nature portfolio

Peer Review File

A ventral pallidum-locus coeruleus-lateral hypothalamus pathway modulates brain states in freely behaving and isoflurane-anesthetized male mice

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The basal forebrain consists of several distinct nuclei that differ in their synaptic inputs and outputs and are implicated in various physiological functions. A detailed examination of individual nuclei could yield a more precise understanding of the neural circuits underpinning the basal forebrain's physiological functions. In this manuscript, Xu et al. explore the role of glutamatergic (Glu) neurons in the ventral pallidum (VP) in elevating arousal levels in both conscious and anesthetized mice. To clarify the neural circuitry responsible for the function of VP Glu neurons, the authors have identified a neural pathway extending from VP Glu neurons to noradrenergic (NA) neurons in the locus coeruleus (LC) and then to the lateral hypothalamus. They have confirmed that this pathway partially mediates VP Glu neurons' arousal-promoting effects in conscious and anesthetized mice. This study enhances our understanding of the neural circuits that regulate arousal levels in conscious and anesthetized mice and suggests that α2 adrenoceptors in the lateral hypothalamus may serve as effective targets for modulating brain arousal in both states. However, the manuscript would benefit from addressing the following concerns:
Major points:
1. Chemogenetic stimulation of VP Glu neurons reduces EEG power in low-frequency bands associated with enhanced movement states. Conversely, chemogenetic inhibition of VP Glu neurons has a minimal effect on EEG, which does not correspond to degraded movement states. Given the variability in the data, the authors should consider increasing the sample size to substantiate these findings.
2. Figure 3H suggests that optogenetic stimulation of VP Glu neurons did not alter the power of delta waves, a finding at odds with typical EEG patterns. The authors should investigate this effect with an expanded sample size.
3. The data in Figure S5 are compelling. In Figures S5B-E, the authors should include a dashed line on each panel to indicate the averaged time points for LORR or RORR and the initiation of isoflurane exposure. These data imply that the activity of VP Glu neurons may be linked to the presence of isoflurane and the induction and emergence from anesthesia. The authors should discuss this in the Results section.
4. Figure S6 indicates that optogenetic stimulation of VP Glu neurons activated several nuclei in conscious mice. Analogous experiments should be conducted in anesthetized mice to investigate the downstream nuclei mediating VP Glu neurons' modulatory effects on anesthesia.
5. In Figure 4, the authors present morphological and functional data supporting the idea that VP Glu neurons innervate LC NA neurons. Their fiber photometry data show that LC NA neurons innervated by VP Glu neurons exhibit inhibition upon air puff, contrasting with the response of VP Glu neurons. These findings suggest that LC NA neurons may not mediate the response of VP Glu neurons to aversive stimulation. The authors should address this discrepancy in the Discussion section.
6. In Figure 5, optogenetic stimulation of the VP-LC Glu projection reduced the power of delta waves during QS and NM but enhanced delta waves during LM. This finding is inconsistent with the expected function of VP Glu neurons and should be explained.
7. Figure 7 shows that stimulation of the VP-LC Glu projection evokes noradrenaline release in the LH by activating LC NA neurons. The authors should examine whether inhibiting LC NA neurons innervated by VP Glu neurons prevents salient stimulus-induced noradrenaline release in the LH and diminishes the change in noradrenaline release during the induction and emergence from anesthesia. These experiments would provide further insight into the role of this pathway.
8. The overall results suggest that the effects of VP Glu neurons on motor activity, brain arousal, and anesthesia are stronger than those of the VP-LC and VP-LC-LH pathways. This implies that VP Glu neurons may exert these effects through other

pathways, such as the VP-LH pathway (Luo et al., 2023). Although the authors cite this article, it is necessary to address whether there is an overlap between LH- and LC-projecting VP Glu neurons.
9. In Line 138, the authors stated: “We observed that intraperitoneal injection of CNO (1 mg/kg) reduced time spent in QS, while increasing time spent in LM and the number of episodes of NM and LM (Fig. 1E-H).” Based on Figures F, G, and H, the authors presented data on % of total time, number of episodes, and duration per episode for three behavioral states (QS, NM, LM) during a 10-20 minute observation period. It appears that data points in Figures F, G, and H should originate from the same mouse within the same time frame. For example, if a mouse has “Numbers of episodes” for LM, it should also have corresponding “Duration per episode” data for LM. However, in the CNO group, the data points (n = 7, although the figure legend states n = 6) in Figure G (Numbers of episodes) and Figure H (n = 6) (Duration per episode) are inconsistent. Please clarify the criteria for data selection.
10. The authors mentioned on line 566 that “The behaviors of the mice were recorded with a camera on the side of the cylinder” and that “The motor states of the mice were classified into three categories: quiet state (QS), when the mouse kept eyes open but was immobile.” However, a single camera may not reliably capture whether the mouse's eyes are open, especially when the mouse's back is turned to the camera. This limitation makes it difficult to distinguish between quiet immobility and a sleep state.

Minor Concerns:
1. The Materials and Methods section lacks the approval number from the Institutional Animal Ethics Committee. Please provide the ethics approval number to ensure compliance with ethical guidelines for animal experimentation.
2. Line 534 states that the animals were “anesthetized with sodium pentobarbital.” However, the method of administration and the dosage used are not specified.
3. In the Results section (Line 184), the authors stated: “We placed the mice in an induction box equilibrated with 0.6-1.2% isoflurane for 15 min and examined latency to loss of righting reflex (LORR) with or without light illumination of the VP in mice (Fig. 2B-D).” To ensure reproducibility, please include detailed information about the dose-response curve for isoflurane-induced anesthesia in the Materials and Methods section. Specifically, provide details on the dosing protocol, including the starting concentration, incremental concentrations, the time interval between each increment, and the specific method used to calculate the dose-response curve. These details will allow other researchers to replicate your study accurately.
4. In the Materials and Methods section, the definitions of LORR (loss of righting reflex) and RORR (recovery of righting reflex) must be described in detail.
5. In the figure legends for Figure 1 and Figure 8, the formatting of the p-values is inconsistent in the final sentences. For consistency, please ensure uniform capitalization (e.g., “P” or “p”) throughout the manuscript.
6. The sample size in Figures 2E and 2F should be stated.

Reviewer #2

(Remarks to the Author)

In this manuscript, the authors conducted a series of experiments to investigate the function of VPGlu-LCNA-LH circuit in regulating brain arousal in both awake and anesthetized mice. They showed that this circuit could modulate arousal in a bidirectional manner: activation of the circuit could promote arousal, whereas its inhibition reduces arousal. Additionally, the authors identified the involvement of α2-adrenoceptors in the lateral hypothalamus (LH) in this process. Technically, the experiments were well designed and uncovered a novel circuit underlying arousal regulation. However, there are some concerns that need to be addressed before publication.

1. The authors showed that optogenetic activation of VPGlu could increase latency to anesthesia and promote anesthesia emergence. It is interesting to know whether this phenomenon is dependent on light intensity. For instance, higher light power may induce a longer latency to LORR and shorter latency to RORR as compared with 4 mW power stimulation ? Furthermore, in line 225, the stimulus pattern is described as 20 Hz, 5 ms, 4 mW, whereas in panel Fig. 3A, the pulse duration was 10 ms. It needs to be verified.

2. In different depths of anesthesia, whether the response of VP-projection LCNA neurons and norepinephrine release in LH were consistent with the activity of VPGlu? It would be valuable to record these responses.

3. As the authors mentioned in Discussion (lines 463-464), VPGlu-LH activation could enhance wakefulness. According to the same reference, LH also receives dense projections from VPGlu. And in lines 256-258, optogenetic activation of VPGlu induced c-fos expression in LHb, VTA, LC, vlPAG and SuM. It is unclear whether c-Fos expression was observed in the LH. Additionally, the authors could provide further insight into the functional role of the VPGlu-LH in anesthesia induction and emergence. And discuss what are the potential differences between the VPGlu-LH and VPGlu-LCNA-LH circuits?

4. Is the VPGlu-LCNA-LH circuit a general mechanism for promoting arousal in anesthetized mice, or is it specific to isoflurane anesthesia? Maybe the authors can prospect it.

Reviewer #3

(Remarks to the Author)
The study conducted by Xu et al. revealed that glutamatergic neurons in the VP play a crucial role in regulating arousal and motor activity. Bidirectional modulation of these VP glutamatergic neurons influences both the induction and emergence from isoflurane-induced general anesthesia. Through anatomical and functional experiments, the researchers identified a specific neural pathway from VP glutamatergic neurons to the LC noradrenergic (NA) system. They further demonstrated that activation of the VP glu-LC NA circuit triggers the release of norepinephrine (NA) in the LH, modulating brain arousal, motor activity, and the dynamics of isoflurane general anesthesia. Additionally, they found that α_2A adrenergic receptors in the LH are involved in this process.
The manuscript is generally well and clearly written and the figures are clear and well executed. The data and statistics are overall of good quality, especially the circuit manipulations and behavior. Overall, the authors conducted extensive research and provided new evidence for both sleep and general anesthesia fields. Although these results are potentially interesting to the field, the depth of the study could be improved with the following recommendations.

1. The authors devote a significant portion of the manuscript to discussing the basal forebrain; however, recent studies do not typically classify the ventral pallidum (VP) as part of the basal forebrain. It is more commonly considered a component of the basal ganglia (Prog Neurobiol. 2015 Jul:130:29-70.). Citation 22 (Neuroscience 79, 1051-1078, 1997) is quite outdated, please replace it. The authors should revise the section discussing the basal forebrain, as their study focuses specifically on the VP.
2. Regarding the anatomical data (Figure 4A-B), the authors should first provide images from a low-magnification objective, showing the VP from rostral to caudal, to ensure that they did not inadvertently affect basal forebrain subregions (such as SI, DBH, and MCPO). The basal forebrain differs significantly from the VP in terms of anatomical structures, projections, and functions. To better define the VP boundaries, the authors could stain for substance P to outline the VP and confirm that their injections were confined to this region (Prog Neurobiol . 2015 Jul:130:29-70.).
3. As mentioned in the Methods, the authors injected a volume of 200 nL of virus into the VP, which may have affected a larger area outside of the VP. For some of the sample images showing virus expression in the VP, the authors should also provide low-magnification images aligned with the higher-magnification images to provide better context and allow for easier comparison.
4. The roles of VP glu neurons in regulating wakefulness and stress have been widely studied. Therefore, the investigation of VP glu neurons in the regulation of isoflurane anesthesia does not offer novel insights. Additionally, the authors dedicate considerable space to measuring changes in various levels of motor activity following modulation of VP glutamatergic neurons. However, the significance of these experiments is not sufficiently explained.
5. The method used to identify motor stages is not highly reliable. For accurate identification of arousal stages, EEG and EMG signals should be analyzed in combination. In Figures 1M-1Q and 5D-5F, the authors should include EMG traces aligned underneath the EEG traces to improve the clarity of motor stage identification. Without the EMG data, it is challenging to accurately discern the motor stage. Additionally, the EEG heatmap and power density of quiet waking (QS) resemble those seen during sleep (Figure 1M). To distinguish between these states, the authors should include a trace of sleep to highlight the differences between sleep and QS, as these two conditions are quite similar. As noted in the manuscript, motor states were identified solely by video and visual inspection, which is not a reliable method, as the authors themselves acknowledge the technical limitations. If a more reliable method for assessing motor states (e.g., Danqian Liu, Science 2020) cannot be used, there may be no need to subdivide the waking state into three levels.
6. In the retrograde and anterograde tracing experiments, retrogradely labeled cells in the VP are sparse (Figure 4H), whereas a larger population is observed in the LPO. Additionally, projections to the LC appear sparse in some images (Figures 5C, 6B-C). This raises concerns about the density of projections from VP glutamatergic neurons (VPglu) to the LC, especially since some studies do not report projections from the VP to the LC (e.g., Brain Structure and functon(2021) 226:1755–1778; Front Neuroanat. 2021 Dec 16:15:801354). To strengthen the argument for the VPglu-LC projection, the authors should provide evidence that rules out the possibility of projections from other regions, such as the LPO, to the LC.
7. As the authors note, the VP glu-LC projection only partially mediates the role of VP glu neurons in regulating brain states, suggesting that the VP glu-LC circuit may not be critically important. This is likely due to the sparse projections from VP glu to the LC. In contrast, VP glu neurons project directly and densely to the LH, and this circuit is likely to play a more significant role in regulating brain states. Since the authors focus on the VPglu-LC circuit and ultimately discuss the LH, a downstream target of the LC, why did they not study the VPglu-LH projection directly?
8. The authors used a relatively complex method, hM4Di, to inhibit VP glu-LC projections. Why did they choose this approach instead of optogenetic inhibition?
9. In Figure 7A, the authors should include images showing that AAV1-DIO-Flp expression is specifically confined to the VP.
10. The LH is a highly heterogeneous nucleus, containing both sleep-promoting and wake-promoting neurons. However, it is not clear which specific neuronal subtypes are innervated by LC neurons. The authors only examine a subset of neurons that are inhibited by an α2A receptor agonist in slices, which does not fully explain the underlying mechanism.
Minor:
1. Figure 1B: The sample images are unclear, with the mCherry+ cell bodies and CaMKII signals not well defined. Please replace them with higher resolution images. Additionally, please include quantification of the percentage of CaMKII+ cells in the VP that are also mCherry+, in order to assess the efficiency of the viral expression. What is the c-Fos expression after CNO application in vivo?
2. Figure 1C: Please add a scale bar to the traces, and include the resting membrane potential (RMP) for the recorded neurons.
3. For the bar plots in Figures 2M, 2O, 3I, N, K, and P, the authors should consider adding the original data points to provide more transparency and context for the results.
4. Figure 4C: Please include images showing ChR2-eYFP fibers in the sample images to better illustrate the experimental setup and validate the expression.
5. For all images showing the LC, please also stain for TH to more clearly delineate the LC region.

6. Figure 7K: Please add the RMP for each trace shown, as it is crucial for interpreting the neuronal activity.
7. Figure 7L: In the sample trace, the baseline appears unstable, showing a decline before Guanfacine application, and there is almost no washback after the drug application is stopped.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript has fully addressed the previous concerns by providing detailed experimental data and clear explanations. The study contributes to the neuroscience mechanism of brain state and anesthesia. I suggest that the manuscript should be accepted.

Reviewer #2

(Remarks to the Author)

The authors are responsive to the original concerns and comments and addressed them by including newly performed experimental results and correcting the mistake. No further comments.

Reviewer #3

(Remarks to the Author)
The authors have addressed most of my comments with new experiments, new data and discussion. My only suggestion is that "brain states" is a fuzzy concept refers to "arousal and motor activity" in the context. May be authors can adjust it.
Congrats to nice work!

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The basal forebrain consists of several distinct nuclei that differ in their synaptic inputs and outputs and are implicated in various physiological functions. A detailed examination of individual nuclei could yield a more precise understanding of the neural circuits underpinning the basal forebrain's physiological functions. In this manuscript, Xu et al. explore the role of glutamatergic (Glu) neurons in the ventral pallidum (VP) in elevating arousal levels in both conscious and anesthetized mice. To clarify the neural circuitry responsible for the function of VP Glu neurons, the authors have identified a neural pathway extending from VP Glu neurons to noradrenergic (NA) neurons in the locus coeruleus (LC) and then to the lateral hypothalamus. They have confirmed that this pathway partially mediates VP Glu neurons' arousal-promoting effects in conscious and anesthetized mice. This study enhances our understanding of the neural circuits that regulate arousal levels in conscious and anesthetized mice and suggests that α2 adrenoceptors in the lateral hypothalamus may serve as effective targets for modulating brain arousal in both states. However, the manuscript would benefit from addressing the following concerns:

Major points:

1. Chemogenetic stimulation of VP Glu neurons reduces EEG power in low-frequency bands associated with enhanced movement states. Conversely, chemogenetic inhibition of VP Glu neurons has a minimal effect on EEG, which does not correspond to degraded movement states. Given the variability in the data, the authors should consider increasing the sample size to substantiate these findings.

Response: We thank the reviewer for raising this point. We increased the sample size to 10 and revised the figure (Fig. S3j). The data show that chemogenetic inhibition of VP Glu neurons significantly enhanced delta wave.

2. Figure 3H suggests that optogenetic stimulation of VP Glu neurons did not alter the power of delta waves, a finding at odds with typical EEG patterns. The authors should investigate this effect with an expanded sample size.

Response: We increased the sample size to 10 and incorporated the data into Fig. 3i. The data show that optogenetic stimulation of VP Glu neurons reduced the power of delta wave.

3. The data in Figure S5 are compelling. In Figures S5B-E, the authors should include a dashed line on each panel to indicate the averaged time points for LORR or RORR and the initiation of isoflurane exposure. These data imply that the activity of VP Glu neurons may be linked to the presence of isoflurane and the induction and emergence from anesthesia. The authors should discuss this in the Results section.

Response: We thank the reviewer for carefully checking the data and helping us improve data illustration. We added time points that LORR and RORR occurred for each mouse in Fig. S6b, d and added average time points for LORR and RORR in Fig. S6c, e. In the Results section, we wrote that "These results indicate that isoflurane reduces the activity of VP Glu neurons in a concentration- and time-dependent manner and induces anesthesia when the activity of VP Glu neurons reduced to a certain level. Therefore, the reduction in the activity of VP Glu neurons may represent the anesthetized states and the depth of anesthesia.".

4. Figure S6 indicates that optogenetic stimulation of VP Glu neurons activated several nuclei in conscious mice. Analogous experiments should be conducted in anesthetized mice to investigate the downstream nuclei mediating VP Glu neurons' modulatory effects on anesthesia.

Response: We thank the reviewer for this insightful viewpoint. We tested the effects

of optogenetic stimulation of VP Glu neurons on neuronal activity in the brain of anesthetized mice. We incorporated the data in Fig. S7. The data showed that stimulation of VP Glu neurons activated neurons in several nuclei in both awake and anesthetized mice.

5. In Figure 4, the authors present morphological and functional data supporting the idea that VP Glu neurons innervate LC NA neurons. Their fiber photometry data show that LC NA neurons innervated by VP Glu neurons exhibit inhibition upon air puff, contrasting with the response of VP Glu neurons. These findings suggest that LC NA neurons may not mediate the response of VP Glu neurons to aversive stimulation. The authors should address this discrepancy in the Discussion section.

Response: We thank the reviewer for this comment. In this study, we show that stimulation of VP Glu neurons enhanced the activity in LC NA neurons; although aversive stimulation increased activity in VP Glu neurons, but reduced the activity in LC NA neurons innervated by VP Glu neurons. In fact, LC NA neurons integrate sensory information in the form of excitatory and inhibitory inputs from multiple upstream nuclei. It should be noted that aversive stimulation increased the activity of VP Glu neurons, while decreased the activity of LC NA neurons receiving projection from VP Glu neurons. This paradoxical observation suggests that LC NA neurons may integrate strong inhibitory synaptic inputs mediating aversive responses, which functionally override the excitatory synaptic inputs from VP Glu neurons. Further investigations are needed to solve this issue.

6. In Figure 5, optogenetic stimulation of the VP-LC Glu projection reduced the power of delta waves during QS and NM but enhanced delta waves during LM. This finding is inconsistent with the expected function of VP Glu neurons and should be explained.

Response: We thank the reviewer for pointing out this confusion. The effect of stimulation on delta wave may be determined by at least two factors: the strength of the

stimulation and the baseline level of delta wave. As the LC is one of downstream nuclei of VP Glu neurons, it is likely that stimulation of the VP^{Glu}-LC projection may exert weaker effect on delta wave than that caused by stimulation of VP Glu neurons.

In the revised manuscript, we wrote "These data indicate that stimulation of the VPGlu-LC projection exerts weaker effect on delta wave than that caused by stimulation of VP Glu neurons. During LM, multiple neural circuits may be recruited, and regulation of the VP^{Glu}-LC^{NA} pathway may not provide a strong driving force to further elevate arousal level. In contrast, stimulation of the VP^{Glu}-LC^{NA} pathway may counteract some neural circuits to reduce their effects on arousal. This may need further investigation to confirm. But this phenomenon clue that the VP^{Glu}-LC^{NA} pathway regulate brain states depending on motor activity states and may be a target to maintain arousal at a certain level."

7. Figure 7 shows that stimulation of the VP-LC Glu projection evokes noradrenaline release in the LH by activating LC NA neurons. The authors should examine whether inhibiting LC NA neurons innervated by VP Glu neurons prevents salient stimulus-induced noradrenaline release in the LH and diminishes the change in noradrenaline release during the induction and emergence from anesthesia. These experiments would provide further insight into the role of this pathway.

Response: We thank the reviewer for pointing out these important experiments. We used viral vectors to transfect hM4Di into LC NA neurons innervated by VP Glu neurons and NE2M (noradrenaline sensor) into the LH in DBH-Cre mice as illustrated in Fig. 7e, f. We then conduced fiber photometry recording of NE2M signal from the LH, and found that inhibition of this LC NA neuron population dramatically attenuated the alterations of NA release in the LH in response to salient stimuli and upon the induction or emergence of isoflurane anesthesia. We added these data in Fig. S12, S13. These data indicate that LC NA neurons innervated by VP Glu neurons play a significant role in providing NA in the LH for executing arousal-related behaviors.

8. The overall results suggest that the effects of VP Glu neurons on motor activity, brain arousal, and anesthesia are stronger than those of the VP-LC and VP-LC-LH pathways. This implies that VP Glu neurons may exert these effects through other pathways, such as the VP-LH pathway (Luo et al., 2023). Although the authors cite this article, it is necessary to address whether there is an overlap between LH- and LC-projecting VP Glu neurons.

Response: We thank the reviewer to raise this important point. To address whether LH- and LC-projecting VP Glu neurons overlap with each other, we injected AAV retro-hSyn-eGFP and AAV retro-hSyn-RFP into the LH and LC, respectively. We observed that LH- and LC-projecting VP neurons were largely separated. We illustrated these data in Fig. S16.

9. In Line 138, the authors stated: "We observed that intraperitoneal injection of CNO (1 mg/kg) reduced time spent in QS, while increasing time spent in LM and the number of episodes of NM and LM (Fig. 1E-H)." Based on Figures F, G, and H, the authors presented data on % of total time, number of episodes, and duration per episode for three behavioral states (QS, NM, LM) during a 10-20 minute observation period. It appears that data points in Figures F, G, and H should originate from the same mouse within the same time frame. For example, if a mouse has "Numbers of episodes" for LM, it should also have corresponding "Duration per episode" data for LM. However, in the CNO group, the data points (n = 7, although the figure legend states n = 6) in Figure G (Numbers of episodes) and Figure H (n = 6) (Duration per episode) are inconsistent. Please clarify the criteria for data selection.

Response: We thank the reviewer for pointing out this inconsistency. We confirmed that 'n = 7' in these figures.

10. The authors mentioned on line 566 that "The behaviors of the mice were recorded with a camera on the side of the cylinder" and that "The motor states of the mice were

classified into three categories: quiet state (QS), when the mouse kept eyes open but was immobile." However, a single camera may not reliably capture whether the mouse's eyes are open, especially when the mouse's back is turned to the camera. This limitation makes it difficult to distinguish between quiet immobility and a sleep state.

Response: We thank the reviewer for pointing out this limitation. We apologize that we made mistake in defining quiet state in writing. The 'quiet state' here is immobile state (IM). That is, the mice have no body movement. The mice may be in awake quiet state and sleep state. In revision, we changed QS into IM.

Minor Concerns:

1. The Materials and Methods section lacks the approval number from the Institutional Animal Ethics Committee. Please provide the ethics approval number to ensure compliance with ethical guidelines for animal experimentation.

Response: We provided the number in the Materials and Methods section.

2. Line 534 states that the animals were "anesthetized with sodium pentobarbital." However, the method of administration and the dosage used are not specified.

Response: We apologize that we wrote the wrong anesthetics here. We conducted the surgery under the anesthesia with isoflurane according to the approved protocols. We corrected this mistake.

3. In the Results section (Line 184), the authors stated: "We placed the mice in an induction box equilibrated with 0.6-1.2% isoflurane for 15 min and examined latency to loss of righting reflex (LORR) with or without light illumination of the VP in mice (Fig. 2B-D)." To ensure reproducibility, please include detailed information about the dose-response curve for isoflurane-induced anesthesia in the Materials and Methods section. Specifically, provide details on the dosing protocol, including the starting

concentration, incremental concentrations, the time interval between each increment, and the specific method used to calculate the dose-response curve. These details will allow other researchers to replicate your study accurately.

Response: In the Materials and Methods section, we wrote "We placed a mouse into an induction box and ventilated mixture of air and increasing concentrations of isoflurane commencing at 0.6% with an increment of 0.1% at 15-min intervals. Before transition to the next concentration, righting reflex was examined by rolling the induction box to let the mouse lay on its back. The conscious mouse has the ability to right itself and stands on feet within seconds. The loss of righting ability refers to loss of righting reflex (LORR). The concentration of isoflurane produced LORR was recorded as the effective anesthetic threshold in each mouse. We calculated the percentage of mice showing LORR at each concentration and drew a concentration-response curve, which was fitted with a nonlinear regression model (GraphPad Prism software)."

4. In the Materials and Methods section, the definitions of LORR (loss of righting reflex) and RORR (recovery of righting reflex) must be described in detail.

Response: In the Materials and Methods section, we wrote "Before transition to the next concentration, righting reflex was examined by rolling the induction box to let the mouse lay on its back. The conscious mouse has the ability to right itself and stand on feet within seconds. The loss of righting ability refers to loss of righting reflex (LORR)." and "After termination of isoflurane exposure, motor function of the mice was scored every two min until the recovery of righting reflex (RORR) 38. RORR refers to restoration of ability for a mouse to right itself after being placed on its back.".

5. In the figure legends for Figure 1 and Figure 8, the formatting of the p-values is inconsistent in the final sentences. For consistency, please ensure uniform capitalization (e.g., "P" or "p") throughout the manuscript.

Response: We corrected “p” to “P” throughout the manuscript.

6. The sample size in Figures 2E and 2F should be stated.

Response: In this figure, n = 8 mice in each group. We added the information in the figure legend.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors conducted a series of experiments to investigate the function of VPGlu-LCNA-LH circuit in regulating brain arousal in both awake and anesthetized mice. They showed that this circuit could modulate arousal in a bidirectional manner: activation of the circuit could promote arousal, whereas its inhibition reduces arousal. Additionally, the authors identified the involvement of α2-adrenoceptors in the lateral hypothalamus (LH) in this process. Technically, the experiments were well designed and uncovered a novel circuit underlying arousal regulation. However, there are some concerns that need to be addressed before publication.

1. The authors showed that optogenetic activation of VPGlu could increase latency to anesthesia and promote anesthesia emergence. It is interesting to know whether this phenomenon is dependent on light intensity. For instance, higher light power may induce a longer latency to LORR and shorter latency to RORR as compared with 4 mW power stimulation ? Furthermore, in line 225, the stimulus pattern is described as 20 Hz, 5 ms, 4 mW, whereas in panel Fig. 3A, the pulse duration was 10 ms. It needs to be verified.

Response: We thank the reviewer for this comment in order to further confirm the link between the activation of VP Glu neurons and alterations in anesthesia induction and emergence. According to our previous experience, higher light power may cause unspecific effect because it may change behaviors in eYFP control mice. Therefore, instead of increasing the light power, we decreased the light power to 1 mW. We observed that 1 mW light significantly prolonged latency to LORR and shortened that to RORR, but the effects were significantly less than that induced by 4 mW light. These data were included in Fig. S5. The light pulse duration is 5 ms. We corrected this mistake.

2. In different depths of anesthesia, whether the response of VP-projection LC-NA neurons and norepinephrine release in LH were consistent with the activity of VPGlu? It would be valuable to record these responses.

Response: We thank the reviewer for this point. We conducted additional fiber photometry experiments to confirm that the activity of LC-NA neurons innervated by VP Glu neurons (Fig. S9) and norepinephrine release in the LH (Fig. S12) altered during the induction and emergence of anesthesia with similar trends as the activity of VP Glu neurons. These data suggest that the VP^{Glu}-LC^{NA}-LH pathway is implicated in anesthesia.

3. As the authors mentioned in Discussion (lines 463-464), VPGlu-LH activation could enhance wakefulness. According to the same reference, LH also receives dense projections from VPGlu. And in lines 256-258, optogenetic activation of VPGlu induced c-fos expression in LHb, VTA, LC, vlPAG and SuM. It is unclear whether c-Fos expression was observed in the LH. Additionally, the authors could provide further insight into the functional role of the VPGlu-LH in anesthesia induction and emergence. And discuss what are the potential differences between the VPGlu-LH and VPGlu-LC NA-LH circuits?

Response: We thank the reviewer to raise these issues which are helpful to understand the role of the VP^{Glu}-LC^{NA}-LH projection in a larger picture. We observed that optogenetic stimulation of VP Glu neurons increased the number of activated LH neurons in both waking and anesthetized mice (Fig. S7). We confirmed that in our experimental condition, ChR2-labeled VP Glu neurons sent monosynaptic innervation to LH neurons and the synaptic connection was strong enough to accelerate firing in LH neurons (Fig. S14a-g). Although optogenetic stimulation of the VP^{Glu}-LH projection reduced delta wave in the QS state (Fig. S14h-p), it did not alter the induction and emergence of isoflurane anesthesia (Fig. S15a-i). Our retrograde tracing data showed that LC- and LH-projecting VP neurons were largely separated (Fig. S16a-e).

Together with the previous study (Luo et al., 2023), our data suggest that the VP^{Glu}-LH and VP^{Glu}-LC^{NA}-LH pathways significantly promote natural arousal, but differ in regulating anesthesia.

4. Is the VPGlu-LCNA-LH circuit a general mechanism for promoting arousal in anesthetized mice, or is it specific to isoflurane anesthesia? Maybe the authors can prospect it.

Response: We thank the reviewer to ask for this thoughtful question. We tested the regulation of isoflurane and propofol anesthesia by stimulation of VP Glu neurons, and observed that VP Glu neurons similarly regulate anesthesia induced by isoflurane and propofol. In this manuscript, we focused on isoflurane anesthesia and included data collected from propofol-anesthetized mice in another manuscript.

Reviewer #3 (Remarks to the Author):

The study conducted by Xu et al. revealed that glutamatergic neurons in the VP play a crucial role in regulating arousal and motor activity. Bidirectional modulation of these VP glutamatergic neurons influences both the induction and emergence from isoflurane-induced general anesthesia. Through anatomical and functional experiments, the researchers identified a specific neural pathway from VP glutamatergic neurons to the LC noradrenergic (NA) system. They further demonstrated that activation of the VP glu-LC NA circuit triggers the release of norepinephrine (NA) in the LH, modulating brain arousal, motor activity, and the dynamics of isoflurane general anesthesia. Additionally, they found that α_2A adrenergic receptors in the LH are involved in this process.

The manuscript is generally well and clearly written and the figures are clear and well executed. The data and statistics are overall of good quality, especially the circuit manipulations and behavior. Overall, the authors conducted extensive research and provided new evidence for both sleep and general anesthesia fields. Although these results are potentially interesting to the field, the depth of the study could be improved with the following recommendations.

1. The authors devote a significant portion of the manuscript to discussing the basal forebrain; however, recent studies do not typically classify the ventral pallidum (VP) as part of the basal forebrain. It is more commonly considered a component of the basal ganglia (Prog Neurobiol. 2015 Jul:130:29-70.). Citation 22 (Neuroscience 79, 1051-1078, 1997) is quite outdated, please replace it. The authors should revise the section discussing the basal forebrain, as their study focuses specifically on the VP.

Response: We thank the reviewer for correcting us in neuroanatomy and providing guidance to revise this manuscript. We changed reference 22 into an updated review article (Morais-Silva et al., 2024). We revised the Introduction and Discussion section accordingly. The opinion about whether the VP belongs to the basal forebrain or the

basal ganglia has not reached a consensus. In (Morais-Silva et al., 2024), the VP is a separate nucleus nestled in the basal forebrain. In (Brain Structure and function 2021, 226:1755–1778) and (Neuroscience 79, 1051-1078, 1997), the VP is regarded as a part of the basal forebrain. In Root et al, 2015, the VP belongs to the basal ganglia. To avoid debate from readers, we wrote "The ventral pallidum (VP), adjacent to the horizontal branch of the diagonal band of Broca and the substantia innominata, contains cholinergic, GABAergic and glutamatergic neurons and have distinct synaptic inputs and outputs 13, 22, 23. However, further investigations are needed to address whether individual neuron types in the VP regulate anesthesia and which neural circuits mediate the regulation.".

2. Regarding the anatomical data (Figure 4A-B), the authors should first provide images from a low-magnification objective, showing the VP from rostral to caudal, to ensure that they did not inadvertently affect basal forebrain subregions (such as SI, DBH, and MCPO). The basal forebrain differs significantly from the VP in terms of anatomical structures, projections, and functions. To better define the VP boundaries, the authors could stain for substance P to outline the VP and confirm that their injections were confined to this region (Prog Neurobiol . 2015 Jul:130:29-70.).

Response: We thank the reviewer for this comment. We provided low magnification images that we did immunostaining for SP antibody and confirmed that injecting 120-150 nl viral vector into the VP at the coordinates mentioned in the Materials and Methods section could transfect the virus in the VP without contamination of surrounding nuclei (Fig. 1b, j; Fig. 4a,c; Fig. 5b; Fig. 6b, c; Fig. 7f; Fig. S1b; Fig. S3b; Fig. S4b; Fig. S7c; Fig. S10b; Fig. S14b).

3. As mentioned in the Methods, the authors injected a volume of 200 nL of virus into the VP, which may have affected a larger area outside of the VP. For some of the sample images showing virus expression in the VP, the authors should also provide low-magnification images aligned with the higher-magnification images to provide better

context and allow for easier comparison.

Response: We thank the reviewer for this correction. We injected 150 nl viral vectors in the VP and provided low and higher magnification images to illustrate virus expression and fiber implantation in the targeted regions (Fig. 1b, j; Fig. 4a,c; Fig. 5b; Fig. 6b, c; Fig. 7f; Fig. S1b; Fig. S3b; Fig. S4b; Fig. S7c; Fig. S10b; Fig. S14b).

4. The roles of VP glu neurons in regulating wakefulness and stress have been widely studied. Therefore, the investigation of VP glu neurons in the regulation of isoflurane anesthesia does not offer novel insights. Additionally, the authors dedicate considerable space to measuring changes in various levels of motor activity following modulation of VP glutamatergic neurons. However, the significance of these experiments is not sufficiently explained.

Response: We agree with the reviewer that in terms of the role of VP Glu neurons in natural arousal, this study is not novel. Beyond this, we provided evidence to prove the existence of a VP^{Glu}-LC^{NA}-LH pathway and demonstrated that activation of this pathway recruited LC noradrenergic neurons and regulated anesthesia and motor states in mice, and in these processes, α2 adrenoceptor in the LH plays an important role. These findings are not mentioned in previous study (Luo et al., 2023).

During revision, we examined the role of the VP^{Glu}-LH projection in regulating arousal and anesthesia. We observed that although stimulating this projection promoted natural arousal (delta wave in IM) (Fig. S14), similar to its effect reported in (Luo et al., 2023), it did not regulate anesthesia induced by either 0.8% or 1.4% isoflurane (Fig. S15). Our retrograde tracing data show that LC- and LH-projecting VP neurons were largely separated.

In this study, we examined the effect of the VP^{Glu}-LC^{NA}-LH pathway on alterations in movement states (including non-locomotor and locomotor movement) and anesthesia states, which are different physiological and pharmacological process from the transition between sleep and wakefulness. The results may extend the scope

of the function and neuroanatomical architecture of VP Glu neurons.

5. The method used to identify motor stages is not highly reliable. For accurate identification of arousal stages, EEG and EMG signals should be analyzed in combination. In Figures 1M-1Q and 5D-5F, the authors should include EMG traces aligned underneath the EEG traces to improve the clarity of motor stage identification. Without the EMG data, it is challenging to accurately discern the motor stage. Additionally, the EEG heatmap and power density of quiet waking (QS) resemble those seen during sleep (Figure 1M). To distinguish between these states, the authors should include a trace of sleep to highlight the differences between sleep and QS, as these two conditions are quite similar. As noted in the manuscript, motor states were identified solely by video and visual inspection, which is not a reliable method, as the authors themselves acknowledge the technical limitations. If a more reliable method for assessing motor states (e.g., Danqian Liu, Science 2020) cannot be used, there may be no need to subdivide the waking state into three levels.

Response: We thank the reviewer for this comment. In this study, we defined the activity state of the mice into immobile state, non-locomotor movement state, and locomotor state. We performed EEG and EMG in a cohort of mice (Fig. S17) and observed that power of delta wave in EEG and EMG power in IM, NM, and LM states differed in the mice. These results support the rationale to classify the motor-related physiological states by movement. We observed that recording EMG from the trapezoid muscle exhibited higher power in NM than in LM. An additional electrode may be used to record EMG from limb muscle to properly evaluate EMG power in NM and LM. Overall, we admit that combination of EEG and EMG (from multiple muscles) is needed in future investigations to provide physiological bases to rationally define motor states.

6. In the retrograde and anterograde tracing experiments, retrogradely labeled cells in the VP are sparse (Figure 4H), whereas a larger population is observed in the LPO.

Additionally, projections to the LC appear sparse in some images (Figures 5C, 6B-C). This raises concerns about the density of projections from VP glutamatergic neurons (VPglu) to the LC, especially since some studies do not report projections from the VP to the LC (e.g., Brain Structure and function (2021) 226:1755–1778; Front Neuroanat. 2021 Dec 16:15:801354). To strengthen the argument for the VPglu-LC projection, the authors should provide evidence that rules out the possibility of projections from other regions, such as the LPO, to the LC.

Response: Guided by the reviewer's second suggestion, we stained coronal slices with SP-antibody to demarcate the VP and confirmed that viral vectors stereotaxically injected according to our protocol transfected neurons within the VP (Fig. 1b, j; Fig. 4a,c; Fig. 5b; Fig. 6b, c; Fig. 7f; Fig. S1b; Fig. S3b; Fig. S4b; Fig. S7c; Fig. S10b; Fig. S14b). Under this experimental condition, we provide morphological and functional evidence to show that VP Glu neurons projected to the LC. By transfecting eYFP or ChR2-eYFP into VP Glu neurons, we observed eYFP-labeled fibers among NA neurons in the LC. We combined optogenetic stimulation and patch-clamp recording to demonstrate that activation of terminals of VP Glu neurons evoked excitatory postsynaptic currents in LC NA neurons (Fig. 4c-e). Using cell-specific retrograde neuronal tracing with rabies virus, we labeled some VP neurons and proved that most of them were stained by CaMKII-antibody. Furthermore, LC NA neurons were labeled by cell-specific transsynaptic viral vector in DBH-Cre mice.

In Brain Structure and function (2021) 226:1755–1778, the authors focus on the projection of VP neurons to the forebrain and midbrain, but did not show images at the brain stem level. In (Front Neuroanat. 2021 Dec 16:15:801354), the authors performed sparse labeling to reconstruct the projections of VP neurons projecting to the mediodorsal nucleus of the thalamus. These studies performed experiments differently from us and may have inconsistent results.

7. As the authors note, the VP glu-LC projection only partially mediates the role of VP glu neurons in regulating brain states, suggesting that the VP glu-LC circuit may not

be critically important. This is likely due to the sparse projections from VP glu to the LC. In contrast, VP glu neurons project directly and densely to the LH, and this circuit is likely to play a more significant role in regulating brain states. Since the authors focus on the VPglu-LC circuit and ultimately discuss the LH, a downstream target of the LC, why did they not study the VPglu-LH projection directly?

Response: We thank the reviewer to raise this issue which is helpful to understand the role of the VP^{Glu}-LC^{NA}-LH projection in a larger picture. We observed that optogenetic stimulation of VP Glu neurons increased the number of activated LH neurons in both waking and anesthetized mice (Fig. S7). We confirmed that in our experimental condition, ChR2-labeled VP Glu neurons sent monosynaptic innervation to LH neurons and the synaptic connection was strong enough to accelerate firing in LH neurons (Fig. S14a-f).

During revision, we examined the role of the VP^{Glu}-LH projection in regulating arousal and anesthesia. We observed that although stimulating this projection promoted natural arousal (delta wave in immobile state) (Fig. S14h-p), similar to its effect reported in (Luo et al., 2023), it did not regulate anesthesia induced by either 0.8% or 1.4% isoflurane (Fig. S15a-i). Our retrograde tracing data show that LC- and LH-projecting VP neurons were largely separated (Fig. S16a-e).

8. The authors used a relatively complex method, hM4Di, to inhibit VP glu-LC projections. Why did they choose this approach instead of optogenetic inhibition?

Response: In Fig. S10, we combined optogenetic stimulation of the VP^{Glu}-LC projection and chemogenetic inhibition of LC NA neurons to address whether LC NA neurons are major components to mediate the effects of the VP^{Glu}-LC projection. In this experiment, if we transfect NpHR into LC NA neurons, when we use blue light to stimulate the VP^{Glu}-LC projection, NpHR in the LC NA neurons can be activated too, resulting in inhibition of LC NA neurons. This may underestimate the behavioral or physiological outcome following stimulation of VP^{Glu}-LC projection. Additionally, it

is hard to deliver blue and yellow light through the same optical fiber. Similar reasons are applicable to examine the contribution of LC NA neurons to NE release in the LH in response to stimulation of VP^{Glu}-LC projection (Fig. 7g-j). Therefore, in these two sets of experiment chemogenetic inhibition of LC NA neurons is better than optogenetic inhibition of LC NA neurons.

9. In Figure 7A, the authors should include images showing that AAV1-DIO-Flp expression is specifically confined to the VP.

Response: As AAV1-DIO-Flp does not carry genes encoding fluorescent protein, we only provided an image with DAPI showing the position of the needle track in the VP. We included images in figures we used AAV1-DIO-Flp (Fig. 4k, Fig. 7A, 7f, Fig. 8A). In Fig. 7f, we injected AAV1-DIO-Flp and AAV-CaMKII-ChR2-eYFP into the VP and ChR2-eYFP did not spread out of the VP. This indirectly suggests that AAV1-DIO-Flp may be contained in the VP.

10. The LH is a highly heterogeneous nucleus, containing both sleep-promoting and wake-promoting neurons. However, it is not clear which specific neuronal subtypes are innervated by LC neurons. The authors only examine a subset of neurons that are inhibited by an α2A receptor agonist in slices, which does not fully explain the underlying mechanism.

Response: We thank the reviewer to instruct us to think and discuss our finding and limitations thoroughly. We agree with the reviewer that finding the neuron types in the LH being innervated by the VP^{Glu}-LC^{NA}-LH pathway is essential to interpret our results. Further investigations are needed to address this issue.

Minor:

1. Figure 1B: The sample images are unclear, with the mCherry+ cell bodies and

CaMKII signals not well defined. Please replace them with higher resolution images. Additionally, please include quantification of the percentage of CaMKII+ cells in the VP that are also mCherry+, in order to assess the efficiency of the viral expression. What is the c-Fos expression after CNO application in vivo?

Response: Figure 1B, we calculated specificity and efficacy of the virus. In the Results section, we wrote "We injected AAV-EF1α-DIO-hM3Dq-mCherry or AAV-EF1α-DIO-eYFP into the VP of Vglut2-Cre mice to transfect hM3Dq into 79.73 ± 2.44% (n = 5 mice) of VP Glu neurons (87.66 ± 2.26% of hM3Dq neurons co-labeled with CaMKII-antibody) (Fig. 1a, b).".

We calculated the percentage of CaMKII neurons labeled with eYFP, and revised Figure 1K.

We observed that chemogenetic stimulation of VP Glu neurons increased c-Fos(+) neurons in the VP. We included the data in Fig. S2c, d.

2. Figure 1C: Please add a scale bar to the traces, and include the resting membrane potential (RMP) for the recorded neurons.

Response: We included scale bar and RMP in Figure 1C.

3. For the bar plots in Figures 2M, 2O, 3I, N, K, and P, the authors should consider adding the original data points to provide more transparency and context for the results.

Response: We added all data points in these panels.

4. Figure 4C: Please include images showing ChR2-eYFP fibers in the sample images to better illustrate the experimental setup and validate the expression.

Response: We replaced the sample image in Figure 4C.

5. For all images showing the LC, please also stain for TH to more clearly delineate the LC region.

Response: We replaced all images.

6. Figure 7K: Please add the RMP for each trace shown, as it is crucial for interpreting the neuronal activity.

Response: We added the RMP for each trace.

7. Figure 7L: In the sample trace, the baseline appears unstable, showing a decline before Guanfacine application, and there is almost no washback after the drug application is stopped.

Response: We conducted patch-clamp recordings from more neurons in the LH. We observed that the firing rate in recorded neurons were stable for up to 25 min without drug application, but was reduced during 5 min perfusion of 100 μM Guanfacine and the inhibition persisted in most neurons (Fig. 7i). The persistent inhibition matches the nature of the G-protein-coupled receptors, whose activation initiates a cascade of signal transduction.