

Comparative specialization of intrinsic cardiac neurons in humans, mice, and pigs

John D. Tompkins, Donald B Hoover, Leif A. Havton, Janaki C. Patel, Youngjin Cho, Elizabeth H. Smith, Natalia P. Biscola, Olujimi A Ajijola, Kalyanam Shivkumar, and Jeffrey Laurence Ardell

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The referees have opted to remain anonymous.

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr Tompkins,

Re: JP-RP-2024-286714 "Comparative specialization of intrinsic cardiac neurons in humans, mice, and pigs" by John D. Tompkins, Donald B Hoover, Leif A. Havton, Janaki C. Patel, Youngjin Cho, Elizabeth H. Smith, Natalia P. Biscola, Olujimi A Ajjola, Kalyanam Shivkumar, and Jeffrey Laurence Ardell

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Please advise your co-authors of this decision as soon as possible.

The referee reports are copied at the end of this email.

Please address all the points raised and incorporate all requested revisions or explain in your Response to Referees why a change has not been made. We hope you will find the comments helpful and that you will be able to return your revised manuscript within 3 months. If you require longer than this, please contact journal staff: jp@physoc.org. Please note that this letter does not constitute a guarantee for acceptance of your revised manuscript.

Your revised manuscript should be submitted online using the link in your Author Tasks: Link Not Available. This link is accessible via your account as Corresponding Author; it is not available to your co-authors. If this presents a problem, please contact journal staff (jp@physoc.org). Image files from the previous version are retained on the system. Please ensure you replace or remove any files that are being revised.

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We look forward to receiving your revised submission.

If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Harold Schultz
Senior Editor
The Journal of Physiology

REQUIRED ITEMS

- Author photo and profile. First or joint first authors are asked to provide a short biography (no more than 100 words for one author or 150 words in total for joint first authors) and a portrait photograph. These should be uploaded and clearly labelled together in a Word document with the revised version of the manuscript. See [Information for Authors](#) for further details.
- You must start the Methods section with a paragraph headed [Ethical Approval](#). A detailed explanation of journal policy and regulations on animal experimentation is given in [Principles and standards for reporting animal experiments in The Journal of Physiology and Experimental Physiology](#) by David Grundy J Physiol, 593: 2547-2549. doi:10.1113/JP270818). A checklist outlining these requirements and detailing the information that must be provided in the paper can be found at: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>. Authors should confirm in their Methods section that their experiments were carried out according to the guidelines laid down by their institution's animal welfare committee, and conform to the principles and regulations as described in the Editorial by Grundy (2015), including an ethics approval reference number. The Methods section must contain a statement about access to food, water and housing, details of the anaesthetic regime: anaesthetic used, dose and route of administration, and method of killing the experimental animals.
- You must start the Methods section with a paragraph headed [Ethical Approval](#). If experiments were conducted on humans, confirmation that informed consent was obtained, preferably in writing, that the studies conformed to the standards set by the latest revision of the Declaration of Helsinki and that the procedures were approved by a properly constituted ethics committee, which should be named, must be included in the article file. If the research study was registered (clause 35 of the Declaration of Helsinki), the registration database should be indicated, otherwise the lack of registration should be noted as an exception (e.g. The study conformed to the standards set by the Declaration of Helsinki, except for registration in a database). For further information see: <https://physoc.onlinelibrary.wiley.com/hub/human-experiments>.
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In summary:

- If $n \leq 30$, all data points must be plotted in the figure in a way that reveals their range and distribution. A bar graph with data points overlaid, a box and whisker plot or a violin plot (preferably with data points included) are acceptable formats.
- If $n > 30$, then the entire raw dataset must be made available either as supporting information, or hosted on a not-for-profit repository, e.g. FigShare, with access details provided in the manuscript.
- 'n' clearly defined (e.g. x cells from y slices in z animals) in the Methods. Authors should be mindful of pseudoreplication.
- All relevant 'n' values must be clearly stated in the main text, figures and tables.
- The most appropriate summary statistic (e.g. mean or median and standard deviation) must be used. Standard Error of the Mean (SEM) alone is not permitted.
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- Please include an Abstract Figure file, as well as the Figure Legend text within the main article file. The Abstract Figure is a piece of artwork designed to give readers an immediate understanding of the research and should summarise the main conclusions. If possible, the image should be easily 'readable' from left to right or top to bottom. It should show the physiological relevance of the manuscript so readers can assess the importance and content of its findings. Abstract Figures should not merely recapitulate other figures in the manuscript. Please try to keep the diagram as simple as possible and without superfluous information that may distract from the main conclusion(s). Abstract Figures must be provided by authors no later than the revised manuscript stage and should be uploaded as a separate file during online submission labelled as File Type 'Abstract Figure'. Please also ensure that you include the figure legend in the main article file. All Abstract Figures should be created using BioRender. Authors should use The Journal's premium BioRender account to export high-resolution images. Details on how to use and access the premium account are included as part of this email.

EDITOR COMMENTS

Reviewing Editor:

Your paper has been reviewed by two experts in the field. Both felt it represents a potentially important addition to the literature. Strengths were the comprehensive comparison of RAGP neurochemical profile, morphology, and function across species, and the inclusion of human data. The human data, however, is superseded to some degree by a more extensive analysis of human ICNS neurons in a (very) recent publication in the Journal of Physiology, so that newly published data should be considered in the context of what is shown in the current manuscript.

There were some major concerns raised by the reviewers that must be addressed before this manuscript can be considered further. This will require an extensive revision and the inclusion of additional data and analysis. The main concerns relate to the depth and quality of the individual analyses. In many cases, especially the measures of cell morphology and electrophysiology, the number of samples or data shown is inadequate to support the conclusions. Also, in places there are claims made that in fact do not appear to be supported by any of the findings. Another concern relates to the health status of the patient population; perhaps that can be (partly) addressed by a comparison with the newly published human data mentioned above.

These major concerns, as well as the others outlined in the Comments for Authors and the attached comments from Review 2, must be addressed in a revision to the manuscript and a point-by-point response to the Reviewers before further consideration.

Please also see 'Required Items' above.

Senior Editor:

Thank you for submission of your research article to the Journal of Physiology for consideration. The article has been reviewed by experts in the field and found to require extensive revision before a decision on publication can be reached. The revision must address all the concerns raised by referees and editors. Please also address the list of requirements or publication in the journal including statistical rigor, and animal and human ethics policies.

1. Please review the journal policy on reporting animal ethics: <https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>

and human subjects: <https://physoc.onlinelibrary.wiley.com/hub/human-experiments>

The Methods section must begin with the subheading "Ethical Approval" wherein authors must state the institutional ethical committee that approved the study and the national guidelines under which the institution operates. The institutional approval code/number should be provided. In addition, the section must include a statement that the investigators understand the ethical principles under which the journal operates and that their work complies with the animal ethics checklist as outlined by the journal and human tissue studies conformed to the standards set by the latest version of the Declaration of Helsinki.

REFeree COMMENTS

Referee #1:

This study evaluates the intracardiac nervous system (ICNS) in mice, pigs, and humans. The novelty of the work is the comparison across species, which has not been done previously. The analysis of human neurons is also somewhat novel, but a new study just published in the Journal of Physiology (I believe after the current manuscript was submitted) carries out a more extensive analysis of human ICNS neurons, comparing neurons from controls with people who have atrial fibrillation. Any additional versions of this manuscript will want to incorporate discussion of the new data just published (J Physiol 2024 Apr 30. doi: 10.1113/JP286278. Online ahead of print), which includes a number of things directly relevant to this study, and which diminishes the impact of the human data presented here.

The authors have tried to cover a lot of ground, generally trying to map out the ICNS (or at least the RAGP) in each species along with comparing neurochemical and peptide profiles, neuron morphology, and functional analyses. The scope of coverage is a strength of the study since this type of thing has not been done side-by-side across species before, and it has some real value. However, the depth and quality of individual analyses have suffered. For many of the analyses, including cell morphology and electrophysiology, a surprisingly small number of cells have been analyzed. This is expected for human cells because gaining access to the tissue is so problematic, but for mouse and pig the "n" for many of the experiments here should be considerably higher than what is presented to support the claims being made. There are also times when things are mentioned in the text that are not clear in the data. Latency measurements are referred to as driving conclusions, but no latency data are shown. sEPSP amplitude and duration data appear to be graphing individual EPSPs rather than the mean value (or gaussian mean) from each cell (figure 10). That is inappropriate. In the EM data several things are called out in the text but not identified in the figures. This includes large dense core vesicles, "protrusions of the somatic cytoplasm into dendritic processes" and "increased complexity of cytoplasmic organelles and a greater partitioning of the membrane in human and pig epicardial neurons". None of these are marked or pointed out in the figures, so it is difficult to see how the data support these statements.

Minor issues

Fig. 5. The formatting of distinct colors for the different species is helpful and appreciated. However, it is almost impossible

to tell the difference between the controls in G,H,I and the drug treatments. Please use different colors to distinguish those groups (and add more cells).

Fig. 7. For the cell morphology what is meant by the "height, length, and depth" of a neuron? Is this about the soma or also dendrites? You already have the measurements people care about - soma volume, dendritic tree size, branch points etc., so I don't understand the purpose of these other items that are unclear.

Fig. 9. I got the impression that SCV size was measured in a single nerve terminal from each species. Is that correct? If so, please measure from at least 2 more neurons in each species.

Referee #2:

See the attached Comments for the Author.

END OF COMMENTS

Confidential Review

19-Apr-2024

General Comments:

This manuscript represents a comprehensive comparative study of the neurons in the intrinsic cardiac ganglia of three mammalian species: mouse, pig, and human. The multidisciplinary approach, histochemistry, morphology, and electrophysiology, is commendable, however, it does not appear that a combination of these techniques has been applied at the single neuron level. Surprisingly, the classic comparative ultrastructural study of mammalian cardiac ganglia by Ellison & Hibbs is not cited (Ellison JP & Hibbs RG, 1976, *J Mol Cell Cardiol.* 8(2):89-101). The experiments are largely well-designed and carried out by an experienced team of researchers in the neurocardiology field. Further details concerning human and animal ethics are most likely required to comply with The Journal's requirements and standards. The experimental methods are relatively well explained and the figures are largely of high quality. In the Results section, it is not clear why the electrophysiology results ('Passive and active membrane properties' and 'Nerve-evoked potentials at intracardiac neurons') are not together but are separated by 'Morphologic properties of single ICNs' and 'ICN ultrastructure'?

The principal strength of the manuscript is the studies on human intrinsic cardiac neurons, however, it also raises concerns. In Table 1 the Organ donor demographic and ECG are given but not the heart transplant time data. Organ donor # 6 appears to be the sole donor with a normal heart rhythm and ECG. A major concern is how physiological are the neurons in the ganglia from donated human hearts, particularly given that several studies report that ganglionic transmission is diminished following myocardial infarction and heart failure (e.g. Bibevski S & Dunlap ME, 1999, *Circulation* 99(22):2958-63; Bibevski S & Dunlap ME, 2004, *Am J Physiol Heart Circ Physiol.* 287(4):H1780-5). Furthermore, extrapolating from inflammation in other tissues, myocardial conditions may well impact the expression of voltage-gated ion channels as reported for HCN1 and HCN2 in rat DRG neurons (Acosta C. et al., 2012, *PLoS One.* 7(12):e504).

Overall, the manuscript provides interesting and valuable new information, particularly on human histochemical, morphological, and electrophysiological properties compared to mouse and minipig intrinsic cardiac ganglion neurons. Following are specific comments pertaining to experimental design and presentation that the authors need to address in any revision of the manuscript.

Specific comments:

Abbreviations: add IHC – ImmunoHistoChemistry

Throughout the manuscript, the term "cells" should be replaced with "neurons"

Line 158: 'paravertebral' does this mean dorsal/posterior root ganglia?

Line 177: "mesoscale" or microscale? Micro- may be preferable.

Line 254: "The amplitude and duration of the action potential upstroke were measured from spontaneous or nerve evoked action potentials".....do they mean action potential upstroke or AP duration? This is not given in the Results or Figures.

In Figure 4G for nerve-evoked action potentials, it is AP ½ width (ms). It will be at different E_m 's, depending on resting membrane potential and AP amplitude e.g. resting E_m is lower for mice compared to minipig and human ICNs. Why not use a fixed membrane potential value for measurement e.g. 0 mV where the inward current should be at its maximum?

Lines 263-264: What is done with data obtained from “stimulus recruitment curves”? No data/figures appear to show plots of “latency of evoked potential against the stimulus current”

Line 266: The latency between intertrain stimuli should be stated.

Line 272: Define IHC in the list of Abbreviations.

Line 280: Clarify “over two nights” Does this mean 48 hours?

Line 292: What are “charged slides”?

Line 402: ‘Despite a similar mean ganglion size...’ is inconsistent with the data presented whereby mouse ganglion area is $54 \pm 45 \text{ mm}^2$ (line 382) whereas the area of minipig ganglia is $168 \pm 200 \text{ mm}^2$ (line 397) and $162 \pm 28 \text{ mm}^2$ in human (line 398)!

Line 432: Instead of “A couple of human ICNs...”, state the number more precisely.

Lines 450-463: Provide the number of observations/neurons (n) for the electrophysiological data given.

In addition, what was the resuscitation time of ganglion preparations before commencing recording? The human ICN preparations would have been in a cardioplegic solution which generally has low Ca^{2+} and high K^+ concentrations.

Figure 4D: It appears that several mouse neurons had a resting membrane potential (E_m) of $< -40 \text{ mV}$. Was a low E_m reflected in input resistance and AP parameters?

Line 457: Does “...correspondingly shorter action potentials” follow less negative E_m ’s of mouse neurons?

Line 460-461: Why not a decimal point for the SD results? significant figures.

Line 491: The 4-AP concentration ($10 \text{ }\mu\text{M}$) used is relatively low although Figure 5G,H shows it works in mouse and minipig ICN. The observation that “4-AP had no effect on excitability in 2 cells from 2 donor hearts” might be attributed to the low concentration of 4-AP. A reference for the effectiveness of low 4-AP concentrations might be helpful (1 mM 4-AP used in previous studies of guinea-pig and rat ICN by Tompkins JD et al., 2016, *Am J Physiol Cell Physiol.* 311(4):C643-C651, and Xi-Moy SX & Dun NJ., 1995, *J Physiol.* 486 (Pt 1):15-31, respectively).

Figure 5 G-I: The Y-axis of plots of No. of APs /500 ms with current injection could be expanded for clarity. Also, please state in the figure legend the concentrations of apamin and 4-AP applied to the neurons.

Lines 509-510: “....projections extended from the basal membrane, while the apical membrane was smooth.” Usually, apical and basal descriptors are associated with polarised cells such as epithelium. It might be preferable to refer to the outer and inner (epicardial) facing sides of the ganglion.

Line 511: “A couple of cells which were deeper within the ganglion....” approximately how many neurons ‘deep’ is the ganglion?

Figure 6 C₂: Considering human ICN, it appears approximately circular in cross-section $\sim 50 \text{ }\mu\text{m}$ diameter, so if the soma is spherical volume given by $4/3\pi r^3$, this gives a volume of $\sim 65,000 \text{ }\mu\text{m}^3$. If the neuron is $\sim 40 \text{ }\mu\text{m}$ in diameter, the soma volume is $\sim 33,500 \text{ }\mu\text{m}^3$ which is at the upper end to outside the range of soma volumes given in Figure 7D. Does this mean that the neurons are prolate spheroids as for guinea-pig cardiac ganglion neurons described in Edwards FR et al., 1995, *J Physiol.* 486:453-471?

Line 734: Reference #2, requires more information. i.e. Edited by Armour J.A. & Ardell, J.L.

EDITOR COMMENTS

Reviewing Editor:

Your paper has been reviewed by two experts in the field. Both felt it represents a potentially important addition to the literature. Strengths were the comprehensive comparison of RAGP neurochemical profile, morphology, and function across species, and the inclusion of human data. The human data, however, is superseded to some degree by a more extensive analysis of human ICNS neurons in a (very) recent publication in the Journal of Physiology, so that newly published data should be considered in the context of what is shown in the current manuscript.

There were some major concerns raised by the reviewers that must be addressed before this manuscript can be considered further. This will require an extensive revision and the inclusion of additional data and analysis. The main concerns relate to the depth and quality of the individual analyses. In many cases, especially the measures of cell morphology and electrophysiology, the number of samples or data shown is inadequate to support the conclusions. Also, in places there are claims made that in fact do not appear to be supported by any of the findings. Another concern relates to the health status of the patient population; perhaps that can be (partly) addressed by a comparison with the newly published human data mentioned above.

These major concerns, as well as the others outlined in the Comments for Authors and the attached comments from Review 2, must be addressed in a revision to the manuscript and a point-by-point response to the Reviewers before further consideration.

Please also see 'Required Items' above.

Dear Reviewing Editor,

Thank you for the overview of reviewer comments and for highlighting the strengths of our study. We are pleased with the positive reviews and have expanded the work as requested to further increase the depth and breadth of the investigation. We include comparison to the datasets presented by Ashton et al. where appropriate. We further discuss some of the differences in this study and this recently published work in response to referee #1 below.

We are grateful for the comprehensive reviews and have responded with extensive revision to the manuscript and figures addressing all input. Additional studies were performed in each species to increase sample sizes for electrophysiology, morphology, and TEM studies. We also discuss the health of the human patients from which the samples were obtained. All changes are documented in the detailed point-by-point response below.

Additional 'Required Items' are included with this resubmission and include: an image to be considered for cover art, graphical abstract, first author profile and photo, a complete point by point response to reviewers, two versions of the manuscript (redlined and clean) and high quality (300dpi) manuscript figures.

The Journal of Physiology special issue 'Cardiac Neurobiology: Concepts to Clinic' would be an excellent home for this work. We hope to have risen The Journal's standards for the opportunity to share our work here. We wish to note that this manuscript represents the effort of multiple institutions over several years culminating in both the written work and the accompanying database of intracardiac neurons with images and recordings from mice, pigs and humans. The database will be made publicly accessible through the NIH SPARC program upon publication of the manuscript. This is the first of its kind database on cardiac neurobiology. We hope other authors in this field contribute to making their data publicly available to help build upon a growing foundation of ground truth data.

Sincerely,
John Tompkins

Senior Editor:

Thank you for submission of your research article to the Journal of Physiology for consideration. The article has been reviewed by experts in the field and found to require extensive revision before a decision on publication can be reached. The revision must address all the concerns raised by referees and editors. Please also address the list of requirements or publication in the journal including statistical rigor, and animal and human ethics policies.

1. Please review the journal policy on reporting animal ethics:

<https://physoc.onlinelibrary.wiley.com/hub/animal-experiments>

and human subjects: <https://physoc.onlinelibrary.wiley.com/hub/human-experiments>

The Methods section must begin with the subheading "Ethical Approval" wherein authors must state the institutional ethical committee that approved the study and the national guidelines under which the institution operates. The institutional approval code/number should be provided. In addition, the section must include a statement that the investigators understand the ethical principles under which the journal operates and that their work complies with the animal ethics checklist as outlined by the journal and human tissue studies conformed to the standards set by the latest version of the Declaration of Helsinki.

Dear Senior Editor,

Thank you for overseeing this manuscript's review. We have made extensive revision to the presented work addressing all concerns raised by the referees and editors, including the list of requirements for publication in the journal including statistical rigor, and animal and human ethics policies.

Regarding animal ethics, we have added the section "Ethical Approval" where both the use of animals and use of human tissue has been expanded to conform with journal policy.

Regarding human tissue, it is important to note that these studies did not enroll any patients. The study used only hearts given for donation after a declaration of patient death. Hearts were donated with consent of patient and patient's family. All had been declined for cardiac transplant due multiple factors,, which is discussed further below in response to referee #2. All data associated with the tissue used was de-identified, and no cells or tissues were maintained or kept by culture.

Sincerely,
John Tompkins

REFEREE COMMENTS

Referee #1:

This study evaluates the intracardiac nervous system (ICNS) in mice, pigs, and humans. The novelty of the work is the comparison across species, which has not been done previously. The analysis of human neurons is also somewhat novel, but a new study just published in the Journal of Physiology (I believe after the current manuscript was submitted) carries out a more extensive analysis of human ICNS neurons, comparing neurons from controls with people who have atrial fibrillation. Any additional versions of this manuscript will want to incorporate discussion of the new data just published (J Physiol 2024 Apr 30. doi: 10.1113/JP286278. Online ahead of print), which includes a number of things directly relevant to this study, and which diminishes the impact of the human data presented here.

We acknowledge the work by Aston et al. on human intracardiac neurons which was accepted for publication while this manuscript was in review. We commend the authors effort and recognize with first-hand experience the many challenges encountered with both recording and imaging of human intracardiac neurons. We have included reference to their data in comparison to this study in the discussion. It is important to note that the intracardiac neurons in that study were isolated from patients undergoing coronary artery

bypass and cannot be considered 'healthy' controls. Future cardiac surgical approaches hopefully attempt to spare these neurons given the correlation of ICN removal and risk for arrhythmia (PMIDs: 37562487, 28128201).

We wish to highlight a few areas where the studies differ. The Ashton et al. work focused on comparison of ICNs from coronary artery bypass patients both with and without atrial fibrillation (AF). We have focused on broad descriptions of ICNs using multiple approaches from a diverse patient population and multiple species. It is challenging to compare 'healthy' to 'disease' neurons, as in the Ashton et al. paper, the membrane excitability of 8 AF intracardiac neurons were compared to 16 non-AF ICNs. The total population of the right atrial ganglionated plexus neurons is estimated to be approximately 6,000 to 12,000 neurons (PMID:33629877). The conclusions must be tempered by the fact that 8 cells from 6 patients is a very poor representation of the total population and has minimal statistical power. Also, for baseline electrophysiology in non-AF patients, the authors state 37.5% of their cells failed to fire action potentials. This was a condition for exclusion of neurons from the current study and draws into question the health of these neurons. For imaging of cellular morphology, while it is the first publication of its kind, 8 neurons were quantified, but the morphology of only two neurons is shown in figure 1 - the same neuron is shown in all three panels (Fig 1A,C&D). For images of whole ganglion morphology, the same ganglion is shown in all 6 panels of Fig 6 C&D. Also while excitability was studied, no effort was made to identify ionic conductances underlying the regulation of excitability in the human ICNs as we have done here.

This manuscript and the accompanying dataset offer a significantly expansion of these published data. The dataset includes whole ganglion 3D imaging from 7 mouse ganglia, 8 pig ganglia, 7 human ganglia, immunohistochemistry for 7 proteins in 36 mouse ganglion sections, 49 pig ganglion sections, and 44 human ganglion sections, intracellular microelectrode recordings from 36 mouse, 44 pig and 22 human ICNs, two pharmacological inhibitors of K⁺ -conductances tested in three species, 3D imaging and manual segmentation of 15 mouse ICNs, 13 pig ICNs and 11 human ICNs, nerve evoked potentials (including latencies) in 9 mouse ICNs, 23 pig ICNs, and 11 human ICNs, and TEM images from 17 mouse ICNs, 19 pig ICNs, and 54 human ICNs. The database describes in detail both similarities and differences between these species and greatly expands upon a foundational dataset of human ICN structure and function. The dataset is also publicly available. We encourage other researchers in the field to openly share data for transparency and collaboration. Importantly both studies have made a significant leap forward in our understanding of human ICNs and in combination represent the first of its kind work to be published anywhere.

The authors have tried to cover a lot of ground, generally trying to map out the ICNS (or at least the RAGP) in each species along with comparing neurochemical and peptide profiles, neuron morphology, and functional analyses. The scope of coverage is a strength of the study since this type of thing has not been done side-by-side across species before, and it has some real value.

We appreciate the acknowledgment and recognition of our efforts to provide a comprehensive overview of these neurons across three species.

However, the depth and quality of individual analyses have suffered. For many of the analyses, including cell morphology and electrophysiology, a surprisingly small number of cells have been analyzed. This is expected for human cells because gaining access to the tissue is so problematic, but for mouse and pig the "n" for many of the experiments here should be considerably higher than what is presented to support the claims being made.

We completed additional experiments in each species. New data was added from six mouse, four pig and three human hearts. Electrophysiology data now includes passive and active membrane properties from 36 ICNs from 14 mice, 44 ICNs from 15 pigs, and 22 ICNs from 13 humans. The morphological data was expanded to include 15 mouse ICNs, 13 pig ICNs and 11 human ICNs. The increased sample size strengthens the conclusions which have largely held true to those of the original work.

There are also times when things are mentioned in the text that are not clear in the data. Latency measurements are referred to as driving conclusions, but no latency data are shown.

We incorporated the latency data into the manuscript. Please see revised figure 7 and its legend. The data presented includes representative stimulus recruitment curves for individual neurons, as well as a frequency histograms of latency values including all measurements from orthodromic inputs. Please see also the expanded discussion of latency results under, "Nerve evoked potentials at intracardiac neurons."

sEPSP amplitude and duration data appear to be graphing individual EPSPs rather than the mean value (or gaussian mean) from each cell (figure 10). That is inappropriate.

We have revised the data to plot mean sEPSP amplitude and duration in each cell measured (see Figure 7).

In the EM data several things are called out in the text but not identified in the figures. This includes large dense core vesicles, "protrusions of the somatic cytoplasm into dendritic processes" and "increased complexity of cytoplasmic organelles and a greater partitioning of the membrane in human and pig epicardial neurons". None of these are marked or pointed out in the figures, so it is difficult to see how the data support these statements.

We have extensively revised figures showing TEM images (Figures 10 and 11). These figures now highlight the presence of large dense core vesicles and show extension of the cytoplasm into dendrites which is representative of the greater membrane partitioning in large mammals. We removed the statement, "increased complexity of cytoplasmic organelles" as this was a qualitative observation with no quantification. The principle focus of the TEM imaging was synaptic organization. We have identified the presence of axo-somatic synapses in mouse ICNs only (of 17 mouse ICNs surveyed) and document the presence of axo-dendritic synapses at pig and human ICNs which never showed axo-somatic connections (19 pig ICNs and 54 human ICNs surveyed). We also quantified the

size of small clear vesicles in multiple synapses of each species ($n = 3$), indicate the presence of large dense core vesicles and reveal the organization of the synaptic specializations in all three mammals. The complete dataset from which all observations were made will be accessible in the public database.

Fig. 5. The formatting of distinct colors for the different species is helpful and appreciated. However, it is almost impossible to tell the difference between the controls in G,H,I and the drug treatments. Please use different colors to distinguish those groups (and add more cells).

We have revised the presentation of the data (please see Figure 6) and have included more cells. It is important to note that we only perform a single antagonist experiment in each preparation (1 animal). These are always done at the end of the experiment to avoid contamination of control recordings. The effects of apamin were both consistent and markedly different in both mice and humans with just three experiments each. In pigs, we performed one additional experiment. There was a trend for a difference in AHP duration after apamin, but it did not reach statistical significance ($p = 0.0659$) after an additional experiment. Pig ICNs clearly responded differently to both drugs in comparison to the mouse or human ICNs.

Fig. 7. For the cell morphology what is meant by the "height, length, and depth" of a neuron? Is this about the soma or also dendrites? You already have the measurements people care about - soma volume, dendritic tree size, branch points etc., so I don't understand the purpose of these other items that are unclear.

The width, height, and depth correspond to the total X,Y, and Z dimensions of the cell soma and dendrites. We have clarified this in the description of the results. We believe these are important measurements as it relays the volume of the dendrites in three axes. It is often reported for neuronal morphologies (e.x. PMID:34616072). We also provide dendritic length; however, dendrites often follow circuitous paths and the length is not reflective of the total volume of dendrite spread from the soma. For example, dendrites do not wrap around the cell body but extend throughout the ganglion. Interestingly the X,Y,Z dimension of soma and dendrites is very similar between human and pig neurons, yet human neurons have greater dendritic length closest to the cell body, as shown by the Sholl analysis.

Fig. 9. I got the impression that SCV size was measured in a single nerve terminal from each species. Is that correct? If so, please measure from at least 2 more neurons in each species.

SCV size was initially quantified from single terminals in each species. We have completed additional TEM imaging identifying more synapses in each species. The figure now shows mean vesicle size from three terminals of each species.

Referee #2:

General Comments:

This manuscript represents a comprehensive comparative study of the neurons in the intrinsic cardiac ganglia of three mammalian species: mouse, pig, and human. The multidisciplinary approach, histochemistry, morphology, and electrophysiology, is commendable, however, it does not appear that a combination of these techniques has been applied at the single neuron level.

It is correct that the data for neuron with are independent with regards to each modality of investigation. Completion of an intersectional approach, i.e. performing multiple methodological analysis on the same cell is far more complex and presents new challenges. The current study provides a population analysis of intracardiac neurons with multiple scientific techniques across three species. The studies have identified proteins of interest, as well as morphological and electrophysiological patterns which we hope to next investigate using an intersectional approach in the attempt to identify specialized populations of intracardiac neurons.

Surprisingly, the classic comparative ultrastructural study of mammalian cardiac ganglia by Ellison & Hibbs is not cited (Ellison JP & Hibbs RG, 1976, *J Mol Cell Cardiol.* 8(2):89-101).

We apologize for the omission. We have reviewed the work and have incorporated the reference into the discussion of TEM results.

The experiments are largely well-designed and carried out by an experienced team of researchers in the neurocardiology field. Further details concerning human and animal ethics are most likely required to comply with The Journal's requirements and standards.

The "Ethical Approval" section has been revised in compliance with The Journal's requirements.

The experimental methods are relatively well explained and the figures are largely of high quality. In the Results section, it is not clear why the electrophysiology results ('Passive and active membrane properties' and 'Nerve-evoked potentials at intracardiac neurons') are not together but are separated by 'Morphologic properties of single ICNs' and 'ICN ultrastructure'?

We have revised the nerve-evoked potentials section to include latency data and have moved this section after passive and active membrane properties.

The principal strength of the manuscript is the studies on human intrinsic cardiac neurons, however, it also raises concerns. In Table 1 the Organ donor demographic and ECG are given but not the heart transplant time data. Organ donor # 6 appears to be the sole donor with a normal heart rhythm and ECG. A major concern is how physiological are the neurons in the ganglia from donated human hearts, particularly given that several studies report that ganglionic transmission is diminished following myocardial infarction and heart failure (e.g. Bibevski S & Dunlap ME, 1999, *Circulation* 99(22):2958-63; Bibevski S & Dunlap ME, 2004, *Am J Physiol Heart Circ Physiol.* 287(4):H1780-5). Furthermore, extrapolating from inflammation in other tissues, myocardial conditions may well impact the expression of voltage-gated ion channels as reported for HCN1 and HCN2 in rat DRG neurons (Acosta C. et al., 2012, *PLoS One.* 7(12):e504).

We have added heart transplant time to Table 1, in addition to the demographics and ECG notes from new hearts added. As indicated, 'transplant time' is defined as time from aortic-cross clamp to organ receipt. We can only access donor hearts which have been declined for heart transplant. Hearts are declined due to many factors including ECG abnormalities, congenital malformations, heart failure, devices, age, drug use, etc. It is unlikely that anyone will have the opportunity to record from healthy 'control' human tissue. In the recently published study by Ashton et al. (PMID: 38687681), ICNs were collected from patients during coronary artery bypass surgery. These recordings are also not from 'healthy' hearts. At this time, given the current sample size and multivariate pathologies of tissues obtained, we have not attempted to correlate cellular structure or function with pathology. While recordings were made from several 'near normal' hearts, the sample size is too small to make comparisons. One thing we can conclude from both our data and that published by Ashton et al. is that all human neurons recorded from thus far are principally (99%) tonically firing, if sufficiently depolarized. As our sample size increases during ongoing studies, we hope to make comparisons between diseased (i.e. MI or AF hearts) and 'near normal' hearts. This was beyond the scope of the current study. We focus here on comparison among species and provide a baseline human dataset (publicly shared). We are currently using animal models (mice, pigs) to investigate impacts of MI on ICN structure and function with and without targeted neuromodulation therapies. We have added to the discussion to discuss the impact of disease on ICNs including reference to the studies you mention.

Overall, the manuscript provides interesting and valuable new information, particularly on human histochemical, morphological, and electrophysiological properties compared to mouse and minipig intrinsic cardiac ganglion neurons. Following are specific comments pertaining to experimental design and presentation that the authors need to address in any revision of the manuscript.

We are grateful for your time spent reviewing this work which has helped to improve the quality of the manuscript.

Specific comments:

Abbreviations: add IHC – ImmunoHistoChemistry

The abbreviation was added.

Throughout the manuscript, the term “cells” should be replaced with “neurons”.

We have made the appropriate edit throughout the manuscript.

Line 158: ‘paravertebral’ does this mean dorsal/posterior root ganglia?

We revised the sentence to read as follows: "This neural network spans the posterior atria and ventricles, and contains nerve fibers from centrally located

preganglionic neurons, postganglionic sympathetic nerves from paravertebral ganglia, sensory fibers from dorsal root and vagal ganglia, as well as the postganglionic neurons residing on the heart."

Line 177: "mesoscale" or microscale? Micro- may be preferable.

We agree "microscale" is more appropriate and have modified the text accordingly.

Line 254: "The amplitude and duration of the action potential upstroke were measured from spontaneous or nerve evoked action potentials".....do they mean action potential upstroke or AP duration? This is not given in the Results or Figures.

The sentence was revised to indicate more clearly that we are measuring the amplitude and duration of the action potential. For duration we are measuring action potential half-width. This is the time between the depolarizing and repolarizing phases of the AP, measured at 50% AP amplitude.

In Figure 4G for nerve-evoked action potentials, it is AP $\frac{1}{2}$ width (ms). It will be at different E_m 's, depending on resting membrane potential and AP amplitude e.g. resting E_m is lower for mice compared to minipig and human ICNs. Why not use a fixed membrane potential value for measurement e.g. 0 mV where the inward current should be at its maximum?

AP half-width is a commonly reported measure for AP duration used to compare properties of neurons (PMID: 17514198). We did not focus solely on the kinetics of the AP upstroke, i.e. the inward current portion of the AP, at this time. Rather AP half-width is the metric of action potential duration spanning both the developing inward and outward currents. We find much greater variability in the measurement of action potential width at 0mV - also for comparison to previous studies and CNS neurons, we have used the AP half-width.

Lines 263-264: What is done with data obtained from "stimulus recruitment curves"? No data/figures appear to show plots of "latency of evoked potential against the stimulus current"

We have revised the presentation of data concerning measurement of latency values. The nerve stimulation data in Figure 7 now shows both frequency histograms of latency values across all cells, and representative plots of stimulus current amplitude and latency at single cells. The results and figure legends were revised appropriately.

Line 266: The latency between intertrain stimuli should be stated.

We added the intertrain interval to the text as follows: "Trains of stimuli were given at 5, 10, or 20 Hz to assess spike following frequency at the post-synaptic neuron, with a two-to three-minute interval between trains."

Line 272: Define IHC in the list of Abbreviations.

The abbreviation was added.

Line 280: Clarify “over two nights” Does this mean 48 hours?

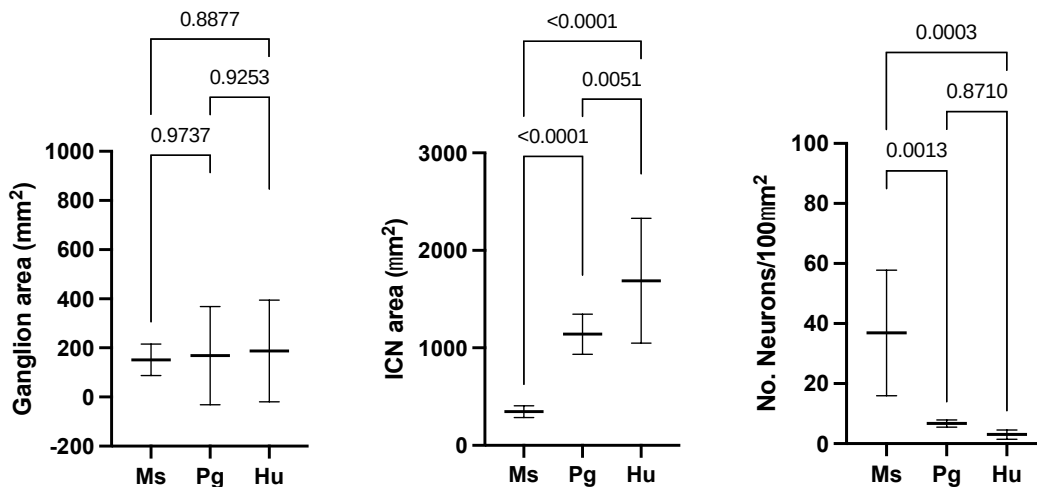
We replaced "over two nights" with "48 hours" to clarify.

Line 292: What are “charged slides”?

We've changed "charged slides" to "adhesion slides". Adhesion slides use electrostatic and chemical binding to bind specimens onto the slide.

Line 402: ‘Despite a similar mean ganglion size...’ is inconsistent with the data presented whereby mouse ganglion area is $54 \pm 45 \text{ mm}^2$ (line 382) whereas the area of minipig ganglia is $168 \pm 200 \text{ mm}^2$ (line 397) and $162 \pm 28 \text{ mm}^2$ in human (line 398)!

Ganglion areas were compared by one-way ANOVA and the means were not significantly different given variability in ganglion size. We increased the number of ganglia quantified (mouse $n = 14$, pig $n = 30$, human $n = 26$). The conclusion holds, that although there is a larger variability in ganglion size observed in pig and human, mean ganglion area is not different between species (one way ANOVA, Tukey post hoc). The data are shown below. We have also revised the sentence to state as follows: "While ganglion size was not significantly different between species, the number of ICNs per unit area was significantly greater in mouse compared to pig ($P = 0.0034$) or human ($P = 0.0054$)."



Line 432: Instead of “A couple of human ICNs...”, state the number more precisely.

We revised this to state, "Two human ICNs out of approximately 100 appeared to exhibit immunoreactivity (IR) to these peptides...".

Lines 450-463: Provide the number of observations/neurons (n) for the electrophysiological data given.

We have provided the number of observations for all electrophysiological data.

In addition, what was the resuscitation time of ganglion preparations before commencing recording? The human ICN preparations would have been in a cardioplegic solution which generally has low Ca^{2+} and high K^{+} concentrations.

All ganglia were dissected from atrial tissue in ice cold physiologic salt solution (PSS) over approximately 30 mins. The tissue was then moved to the microscope and was continuously superfused with warmed (34 C) PSS. The tissue was allowed to equilibrate for at least 1 hr before recording started. We added the following to the text: "The ganglion preparations equilibrated for at least 1 hr in PSS before commencement of recording."

Figure 4D: It appears that several mouse neurons had a resting membrane potential (E_m) of < -40 mV. Was a low E_m reflected in input resistance and AP parameters?

With the addition of new experiments, we have chosen to exclude cells with low E_m (< -40) or cells which did not produce an action potential. The following exclusion criteria were added to the EP data: "Cells with RMPs less negative than -40 mV or cells that did not produce action potentials were excluded from the analysis."

Line 457: Does "...correspondingly shorter action potentials" follow less negative E_m 's of mouse neurons?

Yes, we expect that the decreased driving force due to a low E_m would attenuate spike amplitude. We have clarified this conclusion by simply stating the following: "The action potentials recorded from mouse ICNs were also smaller in amplitude (59 ± 9 mV, $n = 24$) than the action potentials recorded from pig (66 ± 8 mV, $n = 24$) or human (68 ± 12 mV, $n = 17$) ICNs (Fig 4F)."

Line 460-461: Why not a decimal point for the SD results? significant figures.

We have added the appropriate significant figures for these results.

Line 491: The 4-AP concentration ($10 \mu\text{M}$) used is relatively low although Figure 5G,H shows it works in mouse and minipig ICN. The observation that "4-AP had no effect on excitability in 2 cells from 2 donor hearts" might be attributed to the low concentration of 4-AP. A reference for the effectiveness of low 4-AP concentrations might be helpful (1 mM 4-AP used in previous studies of guinea-pig and rat ICN by Tompkins JD et al., 2016, *Am J Physiol Cell Physiol.* 311(4):C643-C651, and Xi-Moy SX & Dun NJ., 1995, *J Physiol.* 486 (Pt 1):15-31, respectively).

We apologize for the typographical error. We did in fact use 1mM 4-AP. This is evidenced by our laboratory notebooks which recorded solution preparation for each experiment. We have revised the drug concentration throughout the manuscript. We have

added data on effects of both 4-AP and apamin on AP half-width, AHP amplitude, and AHP duration. We created a new figure for these data (Figure 6).

Figure 5 G-I: The Y-axis of plots of No. of APs /500 ms with current injection could be expanded for clarity. Also, please state in the figure legend the concentrations of apamin and 4-AP applied to the neurons.

We added the drug concentrations and have expanded the pharmacological data now shown in Figure 6.

Lines 509-510: "...projections extended from the basal membrane, while the apical membrane was smooth." Usually, apical and basal descriptors are associated with polarized cells such as epithelium. It might be preferable to refer to the outer and inner (epicardial) facing sides of the ganglion.

We concur with your suggestion and have revised accordingly. It now reads, "Typically, these projections extended from the inner (epicardial) facing side. A few neurons located deeper within the ganglion had projections extending from both inner and outer membranes."

Line 511: "A couple of cells which were deeper within the ganglion...." approximately how many neurons 'deep' is the ganglion?

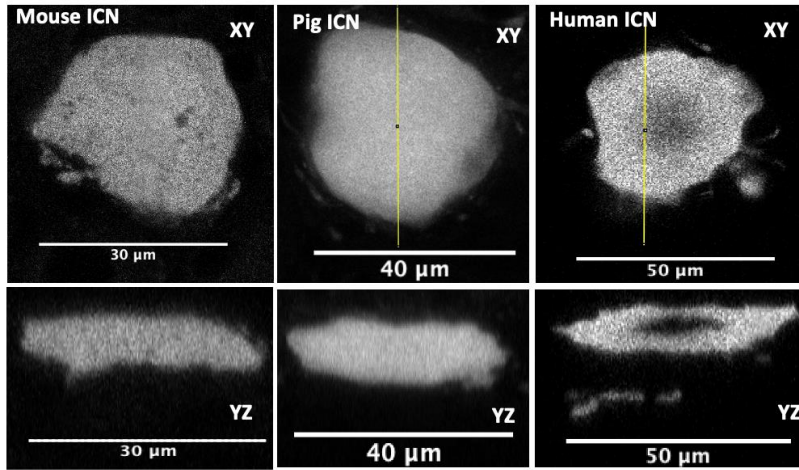
Ganglion depth varied greatly depending on ganglion size averaging 1-7 neurons 'deep'. Smaller ganglia would include one or two layers of neurons, while larger ganglia were seven neurons, with greatest neuronal mass at the center of the ganglion. We did not quantify ganglion depth for all imaged ganglia.

The following sentence was added to the manuscript: "Ganglion depth varied from 1 to 2 layers of neurons to a maximum of 7 layers of neurons with greatest neuronal mass concentrated in the center of the ganglion."

Figure 6 C2: Considering human ICN, it appears approximately circular in cross-section ~50 μm diameter, so if the soma is spherical volume given by $\frac{4}{3}\pi r^3$, this gives a volume of ~65,000 μm^3 . If the neuron is ~40 μm in diameter, the soma volume is ~33,500 μm^3 which is at the upper end to outside the range of soma volumes given in Figure 7D. Does this mean that the neurons are prolate spheroids as for guinea-pig cardiac ganglion neurons described in Edwards FR et al., 1995, *J Physiol.* 486:453-471?

The neurons are not spherical, as you have demonstrated; however, rather than being described as prolate spheroids (i.e. spindle shaped, having longer polar radius), ICN shape is best described as oblate spheroid (i.e. lentil shaped, having shorter polar radius). The volume equation would therefore be $V = \frac{4}{3}\pi b^2 c$ where b is the radius of the major axis and c is the radius of the minor axis. We show the XY and YZ planes of representative ICN somas from each species below. Note that neuron depth (Z axis) is significantly less than XY axis dimensions. All data, including 3D images of traced neurons, will be available for download at <https://sparc.science/> after publication.

We added the following to the manuscript: "Soma shapes, in all three species, are best described as oblate spheroids, having a shorter minor axis (Z axis) relative to the major axis (X,Y axis)."



Line 734: Reference #2, requires more information. i.e. Edited by Armour J.A. & Ardell, J.L.

The reference was corrected.

Dear Dr Tompkins,

Re: JP-RP-2024-286714R1 "Comparative specialization of intrinsic cardiac neurons in humans, mice, and pigs" by John D. Tompkins, Donald B Hoover, Leif A. Havton, Janaki C. Patel, Youngjin Cho, Elizabeth H. Smith, Natalia P. Biscola, Olujimi A Ajijola, Kalyanam Shivkumar, and Jeffrey Laurence Ardell

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We look forward to receiving your revised submission.

If you have any queries, please reply to this email and we will be pleased to advise.

Yours sincerely,

Harold Schultz
Senior Editor
The Journal of Physiology

REQUIRED ITEMS

- The reference list must be in alphabetical order, rather than numbered, to comply with our [Journal format](#).

EDITOR COMMENTS

Reviewing Editor:

The reviewers appreciate the substantial additional work done by the authors in response to their review. Before final acceptance, please see the additional minor revisions suggested by Reviewer 2 in their Comments for the Author and revise the manuscript accordingly, along with a response describing the changes made.

Please also amend your references to align with journal style (see Required Items above).

Senior Editor:

Thank you for submission of your revised research article to the Journal of Physiology for consideration. The article has been reviewed by the referees and found to be markedly improved and acceptable for publication pending addressing suggestions and corrections from referee 2. Please address all comments from the external referee as well as addressing the list of requirements for publication in the journal.

REFeree COMMENTS

Referee #1:

The authors are to be commended for the significant amount of additional work and data that are reflected in this revised manuscript. The data are now sufficient to support the conclusions drawn by the authors. Additional revisions to the text and figures clarify issues that were previously ambiguous. This revised and substantially improved study will be a significant resource for the community.

Referee #2:

The authors have adequately addressed the reviewers' comments and criticisms in the revised manuscript. The additional

experiments conducted on all three species have strengthened the conclusions drawn and improved the manuscript. The authors have also provided useful new data in response to the reviewers' questions and suggestions. While it is beyond the scope of this study, it would be helpful to inform the reader about the potential effects of the different recording methods on the electrophysiological properties of intracardiac neurons, particularly in relation to discharge activity and the absence of I_h and I_M with cell dialysis during whole-cell patch-clamp recordings (see Ashton et al., 2024, J. Physiol).

Following are minor comments largely pertaining to the presentation:

Line 162: Is "paracrine" the best descriptor? Neuroeffector junction maybe more suitable.

Line 172-3: "Novel clinical approaches have arisen to selectively influence organ function by manipulation of the peripheral nerves innervating organs" - the cited references refer to the regulation of cardiac function....Why not state that?

Line 215: D-glucose is 8 mM, why a lower concentration than normally used in *in vitro* studies?

Line 240: Given the flow rate is stated, provide the chamber volume.

Line 248: What were the data acquisition rates?

Line 252: Best to use small hyperpolarizing current steps to calculate input resistance, to minimize activation of hyperpolarization-activated channels

Line 262: It's the duration of the action potential not its upstroke that is being measured

Line 267-8: Correct sentence to the following "Neurons with RMPs less negative..... or neurons that did not produce..."

Line 394: Remove "total" i.e. "....between 1000 and 2400 neurons"

Line 400: "...thick layer of enveloping subepicardial fat." Is it overlying rather than enveloping?

Lines 515-6: Statistical analysis of data for n=3 is problematic!

Line 558: "...evoked potentials elicited spikes nearly 100% of the time, and at human ICNs multiple spikes were observed with each depolarization giving greater than 100% synaptic efficacy." It would be useful to have an electrophysiological record illustrating this behaviour but not necessary.

Line 800: Correct reference of the book: Editors: Dun, N.J., Machado, B.H., Pilowsky, P.M. Neural Mechanisms of Cardiovascular Regulation. (2004) Springer New York, NY. doi: 10.1007/978-1-4419-9054-9

Lines 817: Refs 12,18,19,22,24-26 and many following references - List all authors of the articles.

Figure 6A, B and C: There is a time but no voltage calibration bar for action potentials. It would also be helpful to have 0 mV line.

END OF COMMENTS

1st Confidential Review

01-Sep-2024

REQUIRED ITEMS

- The reference list must be in alphabetical order, rather than numbered, to comply with our [Journal format](#).

The reference list has been revised according to Journal instructions.

EDITOR COMMENTS

Reviewing Editor:

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Please also amend your references to align with journal style (see Required Items above).

We have addressed both the additional minor comments and references.

Senior Editor:

Thank you for submission of your revised research article to the Journal of Physiology for consideration. The article has been reviewed by the referees and found to be markedly improved and acceptable for publication pending addressing suggestions and corrections from referee 2. Please address all comments from the external referee as well as addressing the list of requirements for publication in the journal.

Please see below our response to each of the reviewer comments.

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The authors are to be commended for the significant amount of additional work and data that are reflected in this revised manuscript. The data are now sufficient to support the conclusions drawn by the authors. Additional revisions to the text and figures clarify issues that were previously ambiguous. This revised and substantially improved study

will be a significant resource for the community.

Thank you for your comments.

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The authors have adequately addressed the reviewers' comments and criticisms in the revised manuscript. The additional experiments conducted on all three species have strengthened the conclusions drawn and improved the manuscript. The authors have also provided useful new data in response to the reviewers' questions and suggestions. While it is beyond the scope of this study, it would be helpful to inform the reader about the potential effects of the different recording methods on the electrophysiological properties of intracardiac neurons, particularly in relation to discharge activity and the absence of I_h and I_M with cell dialysis during whole-cell patch-clamp recordings (see Ashton et al., 2024, J. Physiol).

Thank you for your comments. It is important to note that whole-cell patch-clamp recordings are susceptible to washout of second messengers. We have added this information to the discussion (line 800).

Following are minor comments largely pertaining to the presentation:

Line 162: Is "paracrine" the best descriptor? Neuroeffector junction maybe more suitable.

We agree with the recommendation and have changed the text to 'neuroeffector junction'.

Line 172-3: "Novel clinical approaches have arisen to selectively influence organ function by manipulation of the peripheral nerves innervating organs" - the cited references refer to the regulation of cardiac function....Why not state that?

The text was revised to focus on cardiac neuromodulation.

Line 215: D-glucose is 8 mM, why a lower concentration than normally used in *in vitro* studies?

The concentration of extracellular dextrose used varies across *in vitro* studies. For example, many use 11mM (PMID: 7473210), 10mM (PMID: 37330538), 8mM (PMID: 12761283) or 5.6mM (PMID: 20620869). Euglycemia in mammals is typically 70-180 mg/dl. 11 mM (200mg/dl) is approaching hyperglycemic levels. 8mM glucose, equivalent to 144 mg/dl, has worked well for our studies on adult peripheral neurons from multiple species (PMID: 14724192, PMID: 36147731).

Line 240: Given the flow rate is stated, provide the chamber volume.

Chamber volume is approximately 5 ml. This was added to the text.

Line 248: What were the data acquisition rates?

Most recordings were sampled at 20kHz. Some early recordings were made at 5kHz. We have stated the acquisition rate was 5 or 20 kHz in the text. Sampling values will be evident in the metadata of the public dataset.

Line 252: Best to use small hyperpolarizing current steps to calculate input resistance, to minimize activation of hyperpolarization-activated channels

We calculated input resistance and tau from the smallest hyperpolarizing steps (-50 or -100pA). We have edited the text to clarify this. More negative steps were used to activate hyperpolarization-activated channels.

Line 262: It's the duration of the action potential not its upstroke that is being measured

This was corrected.

Line 267-8: Correct sentence to the following "Neurons with RMPs less negative..... or neurons that did not produce..."

This change was made.

Line 394: Remove "total" i.e. "....between 1000 and 2400 neurons"

Removed.

Line 400: "...thick layer of enveloping subepicardial fat." Is it overlying rather than enveloping?

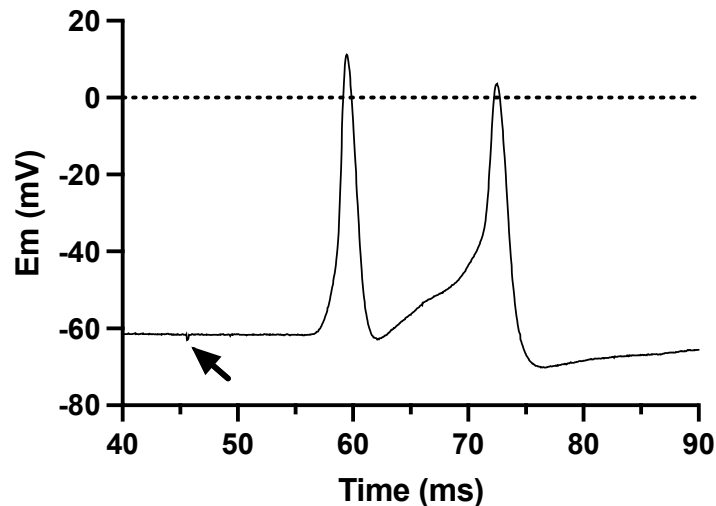
We believe 'enveloped' is accurate as the ganglia in pig and human hearts are embedded within the subepicardial fat. The ganglia may lie on top, beneath or within this adipose tissue.

Lines 515-6: Statistical analysis of data for n=3 is problematic!

Agreed. Although the effect size was substantial in most cases, with the differences between means greater than two standard deviations (Cohen's $d \geq 2$).

Line 558: "...evoked potentials elicited spikes nearly 100% of the time, and at human ICNs multiple spikes were observed with each depolarization giving greater than 100% synaptic efficacy." It would be useful to have an electrophysiological record illustrating this behaviour but not necessary.

At human ICNs, multiple spikes were observed but not with every depolarization as stated. We deleted the phrase, "with each depolarization." Though difficult to see, in Figure 7C, the first EPSP during train stimulation at 5, 10 or 20 Hz always evoked 2 APs. The tracing is expanded below to show these APs in response to a single preganglionic shock (arrow indicates stimulation artifact).



Line 800: Correct reference of the book: Editors: Dun, N.J., Machado, B.H., Pilowsky, P.M. Neural Mechanisms of Cardiovascular Regulation. (2004) Springer New York, NY. doi: 10.1007/978-1-4419-9054-9

This reference was corrected.

Lines 817: Refs 12,18,19,22,24-26 and many following references - List all authors of the articles.

We have revised all references in accordance with the journal style.

Figure 6A, B and C: There is a time but no voltage calibration bar for action potentials. It

would also be helpful to have 0 mV line.

We have added both a voltage calibration bar and 0 mV line to each action potential in figure 6A, B and C.

END OF COMMENTS

Dear Professor Tompkins,

Re: JP-RP-2024-286714R2 "Comparative specialization of intrinsic cardiac neurons in humans, mice, and pigs" by John D. Tompkins, Donald B Hoover, Leif A. Havton, Janaki C. Patel, Youngjin Cho, Elizabeth H. Smith, Natalia P. Biscola, Olujimi A Aijola, Kalyanam Shivkumar, and Jeffrey Laurence Ardell

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Yours sincerely,

Harold Schultz
Senior Editor
The Journal of Physiology

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EDITOR COMMENTS

Reviewing Editor:

Thank you for the extensive revisions of this paper. It will be an excellent addition to the literature.

Senior Editor:

The editors wish to thank the authors for these final adjustments to the manuscript. The article is now accepted for publication. Congratulations for an interesting and insightful study. Please consider the Journal of Physiology for your future studies.

REFEREE COMMENTS

Referee #2:

The authors have satisfactorily revised the manuscript, adequately addressing all specific comments. They should be commended for the substantial amount of work and data reflected in the study, which will serve as a valuable resource for physiologists in the cardiovascular and neuroscience fields.

2nd Confidential Review

08-Oct-2024
