Dear Reviewers:

Thanks for your comments concerning our manuscript entitled “The caudal nucleus of the solitary tract mediates visceral hypersensitivity induced by chronic pancreatitis in rats” (Manuscript ID: 50316). Your comments are very helpful for improving our paper, and are of important guiding significance to our researches. We have studied the comments carefully and made revisions which we hope to meet with approval. The revised parts related to your comments are marked by track changes. In addition, we also corrected some inappropriate expressions you did not mention and add the part of “**ARTICLE HIGHLIGHTS”** and “**ABBREVIATIONS”** according to the style of the journal in this revised manuscript. These changes will not influence the content and framework of the paper. The main responses to the reviewer’s comments are as following:

Reviewer #1: The study by Yang Bai et al provides evidence of the role of the nucleus of the solitary tract in the visceral hypersensitivity induced by chronic pancreatitis. It is a well-conceived study that respects all major regulatory and scientific rigors. The methodology is sound and statistics are appropriate. The number of study animals is sufficient. Experiments were done properly and results are satisfactory. The manuscript is well written, grammar and style are sufficient. Minor corrections can be made (for instance, one word in the core tip has an extra "s").

Answer: Thanks for your kind advice. In this newly-revised manuscript, we tried to correct all typos, grammar mistakes as well as appropriate expressions. In addition, to make the manuscript clear and concise, we deleted some redundant sentences, which will not influence the quality of the paper.

Minor points: Was chronic pancreatitis achieved in all treated rats (i.e. histologically-proven pancreatic lesions)?

Answer: Thanks for your kind advice.

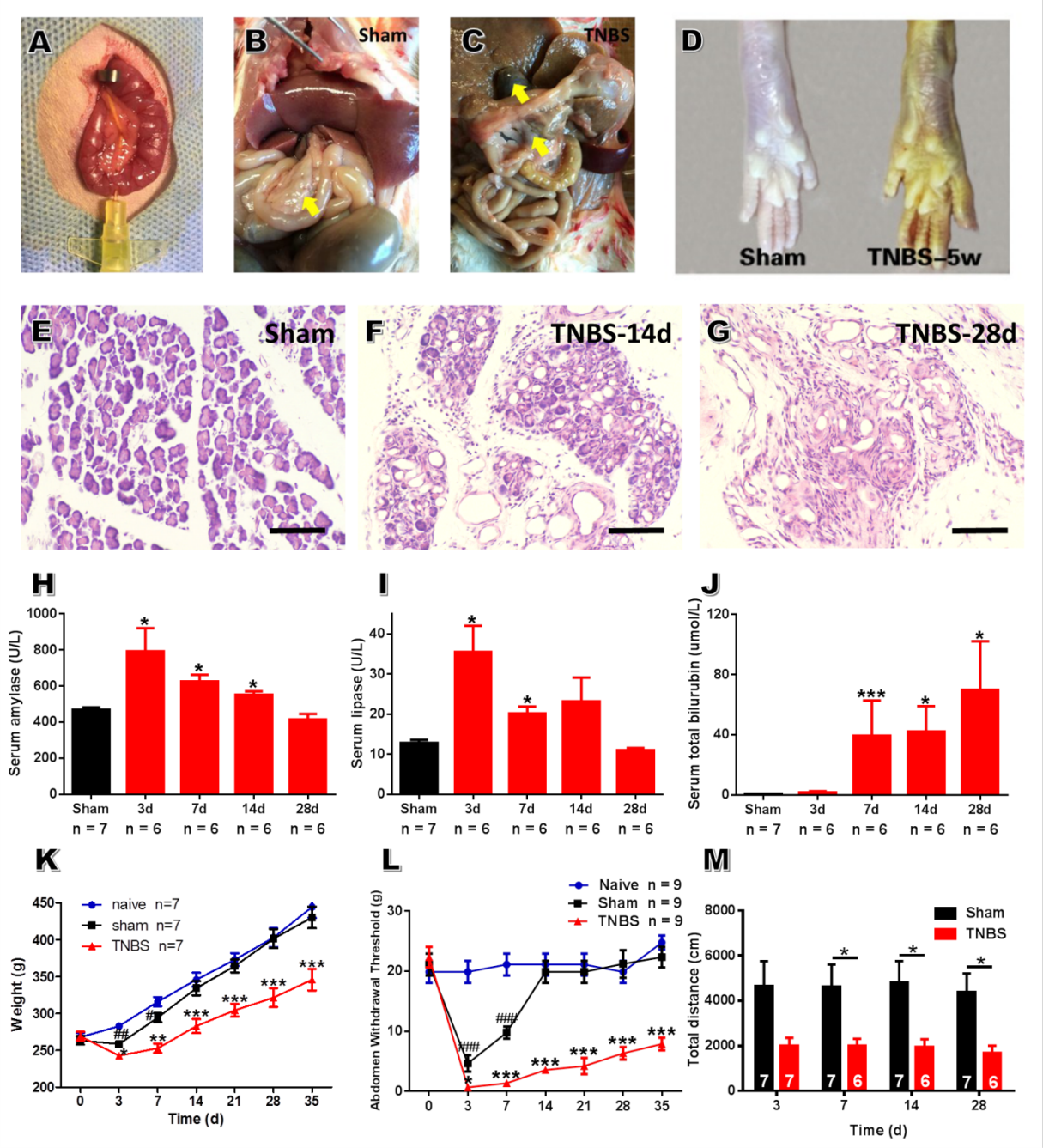
In the present study, chronic pancreatitis was induced in rats by infusion of trinitrobenzene sulfonic acid (TNBS) into the pancreatic duct. This method possesses the advantages of high reliability, easy manipulation and good reproducibility (1, 2).

In our previous study concerning the role of insular cortex in the development of chronic pancreatitis pain entitled “Anterior insular cortex mediates hyperalgesia induced by chronic pancreatitis in rats” (accepted by Molecular Brain, https://doi.org/10.1186/s13041-019-0497-5), we have verified the successful establishment of TNBS-induced CP model. In the study, the rats were exposed to intraductal TNBS or saline treatment. Body weight, abdomen mechanical threshold and locomotion behavior in open field were monitored from POD 3 to POD 35. After behavioral tests, rats were sacrificed. Heart blood was

sampled and serum specimens were measured by amylase, lipase and total bilirubin activity assay kits. Pancreatic tissues were sampled for H&E staining.

As shown in the following figure, the validity of CP model was evidenced by the following evidence: (1) Postmortem examination showed pancreatic atrophy, pancreas cystic duct dilatation and cholestasis in TNBS-treated rats on POD 28 (Figs. A-C); (2) Nearly 50 % of TNBS-treated rats exhibited jaundice in the skin on POD 28 (Fig. D); (3) All TNBS-treated rats exhibited histopathological changes in pancreatic tissue, such as acinar atrophy, inflammatory infiltration and stromal fibrosis (Figs. E-G); (4) Owing to the damage of acinar cells, TNBS-treated rats exhibited increased contents of serum amylase and lipase on POD 3, which decreased along the course of CP and returned to baseline on POD 28 (Figs. H-I); (5) TNBS-treated rats exhibited high level of serum total bilirubin from POD 7 to 28 (Fig. J); (6) TNBS-treated rats exhibited long-term body weight loss along the course of CP (Fig. K); (7) Behavioral assays showed that comparing to sham group, TNBS-treated rats displayed a prolonged decrease in AWT and hypolocomotion along the course of CP (Figs. L-M). All these changes mimicked those appeared in human chronic pancreatitis (3).

All these suggest the successful establishment of CP model in our study. Since these data have been published in **Molecular Brain**, we did not add these data in the present manuscript.



(*P < 0.05, **P < 0.01, ***P < 0.001, TNBS vs sham; #P < 0.05, ##P < 0.01, ###P < 0.001, sham vs naive)

Regarding the time-frame of the experiments, in particular experiment 3: Have the authors considered following the values after these 28 days?

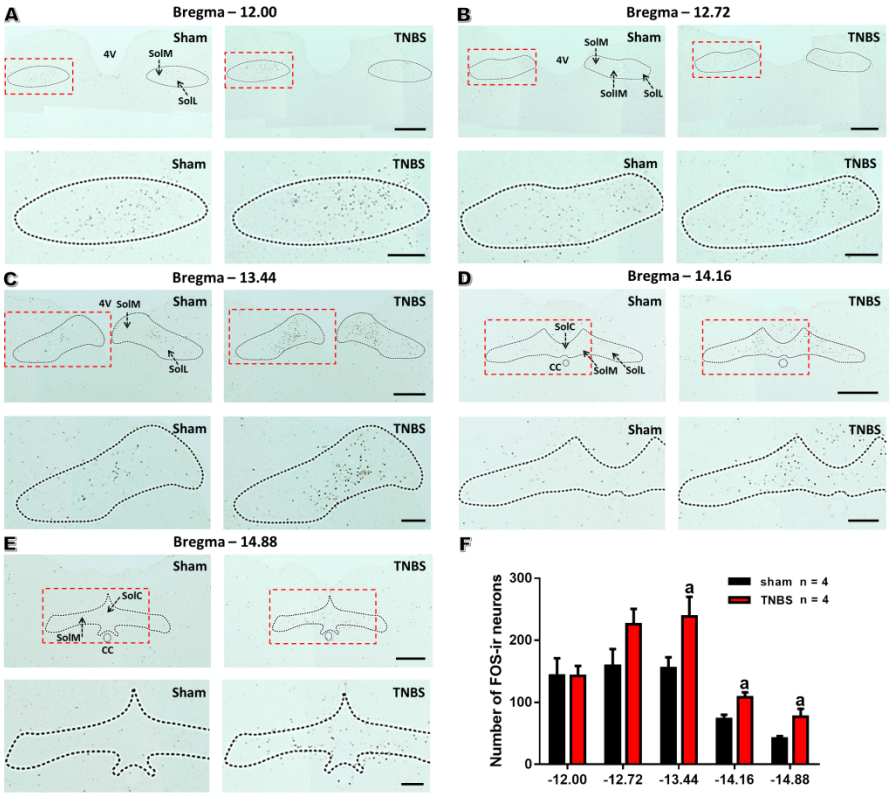
Answer: Thanks for your kind advice. Reviewer 3 also asked the question concerning the time point. Please check the responses in **Page 5, Line 7**.

Reviewer #2: The following points should be corrected: 1) The article is written well but it is too long to read; therefore the text should be summarized, especially in the methods part. 2) Small typing mistakes should be corrected like on page 6 (celiac lexus not plexus) and (An increasing not A increasing). 3) A list of Abbreviations is missing.

Answer: Thanks for your kind advice. In this newly-revised manuscript, we tried to correct the typos, grammar mistakes, and appropriate expression. To make the manuscript clear and concise, we deleted some redundant sentences, especially in the methods part as you mentioned, which will not influence the quality of the paper. Moreover, a list of abbreviations was attached in **Page 26** in the revised manuscript.

4) Graphic 1 shows multiple Brain slices with immunochemical staining, but the small pictures are not as informative as the bar graph, this needs modification.

Answer: Thanks for your kind advice. In this newly-revised manuscript, we added higher power images to facilitate reader's observations. Please check the newly-revised Fig. 1.

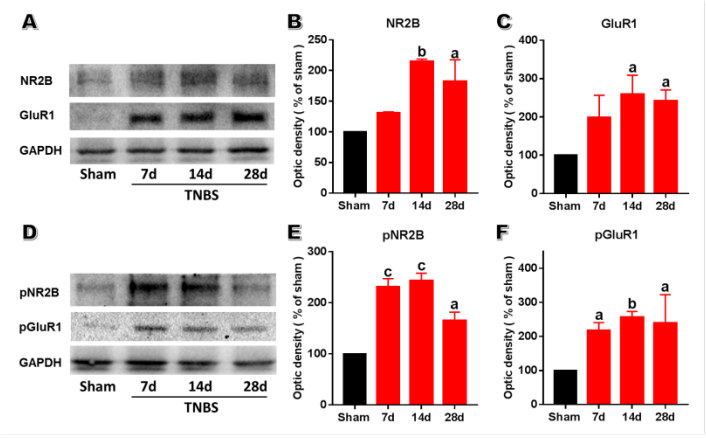


Reviewer #3: In this manuscript entitled "The caudal nucleus of the solitary tract mediates visceral hypersensitivity induced by chronic pancreatitis in rats", the authors showed that some neuroplastic changes in the caudal nucleus of the solitary tract contributed to chronic pancreatitis-induced pain

syndrome. The finding is interesting and the paper is well written. However, some issues need to be addressed. (1) In chronic pancreatitis, the progressive impairment of pancreatic tissues lead to the development of a chronic pain syndrome. However, the longest time point of studying NR2B and GluR1 expressions in Fig 4 is 4 weeks, which already have a trend of down-regulation. What are the expression levels of NR2B and GluR1 at a later time point? If their expressions are further decreased at a later time point, how can we explain the role of NR2B and GluR1 in the chronic pain syndrome?

Answer: Thanks for your kind advice.

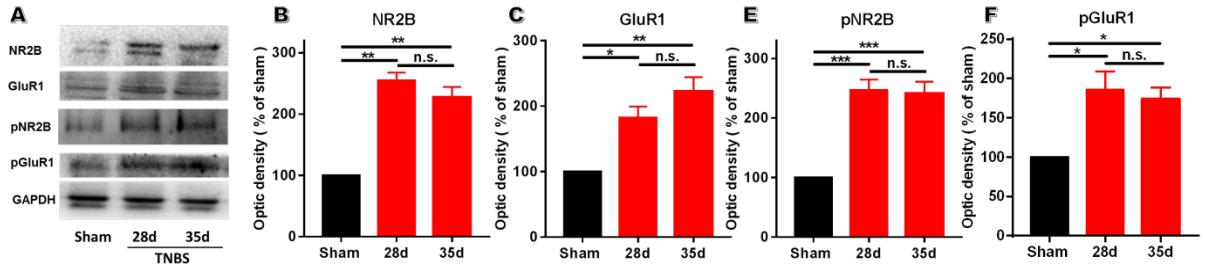
Firstly, we are sorry that we made a mistake in the Figs. 3 and 4 since the results were expressed as the means ± SD. In this new manuscript, we modified the figures by presenting the data as the means ± SEM as described in the "Data analysis" part. Here is the revised Figure 4.



($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$)

Secondly, as you mentioned, there seemed a trend of down-regulation in the expression of these molecules, especially NR2B and pNR2B along the course of CP. We further performed subgroup analyses of the expression of these molecules. Concerning the expression of NR2B, GluR1 and pGluR1, there was no significant change between POD 14 and POD 28. However, there was a significant decrease in the expression of pNR2B on POD 28 comparing to POD 14.

Considering these, we further performed immunoblot analysis of the expression of these glutamate receptors at POD28 and POD 35 by the brain tissues at hand. The result showed that comparing to POD 28, there was no significant changes of the expression of these glutamate receptors on POD 35. In light of these, we speculated that the upregulation of these receptors has reached a plateau after TNBS treatment at this time point.



($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Concerning the decreased expression of pNR2B on POD 28 in the present study, a similar decreased trend of NR2B was also seen within the insula (another pivotal pain-related area) under the condition of neuropathic pain in Qiu's study (4). Considering these, the upregulation of NR2B in pain-related brain areas may play a more important role in the induction of chronic pain than in its maintenance phase.

Another interesting phenomenon is that comparing to POD 14, there was a significant increase in the AWT of CP rats on POD 28 ($P < 0.05$; Fig. 1L). This pain relief after the establishment of experimental chronic pain was also seen in SNI-related neuropathic pain and CFA-induced inflammatory pain based on our prior observations. Considering these, we propose that the downregulation of pNR2B on POD 28 may be correlated with the alleviation of pancreatic pain along the natural course of CP, which merits further investigations in our future researches.

(2) The authors showed that deactivating excitatory neurons within NTS alleviated the pain in chronic pancreatitis. This approach, however, could block the pain caused by peripheral sensitization and pancreatic neuropathy. This should be discussed.

Answer: Thanks for your kind advice. We are sorry that we did not explain this point in the manuscript.

To be frank, the definition of "visceral hypersensitivity (i.e., pain caused by pancreatic neuropathy)" is different from that of "abdomen hyperalgesia (i.e., pain caused by peripheral somatic sensitization)" under the condition of CP. In 2005, Pasricha et al. utilized two methods to measure chronic pancreatitis induced hyperalgesia in a rat model of CP. The first one is to measure the sensitivity of the abdomen to mechanical stimulation, an assay for referred abdominal mechanical hypersensitivity when pancreatic inflammation invades the peritoneum. **Somatic responses to pressure on the upper abdominal wall are an indirect marker of visceral sensitization and do not necessarily implicate the pancreas.** The second one is to record the number of nocifensive behaviors in response to electrical pancreas stimulation, which could directly reflect visceral pain induced by pancreatic neuropathy. In the study, they observed

that rats with pancreatitis exhibited significant increases in the sensitivity to mechanical probing of the abdomen as well as the sensitivity to noxious electrical stimulation of the pancreas (5, 6).

Since VFF probing is much easier to achieve than that of inserting intra-abdominal electrodes, it is more adopted in preclinical studies of chronic pancreatitis pain. In the present study, we only used VFF probing to examine chronic pancreatitis related pain, so we could only conclude that deactivating excitatory neurons within NTS alleviated the pain caused by peripheral somatic sensitization, instead of the pain caused by pancreatic neuropathy. **Thus, observing pancreas stimulation induced defensive behaviors via intra-abdominal electrodes may be a better choice in our future studies to evaluate the sensory aspect of pancreatitis pain.** We have admitted this is a limitation of this study and we have briefly introduced our thoughts mentioned here in the discussion. **Please check Page 24, lines 28 in the revised manuscript.**

(3) In has been shown that 80% of TNBS-treated rats exhibit morphological changes mimicking features of chronic pancreatitis in humans. The percentage of TNBS-treated rats that have had the pathological changes mimicking chronic pancreatitis in this study should be shown.

Answer: Thanks for your kind advice. Reviewer 1 also asked this question concerning the verification of the CP model. **Please check Pages 1-3 in the response letter**.

Reviewer #4: Valuable insight into mechanisms of processing of pancreatic pain in chronic pancreatitis in rats. Potential use of results includes targeting caudal NTS for the treatment of pain in chronic pancreatitis.

Answer: Thanks for your encouragements and recognitions.

In this newly-revised manuscript, we tried to correct all typos, grammar mistakes as well as appropriate expressions. To make the manuscript clear and concise, we deleted some redundant sentences, which will not influence the quality of the paper. In additions, in order to answer other reviewers' concern, we modified Figures 1, 3 and 4. Please check corresponding responses in this letter, and we hope these modifications will meet with your approve.

Reviewer #5: Thank you for this interesting basic science paper, study and review.

Answer: Thanks for your encouragements and recognitions.

In this newly-revised manuscript, we tried to correct all typos, grammar mistakes as well as appropriate expressions. To make the manuscript clear and concise, we deleted some redundant sentences, which will not influence the quality of the paper. In additions, in order to answer other reviewers' concern, we modified Figures 1, 3 and 4. Please check corresponding responses in this letter, and we hope these modifications will meet with your approve.

Reviewer #6: Please specify the current meaning of "sensitization" and of "hypersensitivity", as used in clinics.

Answer: Thanks for your kind advice.

Under the condition of chronic pain, pain arises spontaneously (spontaneous pain), can be elicited by normally innocuous stimuli (allodynia), is exaggerated and prolonged in response to noxious stimuli (hyperalgesia), and spreads beyond the site of injury (secondary hyperalgesia). Considering these, **hypersensitivity (i.e., hyperalgesia) is a prominent characteristic of behavioral manifestations of chronic pain** (7). Similarly, visceral hypersensitivity is the term describing the experience of pain within the inner organs (viscera) at a level that is more intense than normal; In other word, it means a lowered threshold for abdominal pain and discomfort in response to pressure, stimulation, or distension within the abdomen, which is a hallmark characteristic of irritable bowel syndrome, non-cardiac chest pain and chronic pancreatitis pain, etc (8).

Behavioral hypersensitivity is thought to be the result of changes to nerve pathways, which cause a person's nerve system to have an overactive response to pain. **These pathophysiological mechanisms underlying the induction and maintenance of chronic pain are called "sensitization".** Sensitization represents an enhancement in the function of pain-related neurons and pathways caused by increased membrane excitability and synaptic efficacy, and reduced inhibition. It is a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation, and neural injury. The net effect of sensitization is to recruit previously subthreshold synaptic inputs to nociceptive neurons, generating an increased or augmented action potential output. Sensitization is responsible for many of the temporal, spatial, and threshold changes in pain sensibility in acute and chronic clinical pain settings and exemplifies the fundamental contribution of the central nervous system to the generation of pain hypersensitivity.

Sensitization is broadly classified into two types. The first is peripheral, and involves changes in the excitability of primary nociceptors. The second is central and can occur at the spinal cord or higher levels, including the brain (9-11).

Previous studies concerning the sensitization mechanisms of painful CP usually center on the thoracic spinal dorsal horn. In general, prolonged stimulation from peripheral sensitization facilitates aberrant excitation of dorsal horn neurons. This process is referred to as central sensitization and results in visceral hypersensitivity in chronic pancreatic pain. Unfortunately, much less focus is directed on the role of the NTS in painful CP. In light of these, we focus on the role of NTS in the condition of pancreatic pain. The present study showed the synaptic plasticity changes (central sensitization) of NTS during pancreatic pain, which contributed to behavioral hypersensitivity (abdomen hyperalgesia) of CP rats.

References:

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Once again, we earnestly appreciate for your warm work, constructive comments and valuable suggestions, and hope that the revised manuscript will meet with approval.

May you be blessed with health and eudemonia.

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