opto-evoked sniffing compared to all other sniffing types. Classifier performance in the SMOTE data set was in fact perfect. This supports our original conclusion that DA-evoked sniffing is a distinct respiratory pattern. We also have included analyses concerning the buzz-evoked sniffing as requested.

8. Experiment regarding co-expression of glutamate: The rationale for the VGlutT2fl/fl x TH-Cre experiment is unclear before the discussion. (It's not explained that the strategy excludes the cells that co-express glutamate.)

We have updated the results to better explain rationale (lines 314-316) to now read, *"Since VTA neurons are known to co-release glutamate in addition to dopamine[26,39,40], and knowing that ventral striatum neurons express glutamatergic receptors, we next sought to isolate the influence of the above effect (**Fig 4**) on DA versus glutamate. We crossed VGluT2 fl/fl mice with mice expressing Cre recombinase in all cells expressing tyrosine hydroxylase (TH-Cre), the rate-limiting enzyme upstream for DA synthesis. This approach ensures that VGluT2 is lacking from tyrosine hydroxylase expressing neurons, thus eliminating the possibility of glutamate co-release from TH+ neurons."*

9. DA tonic/phasic release: The statement "This suggests that DA release into the ventral striatum is more so to initiate and possibly invigorate sniffing than to maintain its tonic display." - would be much more understandable if the authors would mention the fact that DA neurons typically fire in short bursts.

Thank you for this mention. We now state, *"Taking into account that VTA DAergic neurons fire in characteristic phasic manners (Schultz et al., 1997; Grace & Bunney, 1984), this suggests that DA release into the ventral striatum is more so to initiate and possibly invigorate sniffing than to maintain its tonic display."*

10. Laser power: In the methods section, the laser power is not mentioned. This is a really important detail given the effects observed in the YFP group.

Thank you for raising this important point - we have included the laser power in the methods.

11. Reconsideration of the phrase: The phrase "anterograde AAV expressing" is misleading in many cases, as the authors use local (somatic) expression.

We have eliminated our use of the word "anterograde" when referencing local AAV expression.

12. Color scale: The heatmaps on Fig. 5 require adjusting scale – currently, they are just blue and provide no information.

We agree that at the current scale, in that one figure wherein mice are sedated with urethane, that they provide little information into the frequency dynamics within those panels. This was intentional though since it allows readers to compare those panels to the panels wherein the mice are awake so the reader can see the substantial lack of any modulation in breathing rates under anesthesia. Keeping the current color scape allows readers to easily compare these data to the heatmaps in **Figures 4C and D** and with this we kindly ask the reviewer to allow this remain as is. We have added a note into the figure legend of **Figure 5B** to state*, "Color scale matches that of **Figures 4C and D**. Little to no change in coloring highlights the substantial lack of any modulation in breathing rate under anesthesia."*

Reviewer #2

The authors carefully measured the relationship between dopamine release into the ventral striatum and sniffing in mice. They found that dopamine release was strongly correlated with sniffing and proceeded to show that dopamine terminal activation could drive sniffing. They also recorded from D1 and D2 striatal neurons, showing that their activity was also strongly correlated with sniffing - and that antagonists reduced sniffing.

This work is well done and will be of interest to a broad audience of neuroscientists. However, I think

that the introduction and discussion of the results focuses too heavily on sniffing itself. As the authors note, "rodents, dogs, and even semi-aquatic vertebrates increase their sniffing frequency while foraging for food". Notably, sniffing is not a very important way that humans direct their attention, learn how to solve new problems, investigate new situations, or shape their social interactions. Instead, humans rely more on their visual system and auditory system (language).

But, since mice are the most important model organism for understanding the mechanisms underlying these processes (learning, memory, attention, social interaction) and learning about diseases that affect these processes - we must have a strong understanding of what sensory information the mouse uses, how the mouse directs its' attention and learns, and how the mouse selects actions.

1. Because olfaction is so crucial for mice when learning, performing social interactions, or doing any task - it is crucial that we understand the systems governing the intake of olfactory information. In this way, the authors have made a very nice contribution to the field. I believe that the discussion should focus more heavily on this aspect - and also discuss how their results could be analogous to dopamine guiding attention in other sensory domains in humans (which do not rely as heavily on olfactory cues).

We fully agree and have elaborated more on the possible importance of this circuit in context of olfaction within both the first and last paragraphs of the Discussion.

2. Experimentally, I believe that the core claims being made by the authors are well supported by the data. However, I would suggest avoiding over-interpreting effects with marginal significance if they are not critical to the main conclusions. For example, I would suggest removing the * asterisk in figure 3I indicating a significant difference between response to the buzz between TuS and NAcSh since the p value is only 0.01 and there are multiple comparisons being made with a relatively low sample size. Whether or not there is a real difference between these two regions matters very little to the overall conclusions that are being drawn related to these regions' relation to sniffing. The effect size and p values for the statistical tests needed to support the core claims in the paper are convincing.

In our original submission we used a 2-way ANOVA for these comparisons instead of repeated measures ANOVA which would had been more appropriate. We have corrected this in **Figure 3H** and have also removed the comparison in question and removed reference of the buzz in reference to **Figure 3H** in the text. We believe now that each of the core claims of the paper are supported by compelling and correct statistical tests.

3. Two key measurements for this study are the measurement of sniffing and the photometry measurement. In figure 1, there is a nice quantification of the number of labeled puncta in each region of each animal. This gives confidence that the photometry measurement is consistent across mice. However, figure 2 shows mostly example traces of the sniff detection quality. The traces from the example mouse show an excellent correlation between intranasal cannula pressure recording and plethysmograph pressure recording. I think this figure would benefit from a quantification of the correlation between these measures across a few mice to demonstrate consistency of the measurement. This could be data from a separate set of mice, since it is only meant to show the reliability of the measurement.

We agree. Please see our response to Reviewer 1, concern #6 who raised a similar point.

4. In terms of methodology, the authors use standard methods for the field. They do note in their

discussion that the ChR2 paradigm likely causes "DA release onto postsynaptic neurons in supraphysiological manners". However, this is a well-known and understood aspect of this type of experiment. The authors do a good job of limiting their interpretation to saying that stimulation of these DA terminals is sufficient to initiate sniffing. It would be very interesting to include any data related to titrating the light delivery in these experiments, or any data showing the range in naturally evoked DA signal in different experimental conditions if available. But I don't think these sorts of experiments are essential to add if the data doesn't already exist.

We appreciate being given the option to include those experimental results. We did not titrate light delivery since optical stimulation of DAT terminals expressing ChR2 has been published before by numerous groups and we based our stimulation paradigm off those, especially the careful work of Wolfgang Kelsch's (Oettl et al., Nature Comm 2020) and Fuqiang Xu's (Zhang et al., eLife 2017) labs in the TuS wherein they used comparable light intensities and stimulation frequencies (40Hz and 20Hz respectively; we used 25Hz as a 'compromise' and also based upon our prior work in the TuS (Gadziola et al., 2020 Cell Reports)).

The methods section of the paper provides enough detail that the experiments could be precisely repeated, and I have no concerns or suggestions for that section.

Thank you for mentioning this.

Reviewer #3

The authors performed a set of experiments designed to test the role of ventral striatal dopamine in sniffing responses to novel odors. By performing fiber photometry in conjunction with whole body plethysmography, they show that novel odors promote both sniffing and dopamine release in the olfactory/tubular striatum (TuS) and nucleus accumbens shell regions (NAcS), which could be recapitulated by optogenetic stimulation of ventral tegmental area (VTA) dopaminergic neuron terminals in these loci. Novel olfactory stimuli also increased the activity of both D1 and D2 striatal medium spiny neurons (MSNs) that receive dopamine input, and pharmacological blockade of these receptors in the NAcS or TuS attenuates different aspects of odor-induced sniffing. The authors conclude that odor-evoked dopamine release acting through D1 and D2 MSNs is a critical neurobiological mechanism mediating sniffing. While the manuscript is generally well written and methodologically sound, there are a few significant weaknesses that could be addressed in a revision.

1. First, the authors don't specifically state what odorants were used as 'novel stimuli' in the body of the manuscript (although some information is in the methods). This is problematic because dopamine may encode the stimulus valence (e.g. aversive vs. appetitive) independent of sniffing. Providing these details will be critical for full interpretation of the results.

Thank you for raising this important point that we failed to articulate in the original submission. Indeed, we selected odors which, based upon prior work, are known to be appetitive/attractive to mice with the logic that this would (hopefully) enhance the occurrence of seeing vigorous odor-evoked sniffing. We now explain our selection of odors in the in the results to allow readers to appreciation this selection of odors: *"Each mouse was presented with pseudorandom trials of odors, including R(+)-limonene, thioglycolic acid, and peanut oil, each five times across a behavior session. These odors were selected based upon their known positive hedonic qualities perceived by mice which we had chosen in order to ideally drive reliable and robust sniffing (Mandairon et al., 2009;*

Jagetia et al., 2018; Midroit et al., 2021).”

2. Second, there are inconsistencies in the methods used. For example, the authors perform photometry in freely moving mice in some experiments but head-fixed mice in others, which is partly to reduce any confounds caused by motion artifact. This is an appropriate rationale for head fixation, however no dead sensor or fluorophore control was used in freely moving mice, making it impossible to tell if data collected in these experiments were influenced by animal movement. Analysis of raw data from the isosbestic photometry channel would probably alleviate this concern, but it is my belief that at least some subset of the experiments should be performed with inert sensor controls.

We have gathered data from an additional 9 mice to address potential movement confounds. To do so, we injected the DA-insensitive control virus, $GRAB_{DA}$-mut in all 3 regions of the ventral striatum (*n*=2-4 mice per region) and recorded dynamics during the same behavioral paradigm as used in **Figures 2 and 3**. As shown in **Supplemental Figure 4**, upon sniffing there is little to no change in the mut sensor fluorescence supporting that the elevations in $GRAB_{DA}$ are not an artifact of movement.

A second example of inconsistent methodology is the use of DAT-Cre mice in some experiments but Th-Cre mice in others. There are well-described differences in the populations of neurons targeted between the two lines, so why change the genetic strategy for targeting dopaminergic neurons between experiments?

Regarding selection of the TH-Cre line for the cross to remove VGluT –please forgive the ‘overly honest methods’ here: TH-Cre mice were selected since that cross (*TH-Cre;VGluT2 fl/fl*) was already generated by our collaborator and co-author (Ada Varga). We did not have access to a similar cross with DAT-Cre mice. We have noted this in the Methods wherein we also acknowledge the caveat that identical cell populations would not have been targeted (lines 931-937). Nevertheless, we wish to emphasize that the *TH-Cre;vGluT2 fl/fl* line does allow for isolating the influence of DA on sniffing from glutamate. Indeed, a wealth of careful synaptic work on the topic of transmitter co-release has been done in this cross by several groups (Hnasko, Edwards, Sulzer) as we now reference in the manuscript.

3. Third, the authors make the point that dopamine release is critical for the initiation of sniffing but not its duration (Figure 4). A more thorough testing of this hypothesis – in my opinion – would involve optogenetic inhibition of dopaminergic terminals precisely at the onset of a spontaneous sniff bout to see if it delays sniffing onset, reduces total bout length, or blocks the bout altogether. While the D1 and D2 receptor antagonist experiments provide some evidence that ventral striatal dopamine is necessary for sniffing, the temporal kinetics of the manipulation is quite long and affects both between tonic and phasic modes of dopamine release.

We performed additional experiments wherein we injected the inhibitory opsin, eNpHR 3.0 bilaterally into the VTA, and implanted bilateral optic fibers in the TuS or NAcSh in separate mice. We targeted TuS or NAcSh for this question since those were the ventral striatum subregions wherein greatest elevations in DA were observed in the photometry experiments. See revised results lines 377-411 and new **Figure 6**.

We first triggered light at discreet time-points to objectively capture whether any effect of inhibition was seen on respiratory behavior which, as well know, consists of periods of time wherein mice will spontaneously transition into and out of bouts of sniffing. Separately, on a different behavioral session, we triggered the light to be delivered immediately prior to odor delivery into the plethysmograph or buzz onset (as described in Methods lines 947-976). We indeed found a modest,

yet significant reduction in spontaneous respiratory frequency. That said, this experiment revealed that suppression of DA release in either region alone had little influence on reducing odor- and buzz-evoked sniffing. While there was a subtle reduction on trial 1 of a novel odor in the NAcSh, it was not a dramatic significant reduction as observed in the noted pharmacology experiments (**Fig 9**). It is worth highlighting that bilateral optical inhibition of DAergic terminals would not be sufficient to inhibit all DAergic terminals innervating the TuS or NAc given their large spatial extent. Nevertheless, we reasoned this was the ideal experiment to get at this reviewer's point since if we would have simply inhibited VTA cell bodies, we may see effects due to changing DA dynamics in all structures recipient of VTA input, but just the TuS or NAc.

Additionally, I have included a more comprehensive list of comments and concerns below that the authors may want to consider if they revise their manuscript:

4. In the introduction, the discussion of spinal circuits that orchestrate sniffing is relatively superficial. It may be helpful to provide more detail if the authors feel that background knowledge of respiratory circuitry is required to contextualize the experimental question explored in the manuscript.

We appreciate this and have added addition background text in the revised introduction (lines 57-69).

5. Similarly, the stated rationale for studying the role of the ventral striatum is not as clear or strong as it could be. I would recommend revising the final paragraph of the introduction to make it more compelling.

This has been revised to now state, *"Electrical stimulation of the ventral tegmental area (VTA) elicits vigorous sniffing[25]."* And *"Likely largely due to its influence on cellular plasticity, DA in the ventral striatum is a major mediator of motivated states and reinforcement with manipulation of DA and D1 and D2 neural activity in the ventral striatum guiding those functions[36,38–40]. Since sniffing is a motivated behavior, it is reasonable to believe ventral striatum's role in motivational control is in-part a regulatory system for orchestrating sniffing."*

6. In the Results, it is stated that it is "well known especially in rats that the ventral striatum receives dense input from mesolimbic dopamine neurons…" However, dopaminergic projection targets, axonal arborization, and monosynaptic inputs have been thoroughly studied in mice (e.g. Beier et al., Cell, 2015). Likewise, one of the citations used to support this statement (30) was a study performed in C57Bl/6J mice. Although the tracing data in Figure 1 is well done and supports the use of the recording sites chosen, it is likely confirmatory and not necessarily novel. It would be helpful to the reader if the authors acknowledged similar previous work and stated if the current findings deviate from those studies in a meaningful way.

Thank you for pointing this out. We have now included mention that this circuit has been studied in both mice and rats and incorporated additional references (*e.g.,* Beier et al., 2015). We have also added clarifying text to acknowledge how our results add to the field (See lines 100-102, 108, 120-123).

7. In Figure 2Bi and 2Biii, the authors state that their approach can distinguish between basal breathing and high frequency sniffing bouts. They then provide quantitative details establishing their criteria for identifying a 'sniff bout'. Their approach seems sound based on provided references; however, I am concerned that only relying on plethysmography data to identify sniff bouts does not capture other motor-related dopamine signals (e.g. head rotation or changes in body position) or

provide a visual ‘sanity check’ to confirm that purposeful odor sampling is occurring (instead of anxiety-related increases in respiratory rate). While not necessary for revision, it might be helpful to video record a subset of animals to capture more information about the behavior being studied (e.g. with pose estimation algorithms). This would both validate the approach and provide a more feature-rich dataset that could be correlated with the GRAB-DA data.

While we appreciate this reviewer noting this is not necessary for resubmission, we had been recording video in the experiments simply to help the experimenter identify in real-time what the mouse was doing and looked through them regarding this interesting idea. While the videos are too low of resolution (640 x 480), frame rate (10Hz), not to mention in partial darkness, to allow for a reliable machine learning approach for pose estimation based on video alone, we had a single observer monitor the videos for this ‘sanity check’ as suggested. We had a single observer watch the videos and cross-reference behaviors observed on video to a list of time-points (provided by a different experimenter) wherein sniffing was observed via the respiratory pressure signal from the plethysmograph. In *every instance* the observer saw snout-directed investigatory behavior on video, it corresponded with an event on the list of sniffing time-points from the respiratory pressure sensor. This supports that plethysmograph data is reliable for identifying sniff bouts (noted now in Methods line 825-832) as, *“In a subset of mice, an observer post-hoc monitored video (10Hz) which was recorded simultaneously with respiratory signal from the plethysmograph to validate that focusing on >6Hz changes in respiration was indicative of ‘active sampling’ behaviors from the mouse (including snout directed investigation and head-turning) and found 1:1 correspondence supporting use of respiratory signals from the plethysmograph as suitable for detecting active investigation (of course in this set-up we could not see minor facial, motor or vibrissal movements).”*

8. In Figure 3, it is not clear from the text or the caption which odorants were used as a ‘novel’ odor. Were all three odorants listed in the Methods used? Or just one? If multiple, in what order were they delivered? Why were these specific compounds chosen? These are critical details, as dopamine responses could represent the valence of the sensory stimulus, their novelty, etc. Furthermore, at the end of the paragraph associated with Figure 3, the authors state that “these results indicate that sniffing corresponds with increases in DA release in two specific sub-regions of the ventral striatum, and that the level of DA correlates to the vigor of sniffing.” I still find these correlations hard to interpret, as the experiments presented do not really explore whether the dopamine response to odor could reflect sensory stimulus novelty (i.e. is dopaminergic habituation a function of sensory prediction error encoding?) or its hedonic value (i.e. does sniffing-related dopamine release only occur when the odor is appetitive? Or aversive?).

Thank you for raising these important points. In our results, we have added additional clarification as to what odors were used (as this reviewer requested in comment #1) and included that odor presentation was randomized across animals. We have also included more description to “novel” odors by defining this as Trial #1 of odor in the text when the word “novel” is used. As we note elsewhere in this response to reviewers, all of the odors we selected would be considered ‘attractive/appetitive’ and thus it is not possible to know if hedonics contributes to the evoked DA – although we certainly are interested in exploring the relationship between stimulus hedonics (predator odor vs food odor for instance) and the sniffing related DA levels.

9. This is a minor point, but the authors refer to injection of an ‘anterograde AAV’. To me, this means they will use an AAV that will transduce downstream neurons when injected into an upstream, synaptically connected site (e.g. the work of Zhang and others using AAV1). Since the authors are

performing terminal activation with an AAV5 ChR2 viral construct, I'd probably delete the word 'anterograde' to avoid confusion.

We have eliminated the use of the word anterograde as also requested by Reviewer 1.

10. Significant changes in sniffing and dopamine release was observed in YFP control mice during optogenetic stimulation in Figure 4. While this could be due changes in arousal state secondary to laser light leakage from the fiber ferrule, light stimuli that are barely perceptible to humans can evoke dopamine release in the ventral striatum (see Gonzalez et al., eLife, 2023). If dopamine release in the ventral striatum drives sniffing, then that could explain the response seen in YFP controls.

To clarify at the onset, we did not look at DA release during optical stimulation in relation to sniffing, just two of the three at a time (sniffing + DA release, or sniffing + optical stimulation of DA release). That stated, this is an interesting point the reviewer raises and we appreciate them bringing the work of Gonzalez et al in 2023 to our attention. We now cite that study's findings of light evoked DA release in the ventral striatum as a possible driver of sniffing in EYFP treated mice (lines 279-281).

Additionally, what was the laser power used for optogenetic stimulation (I didn't see it in the Methods)? This is relevant given the possibility of heating artifacts. Overall, additional discussion about the results in 4D and E is probably warranted.

Please see our response to Reviewer #1, issue 10.

11. In Supplementary Figure 3, why was the Th-Cre mouse line used instead of the Dat-Cre mouse line? Given the controversy surrounding the use of the Th-Cre mouse to study VTA dopamine neurons (see Lammel et al., Neuron, 2016), this seems like a strange choice of mouse line. While it likely does not change the results of the experiment, the inconsistency is worth noting and, perhaps, justifying.

Please see our response to your concern #2 wherein we address this choice of mouse line and also acknowledge the findings of the Lammel group in this response.

12. Regarding the experiments in Figure 4H, the authors state that the results of their optogenetic excitation experiments "indicate that DA release into the ventral striatum initiates sniffing, but that it does not persistently driving a sniff bout." I'm of the opinion that you can't fully substantiate this claim without optogenetically inhibiting dopamine release precisely at the onset of a spontaneous sniff bout to show that transiently reducing dopamine release causally suppresses sniffing behavior.

Please see our response to your concern #3 wherein we discuss our opto-inhibition experiments (See revised results lines 377-411 and new **Figure 6)**. While we believe our finding that DA release can initiate a sniffing bout is strongly supported by our multiple lines of evidence, in three different subregions of the ventral striatum, we also believe your point about the need to consider how inhibition of DA release fits into that model is warranted.

13. In Figure 6, why was head-fixation necessary to limit motion artifacts for photometry experiments, whereas it wasn't for Figures 2 and 3? This begs the question: if motion artifacts were truly a concern, why didn't the authors use a dead sensor control for GRAB-DA experiments? Or a GFP control for the GCaMP experiments? These concerns may be addressable by analysis of the raw isosbestic signal during freely moving behavior. However, repeating at least a subset of the photometry with an inert sensor control would probably strengthen the robustness of the approach.

Please see our response to your concern #2.

14. Also in Figure 6, which odorants were used as the ‘novel odors’? Without knowing this, it’s impossible to fully interpret changes in the dopaminergic response to these odors, especially if those odors are pleasurable, aversive, or neutral.

Please see our response to your concern #8.

Response to Reviewers:

We thank the Reviewer's for their time in rereviewing this manuscript. We appreciated the requests for additional clarifications and information from Reviewer 1 and have incorporated their requests in our revised manuscript as outlined below. We also made the requested edits from the Editor, as outlined in the decision letter, including being sure to plot individual animal data in plots wherein less than 10 mice are present.

Reviewer 1:

We appreciate the authors' thorough revisions. The new experiments and analyses have greatly improved the manuscript, especially in clarifying the relationship between sniffing, dopamine, and neuron activity. The added figures and updated text are beneficial.

We are grateful this reviewer found the new analyses improved the manuscript.

However, we have a few minor suggestions for further enhancing the single-unit data presentation. Specifically, we have some comments regarding analyses shown on the following panels:
Figure panel 7B:

It is unclear if the data comes from 'Odor' or 'Spontaneous' periods.

*We have better clarified and specified what data were used for all analyses in the results section pertaining to the single unit analyses and in the corresponding **Figure 7.***

We suggest removing the spike waveforms from the plot, especially because they come from a previously published data set.

These are removed.

- Instead, we propose to show some standard peri-event time histograms (PSTH). The accompanying analysis clearly shows that most spikes are emitted after the initiation of sniffing (which is expected). Adding PSTH would allow the readers to understand the dynamics of neuronal activity better.

*We agree and have incorporated a PSTH of single-unit activity (**Fig 7Cii**) to complement the raster plots (**Fig 7Ci**) and additional analyses on the unit data. We want to emphasize for this Reviewer that for the unit data we detected not just bouts of sniffing as in other figures but also moments of short respiratory cycles since we identified in these data that they also were accompanied by spiking of ventral striatum units. To capture both sniffing bouts (defined as 6Hz sniffing for a prolonged period of time as in all other figures in this paper; see methods) and brief moments of short respiratory cycles we used a sniffing burst detect method based on RMS*

*and Z-score therefore the time 0 represents where the z score crosses the threshold. With this in mind, as illustrated in **Figure 7ai**, the RMS detected increase in respiratory frequency may have some jitter (upon crossing of threshold) relative to the visually absolute increase. We have been careful to describe this in the Results and elsewhere.*

Figure panel 7C:
- Please present this panel before 7B. Now, the order of panels does not correspond to the main text, which is very confusing (breathing rate is discussed in the text first, but in the figure, it is shown only after some spike analysis).

We agree. This order is now corrected. Specifically, we moved the original Figure 7C which displays distributions of respiratory frequencies up prior to analyses of the units (to now be Fig 7B).

Figure panel 7D:
- The main text says: 'Among these, they were more likely to fire within a short respiratory cycle during the inter-trial interval when the background respiratory rate is slower (Fig 7D, mixed ANOVA, $F(3, 76)=31.48$, $p<0.0001$).' This sentence is confusing for several reasons: a) Did the authors mean faster, not slower? b) Does the 'inter-trial interval' refer simply to the 'Spontaneous' periods? If yes, we would suggest sticking to the same language in the main text and figures for clarity.

*We apologize for the confusion the wording caused. We agree the wording should be more consistent and have adjusted wording throughout this section to help readability. This section now reads, "As shown in **Fig 7Cii**, there was a pronounced increase in the firing rate of these units surrounding onset of sniffing bouts. Among these units, they were more likely to fire during spontaneous short respiratory cycles when otherwise the background respiratory rate was slower, in comparison to respiratory cycles during odor wherein the background respiratory rate is faster (**Fig. 7Dii**, mixed ANOVA, $F(3, 48) = 31.48$, $p < 0.0001$). This relationship is observed even among individual neurons (**Fig 7Di).**"*

- The fact that the distribution of spiking relative to breathing frequency is completely flat during 'Odor' periods is very interesting. The authors explain this observation simply as an effect of insufficient trials with low-frequency breathing (which, by the way, could probably be tested by plotting scatter plots with individual trials). However, an alternative explanation - supported by the results of inhibition of the DA terminals - would be that odor-evoked sniffing is controlled by slightly different mechanisms than the spontaneous one. Do the authors find such an interpretation plausible?

*We appreciate this suggestion and have created scatter plots of firing relative to breath frequency for all units during both odor and spontaneous periods from individual trials as shown in the new **Figure 7Di**. Across 168 trials from this unit, it is clear (and significant) that spiking relative to breathing frequency is largely flat during odor periods. This agrees with the*

*population-level and across trials data we originally presented in **Fig 7Dii**. We agree that this possibly may mean that odor-evoked sniffing is controlled by slightly different mechanisms than spontaneous ('internally-generated') sniffing and mention this now in the Discussion (lines 708-710).*

Figure panel 7E: In the 'Odor' condition, there seems to be a trend for increasing breathing frequency already during the first spikes. From our experience, normalizing breathing frequency by simply subtracting it from each trial's baseline is a great way to improve the power of statistical tests (otherwise, there is much variability coming from differences between animals). Would the results remain the same after such a normalization?

*We appreciate this suggestion and now normalized the respiratory frequency by subtracting the mean frequency over all spontaneous period for each recording. Normalization did remove the main effect of animals in the ANOVA, but other statistical outcomes remained the same, including the increase in respiratory frequency relative to the first spike. Given this we now report this normalized figure (new **Figs 7ei and 7eii**).*