Peer Review File

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Reviewer A:

This article explores the neural circuits of pain associated with pancreatic cancer in a syngeneic grafted mouse model. The authors focused on glutamatergic neurons in the paraventricular nucleus of the hypothalamus (PVN), which they found to be multi-synaptically connected to the pancreas. Using slice electrophysiology and in vivo calcium imaging, the authors show an increase in activity of these neurons in animals with pancreatic tumors. The authors then claim that chemogenetic inhibition or ablation of these neurons leads to a reduction in behaviors that may be indicative of cancer-associated pain. However, this conclusion is not supported by the data presented.

The question raised here is of great interest given the current limited understanding of the central mechanisms involved in cancer pain. However, the article, in its current form, unfortunately lacks key information that is essential for a thorough evaluation of the experimental protocols and a full understanding of the results presented. In order to improve the quality of the manuscript, the authors should consider a comprehensive revision.
Although the list of necessary changes is quite extensive, addressing the main points mentioned here would be a significant step in the right direction.

Reply: Thank you for your comments regarding our article on neural circuits of pain associated with pancreatic cancer in a mouse model. We appreciate your insights and concerns about the study's methodology and conclusions.
It is important to have a thorough evaluation of experimental protocols and results presented in scientific research, especially when it comes to understanding central mechanisms involved in cancer pain. While the question raised by this study is of great interest, we agree that there are key information gaps that need to be addressed for a comprehensive evaluation.
We understand that addressing all necessary changes may require an extensive revision, but taking steps towards improving the quality of the manuscript would be beneficial. Thank you again for bringing attention to these issues.

References
There appears to be an issue with the references cited in the manuscript. For instance, in the Introduction section, the authors reference Chen et. al. (Dysfunctions of the paraventricular hypothalamic nucleus induce hypersomnia in mice, eLife, 2021) and Scammell, et al. (Neural Circuitry of Wakefulness and Sleep. Neuron, 2017), as a basis for their hypothesis that the PVN innervates pancreatic beta-cells and is involved in visceral pain.
Both references seem to be unrelated to the topic under investigation.
In addition, to the best of my knowledge, the claim that PVN neurons innervate pancreatic beta cells is not based on any existing data.
I recommend reviewing and cross-checking all references to ensure they are pertinent to the research topic and accurately cited. This will significantly improve the quality of the manuscript.
Reply: I appreciate the recommendation to review and cross-check all references and we have

updated the references in order to ensure their relevance to the research topic and accurate citation in revised manuscript. Besides, Rosario et. al found that pancreatic beta cells were innervated by efferent circuits that emanate from the hypothalamus by pseudorabies virus retrograde tracing (Rosario, W. et al. The brain-to-pancreatic islet neuronal map reveals differential glucose 372 regulation from distinct hypothalamic regions. Diabetes 65, 2711-2723 (2016)).
Changes in the text: We have modified our text as advised (see ' References' section, highlighted in red font)

Materials and methods
1) The 'Materials and methods' section of the manuscript lacks essential details, making it extremely difficult to replicate the experiments and fully understand how they were carried out. The experiments appear to deviate from current standards for animal experimentation and reporting. Critical information such as the number of animals tested, their sex, age, housing conditions and pre- and post-operative care is conspicuously absent. Assessing the reliability and validity of experimental procedures and results becomes a difficult task without this essential information.
Reply: Thank you for your comments regarding the "Materials and methods" section of our manuscript. We appreciate your concerns about the lack of essential details, which may hinder replication of the experiments and a comprehensive understanding of their execution.
We apologize for any inconvenience caused by this omission. We understand that critical information such as the number of animals tested, their sex, age, housing conditions, and pre- and post-operative care is crucial in assessing the reliability and validity of experimental procedures and results. Rest assured that we take these aspects seriously.
In response to your valuable input, we will revise our manuscript to include all necessary details in the "Materials and methods" section. By doing so, we aim to enhance transparency in reporting our research methodology while ensuring compliance with current standards for animal experimentation.
Once again, thank you for bringing these concerns to our attention. Your feedback helps us improve both the quality of our work and its reproducibility.
Changes in the text: We have modified our text as advised (see 'Materials and methods' section, highlighted in red font)

2) Abdominal mechanical hyperalgesia test: Following abdominal stimulation, the authors measured “positive responses”, defined as the presence of the following behaviors: “lifting, scratching, licking the abdomen, moving or jumping immediately”.
It seems that virtually any movement is considered a 'positive response'. However, it remains unclear what is specifically referred to as a 'negative response'. What establishes these 'positive responses' as indicators of pain? Conversely, does the lack of a 'positive response' mean that there is no pain, or could it be that the tumor-bearing mice are too debilitated to respond?
Reply: Thank you for your comments. Behavioral analyses were performed as described previously with some modifications (1. Selvaraj D, Hirth M, Gandla, J. A mouse model for pain and neuroplastic changes associated with pancreatic ductal adenocarcinoma. Pain. 2017; 158 (8): 1609-1621. 2. Yu, D, Zhu, J, Zhu, M, et al. Inhibition of Mast Cell Degranulation Relieves Visceral Hypersensitivity Induced by Pancreatic Carcinoma in Mice. J Mol Neurosci. 2019; 69 (2): 235-245). In this study, the absence of significant response is specifically referred to as a 'negative response'. The comment you raised about

whether the tumor-bearing mice would not respond to pain due to their weakness is an excellent one. Therefore, in this experiment, we selected relatively short periods of time after tumor inoculation (12, 15, and 18 days) to assess behavioral studies in order to avoid excessive weakness that could affect the evaluation of pain response. Additionally, in order to increase credibility, we simultaneously employed another method for evaluating pain known as Hunching Score.

Changes in the text: We have modified our text in the revised manuscript (see ' Abdominal mechanical hyperalgesia test ' section, highlighted in red font)

3) Hunching Score:

"The scoring factors for hunching behavior were as follows. (…) 2: Severe round back posture, marked by a slight reduction in exploratory sexual activity, mild ventral erections and intermittent abdominal contractions (…)."

It is likely that the authors intended to refer to 'piloerection' and not 'ventral erections', and they probably did not intend to study 'exploratory sexual activity' in this test.

Some important details are missing: How was the evaluation done (double blind)? Under what conditions were the mice placed (e.g. open field) and for how long? Over how many days were these observations made? Finally, how is the final score calculated?

Reply: Thanks for your suggestion. We have made revisions in the revised manuscript, replacing 'ventral erections' with 'piloerection', and using 'exploratory behavior' instead of 'exploratory sexual activity'." In addition, based on your suggestions, we have included some experimental details as follows: The hunch score was utilized as a means of evaluating spontaneous visceral pain and was examined as described previously with some modifications (Sevcik MA, Jonas BM, Lindsay TH, (2006). Endogenous opioids inhibit early-stage pancreatic pain in a mouse model of pancreatic cancer. Gastroenterology 131 (3): 900-10). The scoring factors for hunching behavior were as follows. 0: Lack of round-back posture, showing exploratory behavior, and normal coat luster. 1: Mild round-back posture, characterized by exploratory behavior and normal coat luster. 2: Severe round-back posture, marked by a slight reduction in exploratory behavior, slight piloerection and intermittent abdominal contractions. 3: Severe round-back posture, marked by significantly reduced exploratory behavior, moderate piloerection, and intermittent abdominal contractions. 4: Severe round-back posture, characterized by little or no exploratory behavior, a full-body piloerection, and head immobility. Mice were placed individually in the center of an open field arena and observed over a 300 second period., and the hunch score was calculated by taking an average. In all cases, observations were performed by two independent observers blinded as to the experimental status of the mouse.

Changes in the text: We have modified our text as advised (see ' Hunching Score ' section, highlighted in red font)

4) Pancreatic pseudorabies virus injection:

What is the source of the viruses or strains used? Which reporter was used? Could the authors specify the amount and injection protocols for the virus?

Reply: PRV vector containing EGFP (5 μl, BrainVTA, China) was injected at the head of the pancreas. Mice that had been injected with the virus were put back into the cage and given plenty of water and food. After the completion of PRV injection, the state of the mice was observed every day. 5 – 7 days after PRV injection, the whole brain was taken out by perfusion and fixed overnight with 4% (w/v) paraformaldehyde liquid. On the second day, the brain was soaked with 30% (w/v)

sucrose followed by the whole-brain imaging using a Zeiss laser confocal microscope to visualize and record data. The average value of each section's data was calculated for each mouse.
Changes in the text: We have modified our text as advised (see ' Pancreatic pseudorabies virus injection ' section, highlighted in red font)

Can the authors clarify what they mean by “When the state of the mice
became worse, the whole brain was taken out “? How was the health of the mice monitored? Does this mean that the time for virus transport was determined based on the health status of the mice and not standardized?
Reply: We sincerely apologize for any inconvenience caused by our description. Due to the high toxicity of PRV, it can lead to fatal outcomes in mice within a short period of time. Therefore, we will closely monitor their condition throughout the experiment. Specifically, brain extraction was performed on three mice after 5 days of pancreatic injection with PRV, and on three mice after 7 days.
Changes in the text: We have modified our text as advised (see ' Pancreatic pseudorabies virus injection ' section, highlighted in red font)

Regarding the statement “followed by the whole-brain imaging“, more information is needed to understand the methodology used to visualize retrogradely labelled neurons and determine their anatomical localization.
Reply: The brain was soaked with 30% (w/v) sucrose. Then, a series of 5-10μm sections were cut and every fifth section analyzed by immunofluorescence and confocal microscopy. (Rosario Wilfredo, Singh Inderroop, Wautlet Arnaud, et al. The brain-to-pancreatic islet neuronal map reveals differential glucose regulation from distinct hypothalamic regions.diabetes.2016;65 (9): 2711-23.).
Changes in the text: We have modified our text as advised (see ' Pancreatic pseudorabies virus injection ' section, highlighted in red font)

5) Immunofluorescence:
There is a lack of information on the references and concentrations of all antibodies used.
Reply: Thank you for your suggestion. We have added information about the antibodies, as follows: The brain sections were then incubated with Anti-c-Fos antibody (mouse, 1:200, Abcam, ab208942), Anti-Glutamate antibody (Rabbit, 1:200, Sigma-Aldrich, G6642), Anti-GABA antibody (Rabbit, 1:200, Sigma-Aldrich, A2052), Anti-Tyrosine Hydroxylase Antibody (Rabbit, 1:200, Sigma-Aldrich, AB152), Anti-Corticotropin Releasing Factor Antibody (Rabbit, 1:200, Thermo Fisher, PA5-102356) at 4°C for 24 h. Alexa Fluor Plus 488 Donkey anti-Mouse (1:500, Thermo Fisher, A32766) and Alexa Fluor Plus 594 Donkey anti-Rabbit (1:500, Thermo Fisher, A32754) were incubated at room temperature for 2 h.
Changes in the text: We have modified our text as advised (see ' Immunofluorescence ' section, highlighted in red font)

“For each mouse, the averages of data from each section were calculated.” This statement does not make it clear which results were analyzed and how.
Reply: We apologize for the confusion caused by our description. We have made revisions based on your suggestions, as follows: For quantification of cells, three sections around the PVN were

counted manually using NIH ImageJ software. The sections used were at the same coordinates for each group.

Changes in the text: We have modified our text as advised (see ' Immunofluorescence ' section, highlighted in red font)

6) The following experimental procedures are not described in the material and methods:

-In vivo fluorescence imaging of the tumor

Reply: Thank you for your suggestion. We have added the relevant information in the revised manuscript, as follows: 7 days after incubation, orthotopic tumor burdens were measured by the In Vivo Imaging System. Mice with similar tumor sizes were selected for further experiments.

Changes in the text: We have modified our text as advised (see ' Mouse model of pancreatic cancer visceral pain ' section, highlighted in red font)

-In vivo calcium imaging

Reply: Thank you for your suggestion. We have added the relevant information in the revised manuscript, as follows: Real-time Ca^{2+} transients were assessed via fiber photometry (ThinkerTech, Nanjing, China) to determine changes in neuronal activity. Depending on the experiment, we microinjected 100 nl of AAV-CaMKIIα-GCaMp6m-EYFP or AAV-CaMKIIα-EYFP into the PVN. An optical fiber (230 μm OD, 0.37 NA, Inper, Hangzhou, China) was implanted over the region injected with GCaMp6s virus and fixed in place with dental cement. Three weeks after viral expression, the calcium activity of the target neurons was monitored. Fiber photometry recordings were conducted on conscious mice that were simultaneously subjected to 0.16 g Von Frey fiber stimulation applied to the left upper abdomen. Fluorescence signals were obtained by reflecting a laser beam from a laser tube (473 nm) onto a dichroic mirror, focusing it with a 103 lens, and then coupling it to an optical commutator. Light was guided from the implanted fiber to the commutator by a 2-m optical fiber. The calcium signals were acquired with data-acquisition software (ThinkerTech, Nanjing, China) and the onset of stimulation was recorded manually. Raw signals were analyzed and processed with a Matlab program developed by Thinkertech. For each trial, the fluorescence variation was calculated as ΔF/F, where ΔF represents the value obtained by subtracting the mean of the baseline signal from the test signal and F represents standard deviation of the basal signal.

Changes in the text: We have modified our text as advised (see ' Fiber photometry ' section, highlighted in red font)

-Ablation of glutamatergic neurons by taCasp3

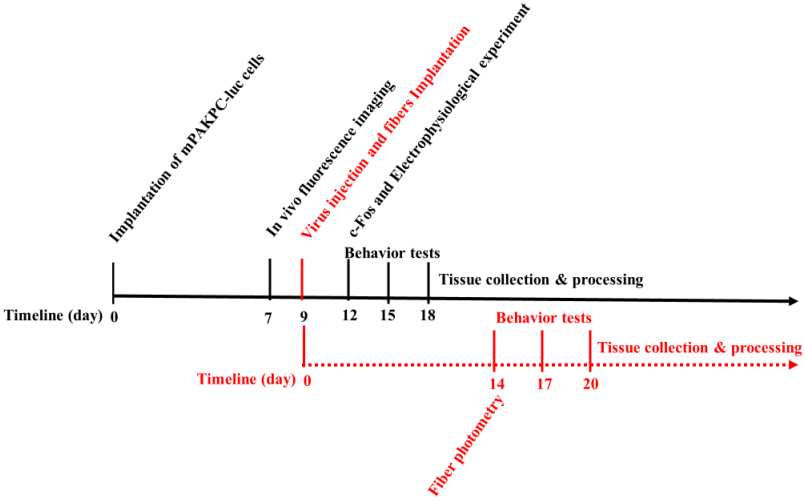
Reply: Thank you for your suggestion. We have added the relevant information in the revised manuscript, as follows: For PVN glutamatergic neuronal selective ablation, rAAV-CaMKIIα-taCasp3-T2A-TEVp-WPREs-pA or rAAV-CaMKIIα-EYFP-WPREs-pA (100 nl, BrainVTA, China) was injected bilaterally into the PVN of anaesthetized model mice. Behavioral assessments were conducted 14, 17, and 20 days after virus injection. After the behavioral test, the distribution of viral fluorescent protein expression in PVN was detected by fluorescence microscopy.

Changes in the text: We have modified our text as advised (see ' Ablation of glutamatergic neurons ' section, highlighted in red font)

-Cre-dependent retrograde trans-monosynaptic rabies virus tracing strategy

Reply: Thank you for your suggestion. We have added the relevant information in the revised manuscript, as follows: Rabies virus-mediated retrograde tracing was employed to investigate the upstream regions of PVN. A mixture (150 nl) of rAAV-CaMKIIa-CRE-WPRE-hGH polyA, rAAV-EF1α-DIO-H2B-EGFP-T2A-TVA-WPRE-hGHpA, and rAAV-EF1α-DIO-oRVG-WPRE-hGH pA in a 1:1:1 ratio was injected into the PVN areas of mice. After two weeks, RV-ENVA-ΔG-dsRed was injected at the same location within the PVN. Mice were sacrificed seven days following rabies virus infection. Retrograde spreading of rabies to presynaptic neurons only occurred in cells expressing both RVG and EnvA cognate receptor TVA. Starter cells were defined as neurons expressing both EGFP (from helper virus) and DsRed (from rabies virus), while input cells were defined as presynaptic partners that expressed only DsRed. (Wickersham, I.R., Lyon, D.C., Barnard, R.J., Mori, T., Finke, S., Conzelmann, K.K., Young, J.A., and Callaway, E.M. (2007). Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. Neuron 53, 639–647.).
Changes in the text: We have modified our text as advised (see ' Cre-dependent retrograde trans-monosynaptic rabies virus tracing strategy ' section, highlighted in red font)

How were these methods combined with tumor implantation and behavioral testing? A schematic of the experimental procedures with a timeline is needed to give a clear overview of how the study was conducted.
Reply: Thank you for your suggestion. A schematic of the experimental procedures with a timeline are shown in the figure below. In this study, 7 days after incubation, orthotopic tumor burdens were measured by the In Vivo Imaging System (IVIS). Mice with similar tumor sizes were selected for subsequent experiments to mitigate the impact of tumor size variability on behavioral assessments. After 9 days of tumor implantation, we conducted c-Fos staining and electrophysiological experiments. At 12, 15, and 18 days after tumor implantation, we performed abdominal mechanical hyperalgesia tests and Hunching Scores before euthanizing the mice. After 9 days of tumor implantation, we injected viruses and implanted optical fibers. After 14 days of virus injection, we conducted Fiber photometry. At 14, 17, and 20 days after virus injection, we performed abdominal mechanical hyperalgesia tests and Hunching Scores before euthanizing the mice.



Result section

7) Tumor model - Figure 1A: How many days after cancer cell transplantation was imaging performed? Given the apparent variability in tumor size between animals, was this variability considered in relation to the behavioral scores and welfare of the mice and, if so, how was it addressed in the current study?

Reply: 7 days after incubation, orthotopic tumor burdens were measured by the In Vivo Imaging System (IVIS). Mice with similar tumor sizes were selected for subsequent experiments to mitigate the impact of tumor size variability on behavioral assessments. When the mice in the experimental process meet the following welfare criteria, euthanasia will be conducted based on animal welfare standards, using excessive inhalation of 95% CO2 to induce death: (1) persistent diarrhea; (2) sluggishness (inability to eat or drink); (3) hunched back and lying on their side; (4) reduced activity and symptoms of muscle atrophy; (5) difficulty breathing; (6) progressive decrease in body temperature; (7) paralysis and convulsions; (8) continuous bleeding; (9) inability for animals to move normally due to large tumors or other reasons; and (10) inability for animals to move normally due to severe ascites or increased abdominal circumference.

Changes in the text: We have modified our text as advised (see ' Mouse model of pancreatic cancer visceral pain ' section, highlighted in red font)

8) Pain assessment: Figure 1B: What was the rationale for choosing the 12, 15, 18 day time points to test the mice? What do the yellow/purple matrices represent? What do the different Trial# represent? How were the 'response' and 'response frequency' shown in the graph calculated?

Reply: Thank you for your comment. 7 days after incubation, orthotopic tumor burdens were measured by the In Vivo Imaging System (IVIS). Mice with similar tumor sizes were selected for subsequent experiments to mitigate the impact of tumor size variability on behavioral assessments. After a two-day rest, behavioral tests were conducted every three days starting from the 9th day after tumor cell implantation to confirm the success of the pain model. Our results showed no significant changes in behavior on the 9th day after tumor cell implantation (hence not shown in Figure 1), but pain started to appear from the 12th day of tumor cell implantation. So, the behavioral results at 12, 15, and 18 time points are presented in Figure 1. The color yellow signifies a positive response, while the color purple denotes a negative response. The x-axis (Trial#) refers to the number of repetitions in the experiment. The Y-axis represents the number of the mice. Response = positive response/12 mice. Response frequency (%) = (Positive response /10 trials) *100.

Changes in the text: We have modified our text as advised (see ' Mouse model of pancreatic cancer visceral pain ' section, highlighted in red font)

9) Pseudorabies virus tracing:

“ Fig. 2: PRV-EGFP fluorescence image of the pancreas innervated by PVN” This title is inappropriate as the images shown are of brain slices, not the pancreas.

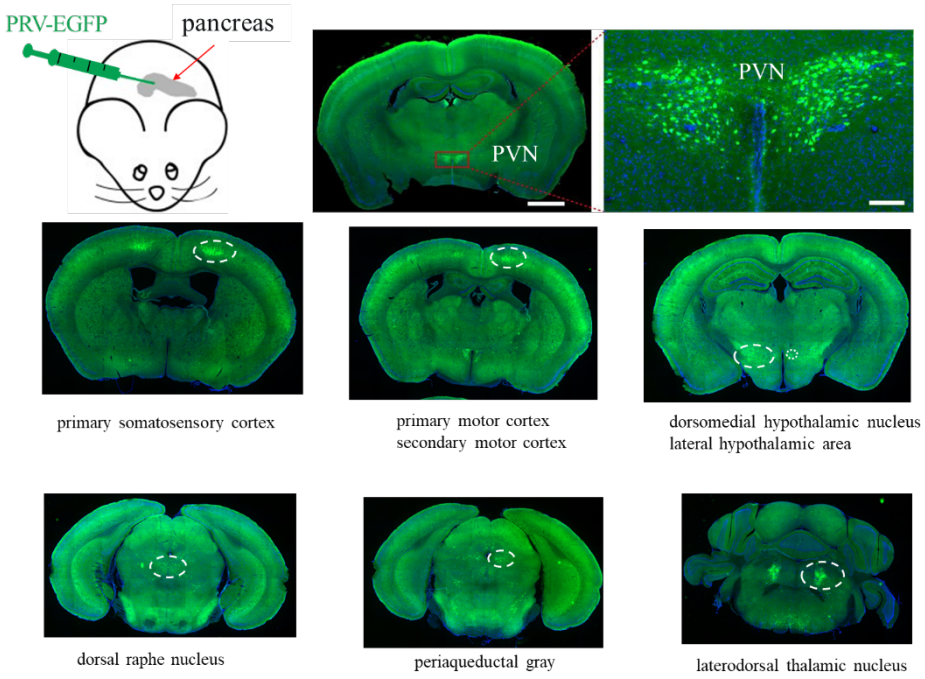
Reply: Thank you for your suggestion. We have revised the title in the revised manuscript, as follows: Fluorescence imaging of PRV-EGFP in the PVN following pancreatic infection.

Changes in the text: We have modified our text as advised (see ' Figure Legends for figure 1 ' section, highlighted in red font)

Could the authors specify what other brain regions were labelled? Furthermore, the number of labelled neurons in the PVN and its different subregions should be quantified.

It is also important to know how many injected mice showed this particular labelling pattern.

Reply: Thank you for your comment. As shown in the figure below, we observed that the brain regions primarily labeled encompassed the paraventricular nucleus of the hypothalamus, primary somatosensory cortex, primary motor cortex, secondary motor cortex, dorsomedial hypothalamic nucleus, lateral hypothalamic area, dorsal raphe nucleus, periaqueductal gray, laterodorsal thalamic nucleus. The PVN is marked by approximately 436 neurons (data obtained from 5 mice), including 265 neurons in the posterior part of the paraventricular hypothalamic nucleus, 109 neurons in the medial magnocellular part of the paraventricular hypothalamic nucleus, and 44 neurons in the anterior parvicellular part of the paraventricular hypothalamic nucleus.



Changes in the text: We have updated Figure 1.

The authors should correct their statement in the Highlight Box and Results section that reads: "The PVN innervates the pancreas". In fact, the PVN does not directly innervate (i.e. supply nerves to) the pancreas. Instead, it is linked to the pancreas by multi-synaptic connections and plays a role in regulating the sympathetic and parasympathetic outflow systems of the pancreas.

Reply: Thank you for your suggestion and we have revised the manuscript, as follows: The PVN is connected to the pancreas through multiple synaptic connections.

Changes in the text: We have modified our text as advised (see ' Highlight Box ' section, highlighted in red font)

10) c-Fos staining:

Fig. 3: The legend is missing from the graph.

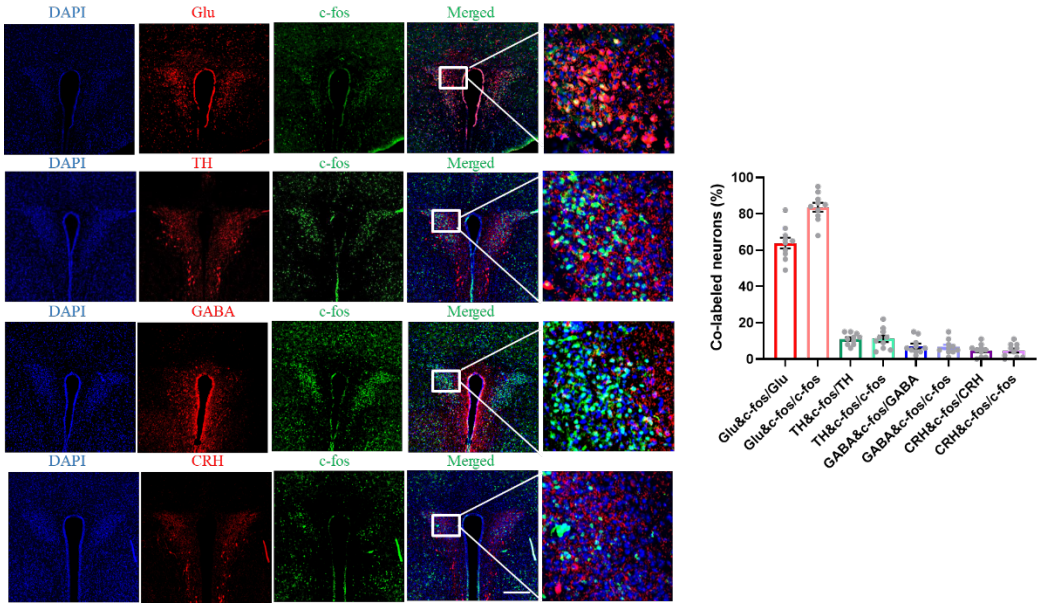
Reply: We apologize for the missing details. We have added the relevant information in the revised manuscript, as follows: Abbreviation: GABA: GABAergic neurons, TH: tyrosine hydroxylase neurons, Glu: glutaminergic neurons, CRH: Corticotropin-releasing hormone neurons. Scale bar: 100 μm.

Changes in the text: We have modified our text as advised (see 'Figure legend for figure 3 ' section,

highlighted in red font)

The c-fos labelling appears inconsistent from image to image. The image quality is poor, making it difficult to assess co-localization between different markers.

Reply: We have provided higher quality images, as shown in the following picture.



Changes in the text: We have provided higher quality images as advised (see 'Figure 3 ')

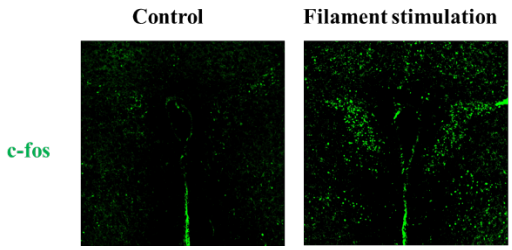
How many days after cancer cell transplantation was c-Fos staining performed? Were there variations in c-Fos staining with tumor growth/size?

Reply: According to Figure 1, we know that mice experience pain symptoms 12 days after tumor implantation. Therefore, we conducted c-Fos staining 12 days after the implantation of tumor cells. We have added the relevant information in the revised manuscript. Since we selected mice with similar tumor sizes for subsequent experiments based on in vivo fluorescence imaging conducted on the 7th day of tumor implantation, we did not observe a correlation between tumor volume and c-Fos staining in this study

Changes in the text: We have modified our text (see 'Immunofluorescence' section, highlighted in red font).

Control experiments with non-transplanted mice should be shown.

Reply: We apologize for not providing clear details about the c-Fos staining experiment. After implanting tumors in mice for 12 days, we stimulated the abdominal pancreas of the mice with a 0.16g filament for approximately 2 seconds, with a 5-minute interval between each stimulation, repeated 10 times. Half an hour later, the mice were euthanized and their brains were collected for PVN c-Fos staining. As shown in the following figure, compared to mice with tumor that did not undergo filament stimulation, there was a significant increase in PVN c-Fos expression.



The authors seem to correlate the increase in c-Fos with visceral pain. However, other conditions can also activate c-Fos in the PVN (e.g. hypoglycemia, restricted feeding), which could be related to the presence of pancreatic tumors. Can the authors comment on these possible associations?

Reply: We apologize for not providing clear details about the experiment. After implanting tumors in mice for 12 days, we stimulated the abdominal pancreas of the mice with a 0.16g filament for approximately 2 seconds, with a 5-minute interval between each stimulation, repeated 10 times. Half an hour later, the mice were euthanized and their brains were collected for PVN c-Fos staining. Compared to mice with tumor that did not undergo filament stimulation, there was a significant increase in PVN c-Fos expression. Therefore, we believe that the increase in c-Fos is associated with visceral pain. While it is possible that other diseases caused by tumors may also be related to changes in PVN c-Fos levels as you mentioned, we consider that in this experiment where filament stimulation exacerbated visceral pain, the increase in c-Fos is primarily associated with visceral pain

Changes in the text: We have added the relevant information in the revised manuscript (see 'Immunofluorescence' section, highlighted in red font).

11) Hyperactivity of PVN neurons:

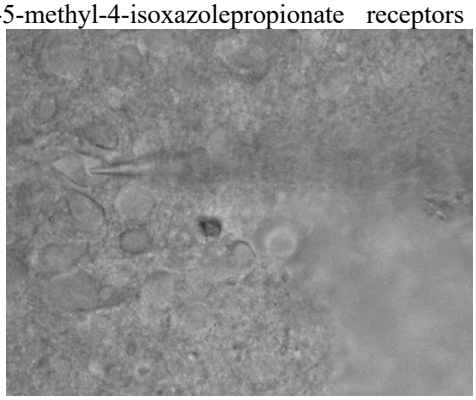
In Figure 4, 'actin potentials' should be corrected to 'action potentials'.

Reply: We apologize for the spelling mistake and have replaced 'actin potentials' by 'action potentials' in revised the manuscript as advised.

Changes in the text: We have modified our text as advised (see 'Figure legend for figure 4 ' section).

In the statement 'We examined the electrophysiological properties of glutamatergic neurons in the PVN using in vitro brain slices', it would be helpful if the authors could specify how the glutamatergic neurons were identified in the brain slices.

Reply: As you know, almost all pyramidal neurons are glutamatergic neurons. Therefore, we can identify glutamate neurons based on the morphology of pyramidal neurons. Pyramidal cells were distinguished from non-pyramidal cells on the basis of pyramidal-like somata and preserved apical and basal (in some neurons) dendrites (Samoilova et al. The open channel blocking drug, IEM-1460, reveals functionally distinct alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors in rat brain neurons.



Neuroscience. 1999; 94 (1): 261-8.). The following image is a typical picture of the glutamatergic neurons we selected during our experiment.

Changes in the text: We have added the relevant information in the revised manuscript (see ' Electrophysiological experiment ' section, highlighted in red font)

"Pancreatalgia induceing hyperactivity of PVN glutamatergic neurons": This title should be corrected.

Reply: We apologize for not providing clear details about electrophysiological experiment. After implanting tumors in mice for 12 days, we stimulated the abdominal pancreas of the mice with a 0.16g filament for approximately 2 seconds, with a 5-minute interval between each stimulation, repeated 10 times. Half an hour later, the mice were euthanized and their brains were collected for electrophysiological experiment. Compared to mice with tumor that did not undergo filament stimulation, there was a significant increase in excitability of glutamatergic neurons. Therefore, we titled it "Pancreatalgia inducing hyperactivity of PVN glutamatergic neurons".

Changes in the text: We have added the relevant information in the revised manuscript (see ' Electrophysiological experiment ' section, highlighted in red font).

What is the evidence that the observed change in PVN hyperactivity is specifically due to pancreatic pain and not to other changes, such as metabolic changes or feeding habits, that may occur in mice with tumor-induced disease?

Reply: We apologize for not providing clear details about electrophysiological experiment. After implanting tumors in mice for 12 days, we stimulated the abdominal pancreas of the mice with a 0.16g filament for approximately 2 seconds, with a 5-minute interval between each stimulation, repeated 10 times. Half an hour later, the mice were euthanized and their brains were collected for electrophysiological experiment. Compared to mice with tumor that did not undergo filament stimulation, there was a significant increase in excitability of glutamatergic neurons. Therefore, we believe that PVN hyperactivity is specifically due to pancreatic pain in this study.

Changes in the text: We have added the relevant information in the revised manuscript (see ' Electrophysiological experiment' section, highlighted in red font).

12) Calcium activity:

Fig. 5; "(C) Comparison of mean calcium activity between the two groups after stimulation (0-2, 0-3, 0-4, 0-5s) (D) Comparison of peak calcium activity between the two groups after Von Frey fiber stimulation (0-2, 0-3, 0-4, 0-5s)."

In section (C), can the authors specify the type of stimulation they are referring to? In addition, were the mice used in these experiments tumor bearing? If so, can they provide information on when the stimulations were performed after cancer cell transplantation?

Reply: We apologize for not providing clear details about fiber photometry. Real-time Ca2+ transients were assessed via fiber photometry (ThinkerTech, Nanjing, China) to determine changes in neuronal activity. After 7 days of tumor implantation, mice with similar tumor sizes were selected for subsequent experiments using in vivo fluorescence imaging. After 9 days of tumor implantation, we microinjected 100 nl of AAV-CaMKIIα-GCaMp6m-EYFP or AAV-CaMKIIα-EYFP into the PVN. An optical fiber (230 μm OD, 0.37 NA, Inper, Hangzhou, China) was implanted over the region injected with GCaMp6s virus and fixed in place with dental cement. two weeks after viral expression, the calcium activity of the

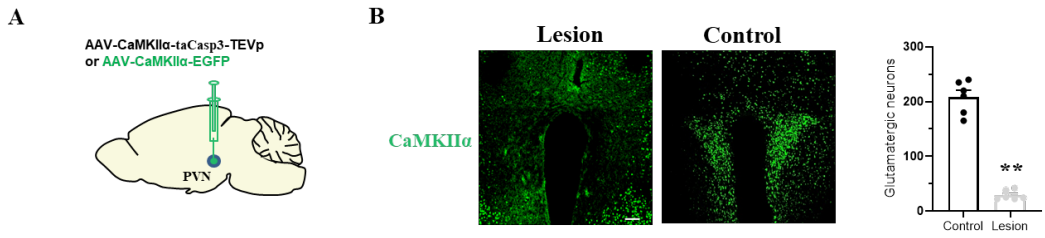
target neurons was monitored. Fiber photometry recordings were conducted on conscious mice that were simultaneously subjected to 0.16 g Von Frey fiber stimulation applied to the left upper abdomen. Fluorescence signals were obtained by reflecting a laser beam from a laser tube (473 nm) onto a dichroic mirror, focusing it with a 103 lens, and then coupling it to an optical commutator. Light was guided from the implanted fiber to the commutator by a 2-m optical fiber. The calcium signals were acquired with data-acquisition software (ThinkerTech, Nanjing, China) and the onset of stimulation was recorded manually. Raw signals were analyzed and processed with a Matlab program developed by Thinkertech. For each trial, the fluorescence variation was calculated as ΔF/F, where ΔF represents the value obtained by subtracting the mean of the baseline signal from the test signal and F represents standard deviation of the basal signal.

Changes in the text: We have added the relevant information in the revised manuscript (see ' Fiber photometry' section, highlighted in red font).

13) Destruction of PVN glutamatergic neurons:

In Figures 6A and 6B: Were the AAV injections unilateral or bilateral? How soon after AAV injection can the death of glutamatergic neurons be detected and what percentage of neurons can be eliminated? It is crucial to provide images and quantified data.

Reply: We apologize for the omission of some details in our manuscript. As you mentioned, AAV was bilaterally injected into the PVN. On the 20th day post-virus injection, we conducted behavioral tests and subsequently euthanized the mice to assess the elimination of glutamatergic neurons. As shown in the figure below, 86.2% of glutamatergic neurons were ablated.



Changes in the text: We have modified our text (see ' Ablation of glutamatergic neurons' section, highlighted in red font).

In Figures 6C, 6E and 6G: Why is the timing of the tests (14, 17, 20 days) different from that in Figure 1?

Reply: Thank you for your comment. Due to the specific experimental procedures being different, we chosen different timing. For figure 6, in order to eliminate the influence of tumor size on the experiment, mice with similar tumor sizes were selected for subsequent experiments using in vivo fluorescence imaging technology on the 7th day after implantation of tumor cells. Then, on the 9th day after implantation of tumor cells, injection of virus was performed for selective ablation of glutamatergic neurons in PVN. As it is usually two weeks after virus injection that behavioral assessment is conducted, we carried out behavioral tests on days 14, 17 and 20 after virus injection. For Figure 1, as it did not involve any virus injection experiments, behavioral testing was conducted two days (resting period) after in vivo fluorescence imaging.

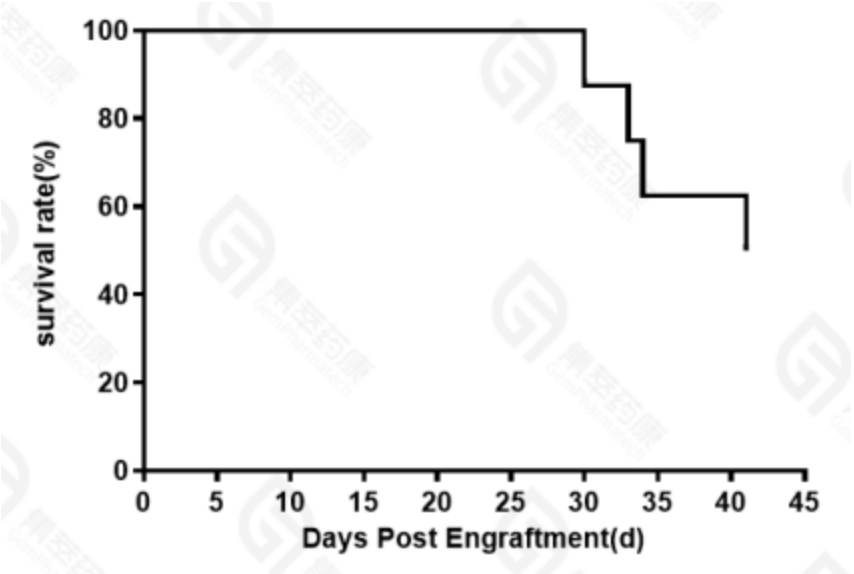
Figures C and E show data relating to the size of von Frey filaments rather than the number of

experiments, making comparison with the results in Figure 6G difficult.

Reply: We appreciate your comment. Using different von Frey stimuli, similar to the methods adopted in sections C and E, is a very good suggestion. However, in order to improve the reliability of behavioral test results, we conducted 10 tests on each mouse. Therefore, we presented this data in Figure 6G, which is different from the x-axis in Figures 6C and E.

The authors conclude that destruction of glutamatergic neurons in the PVN reduces cancer-induced visceral pain. However, the hunching score and mechanical sensitivity results after 20 days show no difference between the conditions, suggesting on the contrary that the destruction has no effect on the behavior of the mice.

Reply: We appreciate your comment. According to Figure 6, we found that the abdominal mechanical sensitivity and Hunch score of mice decreased 14 and 17 days after virus injection. Therefore, we believe that ablation of glutamatergic neurons can alleviate visceral pain. Based on the survival curve (as shown in the figure below, data obtained from Gempharmatech), we know that mice started dying gradually 30 days after tumor cells injection. This indicates that the condition of mice deteriorated 20 days after virus injection (i.e., 29 days after tumor cells injection), possibly due to health factors caused by tumors which prevented effective response to mechanical stimulation. In summary, we believe that ablation of glutamatergic neurons can alleviate visceral pain in pancreatic cancer.



Changes in the text: We have modified our text (see 'Discussion' section, highlighted in red font).

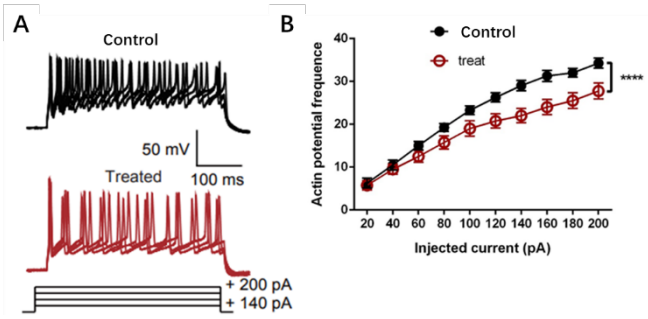
14) Chemogenetic inhibition of PVN glutamatergic neurons:

There is a discrepancy between the Materials and Methods section, which mentions the use of AAV-CaMKIIα-hM4Di-mCherry for PVN injection and administration of CNO or saline, and Figure 7, which shows data from mice injected with AAV-CaMKIIα-hM4Di-mCherry or AAV-CaMKIIα-mCherry virus.

Reply: We sincerely apologize for the errors made during the preparation of the manuscript. In fact, as depicted in Figure 7, for selective inhibition of PVN glutamatergic neurons, the PVN of mice was injected with AAV-CaMKIIα-hM4Di-mCherry or AAV-CaMKIIα-mCherry after 9 days of

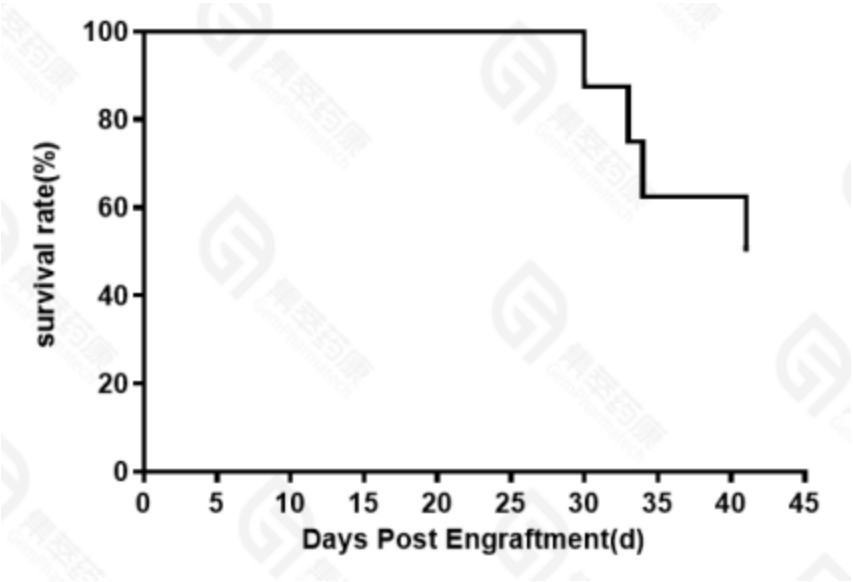
tumor implantation. Behavioral assessments were conducted 14, 17, and 20 days after virus injection. 40 min prior to behavioral assessments, both groups of mice received an intraperitoneal injection of 0.33 mg/ml CNO (0.2 ml/20 g). Relevant information has been added in the revised manuscript.
Changes in the text: We have modified our text (see ' Chemogenetics inhibition of glutamatergic neurons ' section, highlighted in red font).

The efficacy of PVN inhibition needs to be demonstrated. In addition, the effect of CNO alone on mechanical hypersensitivity should be tested.
Reply: As shown in the figure below, the excitability of glutamatergic neurons is significantly inhibited. We sincerely apologize for the errors made during the preparation of the manuscript. In fact, as depicted in Figure 7, for selective inhibition of PVN glutamatergic neurons, the PVN of mice was injected with AAV-CaMKIIα-hM4Di-mCherry or AAV-CaMKIIα-mCherry after 9 days of tumor implantation. Prior to behavioral assessments, both groups of mice received an intraperitoneal injection of 0.33 mg/ml CNO (0.2 ml/20 g). Since we used CNO in both groups, we think there was no need to test the effect of CNO alone on mechanical sensitivity.



Changes in the text: We have modified our text (see ' Chemogenetics inhibition of glutamatergic neurons ' section, highlighted in red font).

As above, inhibition has no effect on the behavior of the mice on day 20, contradicting the authors' assumption that it alleviates visceral pain.
Reply: We appreciate your comment. According to Figure 7, we found that the abdominal mechanical sensitivity and Hunch score of mice decreased 14 and 17 days after virus injection. Therefore, we believe that inhibiting glutamatergic neurons can alleviate visceral pain. Although there was no statistically significant difference in mechanical sensitivity and Hunch score between the two groups 20 days after virus injection, repeated experiments conducted ten times consistently showed a potential decreasing trend in mechanical sensitivity. In addition, based on the survival curve (as shown in the figure below, data obtained from Gempharmatech), we know that mice started dying gradually 30 days after tumor cells injection. This indicates that the condition of mice deteriorated 20 days after virus injection (i.e., 29 days after tumor cells injection), possibly due to health factors caused by tumors which prevented effective response to mechanical stimulation. In summary, we believe that inhibiting glutamatergic neurons can alleviate visceral pain in pancreatic cancer.

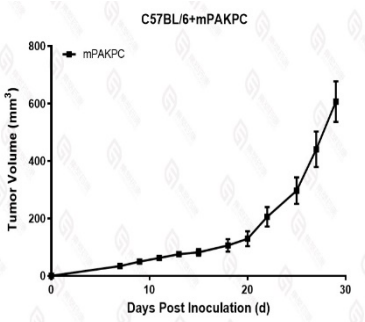


Changes in the text: We have modified our text (see 'Discussion' section, highlighted in red font).

Reviewer B:

Comments to the authors:

- Figure 1
  - Tumor growth curves would be helpful to determine if changes in visceral pain correlated with changes in tumor size.
  - Reply: Thank you for your excellent suggestion. According to the tumor growth curve (as shown in the figure below, data from Gempharmatech), it can be observed that the tumor grows relatively slowly and steadily between 12-18 days after implanting tumor cells. Additionally, according to Figure 1, the severity of visceral pain also increases correspondingly



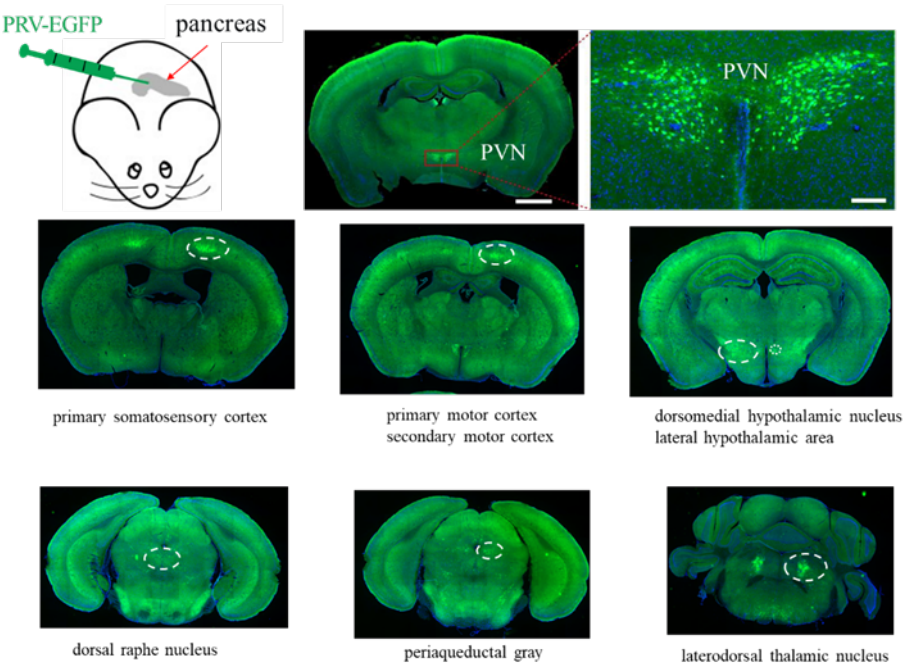
  -
  - A key or interpretation in the text needs to be included for the mechanical hyperplasia grid.

    Reply: We sincerely apologize for the omission of certain details in our manuscript. The color yellow signifies a positive response, while the

color purple denotes a negative response. The x-axis refers to the number of repetitions in the experiment. The Y-axis represents the number of the mice.

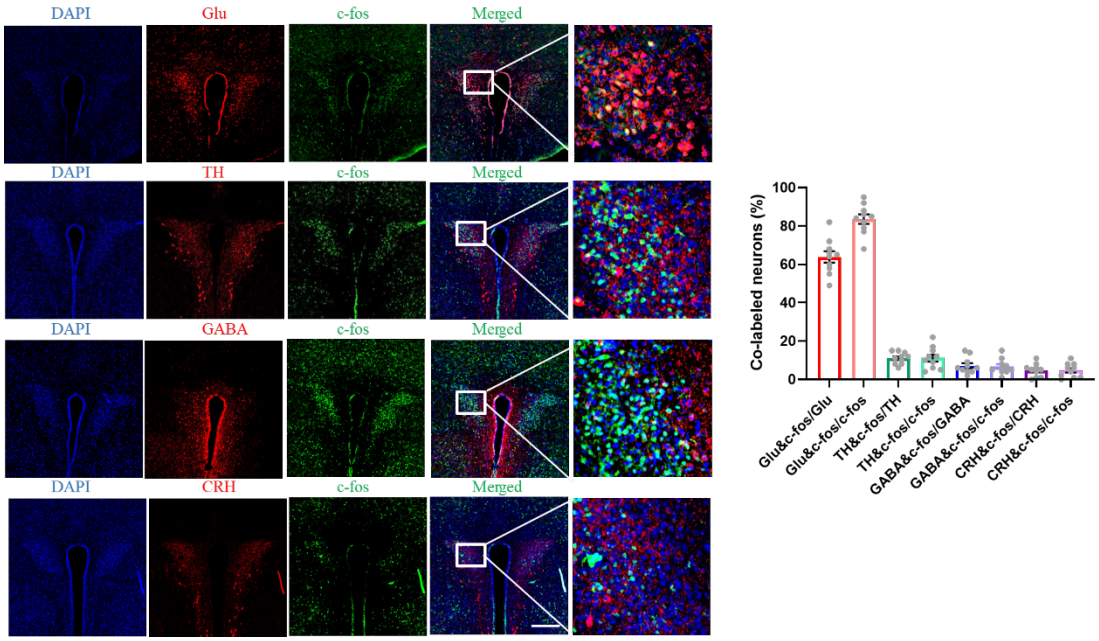
Changes in the text: We have modified our text (see ' Figure legend for figure1 ' section, highlighted in red font).

-
    - How pain is measured needs to be clarified either in the results section or the methods.
    - Reply: We apologize for the missing experimental details in our manuscript. We have revised the paper to add relevant information.
    - Changes in the text: We have modified our text as advised (see 'Behavioral analysis' section and 'Result' section, highlighted in red font).
    -
    - Bars to denote comparisons on graphs, labeled with either stars or a p-value are necessary.
    - Reply: We sincerely apologize for the omission of certain details in our manuscript. We have added stars in figure 1.
    - Changes in the text: We have modified our text as advised (see 'figure1' section).
    -
- Figure 2
    - Address what other areas of the brain were also labeled with EGFP following injection.
    - Reply: Thank you for your comment. As shown in the figure below, we observed that the brain regions primarily labeled encompassed the paraventricular nucleus of the hypothalamus, primary somatosensory cortex, primary motor cortex, secondary motor cortex, dorsomedial hypothalamic nucleus, lateral hypothalamic area, dorsal raphe nucleus, periaqueductal gray, laterodorsal thalamic nucleus.
    -



- Changes in the text: We have updated Figure 2.
- Were the authors able to confirm that the rabies virus is passing through pancreatic beta cells as previous literature suggests?
- Reply: We have found overlapping brain regions with the literature, such as PVN, as well as non-overlapping brain regions, so we cannot determine whether the pseudo-rabies virus is transmitted to the central nervous system through β cells in our study.

- Figure 3
  - The figures are low resolution, but it does not appear that anything is stained with CRH. A positive control is necessary.
  - Reply: As shown in the figure below, a high-resolution image has been uploaded.



- Changes in the text: We have updated Figure 3.

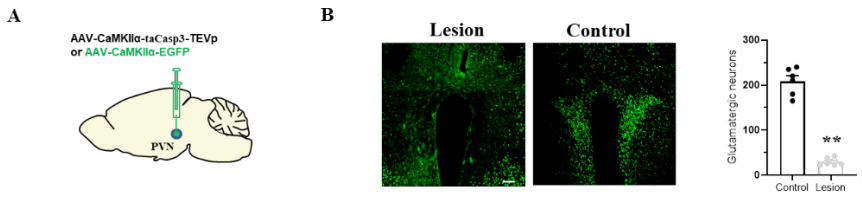
- An orthogonal approach to justify investigating glutamatergic neurons would strengthen the manuscript. As is, the reasoning to investigate glutamatergic neurons is weak.
- Reply: We agree with your point. There is an important background information that we failed to mention in our paper: More than 90% of the PVH consists of glutamatergic neurons, while GABAergic neurons are less represented.[1-3]. Additionally, in this experiment, we found a significant increase in c-fos when stimulating the pancreatic region of mice with filament, and it was mainly co-labeled with glutamatergic neurons. Therefore, we hypothesize that visceral pain may exhibit a strong association with glutamatergic neurons. Subsequent experiments were specifically designed to investigate the role of glutamatergic neurons in this context. We have added relevant content in the results section stating 'c-fos being mainly co-labeled with glutamatergic neurons'.
- Reference:
- 1. Vong L, Ye C, Yang Z, Choi B, Chua S, Jr., Lowell BB. Leptin action on GABAergic neurons 536 prevents obesity and reduces inhibitory tone to POMC neurons. Neuron. 2011;71(1):142-54.
- 2. Xu Y, Wu Z, Sun H, Zhu Y, Kim ER, Lowell BB, et al. Glutamate

mediates the function of melanocortin receptor 4 on Sim1 neurons in body weight regulation. Cell Metab. 539 2013;18(6):860-70.

- 3. Ziegler DR, Cullinan WE, Herman JP. Organization and regulation of paraventricular nucleus glutamate signaling systems: N-methyl-D-aspartate receptors. J Comp Neurol. 542 2005;484(1):43-56.
- Changes in the text: We have added relevant content in rervised manuscript (see results section stating 'c-fos was mainly co-labeled with glutamatergic neurons').

- Figure 5
  - Clarify if both EYFP and GCaMP6m groups were with pancreatic pain mice or if there were also control mice.
  - Reply: Both EYFP and GCaMP6m groups were with pancreatic pain mice. Relevant information has been added in the revised manuscript.
  - Changes in the text: We have modified our text as advised (see ' Chemogenetics inhibition of glutamatergic neurons ' section and 'figure legends' section, highlighted in red font)

- Figure 6
  - The IF should also include a glutamatergic neuron marker in addition to the taCasp3.
  - Reply: In figure 6B, as immunofluorescence of CaMKIIα showed, PVN glutamatergic neurons were ablated by AAV-CaMKIIα-taCasp3-TEVp in mice compared with the control only infected by AAV-CaMKIIα-EGFP.



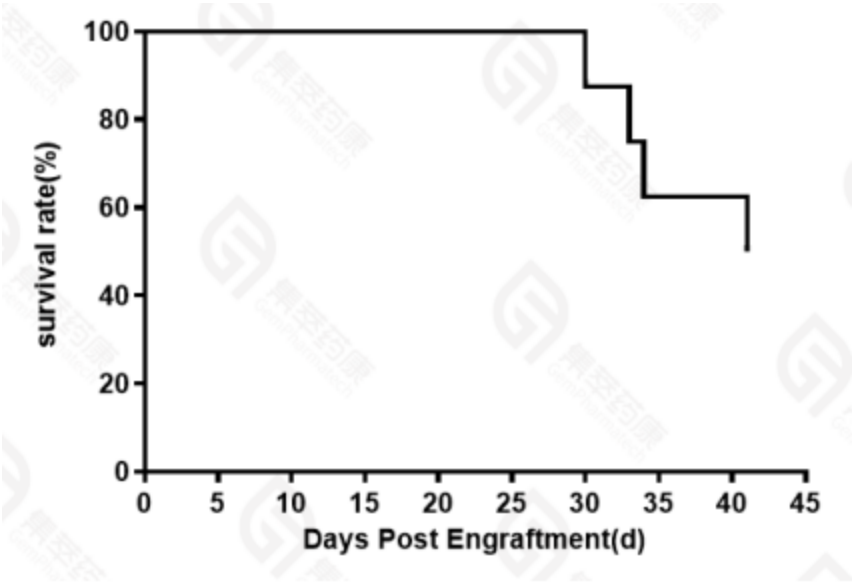
  - Changes in the text: We have modified our text (see ' Figure legend for figure 6 ' section, highlighted in red font).
  - A key or interpretation in the text needs to be included for the mechanical hyperplasia grid.

    Reply: We sincerely apologize for the omission of certain details in our

manuscript. The color yellow signifies a positive response, while the color purple denotes a negative response.

- Changes in the text: We have modified our text (see ' Figure legend for figure 6 ' section, highlighted in red font).
-

- Figure 7
  - The IF should also include a glutamatergic neuron marker in addition to the taCasp3.
  - Reply: Thank you for your comment. However, we have noticed that this comment is identical to the one in Figure 6.
  - A hypothesis on why the control mice and treated mice have more similar responses on day 20 but not earlier would be helpful.
  - Reply: We appreciate your comment. According to Figure 7, we found that the abdominal mechanical sensitivity and Hunch score of mice decreased 14 and 17 days after virus injection. Therefore, we believe that inhibiting glutamatergic neurons can alleviate visceral pain. Although there was no statistically significant difference in mechanical sensitivity and Hunch score between the two groups 20 days after virus injection, repeated experiments conducted ten times consistently showed a potential decreasing trend in mechanical sensitivity. In addition, based on the survival curve (as shown in the figure below, data obtained from Gempharmatech), we know that mice started dying gradually 30 days after tumor cells injection. This indicates that the condition of mice deteriorated 20 days after virus injection (i.e., 29 days after tumor cells injection), possibly due to health factors caused by tumors which prevented effective response to mechanical stimulation. In summary, we believe that inhibiting glutamatergic neurons can alleviate visceral pain in pancreatic cancer.



- Changes in the text: We have modified our text (see 'Discussion' section, highlighted in red font).

- Figures 6 and 7 both inactivate glutamatergic neurons, but an opposing experiment activating glutamatergic neurons would further support the hypothesis.

Reply: We appreciate your suggestion. As you mentioned, validating the hypothesis from both the activation and inhibition of neurons can provide more robust evidence. However, we believe that it is not in line with animal welfare to subject animals to additional pain during animal experiments. Therefore, in this experiment, we have employed two methods to inhibit neurons instead of simultaneously activating and inhibiting them.

Reviewer C:

The authors performed an interesting analysis unravelling the mechanism of visceral pain in pancreatic cancer. The study has several strengths, including the high technical quality and a clear strategy. Overall, I enjoyed reading your manuscript and think it is an important contribution in the filed. However, I have some suggestions for improvement.

Reply: Thank you for your comments on my manuscript. I'm glad to hear that you found the analysis of visceral pain in pancreatic cancer interesting and appreciated the technical quality and clear strategy employed in the study. Your positive remarks regarding its importance as a contribution to the field are also encouraging. We value constructive criticism and believe it can help enhance the overall quality of our work. We have made revisions to the manuscript based on your suggestions, and we hope to earn your approval. Once again, thank you for taking the time to review my manuscript.

Major:

- The description of the experiments and the statistical analysis are not precise enough at serval

points. The way you present your data currently is inadequate. E.g.

o line 134: which filaments did you use?
Reply: In figure 1B, D, and F, as well as in figures 6G and 7G, 0.16g filament was utilized. In figures 6C and E, as well as in figures 7C and E, 0.008 g, 0.02 g, 0.04 g, 0.07 g, 0.16 g and 0.4 g filaments were employed. Relevant information has been incorporated into the figure legends.
Changes in the text: We have modified our text as advised (see ' figure legends ' section, highlighted in red font)

o line 300 and following: you performed pain analysis 14/17/20 days after virus injection. But when was the cancer cell injection? At the same day as the virus injection? Please clarify.
Reply: After 9 days of tumor implantation, we injected viruses and implanted optical fibers. After 14 days of virus injection, we conducted Fiber photometry. At 14, 17, and 20 days after virus injection, we performed abdominal mechanical hyperalgesia tests and Hunching Scores before euthanizing the mice
Changes in the text: We have modified our text as advised (see 'Ablation of glutamatergic neurons' section and 'Chemogenetics inhibition of glutamatergic neurons' section, highlighted in red font)

o Fig 1B-F: which filament did you use?
Reply: We used 0.16 g filament in Figure 1.
Changes in the text: We have modified our text (see 'Figure legends for figure 1' section, highlighted in red font)

o Figure 1 B, D, F: third panel each. I would recommend quantifying the mechanical hyperalgesia by showing the response rate to different filaments, as you did in Figure 6C and 6E. Then you can calculate area under the curves to quantify the overall differences in mechanical hypersensitivity.
Reply: We appreciate your suggestion. Quantifying the mechanical hypersensitivity by assessing the response rate to different filaments is indeed a valuable idea, and we have also considered employing various filaments for this purpose. Previous animal studies have demonstrated that using different filaments ranging from 0.008g to 0.16g can elicit pain responses (1. Hirth, M, Xie, Y, Höper, C, et al. Genetic Mouse Models to Study Pancreatic Cancer-Induced Pain and Reduction in Well-Being. Cells. 2022; 11 (17). 2. Yu, D, Zhu, J, Zhu, M, et al. Inhibition of Mast Cell Degranulation Relieves Visceral Hypersensitivity Induced by Pancreatic Carcinoma in Mice. J Mol Neurosci. 2019; 69 (2): 235-245), thereby indicating the severity of pancreatic cancer-induced pain in animals as well. After confirming successful tumor implantation through in vivo fluorescence imaging, we anticipated the presence of pain; therefore, we selected only the maximum filament weight of 0.16g to verify its existence without subjecting animals to additional discomfort caused by using a wider range of filaments – this approach aligns with animal welfare policies. However, when it comes to subsequent chemogenetics or taCasp3 regulation of glutamatergic neurons, we cannot predict whether successful alleviation of pain will occur or determine its extent accurately. Hence, based on previous literature reports, we have opted to utilize different filaments.
Changes in the text: We have modified our text in the revised manuscript (see ' Abdominal mechanical hyperalgesia test ' section, highlighted in red font)

o Figure 6G right panel: The x-axis is different from Fig. 6 C and E. Why did you change the test

protocol? What does the x-axis mean? Please use different von Frey stimuli, just as you did in C and E.

Reply: Thank you for your comment. The x-axis refers to the number of repetitions in the experiment. Using different von Frey stimuli, similar to the methods adopted in sections C and E, is a very good suggestion. However, in order to improve the reliability of behavioral test results, we conducted 10 tests on each mouse. Therefore, we presented this data in Figure 6G, which is different from the x-axis in Figures 6C and E.

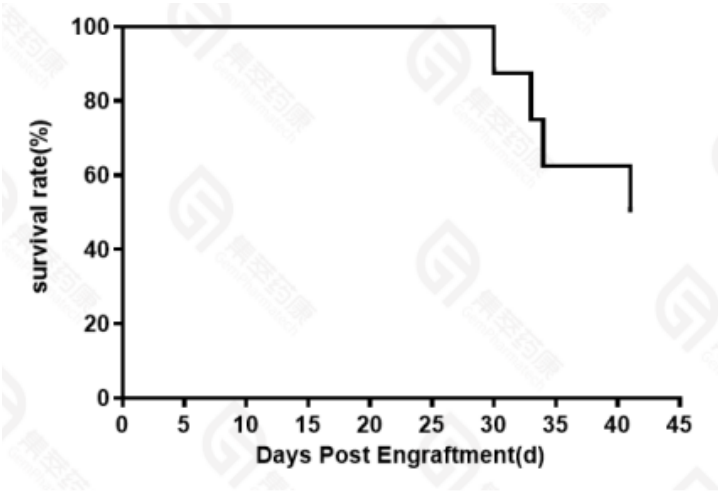
o Please provide proper information on the statistical analysis you performed in each experiment. E.g. Figure 4B. To me it looks like an ANOVA of repeated measures, but in the methods you did not mention ANOVA of repeated measures. Please provide these information for all figures in all figure legends.

Reply: Thank you for your comment. We have now added information on the statistical methods used in each figure legends. For Figure 4B, in order to compare whether there were statistically significant differences between the two groups, we employed a two-way analysis of variance (ANOVA) followed by post hoc Bonferroni instead of using an ANOVA of repeated measures. The results indicated significant statistical differences in action potential frequency between the two groups when injected current ranged from 140-200 pA.

Changes in the text: We have modified our text as advised (see ' Figure legends' section, highlighted in red font).

- Even though other models of orthotopic pancreatic cancer injection models exist, you have established a new model. Thus, please provide more information on this model. How long do the mice survive? Please provide Kaplan-Maier survival curves. What is the rational of the chosen time points for the pain analysis?

Reply: We appreciate your comment. This model is indeed novel; however, it has been previously employed by Wang et al. (Wang et al. (2022). Pyroptosis Remodeling Tumor Microenvironment to Enhance Pancreatic Cancer Immunotherapy Driven by Membrane Anchoring Photosensitizer. Adv Sci (Weinh), 9 (29), e2202914.) to investigate immunotherapy for pancreatic cancer. Kaplan-Maier survival curves were provided by Gempharmatech, where tumor cells purchursed (as shown in the figure below). We apologize for our oversight in not observing the survival time and euthanizing them after behavioral testing. Moving forward, we will heed your suggestion and incorporate observation of animal survival time into this model.



Changes in the text: We have modified our text as advised (see ' Mouse model of pancreatic cancer visceral pain ' section, highlighted in red font)

Minor:

- Please avoid abbreviations in the title

Reply: Thank you for your suggestion and we have modified the title in the revised manuscript, as follows: Glutamatergic neurons in the paraventricular nucleus of the hypothalamus participate in the regulation of visceral pain induced by pancreatic cancer in mice.

Changes in the text: We have modified our text as advised (see ' Title ' section, highlighted in red font)

- Improvement of English language is recommended. Please ask for linguistic revision by a native speaker

Reply: We appreciate your suggestions, and we have sought the assistance of professional linguists to polish our manuscript. We hope to earn your approval for improvement of English language.

- In which part of the pancreas did you inject the cancer cells?

Reply: mPAKPC-luc cells were injected at the head of the pancreas to build an orthotopic tumor model.

Changes in the text: We have modified our text as advised (see ' Mouse model of pancreatic cancer visceral pain ' section, highlighted in red font)

- Lines 153 – 154: use anatomic correct descriptions. “upper end of the pancreas” = pancreatic tail? “middle part” = corpus? “lower part” = pancreatic head?

Reply: We deeply apologize for the errors in manuscript preparation. Specifically, due to the injection of tumor cells into the pancreatic head region, the PRV injection site was also located around this area.

Changes in the text: We have modified our text as advised (see ' Pancreatic pseudorabies virus (PRV) injection ' section, highlighted in red font)

- Line 263 and following: replace “pancreatalgia” by pancreatic cancer induced pain

Reply: Thank you for your suggestion and we have modified the title in the revised manuscript.

Changes in the text: We have modified our text as advised (see ' Result ' section, highlighted in red

font)

- Line 268 and following: please “model mice” by orthotropic pancreatic cancer mouse model.
Reply: Thank you for your suggestion and we have replaced “model mice” by “orthotropic pancreatic cancer mouse model” in the revised manuscript.
Changes in the text: We have modified our text as advised (see ' Results ' section, highlighted in red font)

- Line 302: replace “Figure 6D” by 7D
Reply: Thank you for your suggestion and we have replaced “Figure 6D” by “Figure 7D” in the revised manuscript, as follows: Glutamatergic neurons in the paraventricular nucleus of the hypothalamus participate in the regulation of visceral pain induced by pancreatic cancer in mice.
Changes in the text: We have modified our text as advised (see ' Chemogenetics inhibition of PVN glutamatergic neurons alleviating visceral pain induced by pancreatic cancer ' section, highlighted in red font)

- Line 313: explain the abbreviations SLI, MPON, BST, PVA, Rt, PVN, DMD
Reply: medial preoptic nucleus (MPON), bed nucleus of stria terminalis (BST), paraventricular thalamic nucleus, anterior part (PVA), reticular nucleus (Rt), paraventricular nucleus of hypothalamus (PVN), dorsomedial hypothalamic nucleus, dorsal part (DMD).
Changes in the text: We have modified our text as advised (see ' Figure legends for figure 8' section, highlighted in red font)

- Figure 1A is too small.
Reply:. We appreciate your valuable suggestion. In accordance with your advice, we have made revisions to Figure 1
Changes in the text: We have modified our text as advised (see ' Figure 1')

- Figure 1B-F: What does the colours mean? Please add information in figure legend.
Reply:. The color yellow signifies a positive response, while the color purple denotes a negative response.
Changes in the text: We have modified our text as advised (see ' figure legend for figures 1, 6 and 7', highlighted in red font).

- Figure 3: give more details in the figure legends, e.g. scale bar = XXX μm
Reply:. We apologize for the loss of detailed information and have followed your suggestion to add it to the revised manuscript. The specific details are as follows: Abbreviation: GABA: GABAergic neurons, TH: tyrosine hydroxylase neurons, Glu: glutaminergic neurons, CRH: Corticotropin-releasing hormone neurons. Scale bar: 100 μm. Scale bar, left: 100 μm.

Changes in the text: We have modified our text as advised (see ' Figure legend for figure 3' section, highlighted in red font)

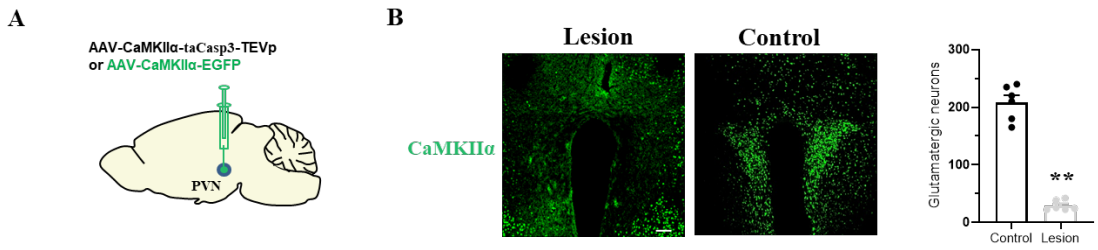
- Line 475: replace “actin” by action

Reply:. We apologize for the spelling error and have made the correction from 'actin' to 'action' as per your suggestion.

Changes in the text: We have modified our text as advised (see ' Figure legends' section, highlighted in red font)

- Fig. 6B and 7B: How many neurons did you infect? Please quantify.

Reply:. According to the statistics, in Figure 6B, 208 glutamatergic neurons were labeled. Using the same method of analysis, in Figure 7B, 245 glutamatergic neurons were labeled.



Changes in the text: We have modified our text as advised (see ' Result' section, highlighted in red font)

Editorial Comments

1. **Comments from Deputy Editor-in-Chief:** The article fails to provide essential information necessary for a full evaluation of the experimental protocols and a full understanding of the results presented. There are several problems with the references cited, the reporting of animal experiments, and the quality of the micrographs. The central conclusion that inhibition or destruction of PVN neurons alleviates cancer pain is a claim that is weakly supported by the available data.

Reply: We appreciate the opportunity you have given us to revise the paper. Your feedback, as well as that of the reviewers, is highly valuable to us. We apologize for any remaining shortcomings in the manuscript and have made revisions based on the reviewer's comments. We hope that the revised manuscript will meet with your requirements.