Peer Review File

Manuscript Title: A neuroanatomical basis for acupuncture to drive the vagal-adrenal axis

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee expertise:

Referee #1: neuro-immune, inflammation

Referee #2: pain and itch circuits

Referee #3: somatosensation

Referee #4: Neuro-immune interactions, sepsis

Referees' comments:

Referee #1 (Remarks to the Author):

The results show that somatosensory neurons marked by Prokr2Cre innervate hindlimb fascial tissues (e.g., periosteum), but not abdominal fascia (e.g., peritoneum). Selective ablation of Prokr2Cre-marked sensory neurons prevents low-intensity electroacupuncture stimulation of hindlimb regions from activating hindbrain vagal efferent neurons and driving catecholamine release from adrenal glands; as such, ES in these mice failed to suppress severe systemic inflammation induced by bacterial endotoxins. In contrast, the spinal-sympathetic axis evoked by high-intensity ES of both hindlimb and abdominal regions was unaffected. Optogenetic stimulation of Prokr2Cre nerve fibers at hindlimb regions was sufficient to drive the vagal- adrenal anti-inflammatory axis, but unable to evoke sympathetic reflexes. Innervation patterns by Prokr2Cre nerve fibers can retrospectively predict effective and non-effective body regions for low-intensity ES to produce anti-inflammatory effects.

The authors conclude "these findings provide a neuroanatomical basis for body region-dependent driving of the vagal- adrenal anti-inflammatory axis, which could help to design optimal bioelectronic stimulation parameters to treat deadly systemic inflammation."

The findings are original and significant, because the authors present a new understanding of sensory neural specialization linked to stimulation of specific reflex outputs.

The data and other findings are clearly presented and discussed, and the methods and statistics appropriately defined and utilized.

The results support the conclusions. The manuscript and abstract are clear, well written, and lucid.

I have only one minor comment:

The authors might consider citing and discussing the work from the Murakami lab on the Gateway reflex, which is likely also activated by the present mechanisms.

Referee #2 (Remarks to the Author):

Summary:

In this work by Shenbin Liu and colleagues, the authors identified a mouse sensory neuron population genetically labeled by Prokr2cre, some of which mediate the low intensity (0.5mA) electric stimulation induced vagal-adrenal anti-inflammatory effect, at a hindlimb acupuncture site, ST36. The authors first characterized the innervation of Prokr2cre marked somatosensory neurons. They found that these genetically labeled neurons are a mix population, including some innervating hair follicles and some innervating the deep tissues, such as the hindlimb fascial tissues. They then showed ablation of these neurons abolished the low-intensity electric stimulation of ST36 induced activation of hindbrain vagal efferent neurons, as indicated by c-fos staining, catecholamine and dopamine release, reduction of inflammatory cytokine, and animal survival. They also demonstrated the optogenetic stimulation of Prokr2Cre nerve fibers at the hindlimb region was sufficient to drive the vagal-adrenal anti-inflammatory axis, but unable to evoke sympathetic reflexes. Lastly, they showed that electric stimulation of the deep but not cutaneous nerve branch at ST36 is necessary for the effect. In contrast, Prokr2Cre labeled fibers don't innervate the abdomen fascial tissues (ST25 acupuncture site) and are dispensable for 3.0mA ST25 electric stimulation (sympathetic output) effects. Overall, this paper is generally interesting and novel, as substrates of acupuncture effects are largely unknown, and the experiment designs and their results are straightforward and clear.

However, the implication of the results, the general significance and context, and whether some results/paradigms are "fair" comparison, are less clear. Some main discussing points are listed below, and then followed by each figure discussion.

1) The title of the paper is "Identification of sensory neurons somatotopically driving the vagal-adrenal anti-inflammatory axis". "Somatotopy" is emphasized in the title and in the manuscript (comparing ST36 and ST25 innervation pattern and effects). However, this reviewer is uncertain whether somatotopy is one of the main novel findings or conceptual advances of this study. First, this distinct hindlimb vs abdomen electric stimulation effects have been reported by the same group before (Somatotopic Organization and Intensity Dependence in Driving Distinct NPY-Expressing Sympathetic Pathways by Electroacupuncture, Neuron, 2020). Second, from the gross neural anatomy point of view, lower segments of the spinal cord (corresponding to the lower body parts such as hind paw) are well known to correlate with parasympathetic system, while middle and higher parts spinal cord segments of the spinal cord (corresponding to the middle or higher body parts, such as abdomen) are well known to correlate with sympathetic system. Thus, "somatotopy" itself doesn't seem to be a novel finding and conceptual advance here.

2) If "somatotopy" itself is not novel, does the genetic strategy, Prokr2Cre, present the novel conceptual advance? The answer still seems to be "no" to this reviewer, as Prokr2Cre allele clearly labels a mix population of DRG sensory neurons based on both molecular markers and morphology, including both cutaneous hair follicle innervating and deeper tissue innervating fibers. The authors presented results that peripheral innervation patterns of this line are different at the belly and hindlimb. However, since the fiscal tissue around and between the limb bones are different from the fiscal tissue at the belly, their sensory innervation should be somewhat different, just like different types of motor neurons innervating the muscles at the belly and the hindlimb. Comparing their sensory innervation difference is like comparing an orange to an apple. What the genetic line helps is to better visualization of the difference, as this particular line recombines in the part of the DRG sensory neurons excluding the main cutaneous non-peptidergic neurons. In addition, with the help of this Prokr2Cre line, the author conducted elegant

genetic loss-of-function and gain-of-function experiments. When combined with peripheral surgery, these experiments allow the authors to conclude that deep innervating sensory fibers are the sensory substrate for low intensity (0.5mA) electric stimulation effect at ST36. This is probably the most exciting conceptual novelty of this manuscript! From the experimental results, it is clear that sensory neurons labeled by the Prokr2Cre line only partially overlap with the sensory substrate of 0.5mA ST36.

3) This reviewer also feels that the current data presented in this manuscript to compare the "somatotopy" differences is incomplete. For the anatomical analysis, the authors mainly presented the peripheral innervation pattern at two different sites. How about DRG neurons and spinal cord at the corresponding levels? Only the lumbar level data were shown in the manuscript. The conclusion for the peripheral innervation, NF200+ ones "only" innervating the deeper tissue but not the hair follicle, doesn't seem to be that convincing based on the presented data. The model/illustration is oversimplified and may be inaccurate. In addition, the authors used low intensity (0.5mA) stimulation for ST36 to show the Prokr2Cre ablation effect, but high intensity stimulation (3mA) for ST25 to show no ablation effect. This is another example of comparing apple to orange.

Discussion figure by figure

1. In Fig 1 and Extended Data Fig. 1, the author characterized the molecular profile of the Prokr2Adv sensory neurons and the peripheral and central innervation of these neurons in the lumbar segments. To really establish the "somatotopic differences", DRGs and spinal cord levels corresponding to ST25 site are also needed. It would also be helpful to conduct triple staining of CGRP, NF200, and Tdt. Based on the numbers and morphology, it seems likely that some Tdt+ neurons may be CGRP and NF200 double positive. In addition, the authors show genetically labeled fibers innervating tibial periosteum membrane. How about the fibula periosteum membrane? It is well known that Pacinian corpuscles are found in the fascial tissues between the tibial and fibula and in the periosteum membrane around the fibula. Does Prokr2Cre line label or spare Pacinian corpuscles? Lastly, there is also no quantification for panels c & d.

2. In Fig 2, the authors performed genetic ablation of Prokr2Adv sensory neurons. They showed that 0.5 mA ST36 electric stimulation (ES) activated DMV neurons and exhibit anti-inflammation effects and that these full effects require the presence of Prokr2Adv sensory neurons. One thing might be missing here is high intensity 3.0mA stimulation, which have even stronger anti-inflammation effect and seem not require the presence of Prokr2Adv sensory neurons. Since the authors mentioned that 3.0mA ST36 ES did not go through the vagal-adrenal axis, it is curious whether 3.0mA ST36 ES condition activate DMV neurons or not (increased c-fos expression in DMV neurons)? If it does, it raises the question of what increased c-fos expression in DMV neurons means? In addition, "WT" mice were used as the control in both Figs. 2 &3. Since Prokr2Adv sensory neurons are intersectional genetic strategies with three different genetic alleles. "WT" mice are not a fair genetic control here. Moreover, the authors inserted the two electric needles punctured through the skin and into both tibialis and fibula anterior muscle and fascial tissues. Is stimulating one sufficient or is the effect of stimulating one vs the other the same or different? Another minor point is In Fig 2d, it is unclear whether LPS treatment is prior or post of the ST36 ES since different ES time point could have different anti-inflammation effect as reported by the same group before (Somatotopic Organization and Intensity Dependence in Driving Distinct NPY-Expressing Sympathetic Pathways by Electroacupuncture, Neuron, 2020).

3. In Fig. 3, the authors used optogenetic stimulation of Prokr2Adv sensory neurons to show that activation of Prokr2Adv sensory neurons is sufficient to trigger the DMV activation (shown by c-fos staining) and the anti-inflammation effect. The sVx manipulation is a nice manipulation to show that the vague nerve is the main efferent output here. For both figures 2 & 3, the evidence to establish a causal relationship between the DMV activation and the anti-inflammation effect is lacking. In addition, since the authors mentioned the ES intensity affects the anti-inflammation axis, does different light intensity lead to different anti-inflammation effects? Some light intensity effect curve will be needed here.

4. In Fig. 4, the authors combine 0.5mA ST36 ES and peripheral nerve surgery to show that deep but not cutaneous innervating nerve is the sensory substrate of this treatment. This is a set of neat experiments. However, the same issue that 3mA ES is not tested exists here. In addition, the results of this figure defeat the "significance" of the Prokr2Adv neurons to some extent: the genetic line labels both cutaneous and deep-innervating sensory fibers. Thus, it is clear that this particular genetic line does not specifically label the acupuncture sensory substrate.

5. In the extended Data Fig. 1 for characterizing DRG and spinal cord recombination pattern of Prokr2Adv neurons, only lumbar spinal cord section stainings were shown. As discussed above, it would be necessary to compare ST25 level to show "somatotopic differences". Though 50% of positive cell bodies are NF200+, majority of the central innervations are in the layer I. This goes back to the point that some Tdt+ neurons may be CGRP and NF200 double positive. The central innervation should be quantified.

6. In the extended Data figures 2-4, peripheral innervations are characterized of Prokr2Adv neurons. Quantification is needed. The model of NF200+ Tdt+ fibers only innervate deep tissue seems over simplified and may be inaccurate (see Fig. S3b and Fig. S4a, need much higher magnification to be sure).

7. In the extended Data Fig. 5, the ablation of cell bodies looks very clean. How about Tuji1 staining at the periosteum membrane? Are all sensory fibers lost there? Showing peripheral nerve staining at the target regions would be helpful.

8. In Extended Data Fig. 6, the authors showed that ablation of Prokr2Adv neurons has no effect of 3.0mA ST25 ES. How about 0.5mA for a fair comparison? In addition, much higher serum NA increase after 3.0 mA ST25 ES compared to 0.5 mA ST36 ES, but a similar anti-inflammation effect as indicated by serum TNF-α and IF-6. What are the potential reasons for the discrepancy? Are the increased NA, A and DA (all of them or one of them) the reasons for the anti-inflammation effect in both ST25 and ST36 ES?

9. In Extended Data Fig. 7, the authors performed optogenetic stimulation of Prokr2Adv fibers at ST36 but didn't see an increased c-fos+ neurons in IML neurons. What is the positive control to show that this optogenetic activation worked? Would ST36 ES (0.5mA and 3.0mA) activate IML neurons or not?

10. In extended Fig. 8, the authors conducted some circuit tracing from the lumbar spinal cord to NTS and from DMV to the adrenal medulla. In panel b, most retrogradely labeled neurons are located in deep layers (layer III to V), so does the c-fos+ neurons. However, quantifications were only done with layer I neurons. Sinc peripheral deep-innervating sensory neurons also innervating deep layers of the spinal cord, it would be more meaningful to quantify the overlapping in deep layers. Related to this point, it would be interesting to conduct c-fos staining after the peripheral nerve branch surgery and ST36 ES (Figure 4 experiment) to determine whether spinal cord activated neurons (indicated by c-fos) from the deep branch stimuli are located in layer I or deep layers. Lastly, this panel d picture looks different from Fig. 2b, which basically is the same experiment but with slightly different marker stainings. In Fig. 2b, the total fos+ neurons are obviously much more in the opotogenetically stimulated mice, but in panel d here, the total c-fos+ neurons look comparable (just the overlap varies). The same issue of whether using "WT" mice is a fair control here.

11. In extended Fig. 9, the authors conducted control ES stimulation at several other sites. It is unclear what these mouse genotypes are, WT, or Tdt labeled line? What are the "positive" evidence that ES at the other sites worked as anticipated?

Referee #3 (Remarks to the Author):

The anti-inflammatory reflex is an endogenous reflex engaging the autonomic nervous system which can dampen systemic and various local inflammatory disorders. Thus, taking advantage of this, nerve stimulation provides a therapeutic benefit for treatment of inflammatory conditions, including sepsis. However, due to the lack of knowledge on the circuits and cells involved, stimulation paradigms are crude and performed by the direct stimulation of dissected nerves without any insights into the cells and pathways which are important for efficacy. Liu et al., have identified a cellular basis for this reflex circuit. A specific type of primary sensory neurons innervating the periosteum connects to a central nervous system circuit ascending to the vagal nucleus which provides parasympathetic motor output initiating anti-inflammation. Thus, their study identifies a specific cell type and the peripheral tissue carrying innervation of this cell type and when activating this cell type, but not other cell types in other tissues, by electrical stimulation, they can engage the anti-inflammatory reflex. Their mapping of this circuit is detailed and thorough, including a number of different controls justifying their conclusions. I find the study very interesting and compelling and expect it to generate interest from scientists across disciplines. I do not have any major issues.

Minor points:
1. In the LPS experiments, only LPS controls are there but not non-LPS controls when measuring TNF and IL6. What are the levels in non-LPS controls?
2. The authors use Prokr2-Cre mice to study the cells and pathway. As they mention, this strategy captures all neurons expressing Prokr2 at any timepoint during development and this is happening in their experiments since most TrkA+ neurons are labeled yet, Prokr2 is persistently expressed in only few DRG neurons in the adult. Among the Prokr2-Cre captured neurons in their study, some project to cutaneous targets while others to periosteum. The first are predicted C-fibers while the latter predicted Ad-nociceptors. The authors do address this by transecting cutaneous versus deep nerves. However, in light of that molecular types of DRG neurons have been identified, it would be interesting to understand the exact neuronal type involved. Interestingly, looking at the expression of Prokr2 using the online resource of single cell RNA-seq data by Sharma et al., 2020 (https://kleintools.hms.harvard.edu/tools/springViewer_1_6_dev.html?datasets/Sharma2019/all) Prokr2 is persistently expressed in a neuronal type called CGRP-eta, but not in the only other type Ad-nociceptor, CGRP-zeta. Consistently, Prokr2 is persistently expressed in the Ad-nociceptors of Zeisel et al., 2018 (http://loom.linnarssonlab.org/dataset/sparklines/Mousebrain.org.level6/L6_Peripheral_sensory_neurons.loom/NrBEoXQGmAGHgEYq2kqi3IExagZjwBYI0R4jkA7AVwBs6UMnt5FZ4lWnUZFtk8VNCEoRGUjHBoKyIlGxMAHDISC8mPgryEtJMnCgBWKLQZNBCthxjyhafD0mjebScErNHwAbADsyIhK~IgAbBh~SkqK~FG6gaRAA) and in Ad-nociceptors of Usoskin et al., 2015 (http://linnarssonlab.org/drg/). A recent study used machine learning to identify the cell correlates between these studies, which confirms that the Prokr2 expressing cell type between these studies/databases in fact represent the same cell type (Kupari et al., Nat communication 2021).
Thus, based on this, it would seem that the neurons specifically engaging the vagal anti-inflammatory reflex are the Prokr2 persistent expressing neurons. I think that it would be very interesting if the authors could evidence a molecular difference between the cutaneous and deep Prokr2-Cre labeled neurons. Is there a Prokr2 antibody? If so, it could be used for example to stain limb nerves and/or the deep tissue fluorgold labeled DRG neurons. I don't think this point is of much interest to the average reader of Nature and it does not change the conceptual advance of the study, however, this might be interesting to try to solve for specialists.
3. This is related to point 2. In extended data fig 2d: As I understand, percent retrogradely traced neurons labeled with Tomato is shown. However, injection of fluorogold is not expected to capture all neurons, so it is not unexcepted that about 39% are labeled. It would be interesting to see how many of the Tomato+ neurons were retrogradely traced.
4. In this study the refinement in identifying the nerves and cell types connecting to the vagal efferent

system is very high. This leads me to wonder if it now would be possible to understand the relation of frequency and current amplitude to the anti-inflammatory reflex. The authors use 10 Hz. Ad-nociceptors can fire at 20Hz. Would the authors expect similar results if keeping current constant and changing frequency and the reverse?

Referee #4 (Remarks to the Author):

This is an innovative and significant study providing critical information about the neuroanatomical basis for ST36 electroacupuncture to activate the vagal-adrenal anti-inflammatory axis. The authors use a wide diversity of approaches to show that ST36 electroacupuncture activates the vagal-adrenal anti-inflammatory axis, modulates systemic inflammation, and improves survival in endotoxemia through somatosensory DRG neurons marker with prokineticin receptor 2 (Prokr2) that innervate the hindlimb fascial tissue and are mostly myelinated CGRP neurons. Depletion of these Prokr2 neurons worsens inflammation and survival in endotoxemia. These results have profound physiological and clinical implications for the neuromodulation of the immune system. Most conclusions are well supported by the clear results, and the results are very significant. However, there are a few concerns that should be addressed for the proper interpretation of the results.
Major concerns:
1. One major concern is the use of wild-type mice as control for the Prokr2Adv-DTR experiments. DTR gene was inserted in the Prokr2Adv to deplete Prokr2 neurons with DTX treatment. However, genetic insertion of DTR can cause alterations by itself even without DTX treatment. Normal control of these experiments is the same DTR mice without DTX treatment. Comparing wild-type mice versus Prokr2Adv-DTR mice with DTX treatment does not rule out unspecific effects induced by genetic insertion of DTR.
2. Figure 2b appears controversial and not very convincing. The differences are not so obvious when comparing ST36 stimulation in mice with Prokr2 depleted cells (Prokr2Adv-Abl; Prokr2Adv-DTR mice with DTX treatment) versus wild-type control (without DTR insertion). The authors may consider analyzing different sections from different mice and performing quantitative analyses in wild-type and Prokr2Adv-DTR mice with and without DTX treatment. These results are critical to show that Prokr2Adv-Abl really abrogates DMV vagal activation and the results are not due to unspecific mechanisms.
3. Although the article claims that deletion of Prokr2 cells (Prokr2Adv-Abl) prevents the production of catecholamines from the adrenal glands and systemic inflammation, but it is not shown that the inhibition of systemic inflammation is due to the adrenal gland. The vagus nerve innervates multiple organs, can Prokr2Adv-Abl prevents vagal regulation of inflammatory cytokines in other organs like lung alveolar macrophages, liver kupffer cells or the spleen?
4. The authors claim that activation of the vagal-adrenal axis is specifically due to the peroneal nerve (Figure 4). However, ST36 acupoint is located at the intersection of the tibial, peroneal, and sural nerve with the literature claiming that the three branches contribute to ST36 electroacupuncture. Thus, the claimed specific role of the peroneal nerve is controversial and will require to perform similar selective activation and transection of the other nerves to confirm that ST36 stimulation is specifically mediated by the peroneal nerve.
5. A main claim of the study is that the location of Prokr2 neurons can predict the effective zones to activate the vagal-adrenal axis and control inflammation. The article shows that "stimulation of the inner tibialis anterior muscle (through the ST36 acupoint) decreased inflammation". However, this stimulation appears to be through the ST36 acupoint. Are Prokr2 neurons only expressed near the ST36 acupoint? This claim will be stronger showing that the vagal-adrenal axis can be activated by stimulating Prokr2 neurons in an independent and far region from the ST36 acupoint.
6. Page 5. The authors claim that "Prokr2 neurons are required selectively for low-intensity ES to drive the vagal-adrenal anti-inflammatory pathway…" because high-intensity ST36 electroacupuncture activates a sympathetic reflex that is not prevented by deleting Prokr2 neurons (data not shown). Can these results be due to the depletion of myeloid neurons around the acupoint regardless of Prokr2? It will be important to show the proportion of myeloid fibers (staining with NF200) after depleting Prokr2

cells in DTR mice with DTX treatment.
7. The article needs some background on prokineticin receptor 2. How can Prokr2 contribute to ST36 stimulation? Any information about Prokr2 knockout mice? What other regions express Prokr2?
Minor Suggestions.
1. The manuscript should be revised for precision as multiple sections are vaguely described and can cause misleading. For instance, the abstract reads: 'the vagal-adrenal anti-inflammatory axis can be evoked by ES of the hindlimb, but not the abdomen' This statement gives the impression that the vagal-adrenal axis can be evoked by performing ES on anywhere in the hindlimb, but this is not true as only stimulation of the acupoint ST36 elicits this pathway. Another example includes '.. low-intensity ES of hindlimb regions lost the capacity…..' Again, previous studies suggest there are no 'regions', and ES activates this pathway only when performed in very specific 'spots' such as the ST36 acupoint.
2. The article is confusing associating early studies of Sato et al on the gastro vagal reflex with the vagal-adrenal axis, when these are two completely different mechanisms. As the study analyzes inflammatory markers, the authors should just focus on the vagal-adrenal anti-inflammatory axis.
3. Page 3 shows the double staining of tdTomato neurons as 82.8%, 57.0%.... These values do not include SEM as indicated in the statistic section. The figure legend reads sample size n = 5. What is the variation between animals?
4. Page 3-4 reads "deep fascial tissues, such as bone periosteum (Fig. 1c) and joint ligaments (data not shown)." This tdTomato staining in the joint ligaments should be shown in the extended data. As the article claims, these regions could be potential zones of stimulation or have clinical implications in joint disorders such as arthritis.
5. Please clarify green and red staining in all the figure legends.

Author Rebuttals to Initial Comments:

Point-by-point addressing of reviewers' comments:

Referee #1 (Remarks to the Author):

The results show that somatosensory neurons marked by Prokr2Cre innervate hindlimb fascial tissues (e.g., periosteum), but not abdominal fascia (e.g., peritoneum). Selective ablation of Prokr2Cre-marked sensory neurons prevents low-intensity electroacupuncture stimulation of hindlimb regions from activating hindbrain vagal efferent neurons and driving catecholamine release from adrenal glands; as such, ES in these mice failed to suppress severe systemic inflammation induced by bacterial endotoxins. In contrast, the spinal-sympathetic axis evoked by high-intensity ES of both hindlimb and abdominal regions was unaffected. Optogenetic stimulation of Prokr2Cre nerve fibers at hindlimb regions was sufficient to drive the vagal-adrenal anti-inflammatory axis, but unable to evoke sympathetic reflexes. Innervation patterns by Prokr2Cre nerve fibers can retrospectively predict effective and non-effective body regions for low-intensity ES to produce anti-inflammatory effects.

The authors conclude "these findings provide a neuroanatomical basis for body region-dependent driving of the vagal- adrenal anti-inflammatory axis, which could help to design optimal bioelectronic stimulation parameters to treat deadly systemic inflammation."

The findings are original and significant, because the authors present a new understanding of sensory neural specialization linked to stimulation of specific reflex outputs.

The data and other findings are clearly presented and discussed, and the methods and statistics appropriately defined and utilized.

The results support the conclusions. The manuscript and abstract are clear, well written, and lucid."

------ Thanks for the positive comments.

I have only one minor comment:

The authors might consider citing and discussing the work from the Murakami lab on the Gateway reflex, which is likely also activated by the present mechanisms.

------ Since 2012, Dr. Murakami proposed that the brain-blood barrier can be broken following modulation of vascular endothelial cells with inputs from sensory and noradrenergic neurons, allowing immune cells to infiltrate into the brain. In particular, they discovered that deep limb muscle stimulation, either via gravity or via prolonged, low intensity (0.1 mA) electric stimulation, can create immune cell infiltration specifically at the L5 spinal level, a phenomenon referred to as gateway reflexes. Our studies suggest that Prokr2^{Cre}-marked sensory neurons mainly drive vagal reflexes, rather than sympathetic reflexes; as such, we decide not to cite this intriguing work. Future studies would be warranted to map sensory afferents driving gateway reflexes.

Referee #2 (Remarks to the Author):

Summary:

In this work by Shenbin Liu and colleagues, the authors identified a mouse sensory neuron population genetically labeled by Prokr2cre, some of which mediate the low intensity (0.5mA) electric stimulation induced vagal-adrenal anti-inflammatory effect, at a hindlimb acupuncture site, ST36. The authors first characterized the innervation of Prokr2cre marked somatosensory neurons. They found that these genetically labeled neurons are a mix population, including some innervating hair follicles and some innervating the deep tissues, such as the hindlimb fascial tissues. They then showed ablation of these neurons abolished the low-intensity electric stimulation of ST36 induced activation of hindbrain vagal efferent neurons, as indicated by c-fos staining, catecholamine and dopamine release, reduction of inflammatory cytokine, and animal survival. They also demonstrated the optogenetic stimulation of Prokr2Cre nerve fibers at the hindlimb region was sufficient to drive the vagal-adrenal anti-inflammatory axis, but unable to evoke sympathetic reflexes. Lastly, they showed that electric stimulation of the deep but not cutaneous nerve branch at ST36 is necessary for the effect. In contrast, Prokr2Cre labeled fibers don't innervate the abdomen fascial tissues (ST25 acupuncture site) and are dispensablefor 3.0mA ST25 electric stimulation (sympathetic output) effects.

Overall, this paper is generally interesting and novel, as substrates of acupuncture effects are largely unknown, and the experiment designs and their results are straightforward and clear.

------ Thanks for positive assessment.

However, the implication of the results, the general significance and context, and whether some results/paradigms are "fair" comparison, are less clear. Some main discussing points are listed below, and then followed by each figure discussion.

1) *The title of the paper is "Identification of sensory neurons somatotopically driving the vagal-adrenal anti-inflammatory axis". "Somatotopy" is emphasized in the title and in the manuscript (comparing ST36 and ST25 innervation pattern and effects). However, this reviewer is uncertain whether somatotopy is one of the main novel findings or conceptual advances of this study. First, this distinct hindlimb vs abdomen electric stimulation effects have been reported by the same group before (Somatotopic Organization and Intensity Dependence in Driving Distinct NPY-Expressing Sympathetic Pathways by Electroacupuncture, Neuron, 2020). Second, from the gross neural anatomy point of view, lower segments of the spinal cord (corresponding to the lower body parts such as hind paw) are well known to correlate with parasympathetic system, while middle and higher parts spinal cord segments of the spinal cord (corresponding to the middle or higher body parts, such as abdomen) are well known to correlate with sympathetic system. Thus, "somatotopy" itself doesn't seem to be a novel finding and conceptual advance here.*

------ Previous studies have suggested the presence of "somatotopy", or called body region specificity or acupoint selectivity-specificity. The novelty of this paper is not to re-prove the presence of somatotopy, but rather to address the underlying neuroanatomical basis. We asked why the vagal-adrenal axis can only be evoked from the hindlimb acupoints, but not from the abdominal acupoints.
------ We respectfully point out that sympathetic reflexes can be evoked throughout the body, including hindlimb, not just the middle or higher body parts. As such, stimulations at hindlimb regions, if performed at high intensity, can drive both vagal and sympathetic reflexes (see the

new Extended Data Fig. 7, originally mentioned as data not shown). To date, the identities of sensory neurons that selectively drive specific autonomic pathways are virtually unknown. Furthermore, we also did not know any sub-region organization within the hindlimb that drives the vagal-adrenal axis, an issue closely linked to the core acupuncture question about the presence of acupoint specificity or not.

2) *If "somatotopy" itself is not novel, does the genetic strategy, Prokr2Cre, present the novel conceptual advance? The answer still seems to be "no" to this reviewer, as Prokr2Cre allele clearly labels a mix population of DRG sensory neurons based on both molecular markers and morphology, including both cutaneous hair follicle innervating and deeper tissue innervating fibers. The authors presented results that peripheral innervation patterns of this line are different at the belly and hindlimb. However, since the ~~fiscal~~ (fascial) tissue around and between the limb bones are different from the fascial tissue at the belly, their sensory innervation should be somewhat different, just like different types of motor neurons innervating the muscles at the belly and the hindlimb. Comparing their sensory innervation difference is like comparing an orange to an apple.*

------ Indeed, the presence of the lateral motor column that selectively innervates limb muscles, combined with expanded representation of proprioceptors in lumbar DRGs[1], represents a major anatomical difference between brachial and thoracic motor systems. Our looking for analogous sensory neurons with differential innervations between limb and abdominal regions was in fact inspired from this anatomical insight. As discussed below, it is quite striking that proprioceptors and Prokr2^{Cre}-marked neurons showed similarly expanded representation in lumbar DRGs compared with thoracic DRGs.

------ For years, the acupuncture field has been debating if stimulation of fascial tissues, which are enriched for fibrous, nervous, vascular, and lymphatic tissues as well as immune cells, couldbe part of the underlying substrates mediating acupuncture effects. However, comparative innervation studies in different fascial tissues have not been extensively studied. Deep fascia associated with bones/muscles and the abdominal peritoneum represent two of largest fascial tissues in our bodies. Our findings that there is a group of sensory neurons that selectively innervate one of these two major fascial tissues should be of great interest for people studying acupuncture and for the emerging fasica research society. Based on this differential innervation, we postulated that Prokr2Cre-marked neurons could be a candidate for driving the vagal- adrenal axis selectively from the hindlimb region. Our subsequent studies supported this hypothesis.

What the genetic line helps is to better visualization of the difference, as this particular line recombines in the part of the DRG sensory neurons excluding the main cutaneous non-peptidergic neurons. In addition, with the help of this Prokr2Cre line, the authors conducted elegant genetic loss-of-function and gain-of-function experiments. When combined with peripheral surgery, these experiments allow the authors to conclude that deep innervating sensory fibers are the sensory substrate for low intensity (0.5mA) electric stimulation effect at ST36. This is probably the most exciting conceptual novelty of this manuscript! From the experimental results, it is clear that sensory neurons labeled by the Prokr2Cre line only partially overlap with the sensory substrate of 0.5mA ST36.

------ Thanks for emphasizing the significance of our major finding. Indeed, Prokr2^{Cre} should only mark a subset of sensory afferents activated by 0.5mA EA of ST36, since only 38% neurons retrogradely labeled from the deep ST36 tissues coexpressed Prokr2^{Adv}-tdTomato. Despite this low representation, these Prokr2^{Cre}-marked sensory afferents are both necessary and sufficient

to drive the vagal-adrenal axis, without having the ability to drive detectable sympathetic reflexes.

3) *This reviewer also feels that the current data presented in this manuscript to compare the "somatotopy" differences is incomplete. For the anatomical analysis, the authors mainly presented the peripheral innervation pattern at two different sites. How about DRG neurons and spinal cord at the corresponding levels? Only the lumbar level data were shown in the manuscript.*

------ Thanks for pointing out this. We have now conducted a new set of experiments to consolidate our conclusion. We showed that Prokr2^{Adv}-marked neurons have much higher representation in brachial DRGs that innervate the forelimbs and hindlimbs, compared to thoracic DRGs (see the new Extended Data Fig.2). For example, Prokr2^{Adv} neurons represent 17.9% of neurons in lumbar DRG (L4-L5), but only 6.4% of neurons in thoracic DRGs (T8-T10) (see revised Fig. 1b). We then found that in lumbar DRGs, there is a significant expansion of Prokr2^{Adv} neurons that express high levels of the NEFH neurofilament and innervate preferentially the deep limb fascial tissues (see revised Fig. 1c and Extended Data Fig.3d). Notably, the Ginty lab recently reported that only few molecular markers show differential representation in lumbar versus thoracic DRGs. One of them is parvalbumin, marking proprioceptors that represent 16% and 6% in lumbar and thoracic DRGs, respectively[1], which are quite similar to 17.9% and 6.4% representation for Prokr2^{Adv}-marked neurons, echoing the aforementioned discussion in Point 1.

The conclusion for the peripheral innervation, NF200+ ones "only" innervating the deeper tissue but not the hair follicle, doesn't seem to be that convincing based on the presented data. The model/illustration is oversimplified and may be inaccurate.

------ We have chosen new images to make such points clearer (in a tight innervation domain, two intertwined fibers sometimes give a pseudo-impression of overlapping).
------ As also raised by reviewer 3 (please see our detailed responses to point 2 by reviewer 3), we have now performed additional studies to characterize distinct identities of Prokr2^{Cre}-marked neurons that innervate the skin versus deep fascial tissues.

In addition, the authors used low intensity (0.5mA) stimulation for ST36 to show the Prokr2Cre ablation effect, but high intensity stimulation (3mA) for ST25 to show no ablation effect. This is another example of comparing apple to orange.

----- Due to space limit, we apparently did not state clearly about our previously reported findings. We reported that 0.5 mA at ST25 cannot induce Fos in either vagal efferents or spinal sympathetic efferents, and accordingly, it is unable to reduce systemic inflammation. We had then reported that 3.0 mA ES can induce spinal sympathetic reflexes, without causing Fos induction in vagal efferents, thereby producing anti-inflammatory effects that depend on NPY^{+} noradrenergic neurons, but not on the vagal-adrenal axis. That was the reason why we used 3.0 mA ES at ST25 to test if Prokr2^{Cre}-marked neurons played any roles in driving spinal sympathetic reflexes.
----- In response to this reviewer's comments, we did test the effects evoked by ES at intermediate intensity (1.0 mA) at ST25. Consistent with our previous report, this ES only produced modest anti-inflammatory effects, reducing TNFa and IL-6 levels by around ~30%. This modest anti-inflammatory effect was again independent of Prokr2^{Adv}-marked neurons, thereby consolidating our conclusion that Prokr2^{Adv}-marked neurons are dispensable for

sympathetic reflexes (see revised Extended Data Fig.6j). We further showed that 3.0 mA ES at ST36 can also drive sympathetic reflexes and produce anti-inflammatory effects in a Prokr2^{Adv} neuron-mdependent manner (see new Extended Data Fig.7).

Discussion figure by figure

1. *" In Fig 1 and Extended Data Fig. 1, the author characterized the molecular profile of the Prokr2Adv sensory neurons and the peripheral and central innervation of these neurons in the lumbar segments. To really establish the "somatotopic differences", DRGs and spinal cord levels corresponding to ST25 site are also needed."*

------ We have addressed this issue (see above, main point 3)

"It would also be helpful to conduct triple staining of CGRP, NF200, and Tdt. Based on the numbers and morphology, it seems likely that some Tdt+ neurons may be CGRP and NF200 double positive. In addition, the authors show genetically labeled fibers innervating tibial periosteum membrane. How about the fibula periosteum membrane? It is well known that Pacinian corpuscles are found in the fascial tissues between the tibial and fibula and in the periosteum membrane around the fibula. Does Prokr2Cre line label or spare Pacinian corpuscles? Lastly, there is also no quantification for panels c & d."

---- We had done triple staining, showing that 50.7% of tdTomato-positive neurons co-expressed NF200 and CGRP. Considering that only 57.2 ± 1.8% tdTomato-positive neurons co-expressed NEFH, we conclude that a majority NEFH-positive tdTomato+ neurons coexpressed CGRP (see revised Extended Data Fig.1c and Extended Data Fig. 4d).
---- We have now shown dense innervation in the fibula periosteum, and no tdTomato$^+$ fibers passing through the Pacinian corpuscles. In examining Pacinian corpuscles (which were not innervated by tdTomato+ fibers), it allowed us to show tdTomato$^+$ fibers in the interosseous membrane between bones, another deep limb fascial tissue (see revised Extended Data Fig. 4c).
---- For quantification, we virtually did not find any innervations in the abdominal peritoneum membrane, and the relative quantification is 25.2 ± 6.6% (the percentage of unit areas containing positive fibers) in the bone periosteum versus 0% in peritonum. Due to space limit, we did not describe such all or none difference with acutal numbers in the text. As described below, we have added quantitative data in different muscles.

2. *In Fig 2, the authors performed genetic ablation of Prokr2Adv sensory neurons. They showed that 0.5 mA ST36 electric stimulation (ES) activated DMV neurons and exhibit anti-inflammation effects and that these full effects require the presence of Prokr2Adv sensory neurons. One thing might be missing here is high intensity 3.0mA stimulation, which have even stronger anti-inflammation effect and seem not require the presence of Prokr2Adv sensory neurons. Since the authors mentioned that <u>3.0mA ST36 ES did not go through the vagal-adrenal axis</u>, it is curious whether 3.0mA ST36 ES condition activate DMV neurons or not (increased c-fos expression in DMV neurons)? If it does, it raises the question of what increased c-fos expression in DMV neurons means?*

------ We would first need to clarify that we did not state that "*<u>3.0mA ST36 ES did not go through the vagal-adrenal axis</u>*". Rather, we reported previously that 3.0mA ST36 ES should not only drive the vagal-adrenal axis (since 0.5 mA can already do), but can also drive spinal sympathetic reflexes and produce <u>redundant, vagal nerve-independent</u> anti-inflammatory

effects. In this revised version, we have now added a new Extended Data Fig.7 (previously described as data not shown), showing that 3.0 mA ES can induce Fos in spinal sympathetic preganglionic neurons and produce anti-inflammatory effects that were independent of Prokr2^{Adv} neurons, as 3.0 mA ES of ST25 did. In contrast, 0.5 mA ES can induce Fos in DMV, but not in spinal sympathetic preganglionic neurons. Without activating redundant sympathetic reflexes that requires high intensity stimulation, it allowed us to reveal the selective driving of the vagal-adrenal anti-inflammatory axis by low intensity ES, in a Prokr2^{Adv} neuron-dependent manner.
------ Addressing this point allows us to re-emphasize that acupoint selectivity or specificity is an operational definition, which depends on stimulation intensities, the depth of needling and the specific outputs you measure (see the revised final summary paragraph). As you can see, if we only performed 3 mA ES and measured the anti-inflammatory effects, without performing 0.5 mA ES, we would have failed to reveal a crucial role of Prokr2^{Adv} neurons in driving the vagal-adrenal anti-inflammatory effects evoked specifically from the hindlimb region by low-intensity ES.

In addition, "WT" mice were used as the control in both Figs. 2 &3. Since Prokr2Adv sensory neurons are intersectional genetic strategies with three different genetic alleles. "WT" mice are not a fair genetic control here.

------ In fact, as described in original Methods section, only breeding littermates were used as control; In Figure Legends, we made a mistake by referring them to as "WT" to save some space. This was also pointed out by reviewer 4, and we have now replaced "WT" with "control littermates" or simply with "control" in all Figure panels.

Moreover, the authors inserted the two electric needles punctured through the skin and into both tibialis and fibula anterior muscle and fascial tissues. Is stimulating one sufficient or is the effect of stimulating one vs the other the same or different?

------ The positive and negative electric needles are separated by 1 mm. For small animals like mice, we do not have sufficient resolution to estimate relative contribution of two adjacent muscles with similar functions. As such, we decided not to pursue this suggested experiment to keep this already quite dense study to be concise.

Another minor point is In Fig 2d, it is unclear whether LPS treatment is prior or post of the ST36 ES since different ES time point could have different anti-inflammation effect as reported by the same group before (Somatotopic Organization and Intensity Dependence in Driving Distinct NPY-Expressing Sympathetic Pathways by Electroacupuncture, Neuron, 2020).

------ ES was performed before LPS. We did not perform ES after LPS, since the focus of this study is to illustrate the neuroanatomical basis driving the vagal-adrenal axis, and LPS-induced inflammation was only used to as a readout for the activation of this axis.

3. *In Fig. 3, the authors used optogenetic stimulation of Prokr2Adv sensory neurons to show that activation of Prokr2Adv sensory neurons is sufficient to trigger the DMV activation (shown by c-fos staining) and the anti-inflammation effect. The sVx manipulation is a nice manipulationto show that the vague nerve is the main efferent output here. For both figures 2 & 3, the evidence to establish a causal relationship between the DMV activation and the anti-inflammation effect is lacking.*

------ We have provided a series of compelling evidence: opto-acupuncture can not only induce c-Fos in DMV but also induced electric discharge in the vagal efferents (we recorded the vagal

nerve with transection to prevent sensory inputs), and both catecholamine release and anti-inflammatory effects can be blocked by sVX. We agree that in the future, it would be nice to determine the identities of DMV neurons and then demonstrate their requirement for driving the vagal-adrenal axis.

In addition, since the authors mentioned the ES intensity affects the anti-inflammation axis, does different light intensity lead to different anti-inflammation effects? Some light intensity effect curve will be needed here.

------ Since 10 mW stimulation already selectively drove the vagal-adrenal anti-inflammatory axis, we decided not to test intensities below 10 mW. We instead performed a new experiment by increasing the power of blue light stimulation to 30 mW (the upper limit for most optogenetic studies), and found that no difference of anti-inflammatory effects in comparison with that induced by 10 mW stimulation, suggesting that 10 mW stimulation may have reached the ceiling effects (see revised Extended Data Fig. 9e). Also, unlike 3 mA ES that can start to recruit sympathetic reflexes, 30 mW still failed to cause an increase in c-Fos induction in spinal sympathetic preganglionic neurons (see revised Extended Data Fig. 8g), consolidating the conclusion that optogenetic stimulation of Prokr2^{Adv} neurons drives selectively the vagal reflexes.

4. *In Fig. 4, the authors combine 0.5mA ST36 ES and peripheral nerve surgery to show that deep but not cutaneous innervating nerve is the sensory substrate of this treatment. This is a set of neat experiments. However, the same issue that 3mA ES is not tested exists here.*

------ As mentioned above, 3 mA ES will activate various anti-inflammatory pathways, which will occlude the revelation of the essential roles of Prokr2^{Adv} neurons for driving vagal-adrenal anti-inflammatory axis. As such, we only chose the 0.5 mA ST36 ES for these lines of studies.

In addition, the results of this figure defeat the "significance" of the Prokr2Adv neurons to some extent: the genetic line labels both cutaneous and deep-innervating sensory fibers. Thus, it is clear that this particular genetic line does not specifically label the acupuncture sensory substrate.

---- We respectfully disagree this assessment. Genetic ablation first revealed that Prokr2^{Adv} neurons, as a group, are necessary for driving the vagal-adrenal axis selectively from the ST36, acupoint. Based on anatomical innervation patterns, we made prediction that this effect is driven from the deep fasica-innervating neurons, and then carried out a series of experiments to support this key conclusion, including differential impacts caused by transection of the cutaneous versus deep branches of the peroneal nerves, and the lack of effects by subcutaneous stimulation at the ST36 acupoints or by sural cutaneous nerve stimulation.

5. *In the extended Data Fig. 1 for characterizing DRG and spinal cord recombination pattern of Prokr2Adv neurons, only lumbar spinal cord section stainings were shown. As discussed above, it would be necessary to compare ST25 level to show "somatotopic differences". Though 50% of positive cell bodies are NF200+, majority of the central innervations are in the layer I. This goes back to the point that some Tdt+ neurons may be CGRP and NF200 double positive. The central innervation should be quantified.*

------ We have addressed the crucial issue on the difference between lumbar versus thoracic DRGs (see above, Main Point 3 from this reviewer, and Point 2 from Reviewer 3). The degree of

the overlapping between CGRP and NF200 has been addressed in Discussion Point 1 (see above), which can be better quantified in DRGs.

6. *"In the extended Data figures 2-4, peripheral innervations are characterized of Prokr2Adv neurons. Quantification is needed."*

------ Thanks for making this important suggestion. We have completed the quantitative data on the innervation densities, revealing 10-fold difference in the inner compartment of the tibialis anterior (TA) muscle in comparison with the outer TA compartment, the gastrocnemius muscle, the abdominal wall muscles, or the semitendinosus muscle, with the latter four showing similar low densities (see revised Extended Data Fig.4h and the Figure legends of Extended Data Fig.11f, i).

"The model of NF200+ Tdt+ fibers only innervate deep tissue seems over simplified and may be inaccurate (see Fig. S3b and Fig. S4a, need much higher magnification to be sure)".

------ We will collectively address this issue (see below, Point 2 from Reviewer 3).

7. *In the extended Data Fig. 5, the ablation of cell bodies looks very clean. How about Tuji1 staining at the periosteum membrane? Are all sensory fibers lost there? Showing peripheral nerve staining at the target regions would be helpful.*

---- As also pointed out by reviewer 4, we have quantified and revealed 46.0% and 72.2% reduction in $TUBB3^+$ and $NEFH^+$ fiber densities within the bone periosteum, respectively.

8. *In Extended Data Fig. 6, the authors showed that ablation of Prokr2Adv neurons has no effect of 3.0mA ST25 ES. How about 0.5mA for a fair comparison?*

--- We have addressed this issue, see above, the main point 3 by this reviewer.

In addition, much higher serum NA increase after 3.0 mA ST25 ES compared to 0.5 mA ST36ES, but a similar anti-inflammation effect as indicated by serum TNF-α and IF-6. What are the potential reasons for the discrepancy? Are the increased NA, A and DA (all of them or one of them) the reasons for the anti-inflammation effect in both ST25 and ST36 ES?

---- We and others reported that 3.0 mA ST25 ES can drive splenic noradrenergic neurons, and can produce anti-inflammatory effects that can be blocked by β2 adrenergic receptor antagonists, whereas the anti-inflammatory effects evoked by 0.5 mA ST36 ES can be blocked by ablation of adrenal chromaffin cells and by the D1 dopamine receptor antagonists[2,3]. Interpretations of any pharmacological data need to be cautious since these drugs can cross the brain-blood barrier. Further addressing how exactly catecholamines (and other peptide transmitters) released from these autonomic cells modulate systemic inflammation is, however, beyond the scope of this study.

9. *In Extended Data Fig. 7, the authors performed optogenetic stimulation of Prokr2Adv fibers at ST36 but didn't see an increased c-fos+ neurons in IML neurons. What is the positive control to show that this optogenetic activation worked?*

----- This same optogenetic stimulation at the ST36 acupoint was sufficient to activate Fos in DMV as well as in spinal lamina I neurons projecting to NTS, and caused vagal-nerve-

dependent release of dopamine and adrenaline and produced anti-inflammatory effects, which should be considered as a positive control.

Would ST36 ES (0.5mA and 3.0mA) activate IML neurons or not?

------ As we reported previously[3], 3.0 mA, but not 0.5 mA ES, can induce c-Fos in IML (see alsothe new Extended Data Fig.7a).

10. *In extended Fig. 8, the authors conducted some circuit tracing from the lumbar spinal cord to NTS and from DMV to the adrenal medulla. In panel b, most retrogradely labeled neurons are located in deep layers (layer III to V), so does the c-fos+ neurons. However, quantifications were only done with layer I neurons. Since peripheral deep-innervating sensory neurons also innervating deep layers of the spinal cord, it would be more meaningful to quantify the overlapping in deep layers.*

------ We have quantified and found that 0.5 mA ES induced c-Fos only in NTS-projecting neurons located in lamina I, but not in deep ventral laminae. The lack of induction in deep laminae has now been described in the Figure legend (see revised Extended Data Fig.9b).

Related to this point, it would be interesting to conduct c-fos staining after the peripheral nerve branch surgery and ST36 ES (Figure 4 experiment) to determine whether spinal cord activated neurons (indicated by c-fos) from the deep branch stimuli are located in layer I or deep layers.

------ We have performed retrograde labeling, showing that c-Fos induction in lamina I NTS-projecting spinal neurons evoked by 0.5 mA ES of ST36 was abolished in mice with the transection of the peroneal nerve (see new Extended Data Fig.10b).

Lastly, this panel d picture looks different from Fig. 2b, which basically is the same experiment but with slightly different marker stainings. In Fig. 2b, the total fos+ neurons are obviously much more in the opotogenetically stimulated mice, but in panel d here, the total c-fos+ neurons look comparable (just the overlap varies). The same issue of whether using "WT" mice is a fair control here.

------ There were variable levels of baseline c-Fos in the NTS and in cells intermingled with $ChAT^+$ vagal efferent neurons in DMV. Fortunately, $ChAT^+$ DMV neurons contain relatively low levels of background c-Fos, allowing us to assess c-Fos induction (including both moderate and high levels) by ES or by opto-acupuncture. We have pointed out this baseline c-Fos in ChAT-negative cells in the revised Figure Legend (see the revised Extended Data Fig.9d).

11. *In extended Fig. 9, the authors conducted control ES stimulation at several other sites. It is unclear what these mouse genotypes are, WT, or Tdt labeled line? What are the "positive" evidence that ES at the other sites worked as anticipated?*

------- For these experiments, only wild type C57BL/6J mice were used, except experiments on ES at LI10, which have used $PROKR^{ADV}$-Abl mice and their control littermates, and we have pointed out in the Methods and in Figure Legends.
------- For "positive" evidence, please see our responses to the same question raised byreviewer 4 (see point 5 by reviewer 4).

Referee #3 (Remarks to the Author):

The anti-inflammatory reflex is an endogenous reflex engaging the autonomic nervous system which can dampen systemic and various local inflammatory disorders. Thus, taking advantage of this, nerve stimulation provides a therapeutic benefit for treatment of inflammatory conditions, including sepsis. However, due to the lack of knowledge on the circuits and cells involved, stimulation paradigms are crude and performed by the direct stimulation of dissected nerves without any insights into the cells and pathways which are important for efficacy. Liu et al., have identified a cellular basis for this reflex circuit. A specific type of primary sensory neurons innervating the periosteum connects to a central nervous system circuit ascending to the vagal nucleus which provides parasympathetic motor output initiating anti-inflammation. Thus, their study identifies a specific cell type and the peripheral tissue carrying innervation of this cell type and when activating this cell type, but not other cell types in other tissues, by electrical stimulation, they can engage the anti-inflammatory reflex. Their mapping of this circuit is detailed and thorough, including a number of different controls justifying their conclusions. I findthe study very interesting and compelling and expect it to generate interest from scientists across disciplines. I do not have any major issues.

------ Thanks for the positive comment.

Minor points:

1. *In the LPS experiments, only LPS controls are there but not non-LPS controls when measuring TNF and IL6. What are the levels in non-LPS controls?*

------ We previously reported virtually no baseline cytokine detected. We performed again in Prokr2^{Adv}-Abl mice and control littermates, and did not detect baseline cytokines (see revised Extended Data Fig.6a). As such, we only compared LPS-treated mice under different conditions, which greatly reduced the number of mice used for this study.

2. *The authors use Prokr2-Cre mice to study the cells and pathway. As they mention, this strategy captures all neurons expressing Prokr2 at any timepoint during development and this is happening in their experiments since most TrkA+ neurons are labeled yet, Prokr2 is persistently expressed in only few DRG neurons in the adult. Among the Prokr2-Cre captured neurons in their study, some project to cutaneous targets while others to periosteum. The first are predicted C-fibers while the latter predicted Ad-nociceptors. The authors do address this by transecting cutaneous versus deep nerves. However, in light of that molecular types of DRG neurons have been identified, it would be interesting to understand the exact neuronal type involved. Interestingly, looking at the expression of Prokr2 using the online resource of single cell RNA-seq data by Sharma et al., 2020 (https://kleintools.hms.harvard.edu/tools/springViewer_1_6_dev.html?datasets/Sharma2019/all) Prokr2 is persistently expressed in a neuronal type called CGRP-eta, but not in the only other type Ad-nociceptor, CGRP-zeta. Consistently, Prokr2 is persistently expressed in the Ad-nociceptors of Zeisel et al., 2018 (http://loom.linnarssonlab.org/dataset/sparklines/Mousebrain.org.level6/L6_Peripheral_sensory_neurons.loom/NrBEoXQGmAGHgEYq2kqi3IExagZjwBYI0R4jkA7AVwBs6UMnt5FZ4lWnUZFtk8VNCEoRGUjHBoKyIlGxMAHDISC8mPgryEtJMnCgBWKLQZNBCthxjyhafD0mjebScErNHwAbADsyIhK~IgAbBh~SkqK~FG6gaRAA) and in Ad-nociceptors of Usoskin et al., 2015 (http://linnarssonlab.org/drg/). A recent study used machine learning to identify the cell correlates between these studies, which confirms that the Prokr2 expressing cell type between these studies/databases in fact represent the same cell type (Kupari et al., Nat communication*

2021).Thus, based on this, it would seem that the neurons specifically engaging the vagal anti-inflammatory reflex are the Prokr2 persistent expressing neurons. I think that it would be very interesting if the authors could evidence a molecular difference between the cutaneous and deep Prokr2-Cre labeled neurons. Is there a Prokr2 antibody? If so, it could be used for example to stain limb nerves and/or the deep tissue fluorgold labeled DRG neurons. I don't thinkthis point is of much interest to the average reader of Nature and it does not change the conceptual advance of the study, however, this might be interesting to try to solve for specialists."

------ Thanks for raising this important point, which was also partly raised by reviewer 2. We have performed new retrograde labeling and co-staining studies, showing that virtually all skin-innervating Prokr2^{Adv}-tdTomato^{+} neurons coexpressed *Bmpr1b* mRNA, and they also expressed low levels of *Nefh* mRNA (which may explain why it is difficult to detect the NEFH protein in the peripheral terminals), thereby indeed corresponding to CGRP-eta neurons (see Extended Data Fig. 3d, 3e). In contrast, a majority of Prokr2^{Adv}-tdTomato^{+} neurons retrogradely labeled from the deep fascia were negative for *Bmpr1b* or low levels of Nefh, and 75.6% of them instead expressed high level of *Nefh* mRNA (which explains the detection of the NEFH protein in their terminals in the bone periosteum and in deep muscles), thereby distinct from CGRP-eta neurons (see Extended Data Fig. 4f, 4g). These additional studies clearly show two distinct populations of Prokr2^{Adv} neurons innervating the skin hair follicles versus the deep fascia tissues(see Extended Data Fig. 4i, 4j). Our genetic ablation, surgical nerve transections and a series ofcontrol stimulation studies jointly support that the subset innervating the deep fascia drives the vagal-adrenal axis.

3. *This is related to point 2. In extended data fig 2d: As I understand, percent retrogradely traced neurons labeled with Tomato is shown. However, injection of fluorogold is not expectedto capture all neurons, so it is not unexcepted that about 39% are labeled. It would be interesting to see how many of the Tomato+ neurons were retrogradely traced.*

------ Apparetnly our writing is misleading. In fact, among Fluro-gold^{+} retrogradely labeled neurons, 39% are tdTomato^{+}; in other words, Prokr2^{Adv} marks about 39% of total neurons innervating the deep tissues. We have revised the text to make it clearer.

4. *In this study the refinement in identifying the nerves and cell types connecting to the vagal efferent system is very high. This leads me to wonder if it now would be possible to understand the relation of frequency and current amplitude to the anti-inflammatory reflex. The authors use 10 Hz. Ad-nociceptors can fire at 20Hz. Would the authors expect similar results if keeping current constant and changing frequency and the reverse?*

---- We have performed a new experiment, and found that under the same 0.5 mA intensity (the focus of this study), 20 Hz stimulation produced similar levels of anti-inflammatory effects in comparison with 10 Hz (see Figure for reviewers only, Fig. R1; we did not include it in the revised manuscript to keep it concise since we cannot provide a clear explanation why 10 Hz and 20 Hz produced similar effects). It is possible that 10Hz stimulation already achieved the ceiling effects. Future studies will be needed to expand the range of stimulation frequencies in modulating distinct autonomic pathways.

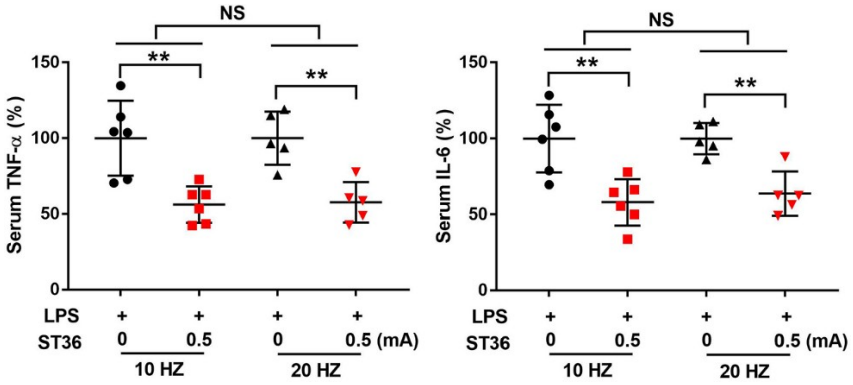

Figure R1. No significant difference in reduction of LPS-induced TNF-α and IL-6 production following 10 Hz versus 20 Hz ES (0.5 mA) of ST36 (two-way ANOVA, n = 5-6mice per group; for TNF-α: $F_{1, 18} = 0.009$; NS, $P = 0.904$; *post-hoc* Tukey test: left: $^{**}P = 0.003$; right: $^{**}P = 0.007$; for IL-6: $F_{1, 18} = 0.160$; NS, $P = 0.694$; *post-hoc* Tukey test: $^{**}P = 0.002$; right: $^{**}P = 0.009$). Data are shown as mean ± SEM.

Referee #4 (Remarks to the Author):

This is an innovative and significant study providing critical information about the neuroanatomical basis for ST36 electroacupuncture to activate the vagal-adrenal anti-inflammatory axis. The authors use a wide diversity of approaches to show that ST36 electroacupuncture activates the vagal-adrenal anti-inflammatory axis, modulates systemic inflammation, and improves survival in endotoxemia through somatosensory DRG neurons marker with prokineticin receptor 2 (Prokr2) that innervate the hindlimb fascial tissue and are mostly myelinated CGRP neurons. Depletion of these Prokr2 neurons worsens inflammation and survival in endotoxemia. These results have profound physiological and clinical implicationsfor the neuromodulation of the immune system. Most conclusions are well supported by the clear results, and the results are very significant. However, there are a few concerns that shouldbe addressed for the proper interpretation of the results.

--- Thanks for the positive comment.

Major concerns:

1. *One major concern is the use of wild-type mice as control for the Prokr2Adv-DTR experiments. DTR gene was inserted in the Prokr2Adv to deplete Prokr2 neurons with DTX treatment. However, genetic insertion of DTR can cause alterations by itself even without DTX treatment. Normal control of these experiments is the same DTR mice without DTX treatment. Comparing wild-type mice versus Prokr2Adv-DTR mice with DTX treatment does not rule out unspecific effects induced by genetic insertion of DTR.*

------ We have addressed this point (see Discussion Point 2 by reviewer 2). Basically, it is a mis-labeling. All “WT” controls were breeding littermates.

2. *Figure 2b appears controversial and not very convincing. The differences are not so obvious when comparing ST36 stimulation in mice with Prokr2 depleted cells (Prokr2Adv-Abl; Prokr2Adv-DTR mice with DTX treatment) versus wild-type control (without DTR insertion). The authors may consider analyzing different sections from different mice and performing*

quantitative analyses in wild-type and Prokr2Adv-DTR mice with and without DTX treatment. These results are critical to show that Prokr2Adv-Abl really abrogates DMV vagal activation and the results are not due to unspecific mechanisms.

---- Thanks for pointing out. We have now shown representative images reflecting quantitative data, which clearly showed a Prokr2^{Adv} neuron-dependent c-Fos induction in DMV.

3. *Although the article claims that deletion of Prokr2 cells (Prokr2Adv-Abl) prevents the production of catecholamines from the adrenal glands and systemic inflammation, but it is not shown that the inhibition of systemic inflammation is due to the adrenal gland. The vagus nerve innervates multiple organs, can Prokr2Adv-Abl prevents vagal regulation of inflammatory cytokines in other organs like lung alveolar macrophages, liver kupffer cells or the spleen?*

---- In our previous studies[3], we had reported that for 0.5 mA ES at ST36, the anti-inflammatory effects are dependent on NPY$^+$ chromaffin cells, which is consistent with the earlier finding by Torres-Rosas et al.[2]. This clarification was added to the text. Future studies will be warranted to determine how the vagal-adrenal axis impacts on inflammation in different organs (the focus of this study was to investigate what drives this vagal-adrenal axis).

4. *The authors claim that activation of the vagal-adrenal axis is specifically due to the peroneal nerve (Figure 4). However, ST36 acupoint is located at the intersection of the tibial, peroneal, and sural nerve with the literature claiming that the three branches contribute to ST36 electroacupuncture. Thus, the claimed specific role of the peroneal nerve is controversial and will require to perform similar selective activation and transection of the other nerves to confirm that ST36 stimulation is specifically mediated by the peroneal nerve.*

------ One major difference is that we used a focal electric stimulation protocol, with a pair of positive and negative electric needles (separated by 1 mm) inserted to each of two ST36 acupoints (four needles in total). In contrast, previous studies by Torres-Rosas et al. used a more diffuse stimulation protocol, with one needle inserted into one leg and another one to the other leg, such that the electric current flew from one leg to another. With the suggestion of this reviewer, we have performed a new study, showing that transection of the tibial nerve did not impact on the anti-inflammatory effects evoked by our "focal" ES stimulation at both left and right ST36 acupoints, different from the redundant contribution from the tibial and peroneal nerves when the "diffuse" electric stimulation mode was performed[2]. We have now cited and concretely pointed out this crucial difference in the text and in the revised Extended Data Fig.10a. Note that focal stimulation also allowed us to reveal difference among hindleg sub- regions, such as a lack of effect by superficial ES at the ST36 acupoint, by ES flanking the suralnerve, or by ES within the gastrocnemius muscle.

5. *A main claim of the study is that the location of Prokr2 neurons can predict the effective zones to activate the vagal-adrenal axis and control inflammation. The article shows that "stimulation of the inner tibialis anterior muscle (through the ST36 acupoint) decreased inflammation". However, this stimulation appears to be through the ST36 acupoint. Are Prokr2 neurons only expressed near the ST36 acupoint? This claim will be stronger showing that the vagal-adrenal axis can be activated by stimulating Prokr2 neurons in an independent and far region from the ST36 acupoint.*

----- Thanks for this crucial suggestion, which was also pointed out by reviewer 2. We have now tested and found that Prokr2^{Adv} neuron-dependent anti-inflammatory effects can also be evoked

by stimulating a forelimb region-the Shousanli (LI10) acupoint (see Extended Data Fig. 11k-m), with electric needle tips close to the deep branch of radius nerve bundle that contains tdTomato$^+$ fibers and that are known to innervate the bones and deep muscles in the foreleg, thereby functionally equivalent to the stimulation of the peroneal nerve via the ST36 acupoint inthe hindleg. This crucial data consolidates our conclusion that innervation patterns of Prokr2^{Adv} neurons (or more precisely, the presence of nerve bundles containing Prokr2^{Adv}-tdTomato$^+$ fibers that innervate deep fascial tissues) can predict the effective sites for low-intensity ES to drive the vagal-adrenal axis.

6. *Page 5. The authors claim that "Prokr2 neurons are required selectively for low-intensity ES to drive the vagal-adrenal anti-inflammatory pathway…" because high-intensity ST36 electroacupuncture activates a sympathetic reflex that is not prevented by deleting Prokr2 neurons (data not shown). Can these results be due to the depletion of ~~myeloid~~ ("myelinated") neurons around the acupoint regardless of Prokr2? It will be important to show the proportion of ~~myeloid~~ ("myelinated") fibers (staining with NF200) after depleting Prokr2 cells in DTR mice with DTX treatment.*

----- We have addressed this point (see Discussion Point 7 by reviewer 2), showing a 72.2% reduction of NEFH$^+$ fiber densities within the bone periosteum.

7. *The article needs some background on prokineticin receptor 2. How can Prokr2 contribute to ST36 stimulation? Any information about Prokr2 knockout mice? What other regions express Prokr2?*

------ This study is more on DRG neurons marked by Prokr2^{Adv} via the intersectional genetic strategy, which include both Prokr2-persistent and -transient DRG neurons. Prokr2 is also known to be expressed in many non-neural tissues (for example, some undefined dermal cells can be marked by Prokr2-Cre alone, but not with intersectional strategy), as well as in a subset of spinal cord neurons (Extended Data Fig. 5e and data not shown). Future studies will be needed to make *Prokr2* conditional knockout in sensory neurons to study its roles in modulating inflammation, particularly if electric stimulation somehow can cause the release of the prokineticin peptide around the acupoint.

Minor Suggestions.

1. *The manuscript should be revised for precision as multiple sections are vaguely described and can cause misleading. For instance, the abstract reads: 'the vagal-adrenal anti- inflammatory axis can be evoked by ES of the hindlimb, but not the abdomen' This statement gives the impression that the vagal-adrenal axis can be evoked by performing ES on anywherein the hindlimb, but this is not true as only stimulation of the acupoint ST36 elicits this pathway. Another example includes '.. low-intensity ES of hindlimb regions lost the capacity…..' Again, previous studies suggest there are no 'regions', and ES activates this pathway only when performed in very specific 'spots' such as the ST36 acupoint.*

------ Thanks for pointing out this. We have revised the abstract to directly include the ST36 and ST25 acupoint names, and we have put more detailed anatomical description of these acupoints in Methods and in various schematics to help non-familiar readers to understand the antomy about these acupoints.

2. *The article is confusing associating early studies of Sato et al on the gastro vagal reflex with the vagal-adrenal axis, when these are two completely different mechanisms. As the study*

analyzes inflammatory markers, the authors should just focus on the vagal-adrenal anti-inflammatory axis.

------ We have now only briefly cited, without concrete elaboration of, the classic Sato work, to make the story more concise. See the revised Introduction.

3. Page 3 shows the double staining of tdTomato neurons as 82.8%, 57.0%.....These values do not include SEM as indicated in the statistic section. The figure legend reads sample size n = 5. What is the variation between animals?

------ We have now presented these data with SEM from the 5 mice we analyzed.

4. Page 3-4 reads "deep fascial tissues, such as bone periosteum (Fig. 1c) and joint ligaments (data not shown)." This tdTomato staining in the joint ligaments should be shown in the extended data. As the article claims, these regions could be potential zones of stimulation or have clinical implications in joint disorders such as arthritis.

------ We have shown the innervation of these neurons in joint ligaments (see Extended Data Fig. 4a).

5. Please clarify green and red staining in all the figure legends.
------ We have done that, directly marking them in Figure panels and/or in Figure legends.

We would like to take this opportunity to thank all four reviewers. Addressing their constructive comments have consolidated and extended our key conclusion on the identification of the neural substrate for acupuncture to drive a specific autonomic pathway.

References

1 Sharma, N. *et al.* The emergence of transcriptional identity in somatosensory neurons. *Nature* **577**, 392-398, doi:10.1038/s41586-019-1900-1 (2020).

2 Torres-Rosas, R. *et al.* Dopamine mediates vagal modulation of the immune system byelectroacupuncture. *Nat Med.* **20**, 291-295 (2014).

3 Liu, S. *et al.* Somatotopic Organization and Intensity Dependence in Driving Distinct NPY-Expressing Sympathetic Pathways by Electroacupuncture. *Neuron* **108**, 436-435,doi:10.1016/j.neuron.2020.07.015 (2020).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

PROKR2Cre-marked sensory neurons innervating deep hindlimb fascial tissues but not abdominal fascia mediate low-intensity ES of ST36 to activate hindbrain vagal efferent neurons and to drive catecholamine release from adrenal glands. The spinal-sympathetic axis, which can be evoked by high-intensity ES at both ST25 and ST36 sites, is not mediated by these neurons. Optogenetic stimulation of PROKR2Cre nerve terminals through the ST36 acupoint drives the vagal-adrenal axis, but not sympathetic reflexes. The authors conclude that this defines a " neuroanatomical basis for the presence

of acupoint selectivity and specificity in driving autonomic pathways. "

The results are timely, original, novel and significant.

The results support the conclusions, and the the manuscript is clearly written and interesting.

Referee #2 (Remarks to the Author):

In the revised manuscript, the reviewer is happy with how most questions raised in the first-round review were answered and addressed. There are still some questions and minor comments.

1. In the revised text, the author reasoned that "Nefhhigh;tdTomato+ neurons represent 6.3% (17.9% x 35.4%) of L4-5 DRG neurons, much higher than 2.0% (30.1% x 6.4%) seen in thoracic DRGs" using multiplication of double staining results. This new paragraph is a little bit confusion, and this indirect method may introduce error. Triple-staining of TUBB3, NEFH and tdTomato would help to directly calculate the percentage.

2. In the Extended Data Fig. 5.c, both the number and morphology of Pacinian corpuscles in the periosteum membrane seems strange. Is it a section or whole mount staining? Which orientation of section? Whole mount staining of Tdt, NF200, and S100 would help to resolve Pacinian corpuscle innervating question.

3. In the Extended Data Fig. 2c and 2d, the authors showed the central innervation of PROKR2ADV-tdTomato+ fibers in the spinal cord. Interestingly, there is obvious innervation of in the lamina III and IV (in addition to lamina I) in the lumbar spinal cord but not in the thoracic spinal cord. The authors seem to assume the deep-innervating PROKR2-Tdt+ fibers innervating layer I. However, based on the known anatomy, peripheral deep tissue innervating, NFH strong, DRG neurons usually have central projections innervating the deep layers. The authors have done retrograde tracing from ST36. Do the retrograde central fibers innervate superficial or deep layer of the spinal cord? Given that the main novelty of this manuscript is to discover a neuronal substrate for an acupuncture site, this is an important question that will need to be cleared out.

4. In the Extended Data Fig. 11, the author showed that ES of LI10 in the forelimb acupoint can evoke PROKR2ADV neuron-dependent anti-inflammatory effects. Since ES in different acupoints or with different density could go through different pathways to generate the anti-inflammatory effect, it is unclear whether ES of this site would also go through the vagal reflexes (though this is the assumption). Evidence either supporting or disapprove the assumption would be nice. In addition, does ES of LI10 and ST36 generate the same effect in human? This should be an interesting point to be included in discussion.

5. In several places, the "lumbar" was misspelled to "lumber". Please check and revise.

Referee #3 (Remarks to the Author):

The authors have fully addressed my concerns. This is a comprehensive and rigorous study of very high interest.

Referee #4 (Remarks to the Author):

Thank you for your answers and the corrections to the article.
The authors have performed the proposed experiments, revised the article as requested, and addressed my concerns.

Author Rebuttals to First Revision:

Point-by-point responses to reviewers' comments:

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The results are timely, original, novel and significant.

The results support the conclusions, and the manuscript is clearly written and interesting.

--- Thanks for the very positive assessment.

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1. In the revised text, the author reasoned that "Nefhhigh;tdTomato+ neurons represent 6.3% (17.9% x 35.4%) of L4-5 DRG neurons, much higher than 2.0% (30.1% x 6.4%) seen in thoracic DRGs" using multiplication of double staining results. This new paragraph is a little bit confusion, and this indirect method may introduce error. Triple-staining of TUBB3, NEFH and tdTomato would help to directly calculate the percentage.

---- Thanks for this suggestion. We have performed triple-staining (tdTomato plus TUBB3 immunostaining combined with in situ hybridization with Nefh; we found Nefh mRNA was better to tell Nefh-high versus Nefh-low neurons). The new data (see the revised Fig. 1b, 1c) were consistent with the results we reported last time, showing significant enrichment of $Nefh^{high}$;$tdTomato^{+}$ neurons in lumbar DRGs compared with thoracic DRGs.

2. In the Extended Data Fig. 5.c, both the number and morphology of Pacinian corpuscles in the periosteum membrane seems strange. Is it a section or whole mount staining? Which orientation of section? Whole mount staining of Tdt, NF200, and S100 would help to resolve Pacinian corpuscle innervating question.

---- Yes, the original Pacinian corpuscle images were acquired from transverse sections. We have now replaced with whole-mount images, which supported our previous conclusion:

PROKR2^{ADV}-tdTomato^{+} fibers are not associated with these mechanoreceptors (see the revised Extended Data Fig.4c)

3. In the Extended Data Fig. 2c and 2d, the authors showed the central innervation of PROKR2ADV-tdTomato+ fibers in the spinal cord. Interestingly, there is obvious innervation of in the lamina III and IV (in addition to lamina I) in the lumbar spinal cord but not in the thoracic spinal cord. The authors seem to assume the deep-innervating PROKR2-Tdt+ fibers innervating layer I. However, based on the known anatomy, peripheral deep tissue innervating, NFH strong, DRG neurons usually have central projections innervating the deep layers. The authors have done retrograde tracing from ST36. Do the retrograde central fibers innervate superficial or deep layer of the spinal cord? Given that the main novelty of this manuscript is to discover a neuronal substrate for an acupuncture site, this is an important question that will need to be cleared out.

----- In fact, in both lumbar and thoracic DRGs, PROKR2^{ADV}-tdTomato^{+} central terminals innervate mainly in the superficial laminae, and minorly in deep laminae. We have replaced with new boxed regions to highlight this point (see the revised **Extended Data Fig. 2c and 2d)**. The Koerber lab had also reported similar innervation patterns for type III (thinly myelinated) muscle afferents, enriched in superficial laminae (J Neurophysiol 109: 2374–2381, 2013). It should also be pointed out that in response to low-intensity ES, PROKR2^{ADV} neuron-dependent c-Fos induction in spino-NTS projection neurons was enriched in lamina I, rather than in deep laminae. Detailed studies on how PROKR2^{ADV} neurons transmit sensory information from the spinal cord to the NTS and then to DMV to produce the vagal-adrenal reflexes are, however, beyond the scope of the current study.

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--- Thanks for this suggestion. We have performed a new set of experiments, and LI10 ES-evoked anti-inflammatory effects were indeed dependent on vagal reflexes (see **Extended Data Fig. 11n)**. We are not aware of any human studies that directly measure the vagal-adrenal axis evoked by low-intensity acupuncture in either LI10 or ST36. Future studies will be highly warranted to test if the principles we draw from animal studies can be applied to humans.

5. In several places, the “lumbar” was misspelled to “lumber”. Please check and revise.

--- Thanks, and we have made the corrections.

Referee #3 (Remarks to the Author):

The authors have fully addressed my concerns. This is a comprehensive and rigorous study of very high interest.

--- Thanks.

Referee #4 (Remarks to the Author):

Thank you for your answers and the corrections to the article.
The authors have performed the proposed experiments, revised the article as requested, andaddressed my concerns.

--- Thanks.

Once again, thank all reviewers for the constructive comments and suggestions. With theserevisions, the paper has been greatly improved. We hope this study will greatly stimulate people's interest in acupuncture and in modern systems physiology approaches in studyingdisease progression and treatment.

Reviewer Reports on the Second Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have addressed all concerns.

Author Rebuttals to Second Revision:

Point-by-point responses to reviewers' comments:

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---- Thanks.