

Descending Enkephalinergic Neurons in Barrington's Nucleus Gate Sex-Biased Control of Micturition

Corresponding Author: Dr Anne Verstegen

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, Klymko et al. reanalyzed published single-nucleus RNA sequencing data and integrated these with ISH datasets to identify six molecularly distinct glutamatergic subpopulations within and surrounding Barrington's nucleus. Among these, they focused on a subset of excitatory neurons expressing proenkephalin, showing that these neurons selectively activate during voiding and contribute to external urethral sphincter (EUS) relaxation, with sex-specific effects. The discovery of a non-Crh excitatory Bar subpopulation and its involvement in micturition control is commendable and advances our understanding of the functional heterogeneity within Barrington's nucleus. However, several key issues require further clarification or discussion prior to acceptance.

Major:

1. A major concern with this study lies in the lack of specificity of Penk expression in Barrington's nucleus. As demonstrated in the authors' previous publication (e.g., Figure 5a–5d in PMID: 38438345), the Allen Brain Atlas, and the current manuscript (e.g., Figures 2d, 3a, and 5a), Penk is not uniquely expressed in Barrington's nucleus. Rather, it is broadly distributed across several surrounding pontine regions, including the MTN (abundant), pre-LC, LC (moderate), and the PCG (abundant). This anatomical distribution challenges the specificity of Penk as a selective genetic marker for Barrington's neurons and, more critically, raises concerns about the interpretation of Bar-specific functional manipulations, particularly those presented in Figure 5, where hM3Dq expression is observed in Bar, MTN, and PCG.

At a minimum, the authors should replicate their key findings (Figures 4–6) using an intersectional genetic approach, such as combining a lumbosacral AAV-retro-Flp injection with a spatially restricted AAV-Con/Fon-DREADD injection into Barrington's nucleus. This strategy would enhance anatomical specificity, particularly since among the adjacent Penk-expressing nuclei, only neurons in the ventral LC are known to project supraspinally.

2. The re-clustering of single-nucleus RNA-seq datasets from regions surrounding Barrington's nucleus does not appear to identify novel markers with strong anatomical specificity for this nucleus. Among the six glutamatergic neuronal populations residing in or near Bar, only Crh, a well-established marker, shows high specificity for Barrington's nucleus (Figures 2 and S2). In contrast, Fgf10 and Foxp2, the latter a known marker for pre-LC and PCG, exhibit minimal expression within Bar and are therefore unsuitable as reliable markers for this nucleus.

This assessment is based on post hoc analysis, and I fully acknowledge the technical challenges associated with microdissecting such a small and anatomically complex region. However, in light of these limitations, I suggest merging Figures 1 and 2 into a single main figure, reorganizing the corresponding Extended Figures accordingly, and including a brief discussion of the limitations of the current approach. This restructuring would streamline the main text and allow greater focus on Penk.

3. Given that both BarPenk and BarCrh supraspinal axons robustly innervate ChAT-expressing pre-ganglionic neurons in lumbosacral IML, the authors should discuss why activation of BarPenk neurons fails to induce bladder contraction, unlike what has been shown for BarCrh neurons.

4. The relative anatomical (i.e. mono-synaptic input) and functional distinctions between BarPenk and BarCrh neurons warrant a more thorough discussion. For example, though molecularly distinct subgroups, both seem to be involved in scent-marking behaviors, as shown in PMID 27662084 for BarCrh neurons.

Minor:

1. "Conditional ablation of the BarPenk neurons did not affect voiding frequency or void sizes in either sex (Fig. 6c-f; ED Fig. 6a-d), in contrast to ablation of the entire glutamatergic Bar population, which leads to severe urinary retention with overflow incontinence." Does this imply that the remaining unclassified excitatory population (40% in Figure 3) is crucial for voiding?

Reviewer #2

(Remarks to the Author)

In 'Enkephalinergic neurons in barrington's nucleus gate sex-specific control of micturition' Klymko et al. analyze transcriptional profiling to identify and study a novel subpopulation of neurons in the Bar expressing Penk1. They use anatomic tracing, neural activity manipulations, and behavior (urination and cystometry) to study this new neural type and find the Penk1+ neurons share some features with the previously studied Esr1+ subset, and contrast with the activity and functional importance of the previously studied Crh+ Bar neurons. They find Penk1 neural activity to be important for driving normal micturition in males, but not in females. Overall, the studies are tightly performed, and the manuscript is well written. The transcriptional analysis in Figure 1 is expected to open up new areas of study for this group and others iterate the series of approaches described here to understand the functional relevance of the repertoire of Bar excitatory neurons. The conclusion that the effects of Penk1+ neurons are sex-specific is quite exciting, however more experiments done in parallel with females (anatomy and cystometry) need to be included in this study to enable the reader to better interpret the behavioral observations. Overall, this is an excellent study that illuminates a new marker in the Bar that will be valuable to advance the study of urination.

Concerns:

- 1) Reading through the results section, the focus seemed to be on characterizing the small subset of Penk1+ neurons by anatomy and behavior compared to the Crh+ subset. However, more of the discussion is focused on comparing the Penk1+ neurons to previously published work on the Esr1+ neurons. Figure 1F shows that most other neurons (88%) express Esr1, but that does not seem to be the case in Figure 1E, and the expression of Penk1 seems to be equally broad in the Figure 6b example. To let the reader evaluate the specificity of the Penk1 compared to the Esr1 neurons, please include Esr1 on the Y-axis of Figure 1D, and evaluate the expression of Esr1 and Penk by a non-genetic reporter means (antibody, RNA in situ) to better quantify. Similar inclusion of Esr1 would be helpful in Figure 2 (A-F anatomy study).
- 2) Please provide more information about the sex dependent male behavior following Penk1+ activation (Figure 5). The text suggests that they are either voiding incompletely or undergoing non-voiding events (leaks). Do you have any data to shed light on what is happening? Please include a temporal analysis of when the large voids were deposited compared to the small voids. If they are 'leaks' they would be expected to occur prior to the large voids when the bladder is full. In contrast, if large voiding events occur first followed by only smaller voids, then this would reflect an increase in urgency irrespective of bladder fullness.
- 3) It would be most informative to use optogenetic manipulations to better correlate neural activity manipulations with urination behavior.
- 4) Did the Penk1+ hm3Dq animals show any overt behavioral phenotypes following CNO activity that may further explain the sex-biased phenotype?
- 5) Do the authors perform staining of descending Bar-Penk, Bar-Crh and Bar-Tac1 projections into the spinal cord across both male and female animals and compare the innervation patterns? This is partially mentioned in the discussion but is not made clear in Fig 2 or in the accompanying text. This is especially relevant given the sexual dimorphism observed in the behavioral consequences of Bar-Penk chemogenetic activation as described in Fig 5.
- 6) Similarly, please highlight and report which individuals in the GCaMP (Figure 4) and cystometry (Figure 7) experiment were males and females that the reader can evaluate the effects of activity in both sexes. If only males were included, it seems premature to focus the study on sex differences (title).

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

During revision, the authors performed a series of experiments to address my concerns and questions. Their efforts are highly appreciated, and I have only a few minor suggestions before acceptance.

1. The revised manuscript now clearly shows that supraspinal Penk neurons in Barrington's nucleus play a crucial role in the reported phenotype. I therefore suggest that the authors consider revising the title to reflect this key finding as "Descending Enkephalinergic Neurons in Barrington's Nucleus Gate Sex-Biased Control of Micturition."
2. Regarding the statement, "Importantly, animals with incomplete or unilateral targeting conditions—more likely to include neighboring Penk-expressing regions—do not exhibit the behavioral phenotype (ED Fig. 6h)," it would be more informative to include a schematic illustration depicting the contours of complete versus incomplete targeting, similar to ED Fig. 6e. In addition, a regression plot correlating anatomical targeting with behavioral function would help further support this conclusion.
3. I appreciate that the authors compared the innervation patterns of BarPenk and BarCrh populations in the lumbosacral spinal cord, potentially reflecting their distinct roles in micturition control. A recent study, consistent with the authors' own observations (PMID: 28340519), showed that BarCrh axons, although sparse, innervate the superficial dorsal horn and contribute to descending pain modulation (PMID: 40132590). This finding highlights functional heterogeneity even within the

same molecularly defined population in Barrington's nucleus. Do BarPenk descending axons show a similar or distinct spinal innervation pattern, e.g., more exclusive innervation of lumbosacral segments? A brief discussion of this point would further strengthen the distinction between these neuronal populations and highlight the functional heterogeneity of this small nucleus.

Reviewer #2

(Remarks to the Author)

The authors have addressed my comments both experimentally and textually. I have no further concerns.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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At a minimum, the authors should replicate their key findings (Figures 4–6) using an intersectional genetic approach, such as combining a lumbosacral AAV-retro-Flp injection with a spatially restricted AAV-Con/Fon-DREADD injection into Barrington's nucleus. This strategy would enhance anatomical specificity, particularly since among the adjacent Penk-expressing nuclei, only neurons in the ventral LC are known to project supraspinally.

Author's response: We thank the reviewer for raising this important concern regarding the anatomical specificity of *Penk* expression in the pontine tegmentum. We acknowledge that *Penk* is not restricted to Barrington's nucleus (Bar) and is expressed in several neighboring structures. At the same time, our original manipulations were anatomically centered in Bar and yielded a consistent set of anatomical, physiological, and behavioral findings. To further strengthen the Bar-specific interpretations, we have performed additional experiments, including intersectional strategies, to selectively target spinally projecting Bar^{Penk} neurons and to replicate our key findings.

First, to restrict recordings to spinally projecting Penk neurons in Bar, we used a retrograde approach by injecting a Cre-dependent GCaMP8s (AAVretro-DIO-GCaMP8s) into the lumbosacral spinal cord of Penk-IRES-Cre mice, followed by an optical fiber placement above Bar. Fiber photometry recordings from this population revealed activity patterns closely matching those observed in the broader Bar^{Penk} population, with robust activation time-locked to void onset (**Fig. 4i, j; ED Fig. 4c**). Moreover, the duration of neural activity strongly correlated with void duration (**Fig. 4k**), indicating that spinally projecting Bar^{Penk} neurons recapitulate the core dynamics of void-specific activity.

Second, to test functional sufficiency, we used an intersectional chemogenetic strategy by injecting AAVretro-DIO-FlpO into the lumbosacral spinal cord, followed by an injection of Flp-dependent DREADDs (AAV8-fDIO-hM3Dq) into Bar, thereby restricting DREADDs expression

to spinally projecting Bar^{Penk} neurons. Activation of this population replicated the main behavioral effects observed with broader Bar^{Penk} stimulation, including increased urinary leak occurrence and reduced urine volume per void, without changes in voiding frequency (**Fig. 5I**). These results demonstrate that the incontinence-like phenotype observed in the original experiments is reproduced by specifically targeting spinally projecting Bar^{Penk} neurons.

Finally, for the ablation experiment, we provide additional evidence supporting anatomical specificity. Analysis of the injection-site overlap shows that effective ablations consistently target the Bar core bilaterally (**ED Fig. 6e**). Importantly, animals with incomplete or unilateral targeting - conditions more likely to include neighboring *Penk*-expressing regions - do not exhibit the behavioral phenotype (**ED Fig. 6h**). This indicates that bilateral targeting of the Bar core, rather than off-target ablation of nearby *Penk* populations, is required for the observed effects.

Taken together, these additional experiments and analyses demonstrate that the key findings of this study are reproduced when selectively targeting spinally projecting Bar^{Penk} neurons, and support the conclusion that the observed effects of functional manipulations arise from *Penk*+ neurons in Bar rather than from neighboring *Penk*-expressing populations.

We have updated the manuscript accordingly by adding new data (**Fig. 4g-k, ED Fig. 4c-d, Fig. 5j-l, ED Fig. 6e, h**) and revising the Results (lines 229-233, 253-257, 273-274) and Discussion (lines 346-348).

2. The re-clustering of single-nucleus RNA-seq datasets from regions surrounding Barrington's nucleus does not appear to identify novel markers with strong anatomical specificity for this nucleus. Among the six glutamatergic neuronal populations residing in or near Bar, only *Crh*, a well-established marker, shows high specificity for Barrington's nucleus (Figures 2 and S2). In contrast, *Fgf10* and *Foxp2*, the latter a known marker for pre-LC and PCG, exhibit minimal expression within Bar and are therefore unsuitable as reliable markers for this nucleus. This assessment is based on post hoc analysis, and I fully acknowledge the technical challenges associated with microdissecting such a small and anatomically complex region. However, in light of these limitations, I suggest merging Figures 1 and 2 into a single main figure, reorganizing the corresponding Extended Figures accordingly, and including a brief discussion of the limitations of the current approach. This restructuring would streamline the main text and allow greater focus on *Penk*.

Author's response: We thank the reviewer for this thoughtful comment and acknowledge that identifying highly specific genetic markers for Bar remains challenging, particularly given its small size and anatomical proximity to adjacent pontine structures.

We appreciate the reviewer's suggestion to merge Figures 1 and 2 and have carefully considered this recommendation; however, we have retained them as separate figures to preserve the distinction between the analyses presented. Figure 1 focuses on transcriptional profiling and clustering of the glutamatergic neuronal populations, whereas Figure 2 shows the anatomical and functional characterization of candidate Bar populations. Maintaining this separation allows the reader to follow the progression from molecular identification to circuit-level analysis more clearly. In addition, with the inclusion of new data requested by other reviewers, combining the figures could reduce readability and make it challenging to present the expanded results clearly.

In response to the reviewer's suggestion, we have extended the Discussion (lines **484-486**) to address the limitations of the sequencing approach more explicitly. Specifically, we note that the use of DroNc-seq, with its relatively low sensitivity, may limit detection of low-abundance transcripts and rare or spatially restricted neuronal subtypes. Additionally, we acknowledge that the lack of highly specific markers likely reflects both technical limitations and the biological heterogeneity of the pontine tegmentum, where transcriptionally related populations span adjacent regions.

3. Given that both BarPenk and BarCrh supraspinal axons robustly innervate ChAT-expressing pre-ganglionic neurons in lumbosacral IML, the authors should discuss why activation of BarPenk neurons fails to induce bladder contraction, unlike what has been shown for BarCrh neurons.

Author's response: We thank the reviewer for raising this important point. In response, we have added a quantitative analysis of the spinal distribution of synaptic boutons in IML and DGC from Bar^{Penk} and Bar^{Crh} neurons (**ED Fig. 3f-j**). As described in the revised Results (lines 209-216), although both populations project to the DGC and IML, their patterns of innervation differ: Bar^{Penk} boutons are consistently enriched in the DGC across the lumbosacral levels, whereas Bar^{Crh} boutons show greater enrichment in the S1 and S2 IML, which contains most of the preganglionic bladder motor neurons. These findings indicate that, despite connecting with the same spinal regions, Bar^{Penk} neurons provide relatively less input to the IML subregions associated with bladder motor output. Accordingly, the lack of bladder contraction following Bar^{Penk} activation is consistent with a projection pattern that is anatomically overlapping with, but not functionally equivalent to, that of Bar^{Crh} neurons.

Consistent with this interpretation, Bar^{Crh} neurons have been linked to detrusor activity (Keller et al., 2019), and our data show that Bar^{Penk} neurons preferentially engage circuits controlling the external urethral sphincter. Together, these findings suggest that shared projection targets can support distinct functions through differential engagement of local downstream circuitry, including potentially distinct neuronal targets within the IML. At present, however, the relevant spinal subregions and circuit elements are not sufficiently defined, highlighting the need for future studies to resolve these mechanisms with greater anatomical and functional resolution.

We have updated the manuscript accordingly by adding new panels to supplemental Figure 3 (**ED Fig. 3f-j**), revising the results, and expanding the Discussion (lines 209-216, 356, 365-368).

4. The relative anatomical (i.e. mono-synaptic input) and functional distinctions between BarPenk and BarCrh neurons warrant a more thorough discussion. For example, though molecularly distinct subgroups, both seem to be involved in scent-marking behaviors, as shown in PMID 27662084 for BarCrh neurons.

Author's response: We thank the reviewer for this insightful comment. We agree that the relative anatomical and functional distinctions between Bar^{Penk} and Bar^{Crh} neurons merit further discussion, particularly in light of prior work showing that Bar^{Crh} neurons also contribute to scent-marking behavior. In revisiting the monosynaptic rabies tracing datasets for Bar^{Crh} neurons (Hou et al., 2016; Verstegen et al., 2019), we note that the major upstream input regions reported are largely similar to those identified here for Bar^{Penk} neurons, including spinally projecting Bar^{Penk} neurons. This suggests that the two populations are unlikely to be controlled by completely

distinct upstream nuclei and instead may represent parallel output channels recruited by overlapping higher-order circuits during voluntary urinary behaviors.

This interpretation is consistent with the behavioral data. Scent marking, like voiding behavior more broadly, requires coordinated bladder contraction and relaxation of the external urethral sphincter. Hou et al. 2016 showed that Bar^{Crh} neurons are important for scent-marking behavior, and our data show that Bar^{Penk} neurons are required as well, as ablation of these neurons significantly impairs this behavior. We therefore view these populations not as redundant or independently controlled pathways, but as functionally complementary components of the Bar circuitry, with Bar^{Crh} neurons primarily contributing to detrusor activity and Bar^{Penk} neurons to EUS relaxation.

We have expanded the Discussion accordingly (lines 446-454).

Minor:

1. “Conditional ablation of the BarPenk neurons did not affect voiding frequency or void sizes in either sex (Fig. 6c-f; ED Fig. 6a-d), in contrast to ablation of the entire glutamatergic Bar population, which leads to severe urinary retention with overflow incontinence.” Does this imply that the remaining unclassified excitatory population (40% in Figure 3) is crucial for voiding?

Author’s response: We thank the reviewer for this thoughtful question. While the lack of effect following Bar^{Penk} ablation could suggest a role for the remaining excitatory populations, we do not interpret this result as evidence that the unclassified (~40%) population is solely responsible for voiding. As noted in the Discussion, ablation of Bar^{Crh} neurons likewise does not abolish voiding, despite their established role in promoting detrusor contractions. Our findings may suggest a presence of functional redundancy within Bar, rather than a strict division in which a single population is necessary for voiding.

An alternative possibility is that multiple Bar subpopulations, including *Penk*- and *Crh*-expressing neurons, act in a coordinated manner, and that ablating any single population is insufficient to disrupt overall function. Combined or more comprehensive targeting of excitatory Bar neurons may be required to impair voiding fully. In addition, genetic targeting based on individual molecular markers may not capture entire functional populations. As the reviewer notes in Comment 2, the small size and anatomical complexity of this region, together with technical limitations in microdissection and sequencing resolution, may constrain the identification of singular markers that fully define each Bar subpopulation.

Reviewer #2 (Remarks to the Author):

In 'Enkephalinergic neurons in barrington's nucleus gate sex-specific control of micturition' Klymko et al. analyze transcriptional profiling to identify and study a novel subpopulation of neurons in the Bar expressing Penk1. They use anatomic tracing, neural activity manipulations, and behavior (urination and cystometry) to study this new neural type and find the Penk1+ neurons share some features with the previously studied Esr1+ subset, and contrast with the activity and functional importance of the previously studied Crh+ Bar neurons. They find Penk1 neural activity to be important for driving normal micturition in males, but not in females. Overall, the studies are tightly performed, and the manuscript is well written. The transcriptional analysis in Figure 1 is expected to open up new areas of study for this group and others iterate the series of approaches described here to understand the functional relevance of the repertoire of Bar excitatory neurons. The conclusion that the effects of Penk1+ neurons are sex-specific is quite exciting, however more experiments done in parallel with females (anatomy and cystometry) need to be included in this study to enable the reader to better interpret the behavioral observations. Overall, this is an excellent study that illuminates a new marker in the Bar that will be valuable to advance the study of urination.

Concerns:

1) Reading through the results section, the focus seemed to be on characterizing the small subset of Penk1+ neurons by anatomy and behavior compared to the Crh+ subset. However, more of the discussion is focused on comparing the Penk1+ neurons to previously published work on the Esr1+ neurons. Figure 1F shows that most other neurons (88%) express Esr1, but that does not seem to be the case in Figure 1E, and the expression of Penk1 seems to be equally broad in the Figure 6b example. To let the reader evaluate the specificity of the Penk1 compared to the Esr1 neurons, please include Esr1 on the Y-axis of Figure 1D, and evaluate the expression of Esr1 and Penk by a non-genetic reporter means (antibody, RNA in situ) to better quantify. Similar inclusion of Esr1 would be helpful in Figure 2 (A-F anatomy study).

Author's response: We thank the reviewer for this thoughtful comment and for highlighting the importance of clearly evaluating the relationship between *Penk*- and *Esr1*-expressing populations in Bar.

We first note that there may have been ambiguity in the presentation of Figure 1e-f. In these panels, we quantified the proportion of *Crh*-expressing neurons that co-express *Esr1*, as detected by immunohistochemistry ($88\% \pm 1\%$), rather than the fraction of all Bar neurons expressing *Esr1*. We agree that this distinction is important, and to avoid confusion, we have revised the Figure organization, labeling, and legends (**ED Fig. 1d, e**) to more clearly indicate the populations being quantified.

To directly address the reviewer's request, we have expanded our analysis of *Esr1* expression using multiple complementary approaches:

- snRNA-seq analysis: We have added a dot plot showing *Esr1* expression across clusters (**ED Fig. 1c**), demonstrating that *Esr1* is not restricted to a single population but is distributed across multiple Bar clusters, with the highest expression in the *Crh*-enriched cluster and expression detected in the *Penk*, *Tac1*, and *Prlr* populations.

- Quantification of *Esr1*+ neurons: We quantified the proportion of *Esr1*-expressing neurons within Bar ($75\% \pm 1.5\%$; **ED Fig. 3c, d**), indicating that *Esr1* marks the majority of the total Bar neuronal population.
- Non-genetic validation: We performed triple-label RNAscope for *Crh*, *Penk*, and *Esr1* (**ED Fig. 3e**), revealing the same broad pattern of *Esr1* expression in Bar and substantial overlap with both the *Crh* and *Penk* populations. In addition, *Esr1* immunohistochemistry (**ED Fig. 1d, f**) independently confirms widespread expression within and around Bar.
- Reporter-based labeling: Injection of Cre-dependent reporter AAVs into adult *Esr1-Cre* mice resulted in the same extensive labeling in Bar (**ED Fig. 1e, ED Fig. 2g**), further supporting widespread *Esr1* expression in adulthood.
- Anatomical projections: Per the reviewer's request, we analyzed projections from *Esr1-Cre* neurons and found dense innervation of both the DGC and IML, comparable to that observed when targeting *Vglut2*-expressing Bar neurons (**ED Fig. 2d, g**).

Together, these data demonstrate that *Esr1* is broadly expressed across multiple Bar subpopulations and does not define a distinct, anatomically restricted neuronal group. Rather, *Esr1* overlaps extensively with both *Crh*- and *Penk*-expressing neurons (as well as other Bar populations). For this reason, we selected *Crh* as the primary point of comparison, as it represents a genetically and anatomically distinct population within Bar, consistent with prior studies. The broad expression of *Esr1* within Bar limits its utility as a reference population for defining discrete Bar subpopulations.

Finally, our findings align with prior functional work showing that activation of *Esr1*-expressing Bar neurons engages both bladder and sphincter components of lower urinary tract control (Keller et al., 2019). In contrast, the *Crh* and *Penk* populations contribute differently to these functions. This further supports the interpretation that *Esr1* marks a broader population spanning multiple functional subtypes.

We have revised the manuscript accordingly by restructuring and clarifying Figure labeling (**ED Fig. 1d, e**), adding new analyses (**ED Fig. 1c, ED Fig. 2d, g, ED Fig. 3c-e**), and expanding the Results (lines 122-123, 173-175, 205-207), to more clearly present these findings.

2) Please provide more information about the sex dependent male behavior following *Penk1*+ activation (Figure 5). The text suggests that they are either voiding incompletely or undergoing non-voiding events (leaks). Do you have any data to shed light on what is happening? Please include a temporal analysis of when the large voids were deposited compared to the small voids. If they are 'leaks' they would be expected to occur prior to the large voids when the bladder is full. In contrast, if large voiding events occur first followed by only smaller voids, then this would reflect an increase in urgency irrespective of bladder fullness.

Author's response: We thank the reviewer for this helpful suggestion. In response, we added a temporal analysis of male voiding behavior during the 2h MVT assay, which plots the timing of regular voids and leaks (non-voiding events) under saline and CNO-treated conditions (**Fig. 5i**).

We realized that our prior labeling may have contributed to ambiguity. To improve clarity, we have replaced the term "abnormal urine spots" in the main text and figures with "leaks" throughout. Importantly, this classification (as described in the Methods) was based on

behavioral criteria rather than urine spot size. Specifically, regular voids were defined as urination events accompanied by the typical voiding behavioral sequence (usually at the cage edge or corner, with the tail lifted and a brief pause). In contrast, leaks were defined as events occurring outside this normal sequence, often in the center of the arena, during locomotion, or while the animal was engaged in other behaviors.

The temporal analysis suggests that bladder fullness may contribute to the leakage intensity, as leak frequency decreased after a regular void in some cases. However, this pattern was inconsistent across animals and recording sessions: leak events were not restricted to pre-void periods and often persisted after regular voids had occurred. Overall, the phenotype is more consistent with an incontinence-like pattern resulting from poorly timed urine release than with a simple overflow-incontinence model. Additionally, we observed a significant reduction in urine volume associated with regular (corner) voids (**Fig. 5d**), indicating that the phenotype is not limited to increased leak events but also affects the structure of normal voiding.

Furthermore, we note that the behavioral phenotype is not entirely uniform across animals. In some mice, agonist treatment produced frequent small corner voids with relatively few leaks, whereas in the majority of cases, the dominant effect was widespread incontinent-like leakage. We therefore interpret the phenotype as reflecting disrupted bladder-outlet coordination, with frequent and mistimed sphincter relaxation likely contributing to the observed leakage. The same disruption may also hinder efficient voiding by altering timing and coordination between bladder contraction and EUS relaxation, leading to reduced void volumes and increased void frequency in some animals.

We have updated the manuscript accordingly by adding a new figure panel (**Fig. 5i**), revising the Results (lines 241-244), and expanding the Discussion (lines 379-388).

3) It would be most informative to use optogenetic manipulations to better correlate neural activity manipulations with urination behavior.

Author's response: We thank the reviewer for this valuable suggestion. We agree that temporally precise manipulations are particularly useful for linking Bar^{Penk} activity to lower urinary tract function. In fact, the original submission already included an optogenetic experiment designed for this purpose (**Fig. 7**), in which Bar^{Penk} neurons were photo stimulated during simultaneous cystometry and EUS-EMG recordings in awake animals. This approach provided direct, time-locked physiological readouts of both bladder pressure and sphincter activity. These experiments showed that optogenetic activation of Bar^{Penk} neurons produced a rapid and reproducible reduction in EUS-EMG activity, while bladder pressure remained unchanged. Specifically, 75% of stimulation epochs were associated with EUS relaxation, whereas increases in bladder pressure were observed in only 6% of trials. Thus, the optogenetic data demonstrate with high temporal precision that Bar^{Penk} activation is sufficient to suppress sphincter activity without directly driving bladder contraction.

In the revised manuscript, we interpret the chemogenetic phenotype in light of these optogenetic findings and the newly added temporal analysis of voids and leaks (**Fig. 5i**). Together, these data support the conclusion that prolonged Bar^{Penk} activation disrupts bladder-outlet coordination through frequent and mistimed EUS relaxation rather than by directly promoting bladder contraction. For this reason, we did not add a separate behavioral optogenetic experiment, as the existing optogenetic CMG/EMG dataset provides more direct mechanistic insight.

4) Did the Penk1+ hm3Dq animals show any overt behavioral phenotypes following CNO activity that may further explain the sex-biased phenotype?

Author's response: We thank the reviewer for this question. We did not observe overt non-urinary behavioral abnormalities in response to agonist administration in hm3Dq-expressing animals of either sex during the MVT assay. Although these behaviors were not systematically quantified, we did not observe any obvious changes in locomotion, grooming, posture, or arousal that would provide an alternative explanation for the sex-biased urinary phenotype. In addition, administration of the agonist to mice lacking viral expression did not produce detectable behavioral or LUT effects, further arguing against a non-specific effect of the agonist itself.

5) Do the authors perform staining of descending Bar-Penk, Bar-Crh and Bar-Tac1 projections into the spinal cord across both male and female animals and compare the innervation patterns? This is partially mentioned in the discussion but is not made clear in Fig 2 or in the accompanying text. This is especially relevant given the sexual dimorphism observed in the behavioral consequences of Bar-Penk chemogenetic activation as described in Fig 5.

Author's response: We thank the reviewer for raising this important point. To clarify the sex composition of the experiments in the publication, we have added Supplemental Table 5, which lists the number and sex of mice used in each experiment.

For the Bar^{Penk} axonal projection analysis, tracing was performed in both males and females (n = 4 per sex). We did not observe an obvious sex difference in the gross projection pattern: in both sexes, Bar^{Penk} neurons innervated the same lumbosacral regions, including the DGC and IML, with no clear differences in overall distribution or labeling intensity.

In addition, because the reviewer raised the possibility of sex-specific differences in spinal innervation, we have examined the distribution of Bar^{Penk} synaptic boutons separately in males and females across lumbosacral levels (4 males, 3 females). We did not detect a significant sex difference in the relative distribution of boutons between the DGC and IML at the L6, S1, or S2 levels. Although this analysis may not detect subtle differences, it suggests further that the sexually dimorphic behavioral effects are unlikely to be explained by a major difference in the overall spinal terminal distribution of Bar^{Penk} neurons. (In **ED Fig. 3f-I**, the male and female data are shown together as a single cohort, n = 7).

For the remaining candidate Bar populations, these experiments were intended primarily as an anatomical screen and were neither designed nor powered for systematic comparisons between the sexes. Although both sexes were represented in some cohorts (Supp. Table 5) and no clear qualitative differences were observed, we did not conduct a dedicated sex-based analysis of spinal innervation for those populations. Thus, while our current data do not support a major sex difference in the descending projection pattern of Bar^{Penk} neurons, it does not rule out more subtle sex-dependent differences in connectivity or function. Together with the absence of sex-specific transcriptomic clustering in Bar, these findings may suggest that the sexually dimorphic behavioral effects of Bar^{Penk} activation instead arise downstream, for example, through differences in spinal circuit organization, postsynaptic receptor composition, or peripheral LUT anatomy. Addressing this question more fully will require future studies specifically designed to resolve sex-dependent differences in connectivity and function across Bar-defined populations.

6) Similarly, please highlight and report which individuals in the GCaMP (Figure 4) and cystometry (Figure 7) experiment were males and females that the reader can evaluate the effects of activity in both sexes. If only males were included, it seems premature to focus the study on sex differences (title).

Author's response: We thank the reviewer for raising this point. To clarify the sex composition across all experiments, we have added Supplementary Table 5 and now explicitly report the sex of animals in each cohort. As noted, the GCaMP recordings in Figure 4 and the awake cystometry/ EUS-EMG recordings in Figure 7 were performed in male animals.

These experiments were designed to define the physiological role of *Penk*-expressing neurons under conditions in which the behavioral phenotype was robustly observed. Chemogenetic activation of Bar^{Penk} neurons produced a clear LUT phenotype in males, including widespread leakage and reduced urine volume per void, whereas females did not exhibit a similar phenotype. In addition, the voluntary scent-marking behavior disrupted by Bar^{Penk} ablation is a male-associated behavioral readout. We therefore focused the mechanistic experiments on males to define how Bar^{Penk} activity relates to voiding and EUS control in the sex in which the phenotype was most clearly expressed.

We did not intend for the title to imply that we studied intrinsic sexual dimorphism of the Bar^{Penk} neurons, or that all mechanistic features were examined equally in both sexes. To better reflect the scope of the data, we have revised the title from “**Enkephalinergic Neurons in Barrington’s Nucleus Gate Sex-Specific Control of Micturition**” to “**Enkephalinergic Neurons in Barrington’s Nucleus Gate Sex-Biased Control of Micturition**.” This revised wording more accurately captures our central finding that Bar^{Penk} neurons contribute to urinary control in a sex-biased behavioral context, including male scent marking and the male-biased effects of Bar^{Penk} activation.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

"During revision, the authors performed a series of experiments to address my concerns and questions. Their efforts are highly appreciated, and I have only a few minor suggestions before acceptance.

1. The revised manuscript now clearly shows that supraspinal Penk neurons in Barrington's nucleus play a crucial role in the reported phenotype. I therefore suggest that the authors consider revising the title to reflect this key finding as "Descending Enkephalinergic Neurons in Barrington's Nucleus Gate Sex-Biased Control of Micturition."

Author's response: We thank the reviewer for the positive assessment of the revised manuscript and for these additional suggestions. We have revised the title as suggested to: "Descending Enkephalinergic Neurons in Barrington's Nucleus Gate Sex-Biased Control of Micturition."

2. Regarding the statement, "Importantly, animals with incomplete or unilateral targeting conditions—more likely to include neighboring Penk-expressing regions—do not exhibit the behavioral phenotype (ED Fig. 6h)," it would be more informative to include a schematic illustration depicting the contours of complete versus incomplete targeting, similar to ED Fig. 6e. In addition, a regression plot correlating anatomical targeting with behavioral function would help further support this conclusion.

Author's response: We have added two new panels to Supplementary Data Figure 6. Supplementary Data Figure 6h shows the contours of AAV-mCherry-DIO-DTA injection sites in animals with incomplete ablation, and Supplementary Data Figure 6j shows the relationship between the proportion of Bar^{Penk} neurons remaining after targeting and marking behavior.

3. I appreciate that the authors compared the innervation patterns of BarPenk and BarCrh populations in the lumbosacral spinal cord, potentially reflecting their distinct roles in micturition control. A recent study, consistent with the authors' own observations (PMID: 28340519), showed that BarCrh axons, although sparse, innervate the superficial dorsal horn and contribute to descending pain modulation (PMID: 40132590). This finding highlights functional heterogeneity even within the same molecularly defined population in Barrington's nucleus. Do BarPenk descending axons show a similar or distinct spinal innervation pattern, e.g., more exclusive innervation of lumbosacral segments? A brief discussion of this point would further strengthen the distinction between these neuronal populations and highlight the functional heterogeneity of this small nucleus."

Author's response: We have expanded both the Results and Discussion to describe dorsal horn innervation by Bar^{Penk} and Bar^{Crh} neurons. The Results now state: "Sparse bouton labeling was also observed in the dorsal horn, with BarCrh boutons preferentially localized to the superficial

laminae and Bar^{Penk} boutons distributed more broadly across the dorsal horn” (lines 211-213). The Discussion now states: “Consistent with recent work [PMID: 40132590], Bar^{Crh} neurons also sparsely innervate the superficial dorsal horn, a sensory region implicated in descending pain modulation, whereas Bar^{Penk} boutons appear more broadly distributed within the dorsal horn” (lines 364-367). We have also moved the panels comparing Bar^{Penk} and Bar^{Crh} innervation of the lumbosacral spinal cord to main Figure 3f-h.

Reviewer #2 (Remarks to the Author):

The authors have addressed my comments both experimentally and textually. I have no further concerns.

Author’s response: We thank the reviewer for confirming that all concerns have been addressed.