nature portfolio

Peer Review File

SST neurons in the periaqueductal gray regulate urination and bladder function

Corresponding Author: Professor Jiwei Yao

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)
These are very well performed studies which identify a subpopulation of periaquaductal gray (PAG) glutamatergic neurons (SST+) which play a critical role in controlling bladder contraction and voiding. The authors use pseudorabiesvirus to link PAG to bladder, and then examine the timing of activation of SST neurons with the void cycle. They go on to show that direct stimulation of these neurons causes bladder contraction, that inhibiting them prevents normal voiding, and that they project to the Pontine Micturition Center. The studies are well performed, well described, and appear to be rigorous and statistically valid. I have only a few concerns:

1. The authors need to describe with a bit more clarity why they chose to look at the SST+ neurons. Are SST+ the only subpopulation of PAG vGlut2 neurons so far identified?
2. The authors should provide some estimate as to what proportion of PAG vGlut2 neurons are SST+.
3. It is not clear what the PRV data really adds here. The pictures are pretty, but we already knew that bladder afferents project to this region of the PAG, and this is the only conclusion that the data they present supports. The authors make the statement that the PRV data supports their Figure 9 map of the neural circuit, but they present evidence at 96h only, when PRV could have jumped many synapses. If they want to develop evidence supporting the actual circuit, they need to examine PRV labeling at earlier time points, such as 24h, 48h and 72h, to see how the virus progresses along the circuits.
4. The authors state that their studies demonstrate that the SST+ neurons project to PMC. It is already known that vGLUT2 PAG neurons project to PMC (see ref 19), and in the article cited the authors performed channel rhodopsin-assisted circuit mapping, demonstrating these connections. What is new here is that the SST+ subpopulation of these PAG glutamatergic neurons reaches PMC.
5. The GCaMP6s and stimulatory optogenetic findings reported here for the SST+ neurons corroborate the results obtained for the entire PAG vGlut2 population as reported in ref 19. Perhaps this should be cited.
6. It is very interesting that the optogenetic stimulation of SST+ neurons contracts the bladder but does not affect the sphincter unless the bladder is partially filled. This point could be emphasized a bit more.
7. The authors might also want to cite a recent paper in Nature Communications by Nardone et al (PMID 38438345) which maps out in detail the different neuron types in the PMC.

Reviewer #2

(Remarks to the Author)
The manuscript from Yan et al. defined a population of SST neurons in the l/vlPAG role in voiding. It is an important addition to the literature and well-written. There are a few points that need to be addressed for clarity prior to publication.
Comments:
• How long after catheter implantation were the awake cystometry experiments performed? Was it the same day?
• SST needs to be defined in the manuscript.
• Line 77. The PAG neurons don't innervate the bladder directly and the EUS but the circuits do. This language should be changed.
• Not sure about the point of Figure 1a-f. How does this support the hypothesis?
• What are the differences in EUS and Bladder PRV expression in the l/vlPAG? Quantifying these differences would be a valuable addition to this paper.
• Why was the diuretic furosemide used?

• Line 122. What is the difference between individual and average urination events? This could use more explanation.
• Why was the data in Fig 2h compared to shuffled data? I don't see the point in this statistical comparison. This data demonstrates the temporal involvement of these neurons, which means there is nothing in randomly shuffled data. The time delay itself is the important part.
• In the opto-stim experiment how many times was each mouse stimulated? Did the repeated stimulation decrease the chances of a void? Seems like voiding would also be dependent on the fill state of the bladder. How was the bladder fill state controlled?
• line 154 – how were the intervals randomized? This seems like a way to introduce bias, why not regular intervals?
• Not sure about the statement SST neuron activation is sufficient to dive voiding contractions.
• In the chemogenetic experiment. Was the total volume of urine expressed altered? This is an important parameter to report, given that more frequent voiding could be the result of increased diuresis as well.
• Reduce the number of abbreviations if possible. There is no need to abbreviate pelvic nerve. LUT, SPN, DGC and ESR1 also could be removed as they are used minimally.
• Cutting the pelvic nerve will result in decreased bladder contractions no matter what your stimuli. The results from this experiment add minimally to the whole work.

Reviewer #3

(Remarks to the Author)
The manuscript entitled SST Neurons in the Periaqueductal Gray Regulate Urination and Bladder Function by Yen et al details extensive experiments exploring the brainstem circuits underlying urination. Urination is an essential homeostatic mechanism which when unregulated can have profound impacts on health and quality of life. Despite this, the brainstem circuits regulating micturition have yet to be fully characterised.

In this study, the authors present compelling evidence that l/vlPAG-SST+ neurons that project to the PMC are an essential component of the brainstem circuitry that regulates micturition via the use of cell type-specific neuronal activity recordings, optogenetic stimulation, and chemogenetic manipulation combined with cystometrograms and EUS electromyograms (EMG). The data from this study complements and extends previous work exploring these networks, providing an understanding of the link between the two major brainstem regions involved in bladder storage, the PAG and PMC. The methodology is mostly comprehensive, the data are well presented, and the discussion is sound. I have only minor comments that I believe would improve the quality of this manuscript.

Line 74 – 'itch' not inch

Line 103 – It is not clear why there are four synaptic connections from the bladder or EUS.

Figure 1: line 105 – labelled neurons are not clear within the SPN in the spinal cord. Out of the whole dorsal horn, this area appears to have the least labelling.
Why was this selected to highlight? There is more extensive labelling with the DGC and across lamina I-V. This is in line with recent studies showing the terminals of sensory axons within the bladder wall (PMID: 30531372). The presence of staining in the MPG confirms that the tracer is non-specific and will label both autonomic and sensory nerves. This should be clarified.

Figure 3: it is intriguing that in ChR2-expressing mice, light-induced EUS busting activity. It would be typical to expect that during the stimulus for micturition there would be EUS relaxation to allow efficient expulsion of urine. Was there a definable relationship between EMG activity and the fill state of the bladder during optogenetic stimulation? Or could it be that there is a different subset of neurons in the PAG or PMC that regulate EUS relaxation? This is partly explored in figure 4 where EUS activity is not seen in the empty bladder.

Fig 4d: Looks like there is one responder in the EMG group which is not in the dot histograms. Is this an artifact?

Line 295 – missing P from PMC

Line 312 – The point being made on lines 311-312 is not clear. You have made it clear in this study that l/vlPAG do receive extensive spinal cord inputs?

Methods:

The methods for the recording of urination are not provided including data transposition. How were the images recorded and then translated into the images that could be analysed and quantified?

Statistical analysis – large differences in groups – clearly identified comparisons.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
These are well performed and well documented studies which examine the role of a novel population of periaquaductal gray (PAG) neurons in bladder and external sphincter function. The authors have responded very thoroughly to the original concerns. Two minor comments remain: 1. On page 2, line 78, I believe the authors mean, "contrast" rather than "contact." 2. More importantly, in the narrative of the pseudorabies virus experiments (page 3 line 112) and in the supplemental figure, it appears that the first time point is 24h and not 48h. If the authors are describing the sequential movement of the virus from the bladder up to the ganglia and than back to the PAG, the ganglion would appear to be labeled at 24h and not 48h, as they describe in the text and the figure legend.

Reviewer #2

(Remarks to the Author)

Minor comment.

The description on why diuretic furosemide used was perfect please add this or an abbreviated version to the methods.

Reviewer #3

(Remarks to the Author)
The authors have comprehensively addressed all my previous comments.

We would like to express our deep appreciation to the editor and the reviewers for their constructive comments on our manuscript (COMMSBIO-24-8218A). In order to fully address their comments, we have conducted additional experiments and performed the new analyses that resulted in the following changes. In the revised manuscript, we highlight the major changes to the text.

Here is an overview of the major changes made in the revised manuscript:

(1) We have added more details in the third paragraph of the Introduction (lines 73-84) to more clearly elaborate on our rationale for focusing on SST^+ neurons in the l/vlPAG.

(2) We have cited reference 19 in the optogenetic results section (lines 291-293). Also, we have added the following sentence "The PMC consists of a variety of cell subtypes that express different kinds of marker genes[55]"at lines 367-368, and cited the literature (Nardone et al., Nature Communications, 2024, PMID 38438345) in this sentence (reference #55 in our revised manuscript).

(3) We have conducted additional experiments to examine PRV labeling at earlier time points. Specifically, PRV tracing data at 48-hour and 72-hour post-PRV injection have been added. The results of these experiments have been included in a new **Supplementary Figure 1**, and the relevant experimental findings have also been integrated into the corresponding parts of the Results section (lines 112-128).

(4) We have added more description of the result ("optogenetic stimulation of SST^+ neurons contracts the bladder but does not affect the sphincter unless the bladder is partially filled") in the Results section (lines 198-199) and in the Discussion section (lines 330-331).

(5) We have clarified the abbreviation by spelling out "Somatostatin (SST)" at its first mention in the text (Introduction, line 73). We have reduced the number of abbreviations and removed the abbreviations LUT and ESR1 as they were used minimally.

(6) We have added more details about the experimental methods in the relevant sections of the revised manuscript based on the reviewer's comments. For instance, we have specified the time point for cystometry experiments after catheter implantation (lines 524-525), explained the reason for using the diuretic furosemide (line 147), detailed the number of stimulations each mouse received (lines 544-546), explained the rationale behind using randomized intervals in the optogenetic activation experiment (lines 554-556), as well as the methods for recording urination (lines 562-567, 575-581), and so on.

(7) We have corrected the unclearly or improperly described sentences (line 85, lines 348-351) and the words with spelling errors (line 81 and line 333) in the revised manuscript.

(8) We have carried out a new analysis focusing on the changes in the total urine volume within the chemogenetic experiment. The findings have been incorporated into the revised manuscript's **Figure 7f** and **Supplementary Figure 2d**, as well as the corresponding part of the Results section (lines 263-274).

(9) Lastly, we've addressed all the other minor points raised by the reviewers.

We hope these revisions meet the reviewers' expectations and contribute to the improvement of our manuscript.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

These are very well performed studies which identify a subpopulation of periaquaductal gray (PAG) glutamatergic neurons (SST+) which play a critical role in controlling bladder contraction and voiding. The authors use pseudorabiesvirus to link PAG to bladder, and then examine the timing of activation of SST neurons with the void cycle. They go on to show that direct stimulation of these neurons causes bladder contraction, that inhibiting them prevents normal voiding, and that they project to the Pontine Micturition Center. The studies are well performed, well described, and appear to be rigorous and statistically valid. I have only a few concerns:

Thank you very much for your valuable and constructive feedback on our manuscript. We deeply appreciate the time and effort you have invested in reviewing our work. In response to your comments, we have made the following revisions to improve the manuscript. Below, we provide a point-by-point response to your suggestions and outline the specific changes made:

1. The authors need to describe with a bit more clarity why they chose to look at the SST+ neurons. Are SST+ the only subpopulation of PAG vGlut2 neurons so far identified?

Thank you for your insightful feedback. We apologize for any lack of clarity in our initial manuscript regarding the rationale for focusing on SST^+ neurons in the PAG. We have revised the manuscript to explicitly address this point and added more details in the third paragraph of the introduction (lines 73-84). The details are as follows: "Somatostatin (SST)-positive neurons are widely distributed throughout the mammalian brain and participate in regulating various innate behaviors like sleep, feeding, and defensive behaviors. Their function and importance in the control of urination, which is also an innate behavior, have not yet been demonstrated. Notably, SST^+ neurons in the l/vlPAG are a specific subpopulation of glutamatergic neurons, in contract to SST^+ neurons in most other brain regions being inhibitory interneurons. Moreover, SST^+ neurons in the l/vlPAG and $Vglut2^+$ neurons in the same area do not have identical roles in regulating some physiological functions (e.g., pain and inch itch processing). Taking into account the PAG's essential role in urination control and the unique characteristics of l/vlPAG SST^+ neurons, we propose that these neurons may be key regulators of urination."

In response to your query, we would like to clarify that SST^+ neurons are not the only subpopulation of PAG $Vglut2^+$ neurons identified so far. In addition to SST^+ neurons, tachykinin 1 (Tac1)-expressing neurons have also been identified as another subpopulation of excitatory PAG neurons (Gao et al., Neuron, 2019, PMID: 30554781).

2. The authors should provide some estimate as to what proportion of PAG vGlut2 neurons are SST+.

Thank you for raising this important point. We have consulted the In Situ Hybridization (ISH) data in the Allen Brain Atlas. Based on the ISH datasets of SST and Vglut2 gene expression throughout the adult mouse brain, we calculated that the proportion of SST^+ neurons to $Vglut2^+$ neurons in the l/vlPAG is approximately 23%. However, since the gene expression results of SST and Vglut2 came from two different mice and the sample size was insufficient, the calculated proportion is not precise enough. Therefore, we did not add this proportion in the revised manuscript. In future research, we plan to conduct in situ hybridization experiments in the l/vlPAG of the same mouse to label the mRNA of SST and Vglut2 with different fluorescence markers respectively. Then we will calculate

the proportion of l/vlPAG Vglut2^{+} neurons that are SST^{+}. The results obtained in this way will be more accurate.

(SST gene expression: https://mouse.brain-map.org/experiment/show/1001)

(Vglut2 gene expression: https://mouse.brain-map.org/experiment/show/73818754)

3. It is not clear what the PRV data really adds here. The pictures are pretty, but we already knew that bladder afferents project to this region of the PAG, and this is the only conclusion that the data they present supports. The authors make the statement that the PRV data supports their Figure 9 map of the neural circuit, but they present evidence at 96h only, when PRV could have jumped many synapses. If they want to develop evidence supporting the actual circuit, they need to examine PRV labeling at earlier time points, such as 24h, 48h and 72h, to see how the virus progresses along the circuits.

Thank you for your valuable comments. We apologize for not providing sufficient PRV experimental data in the previous version, which failed to fully highlight the significance of this part of the content. In response to your suggestion, we have carried out additional experiments to examine PRV labeling at earlier time points. Specifically, data at 48-hour and 72-hour post-PRV injection have been added. These data provide a clearer view of the trans-synaptic progression of the virus and the sequential infection of brain regions, which better support our Figure 9 map of the neural circuit. The results of these additional experiments have been included in a new **Supplementary Figure 1**, and the relevant experimental findings have also been integrated into the corresponding parts of the **Results** section (lines 112-128). Pseudorabies virus (PRV), as a neurotropic retrograde multi-transsynaptic tracer, is widely employed to map the descending central neural networks that innervate peripheral organs (Fan et al., J Neurosci Methods, 2020, PMID: 32371062). In future studies, we intend to utilize the anterograde trans-synaptic tracer herpes simplex virus (HSV) to identify CNS regions (including the PAG) that receive bladder afferent inputs.

4. The authors state that their studies demonstrate that the SST+ neurons project to PMC. It is already known that vGLUT2 PAG neurons project to PMC (see ref 19), and in the article cited the authors performed channel rhodopsin-assisted circuit mapping, demonstrating these connections. What is new here is that the SST+ subpopulation of these PAG glutamatergic neurons reaches PMC.

Thank you for your insightful comment. We agree that the projection of PAG Vglut^{2+} neurons to the PMC has been previously reported in reference 19. Nevertheless, it's crucial to recognize that this prior finding doesn't automatically signify that all subtypes of PAG Vglut2^{+} neurons have projections to the PMC. Given that the PAG modulates various important physiological functions of the organism, it's highly probable that different subtypes of PAG Vglut2^{+} neurons may exhibit distinct projection patterns to regulate various physiological processes. Prior to our study, it was uncertain whether PAG SST^{+} neurons project to the PMC. Our study has now unveiled that PAG SST^{+} neurons do indeed project to the PMC at the anatomical level. Moreover, functionally, these projections are primarily responsible for controlling bladder contraction, which highlights their unique role within this circuit. By specifically identifying the involvement of the SST^{+} subpopulation, our findings offer a more refined and comprehensive understanding of the PAG-PMC circuit. They not only add to the existing body of knowledge but also shed new light on the particular subtypes of glutamatergic neurons engaged in this projection, emphasizing their distinct functional contributions.

5. The GCaMP6s and stimulatory optogenetic findings reported here for the SST+ neurons

corroborate the results obtained for the entire PAG vGlut2 population as reported in ref 19. Perhaps this should be cited.

Thank you for this insightful suggestion. We have now cited reference 19 in the optogenetic results section. As mentioned in lines 291-293, "similar to the results obtained by photoactivating axon terminals of the entire l/vlPAG Vglut2$^+$ neurons in the PMC in previous work[19]". However, since the GCaMP6f data in reference 19 were recorded from PMC Vglut2$^+$ neurons, which represent a distinct population from the PAG neurons examined in our study due to their differing anatomical locations, we believe it would be more appropriate to restrict the citation to the optogenetic stimulation context where the comparison is directly applicable.

6. It is very interesting that the optogenetic stimulation of SST+ neurons contracts the bladder but does not affect the sphincter unless the bladder is partially filled. This point could be emphasized a bit more.

Thank you for your valuable and constructive comment. We appreciate your interest in the findings related to the optogenetic stimulation of SST$^+$ neurons. We have emphasized this point further in the revised manuscript. Specifically, we have added more description of this result in the **Results** section (lines 198-199, "These findings confirm that activation of l/vlPAG SST$^+$ neurons triggers bladder contractions but does not affect the EUS unless the bladder is partially filled"), and in the **Discussion** section (lines 330-331, "bladder contraction but did not affect the EUS unless the bladder is partially filled in anesthetized mice").

7. The authors might also want to cite a recent paper in Nature Communications by Nardone et al (PMID 38438345) which maps out in detail the different neuron types in the PMC.

Thank you for highlighting this important reference. The detailed information on neuronal types in the PMC provided by this paper is indeed very valuable. To properly cite this literature, we have added the following sentence "The PMC consists of a variety of cell subtypes that express different kinds of marker genes[55]" at lines 367-368, and cited this literature in this sentence (reference #55 in our revised manuscript).

Reviewer #2 (Remarks to the Author):

The manuscript from Yan et al. defined a population of SST neurons in the l/vlPAG role in voiding. It is an important addition to the literature and well-written. There are a few points that need to be addressed for clarity prior to publication.

We sincerely appreciate the reviewer's positive assessment of our manuscript and their constructive feedback to enhance clarity. We have meticulously addressed each of the reviewer's concerns and implemented the necessary revisions in the updated manuscript. Below, we provide a detailed, point-by-point response to the reviewer's comments.

Comments:

1.How long after catheter implantation were the awake cystometry experiments performed? Was it the same day?

Thank you for your important question. We sincerely apologize for the insufficient clarity in the original manuscript regarding the experimental timeline. To clarify, the cystometry experiments were performed on the postoperative day 1, rather than on the same day of surgery. This allowed the animals to recover from the surgical procedure and minimized potential confounding effects from acute surgical stress on urodynamic measurements. We have added this detail to the **Methods**

section (lines 524-525, “mice were allowed to recover for one day before the subsequent experiments”) of the revised manuscript.

2.SST needs to be defined in the manuscript.

Thank you very much for raising this point. We have now clarified the abbreviation by spelling out “Somatostatin (SST)” at its first mention in the text (Introduction, line 73). We apologize for any confusion this oversight may have caused and appreciate your attention to this detail.

3.Line 77. The PAG neurons don’t innervate the bladder directly and the EUS but the circuits do. This language should be changed.

Thank you very much for your thoughtful comment. We wholeheartedly agree with your point and sincerely apologize for this oversight in our initial manuscript. We appreciate your attention to this detail and have rephrased this sentence in the text (line 85, “PAG neurons involved in controlling the bladder and external urethral sphincter”) to convey the correct information.

4.Not sure about the point of Figure 1a-f. How does this support the hypothesis?

Thank you very much for your insightful comment. The results in Figure 1a-f are indeed crucial for supporting our hypothesis. As you may know, pseudorabies virus (PRV) is a neurotropic multi-transsynaptic tracer widely used to map the central neural networks that innervate peripheral organs (Fan et al., J Neurosci Methods, 2020, PMID: 32371062). For PRV to effectively trace neural circuits, it must be injected into areas where nerve fibers are distributed. These data in Figure 1a-f provides detailed information on the nerve fiber distribution in the target organ, which is essential for guiding PRV injection into the specific sites where nerve fibers are distributed. This ensures that the PRV can effectively infect the neurons and trace the neural circuits, thereby increasing the success rate of the experiment and providing strong support for our hypothesis. We have added the sentence “These data provide detailed information on the nerve fiber distribution in the bladder and EUS, which is essential for guiding pseudorabies virus (PRV) injection into the specific sites (muscular layers) in the following retrograde trans-synaptic tracing experiments” in the **Results** section (lines 106-109) to emphasize this point.

5.What are the differences in EUS and Bladder PRV expression in the l/vlPAG? Quantifying these differences would be a valuable addition to this paper.

Thank you for raising this important question. We have tried to compare the distribution of PRV-infected neurons in the l/vlPAG following PRV injections into the bladder and the external urethral sphincter. No significant differences in the spatial distribution of PRV-infected neurons were observed between the two groups. However, due to the different volumes of PRV injected into the bladder and EUS, a direct comparison of the number of infected neurons is not meaningful. Therefore, quantifying these differences is not feasible in the current study. In future work, we plan to inject the same amount of two distinct fluorescent PRVs (e.g., PRV-GFP and PRV-mRFP) into the bladder and EUS of the same animal, respectively. This approach will enable a more accurate and meaningful quantitative analysis of the number and spatial distribution of infected neurons in the l/vlPAG.

6.Why was the diuretic furosemide used?

Thanks a lot for this important question. Under normal circumstances, adult mice urinate approximately 20 times a day (Negoro et al., the Journal of Urology, 2012, PMID: 22982423), making it challenging to record multiple urination events during a 40-60-minute fiber recording session. To address this, we used the diuretic furosemide to increase the urination frequency of the mice, enabling us to capture multiple urination events within the recording period. We have

incorporated the reason for administering the diuretic furosemide into line 147 ("which increased the number of urination events during the recording period") of the revised manuscript. This approach is consistent with a previous study (Hou et al., Cell, 2016, PMID: 27662084) that utilized furosemide to enhance the number of urination events in fiber recording experiments. Additionally, we administered the diuretic before optogenetic experiments in conscious and freely moving mice, ensuring that a larger volume of urine was stored in the bladder over a short period, which facilitated more robust data collection.

7.Line 122. What is the difference between individual and average urination events? This could use more explanation.

Thank you for your valuable comment. The sentence at line 122 was intended to convey that the pattern of Ca^{2+} fluorescence intensity changes observed in individual and average urination events was the same. We apologize for the unclear phrasing in our original manuscript, which may have caused confusion. We have revised the sentence (lines 148-151, "In both the average and individual urination events, the Ca^{2+} fluorescence intensity from GCaMP6f-expressed l/vlPAG SST^{+} neurons in experimental mice robustly increased during urination, and the fluorescence increase began prior to the onset of each urination event") to clarify this point.

8.Why was the data in Fig 2h compared to shuffled data? I don't see the point in this statistical comparison. This data demonstrates the temporal involvement of these neurons, which means there is nothing in randomly shuffled data. The time delay itself is the important part.

Thank you for your important question. We compared the data in Fig 2h to shuffled data to establish a baseline for random associations between photometry data and urination events. This approach allows us to determine whether the observed temporal correlations are significant and not attributable to chance. The method of shuffling the data preserves the natural statistics of each signal on the relevant timescale of the analysis, as previously reported in the literature (Hou et al., Cell, 2016, PMID: 27662084). By preserving the natural statistics of the signals while disrupting their temporal alignment, shuffled data serve as a rigorous control for random fluctuations. This comparison is critical for demonstrating that the observed neuronal involvement is meaningful rather than a result of stochastic variability. We hope this clarifies our rationale.

9.In the opto-stim experiment how many times was each mouse stimulated? Did the repeated stimulation decrease the chances of a void? Seems like voiding would also be dependent on the fill state of the bladder. How was the bladder fill state controlled?

Thank you for your insightful questions. In the optogenetic stimulation experiment with freely moving mice, each mouse underwent light stimulation over two consecutive days, with one 40-minute photostimulation session per day (no more than 10 stimulations per day). We have added this detail to the Methods section (lines 544-546). As depicted in Figure 3f-l, voiding is indeed dependent on the bladder's fill state. To control the bladder fill state, we administered the diuretic via intraperitoneal injection prior to the photostimulation experiment. This ensured that the bladder could store a larger volume of urine within a short period and remained effective throughout the 40-minute testing window. Consequently, the repeated stimulation did not decrease the chances of voiding, as the diuretic maintained an appropriate bladder fill state for effective voiding responses.

10.Iine 154 – how were the intervals randomized? This seems like a way to introduce bias, why not regular intervals?

Thank you for raising this important question. The intervals were randomized using MATLAB software, which sorted the 20 s or 40 s stimulation intervals in a random sequence. We chose

randomization to prevent the possible entrainment of bladder contracture, as reported in previous literature (Hou et al., Cell, 2016, PMID: 27662084). Regular intervals might lead to entrainment, affecting the bladder's response to light stimulation. Randomized intervals reduced this risk, allowing the bladder to respond more naturally and providing more reliable data.

11.Not sure about the statement SST neuron activation is sufficient to dive voiding contractions.

Thank you for bringing this to our attention. We have carefully reviewed our manuscript and confirmed that the statement "SST neuron activation is sufficient to drive voiding contractions" does not appear in our manuscript. We understand the importance of clarity in our statements. In our text, we used the term "bladder contractions" rather than "voiding contractions". Our experimental results, as shown in Figure 3f-i, demonstrate that activation of SST^+ neurons reliably induced bladder contractions (pressure increases) in every trial. However, voiding contractions, which are part of the reflex voiding process, only occurred in some trials when the bladder was sufficiently filled (Figure 3f-i). Therefore, we believe that using "bladder contractions" is more accurate in this context. We appreciate your attention to detail and will make sure to address any potential ambiguities in our manuscript.

12.In the chemogenetic experiment. Was the total volume of urine expressed altered? This is an important parameter to report, given that more frequent voiding could be the result of increased diuresis as well.

Thank you for your important question. We have conducted a new analysis focusing on the changes in the total urine volume within the chemogenetic experiment and have incorporated the findings into the revised manuscript's Figure 7f and Supplementary Figure 2d, as well as the corresponding part of the **Results** section (lines 263-274). Our results indicated that the pharmacogenetic activation of PMC-projecting l/vlPAG SST^+ neurons led to a significant increase in the total urine area (which served as an indicator of total urine volume) during the 2-hour observation period when compared to the saline trials (as shown in the revised Figure 7f). By contrast, in the control mice with mCherry expressed in PMC-projecting l/vlPAG SST^+ neurons, there were no changes in the total urine area after CNO administration (as depicted in the revised Supplementary Figure 2d). You are quite right that more frequent voiding could potentially be a consequence of increased diuresis, and this might indeed be one of the underlying reasons for the observed phenomena.

13.Reduce the number of abbreviations if possible. There is no need to abbreviate pelvic nerve. LUT, SPN, DGC and ESR1 also could be removed as they are used minimally.

Thank you for your valuable suggestions. We agree that reducing the number of abbreviations can enhance the readability of the manuscript. We have carefully reviewed the text and removed the abbreviation for pelvic nerve. Additionally, we have also removed the abbreviations LUT and ESR1 as they were used minimally. Since we have incorporated new PRV tracing experiments and revised the corresponding experimental results in the Results section (lines 112-128), where SPN and DGC are frequently referred to, we have chosen to retain these two abbreviations.

14.Cutting the pelvic nerve will result in decreased bladder contractions no matter what your stimuli. The results from this experiment add minimally to the whole work.

Thank you for your important comment. We fully agree with your opinion. The results of this experiment indeed have a minimal contribution to the overall work. However, since the experiment was conducted in the same animals before and after cutting the pelvic nerve, it could provide a small but additional piece of evidence that SST^+ neurons primarily control bladder contractions. This also allows us to emphasize in the working model (Figure 9) that the regulation of bladder contractions

by SST neurons ultimately depends on the pelvic nerve.

Reviewer #3 (Remarks to the Author):

The manuscript entitled SST Neurons in the Periaqueductal Gray Regulate Urination and Bladder Function by Yen et al details extensive experiments exploring the brainstem circuits underlying urination. Urination is an essential homeostatic mechanism which when unregulated can have profound impacts on health and quality of life. Despite this, the brainstem circuits regulating micturition have yet to be fully characterised.

In this study, the authors present compelling evidence that l/vlPAG-SST+ neurons that project to the PMC are an essential component of the brainstem circuitry that regulates micturition via the use of cell type-specific neuronal activity recordings, optogenetic stimulation, and chemogenetic manipulation combined with cystometrograms and EUS electromyograms (EMG). The data from this study complements and extends previous work exploring these networks, providing an understanding of the link between the two major brainstem regions involved in bladder storage, the PAG and PMC. The methodology is mostly comprehensive, the data are well presented, and the discussion is sound. I have only minor comments that I believe would improve the quality of this manuscript.

We sincerely appreciate the reviewer's positive feedback and the constructive comments that are essential for improving our paper. We completely agree with the comments and suggestions. In the revised manuscript, we have addressed each of the minor comments and suggestions you provided.

1.Line 74 – 'itch' not inch

Thank you so much for pointing out this typo. We sincerely apologize for this oversight. We have promptly made the correction in the manuscript (line 81). We are grateful for your attention to detail, and we appreciate your help in ensuring the accuracy of our work.

2.Line 103 – It is not clear why there are four synaptic connections from the bladder or EUS.

Thank you for your crucial feedback. We apologize for not providing sufficient PRV tracing experimental data in the previous manuscript to clearly confirm four synaptic connections between the PAG and the bladder. To better illustrate this conclusion, we have added extra PRV tracing experiments to compare the distribution of PRV-labeled neurons at different time points after PRV injection. Specifically, PRV tracing data at 48-hour and 72-hour post-PRV injection have been added. These data provide a clearer view of the trans-synaptic progression of the virus and the sequential infection of brain regions. The corresponding results were added into **Supplementary Figure 1**, and the corresponding text descriptions were added to the corresponding positions in the **Results** section (lines 112–128). The details are as follows: "At 48 hours post-injection into the bladder, PRV-labeled neurons were found only in the major pelvic ganglion (MPG) of the peripheral nervous system and the sacral parasympathetic nucleus (SPN) within the spinal cord (Supplementary Fig. 1a). Neurons in the SPN send parasympathetic preganglionic fibers to MPG parasympathetic postganglionic cells, which subsequently innervate the bladder. At 48 hours post-PRV injection into the EUS, PRV-labeled neurons were only observed in the Onuf's nucleus and the dorsal gray commissure (DGC) in the spinal cord (Supplementary Fig. 1b). Somatic motor neurons in the Onuf's nucleus receive dense inputs from the interneurons in the DGC and then send their axons to directly control EUS activity. Based on our results and these previous reports,

the spinal cord neurons in the SPN and DGC act as the second-order neurons for controlling bladder and EUS, respectively. By 72 hours post-injection, viral labeling progressed to the pontine micturition center (PMC), as seen in both the bladder and EUS injection groups (Supplementary Fig. 1c-d). It is known that PMC neurons directly project to the SPN and DGC, indicating that they are the third-order neurons. At 96 hours post-injection into the bladder and EUS, PRV-labeled neurons appeared in the l/vlPAG (Fig. 1h, 1j), while they were not present at earlier time points (Supplementary Fig. 1a-d). This suggests retrograde trans-synaptic transport of PRV through at least four synaptic connections from the bladder or EUS to the l/vlPAG."

3.Figure 1: line 105 – labelled neurons are not clear within the SPN in the spinal cord. Out of the whole dorsal horn, this area appears to have the least labelling. Why was this selected to highlight? There is more extensive labelling with the DGC and across lamina I-V. This is in line with recent studies showing the terminals of sensory axons within the bladder wall (PMID: 30531372). The presence of staining in the MPG confirms that the tracer is non-specific and will label both autonomic and sensory nerves. This should be clarified.

Thank you for your valuable comment. PRV is a retrograde trans-synaptic tracer widely used to map the central neural networks that innervate peripheral organs (Fan et al., J Neurosci Methods, 2020, PMID: 32371062). When injected into the bladder, it mainly infects the efferent pathways innervating the bladder. In the revised manuscript, our newly added data shows that at 48 hours after PRV injection into the bladder, the SPN neurons were the first to be infected by PRV in the spinal cord (Supplementary Fig. 1a). At 72 hours after PRV bladder injection, the SPN neurons labeled by PRV could also be clearly seen (Supplementary Fig. 1a). The neurons in the DGC and lamina I-V also send projections to the SPN (Fowler et al., Nat Rev Neurosci, 2008, PMID: 18490916), so these neurons can be labeled by PRV as well. At 96 hours post-injection, the number of PRV-labeled neurons in the DGC and lamina I-V increased significantly, exceeding the number of PRV-labeled neurons in the SPN. Notably, the parasympathetic postganglionic cells in the MPG receive dense inputs from the parasympathetic preganglionic bladder motoneurons in the SPN and then send their axons to directly excite the bladder (Fowler et al., Nat Rev Neurosci, 2008, PMID: 18490916; Wanigasekara et al., Cell Tissue Res, 2003, PMID: 12596037). Moreover, PMC neurons directly project to the SPN to control bladder contraction (Hou et al., Cell, 2016, PMID: 27662084). Therefore, the neurons in the SPN are the most important efferent motoneurons in the spinal cord that control bladder function, which is why we chose to highlight them.

Regarding the PRV staining in the MPG, it's important to note that the MPG are mixed autonomic ganglia that contain both postganglionic sympathetic and parasympathetic neurons. These neurons innervate pelvic organs, including the bladder, and receive synaptic inputs from preganglionic neurons located in the lumbar and sacral cord (i.e., SPN neurons) (Wanigasekara et al., Cell Tissue Res, 2003, PMID: 12596037; Bertrand et al., J Vis Exp, 2020, PMID: 32202526). Therefore, it is understandable that our results showed PRV-labeled neurons in the MPG and SPN at 48 hours post-injection into the bladder (Supplementary Fig. 1a), which is consistent with these reported anatomical theories (Nadelhaft et al., Brain Res, 2001, PMID: 11382385; Wanigasekara et al., Cell Tissue Res, 2003, PMID: 12596037; Fowler et al., Nat Rev Neurosci, 2008, PMID: 18490916). It's well-known that the MPG also provide the route by which lumbar and sacral sensory axons reach the pelvic organs. Even if PRV infects sensory neurons through the MPG, it will not interfere with the results. This is because PRV is a retrograde trans-synaptic tracer that infects neural circuits in the reverse direction of synaptic transmission. The sensory neurons that innervate the

bladder do not have upstream input neurons, so PRV cannot be transmitted retrogradely from these sensory neurons to their input neurons.

4.Figure 3: it is intriguing that in ChR2-expressing mice, light-induced EUS busting activity. It would be typical to expect that during the stimulus for micturition there would be EUS relaxation to allow efficient expulsion of urine. Was there a definable relationship between EMG activity and the fill state of the bladder during optogenetic stimulation? Or could it be that there is a different subset of neurons in the PAG or PMC that regulate EUS relaxation? This is partly explored in figure 4 where EUS activity is not seen in the empty bladder.

Thank you for your crucial comment. In our experiment, we induce reflex voiding by perfusing saline into the bladder at a constant rate. This allows us to observe periodic increases in bladder pressure along with EUS bursting muscle patterns, which are correlated with voiding and subsequent decreases in bladder pressure. During voiding in mice, the EUS shows a unique activity pattern, with bursting contractions interspersed with periods of muscle relaxation. This EUS bursting activity is characterized by clusters of high-frequency spikes (active periods) separated by low tonic activity (silent periods), and results in rhythmic contractions and relaxations of the EUS, which are believed to act like a pump, enabling efficient urine flow through the narrow rodent urethra (as described in the study by Kadekawa et al., Am J Physiol Regul Integr Comp Physiol, 2016, PMID: 26818058). In contrast, in humans, during urination, the EUS transitions from a state of sustained contraction to complete relaxation, with no EUS-EMG activity detected.

The light-induced EUS bursting activity we recorded is consistent with the results reported in previous studies (Keller et al., Nat Neurosci, 2018, PMID:30104734; Ito et al., Elife, 2020, PMID:32347794). Optogenetic stimulation of PMC $ESR1^{+}$ neurons, which mainly control the EUS, has been shown to induce EUS bursting activity regardless of whether the bladder is full or empty, according to the previous report (Keller et al., Nat Neurosci, 2018, PMID:30104734). However, when it comes to optogenetic stimulation of neurons involved in the regulation of bladder function, such as the PAG SST^{+} neurons reported in our article and the CRH^{+} neurons in the PMC reported previously (Ito et al., Elife, 2020, PMID:32347794), the situation is different. Light stimulation of these neurons stably induces bladder contraction but does not affect the sphincter unless the bladder is partially filled. We speculate that there may be other subtypes of neurons in the PAG that are responsible for controlling the EUS. In our future research, we plan to further investigate these specific PAG neuron subtypes to better understand the neural circuits involved in urinary continence and voiding.

5.Fig 4d: Looks like there is one responder in the EMG group which is not in the dot histograms. Is this an artifact?

Thank you for your important comment. We carefully examined the EMG data of all experimental animals in Fig 4d and did not observe any light-evoked EUS-EMG bursting activity during the 5-second optogenetic stimulation period (under the blue bar). What we quantitatively analyzed in Fig 5g is the success rate of light-induced EUS-EMG bursting activity during the 5-second light stimulation period. Because the one responder showed weak EMG activity outside the light stimulation period, we did not add it into the dot histograms. We further analyzed the EMG data of the one responder and found that the characteristics of these weak EMG signals were different from those of EUS-EMG bursting activities during urination or during 5-second light stimulation period. These EMG signals are likely to be artifacts caused by the mouse's limb twitching due to the decreased depth of anesthesia, which pulled on the recording electrodes.

6.Line 295 – missing P from PMC

Thank you very much for bringing this to our attention. We sincerely apologize for the omission of the "P" in "PMC" at line 333. This was an oversight on our part. We have now corrected it in the manuscript. We appreciate your thorough review and are grateful for your help.

7.Line 312 – The point being made on lines 311-312 is not clear. You have made it clear in this study that l/vlPAG do receive extensive spinal cord inputs?

Thank you for your important comment. We apologize for the confusion caused by the unclear statement. In fact, our study did not investigate whether l/vlPAG neurons receive extensive spinal cord inputs. Pseudorabies virus (PRV), as a neurotropic retrograde multi-transsynaptic tracer, is widely employed to map the descending central neural networks that innervate peripheral organs (Fan et al., J Neurosci Methods, 2020, PMID: 32371062). We have revised the sentences (lines 348-351) as follows: "First, our transsynaptic retrograde tracing data indicate that a subset of l/vlPAG neurons participate in controlling the motor functions of the bladder and EUS via descending neural pathways, probably sending out signals that promote urination. This is in contrast to the previous notion that l/vlPAG neurons receive ascending inputs from the spinal cord, which might carry sensory information from the bladder." We hope this clarifies the point and addresses your concern.

Methods:

8.The methods for the recording of urination are not provided including data transposition. How were the images recorded and then translated into the images that could be analysed and quantified?

Thank you for bringing up this important point. We apologize for the oversight in not detailing the methods for recording urination events and the quantitative analysis of urine spot area in the initial **Methods** section. We have now included a comprehensive description of the urination recording process and the data transposition procedures in the **Methods** section (lines 562-567, lines 575-581). We believe this additional information will provide a clearer understanding of our approach and hope it addresses your concerns satisfactorily.

9.Statistical analysis – large differences in groups – clearly identified comparisons.

Thank you for your important comment. We have carefully reviewed and improved the statistical analysis in our manuscript. Specifically, we have clearly identified the comparisons in the figure legends.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):
These are well performed and well documented studies which examine the role of a novel population of periaquaductal gray (PAG) neurons in bladder and external sphincter function. The authors have responded very thoroughly to the original concerns.
We sincerely thank you for your professional insight and critical review of our manuscript. The constructive feedback you provided has significantly improved the scientific rigor and quality of our research. In the revised manuscript, we have addressed each of the minor comments you provided. Below, we respond to the specific points raised:

Two minor comments remain:
1. On page 2, line 78, I believe the authors mean, "contrast" rather than "contact."
Responses: Thank you for your careful review. We sincerely apologize for the oversight. We have corrected “contact” to “contrast”.

2. More importantly, in the narrative of the pseudorabies virus experiments (page 3 line 112) and in the supplemental figure, it appears that the first time point is 24h and not 48h. If the authors are describing the sequential movement of the virus from the bladder up to the ganglia and than back to the PAG, the ganglion would appear to be labeled at 24h and not 48h, as they describe in the text and the figure legend.
Responses: Thank you for raising this important point. We sincerely appreciate your attention to the time-ordered course of infection after PRV injection into the bladder in our experiments. We agree that the major pelvic ganglion (MPG), which contains postganglionic neurons directly innervating the bladder, would theoretically be labeled first at 24 hours post-injection. However, our experimental design intentionally focused on later time points (48 h, 72 h, and 96 h) to track the sequential spread of the virus beyond the ganglia. We did not include a 24 h time point in our experimental design.

At 48h post-injection, we observed PRV-labeled neurons in both the MPG and the sacral parasympathetic nucleus (SPN) within the spinal cord, as detailed in the text and Supplemental Figure 1a. This observation aligns with prior research showing that PRV spreads from the bladder to the spinal cord by 48 hours (Nadelhaft et al., Neuroscience Letters, 1992, PMID 1331903; DeFinis et al., Neurotrauma Reports, 2021, PMID 35018366; Yu et al., Urology, 2003, PMID 14550467). The fact that neurons in the SPN send parasympathetic preganglionic fibers to MPG parasympathetic postganglionic cells, which subsequently innervate the bladder, further supports this timeline.

Reviewer #2 (Remarks to the Author):

We sincerely appreciate your dedicated efforts in ensuring the quality of our work. Your meticulous review and suggestions have significantly contributed to making our study more comprehensive and meaningful.

Minor comment.

The description on why diuretic furosemide used was perfect please add this or an abbreviated version to the methods.

Responses: Thank you for your valuable comment. We have added the reason for administering the diuretic furosemide to the Methods section (lines 488-490: “In order to increase the number of urination events during the recording period, each mouse was administered furosemide via intraperitoneal injection”; lines 530-532: “Each animal was treated with furosemide before optogenetic stimulation to ensure that a larger volume of urine could be stored in the bladder within a short period”).

Reviewer #3 (Remarks to the Author):

The authors have comprehensively addressed all my previous comments.

Responses: We sincerely appreciate your positive feedback. We are pleased to hear that we have fully addressed all your previous comments. We truly value the time and effort you have invested in reviewing our manuscript. Your professional evaluation and insightful comments have significantly enhanced the quality of our research.