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

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Abstract

Intrinsic cardiac neurons (ICNs) play a crucial role in the proper functioning of the heart; yet a paucity of data pertaining to human ICNs exists. We took a multidisciplinary approach to complete a detailed cellular comparison of the structure and function of ICNs from mice, pigs, and humans. Immunohistochemistry of whole and sectioned ganglia, transmission electron microscopy, intracellular microelectrode recording and dye filling for quantitative morphometry were used to define the neurophysiology, histochemistry, and ultrastructure of these neurons across species. The densely packed, smaller ICNs of mouse lacked dendrites, formed axosomatic connections, and had high synaptic efficacy constituting an obligatory synapse. At Pig ICNs, a convergence of subthreshold cholinergic inputs onto extensive dendritic arbors supported greater summation and integration of synaptic input. Human ICNs were tonically firing, with synaptic stimulation evoking large suprathreshold excitatory postsynaptic potentials like mouse, and subthreshold potentials like pig. Ultrastructural examination of synaptic terminals revealed conserved architecture, yet small clear vesicles (SCVs) were larger in pigs and humans. The

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{Donald B. Hoover} Design and direction of sectioned tissue IHC work.

{Leif A. Havton} Design and direction of TEM work.

{Janaki Patel} Assisted JDT with analysis of electrophysiological data as well as single cell segmentation and morphological analysis.

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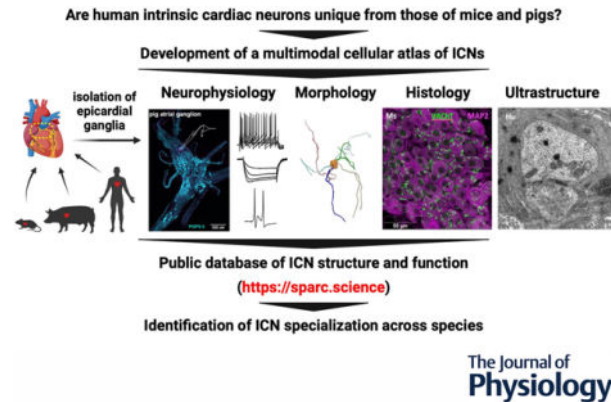
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presence and localization of ganglionic neuropeptides was distinct, with abundant VIP observed in human but not pig or mouse ganglia, and little SP or CGRP in pig ganglia. Action potential waveforms were similar, but human ICNs had larger after-hyperpolarizations. Intrinsic excitability differed; 95% of human neurons were tonic, all pig neurons were phasic, and both phasic and tonic phenotypes were observed in mouse. In combination, this publicly accessible, multimodal atlas of ICNs from mice, pigs, and humans identifies similarities and differences in the evolution of ICNs.

Graphical Abstract



Neurons clustered into ganglia at the heart's surface, referred to as intrinsic cardiac neurons (ICNs), supply innervation to the heart and control cardiac function. While studied in mammalian and non-mammalian species, data from human ICNs is largely unavailable. To fill this gap, we conducted a comprehensive study of ICNs from mice, pigs, and humans using a combination of biological techniques. Intracellular recording, dye filling of single neurons, immunohistochemistry of whole and sectioned ganglia, and transmission electron microscopy of single cells were used to generate a dataset encompassing the physiology, morphology, histochemistry, and ultrastructure of these neurons from each species. The resulting publicly available database identifies both similarities and differences in ICNs across mammals, highlighting structural and functional properties unique to humans.

Keywords

cardiac physiology; autonomic ganglia; neurophysiology; neurocardiology; neuromodulation; intracardiac neuron; ganglionic neurotransmission; homo sapiens

Introduction

The network of nerves on the heart's surface is essential to the control of cardiac function. At the heart's hilum, extrinsic nerves enter and splay across the epicardium, intermingling with neurons within epicardial ganglia, forming collectively an 'intrinsic cardiac nervous system' (ICNS)(Kuntz, 1934; Pauza *et al.*, 2000; Ardell & Armour, 2004). 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(Pauza *et al.*, 2002; Parsons & Dun, 2004; Wake & Brack, 2016). Functional interconnection of the ganglionic neurons is observed; a network interaction that may be subserved by local circuit neurons(Thompson *et al.*, 2000; Dusi & Ardell, 2020). The epicardial neurons in turn project to cells and tissues of the heart to regulate cellular function via neuroeffector junctions. This network constitutes the ultimate phase of cardiac autonomic control, encompassing neural components that provide final modulation of regional cardiac function(Randall & Wurster, 1994; Armour, 1999; Ardell, 2001; Parsons & Dun, 2004). Activity within the network modulates the initiation of each heartbeat at the sinoatrial node (SAN), the rate of impulse transmission through the atrioventricular (AV) node, and the amplitude of calcium transients within cardiac myocytes and vascular smooth muscle cells(Ardell *et al.*, 2016). This modulation maintains a life-sustaining cardiac output in alignment with both organ-specific homeostatic demands and the overall needs of the organism.

Dysfunction in this regulatory neural network, whether initiated by disease or injury, is detrimental to cardiac electrical and mechanical function and increases the risk for sudden cardiac death(Scherlag *et al.*, 2006; Vaseghi & Shivkumar, 2008; Florea & Cohn, 2014; Shen & Zipes, 2014; Wu & Vaseghi, 2020). Novel clinical approaches have arisen to selectively modulate cardiac neural control through manipulation of peripheral nerves (Shivkumar *et al.*, 2016; Hadaya & Ardell, 2020; Hadaya *et al.*, 2023). The epicardial nerves of the heart have been targeted using electrical, chemical, and/or genetic strategies(Herring *et al.*, 2019; Hadaya & Ardell, 2020).

An understanding of the underlying cell structure and diversity among species is essential for developing an informed approach for neuromodulation strategies targeting epicardial neurons. Here, we present a structural and functional atlas of intrinsic cardiac neurons (ICNs) at macro-, micro- and nanoscales from three species: mice, pigs, and humans. Our focus was on clusters of epicardial neurons within the right atrial ganglionated plexus (RAGP), which contain neurons essential to the regulation of heart rate at the SAN(Bluemel *et al.*, 1990; Randall *et al.*, 1998; Hanna *et al.*, 2021*a*). Through our investigation, we identified both conserved and derived attributes of these neurons among species. Adaptations in synaptic function, neuronal morphology, and neurochemistry revealed specificities in ICN structure and function across mammals, highlighting divergent specifications of human ICNs from those of mice and pigs. This study represents one of the first efforts to characterize human ICN membrane physiology. The research was made possible through collaboration among a multidisciplinary team and funded from the National Institutes of Health, specifically the Common Fund Program for Stimulating Peripheral Activity to Relieve Conditions (SPARC). Portions of this work were previously shared in poster and abstract form (Tompkins *et al.*, 2022*c*, 2022*a*).

Methods

Ethical Approval

Animal experiments were conducted in accordance with United States Federal Regulations as set forth in the Animal Welfare Act, the 2011 Guide for the Care and Use of Laboratory Animals (1), the Public Health Service Policy for the Humane Care and Use of Laboratory

Animals, the policies and procedures established by the University of California - Los Angeles (UCLA) Animal Research Committee (ARC) (UCLA ARC protocol #s 2016–033, 2016–084, 2016–085), These studies also conform to the principles and regulations as described in the editorial by Grundy(Grundy, 2015). Animals were provided ad libitum access to food and water, kept under a 12-hr light/dark cycle, and were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility.

Experiments using human tissue conformed to the standards set by the latest revision of the Declaration of Helsinki, except for registration in a database, and were approved by the UCLA Institutional Review Board (IRB). Tissues were procured consistent with organ donation preferences indicated by the donor and/or after signed consent was obtained from family members in compliance with the bioethical laws of the United States of America, the Uniform Anatomical Gift Act (2006), as well as UCLA policies and procedures for use of human tissue.

Isolation of epicardial ganglia

Adult C57BL/6J (Jackson Labs) mice ($n = 18$; 10 male, 8 female, age 10 ± 2 wks) were anesthetized deeply with isoflurane (5%) and exsanguinated. The thoracic cavity, including ribs, heart, and lungs, was removed and placed in ice cold physiologic salt solution (PSS) containing (in mM): 121 NaCl, 5.9 KCl, 1.2 NaH_2PO_4 , 1.2 MgCl_2 , 25 NaHCO_3 , 2 CaCl_2 , 8 D-glucose; pH 7.4 maintained by 95% O_2 - 5% CO_2 aeration. The heart was excised from the thorax and observed beneath a stereomicroscope to isolate epicardial ganglia by fine dissection. Connective tissue enveloping isolated ganglia, which were devoid of contracting myocardium, was pinned to the SylGard (Dow Corning) floor of a glass bottom petri dish.

Adult Yucatan minipigs (Premier Biosource; $n = 15$; 9 castrated male, 6 female; age 6 to 8 mo) were sedated with intramuscular telazol (4–6 mg/kg), intubated, and mechanically ventilated. General anesthesia was maintained with inhaled isoflurane (1.5–2.5%) and intravenous boluses of fentanyl (total: 10–30 $\mu\text{g/kg}$) during surgical preparation by way of median sternotomy to expose the heart. Major vessels were clamped, the heart was removed, rinsed in PSS, and the dorsal right atrium, at the junction of the superior vena cava (SVC) and the inferior vena cava (IVC), was dissected for subsequent dissection of epicardial ganglia beneath a stereomicroscope.

Human hearts ($n = 21$; 15 male, 6 female; aged 31 to 72yrs) were procured from deceased organ donors following a formal declaration of brain death. Hearts received were designated for biomedical research after being declined for cardiac transplantation. Deidentified organ donor demographic data is provided in Table 1. The hearts were transported in ice cold crystalloid cardioplegia (University of Wisconsin solution) and were received within six hours after aortic cross clamp. Upon receipt, a portion of the posterior right atrium containing the RAGP (Figure 1C₁) was immediately excised and the epicardial ganglia were isolated under a stereomicroscope by fine dissection. The isolated ganglia were pinned to the SylGard floor of a glass bottom petri dishes using methods identical to those for pig and mouse tissue.

Isolated ganglion preparations were transferred to the stage of an upright compound microscope (Zeiss AxioExaminer) equipped with differential interference contrast optics, an Axiocam camera, and a 5X air and a 40X water-immersion objective. Ganglia were superfused (6–7 ml/min; 5 ml chamber volume) continuously with PSS maintained at 34 ± 2 °C.

Intracellular microelectrode recording

Visually identified neurons were impaled with borosilicate glass microelectrodes filled with 2% neurobiotin (Vector Labs, Burlingame, CA) in 2.0 M KCl. Electrode tip resistances measured between 60 and 120 M Ω . Recordings of membrane potential were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in current clamp configuration. Data was acquired using a Digidata 1550B (Molecular Devices) analog-to-digital converter at a sampling rate of 5 or 20 kHz. The amplifier and data acquisition were controlled with pCLAMP software (Version 10, Molecular Devices).

Depolarizing and hyperpolarizing currents were injected through the recording electrode to determine responsiveness of the neuronal membrane. Small amplitude hyperpolarizing current steps (500 ms duration; –50 to –100 pA) were used to measure whole cell input resistance and the membrane time constant (τ). τ was measured by single exponential fit to the membrane potential change in response to the hyperpolarizing current (–50 or –100 pA). The instantaneous and steady-state membrane potential changes in response to large hyperpolarizing steps (500 ms duration; –200 to –500 pA) were also measured. Depolarizing current steps (500 ms duration; 50 to 600 pA) of increasing intensity were used to assess membrane excitability. Excitability curves were produced by plotting the number of action potentials evoked by depolarizing currents (Tompkins *et al.*, 2018). While the data denotes a continuous variable (spikes/time), they are presented as spike counts for both clarity and simplicity. Neurons were classified as either phasic (firing ≤ 4 spikes) or tonic (firing > 4 spikes continuously) based on the number of action potentials evoked by long-duration, supramaximal depolarizing currents (500 ms). The amplitude and duration of the action potential were measured from spontaneous or nerve evoked action potentials. The amplitude and duration of the after-hyperpolarizing potential was measured from action potentials evoked by brief intracellular current injection (5 ms duration). Cumulatively, the following membrane properties were assessed: resting membrane potential, rheobase, whole cell input resistance, τ , AP 1/2 width, AP duration, AHP amplitude, 2/3rds AHP duration. Neurons with RMPs less negative than –40 mV or neurons that did not produce action potentials were excluded from the analysis.

Synaptic potentials were evoked within intact ganglion preparations by focal stimulation of interganglionic nerves using extracellular, concentric bipolar electrodes (FHC; Bowdoin, ME). Graded shocks (50–800 μ A; 100 μ s) were delivered by an AMPI Master 8 and IsoFlex optical Stimulus Isolation Unit. Stimulus recruitment curves were generated by plotting the latency of the evoked potential against the stimulus current. Five to 20 stimuli were delivered at each stimulus intensity, at an interval of three seconds between stimuli. Latency was calculated from the onset of the command pulse to the upstroke of the evoked potential. 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. The amplitudes of spontaneous excitatory post-synaptic potentials (sEPSPs) were quantified using MiniAnalysis software (Synaptosoft, Inc.). The following synaptic properties were assessed: latency, spike following frequency, sEPSP amplitude.

Tissue fixation and IHC

After intracellular recording, ganglia were fixed by overnight immersion in 4% paraformaldehyde at 4 °C. The fixed tissue was rinsed in phosphate buffered saline (PBS) and stored in PBS + 0.02% sodium azide at 4 °C until subsequent immunohistochemical processing. For immunostaining of ganglion whole mounts, tissue was blocked in 0.01M PBS, 0.02% sodium azide, 0.1% Triton X-100, and horse serum for four hours at room temperature with agitation. The tissue was then incubated in primary antibodies, in a solution of 0.01M PBS, 0.02% sodium azide, and 0.1% Triton X-100, with agitation over two nights. Tissues were rinsed in a solution of 0.01M PBS, 0.02% sodium azide (1hr rinse, 3 rinses) and incubated in secondary antibodies, diluted in 0.01M PBS with 0.1% Triton X-100 and 0.02% sodium azide, over 48 hours at room temperature with agitation. Stained tissue received a final rinse in 0.01M PBS, 0.02% sodium azide (1hr rinse, 3 rinses) and was mounted on glass slides in anti-fade media (Citifluor, Electron Microscopy Sciences) and coverslipped. The source and concentration of primary and secondary antibodies is given in Table 2.

IHC of sectioned tissue

Tissue blocks of the RAGP fat pad were frozen on dry ice and stored at -80 °C until sectioning. Blocks were attached to specimen plates, cut at -20 to -25°C using a Leica CM3050S cryostat (Leica Microsystems Inc.), and 30 µm thick sections collected on adhesion slides. Tissues were sectioned in a plane parallel to the epicardial surface, and sections were collected in a sequence that yielded eight sets of sections that each spanned the entire thickness of the specimen. Each set of tissue sections was stored at -20 °C until further processing. Slide-mounted tissue sections were immunostained at room temperature for markers using standard methods of fluorescence IHC, as described previously (Kaestner *et al.*, 2019). Briefly, slides were rinsed with 0.01 mol/L PBS (pH 7.3), incubated for 10 minutes in 0.01 mol/L PBS containing 0.4% Triton X-100 and 0.5% bovine serum albumin (BSA), and blocked for 2 hours in 0.01 mol/L PBS containing 1% BSA, 0.4% Triton X-100, and 10% normal donkey serum (Jackson ImmunoResearch Laboratories). Each section was then incubated overnight in blocking buffer containing one or more of the primary antibodies listed in Table 2. Sections were washed again and incubated in blocking buffer before application of secondary antibodies. Species-specific donkey secondary antibodies conjugated to Alexa Fluor 488, 555, 594 or 647 (Jackson ImmunoResearch Laboratories) were applied at a 1:200 dilution in 0.01 mol/L PBS containing 0.4% Triton X-100 and 1% BSA, and sections were incubated for 2 hours before final washing in 0.01 mol/L PBS. After final washes with 0.01 mol/L PBS, cover glasses were applied with Citifluor (Ted Pella, Inc.) or SlowFade Gold antifade reagent (Life Technologies Corporation). Specific staining did not occur in negative control sections processed without the addition of the primary antibodies.

Slides were viewed under fluorescence illumination with an Olympus BX41 microscope equipped with an Olympus DP74 digital camera and cellSens software (Olympus America Inc., Center Valley, PA, RRID:SCR_016238). After localizing ganglia within sections, they were evaluated with a Leica TCS SP8 Confocal Microscope with 10x, 20x and 40x objective lenses (Leica Microsystems Inc.). Confocal images were collected at a resolution of 1024×1024 using 488 and 552nm laser lines. Stacks spanned tissue thicknesses of 25–33 μ m unless otherwise noted. Figures were created using maximum intensity projection (MIP) images for individual channels and merged images.

Whole ganglia were imaged with a confocal microscope (Zeiss LSM880) by tile-scanning in X, Y, and Z planes using a 63X objective (Zeiss Plan-Apo, 1.4NA). Z-stack images were acquired at step sizes consistent with Nyquist sampling in relation to the numerical aperture of the objective. Three-dimensional scans of whole ganglion neuroanatomy were acquired with 10X objectives. Images were quantified using ImageJ to measure neuronal cross-sectional areas (measured at Z-plane containing nucleus), and total cell counts per ganglia were quantified with ImageJ (FIJI distribution).

Quantification of cellular morphology

Neurobiotin filled neurons were serially imaged to visualize cell structure. For cases in which a neurobiotin labeled soma could not be located, or there were not enough dendrites labeled in the volume, the cell was omitted from further analysis. Neuronal structures were segmented, and quantified, using Neurolucida 360 and Neurolucida Explorer (MBF Bioscience). The file format of the traced structures was converted from .dat to .swc using HBP Neuron Morphology Viewer (Bakker *et al.*, 2017). SWC files were further quantified using the ImageJ plugin SNT (Arshadi *et al.*, 2021). Quantified values include the following: cell soma and dendrite area in X, Y and Z planes, soma volume, number of dendrite stems (originating branches from soma), number of dendrite bifurcations, number of dendrite branches, number of dendrite tips, total dendrite length, mean dendrite length, total dendrite surface area, total dendrite volume.

Transmission electron microscopy

Samples of whole-mount right atrial ganglion plexi (RAGPs) intended for electron microscopy (EM) analysis were carefully isolated from the heart using a dissection microscope and then submerged in a solution containing 2% paraformaldehyde (PFA) and 2.5% glutaraldehyde overnight at 4°C in a 0.12 mol/L Millonigs buffer (MB). After thorough washing with Millonigs buffer and double-distilled water (ddH₂O), the tissues underwent fixation in a 1% osmium solution diluted in ddH₂O, followed by a series of ethanol (EtOH) and propylene oxide dehydration steps. Subsequently, the tissues were embedded in a plastic resin (Epon, Ted Pella). Semi-thin cross-sections (0.5 μ m thick) were prepared and stained with a 1% toluidine blue solution diluted in ddH₂O to enable a general overview of RAGP organization under a light microscope (Nikon Eclipse E600 microscope equipped with a Nikon DS-Fi3 camera). Ultra-thin sections (70 nm thickness) were then mounted onto formvar-coated grids (Ted Pella), stained with uranyl acetate and lead citrate for contrast enhancement, and examined in a Tecnai G2 Spirit Twin transmission electron microscope (FEI, ThermoFisher Scientific) operating at 80 kV. Detailed characterization of

epicardial ganglion organization was performed, and images were captured using a Gatan Orius SC 1000B digital camera (Gatan) and SerialEM software (Schorb *et al.*, 2019).

Statistical analysis

Statistical analysis was performed using GraphPad Prism statistical software (version 9.2; La Jolla, CA). Data are presented as mean $\pm$ SD. Means of two independent groups were compared with an unpaired t test. Grouped data with two categorical variables and one continuous dependent variable were compared by two-way ANOVA and Šidák's multiple comparisons test. Means between three groups were compared with one-way ANOVA and Tukey's post hoc analysis. For datasets with missing values a mixed-effects analysis model was used. Values were considered statistically significant at $P \leq 0.05$.

Data Availability

Data associated with this study were collected as part of the Stimulating Peripheral Activity to Relieve Conditions (SPARC) program and are available through the SPARC Portal (<https://sparc.science/>) (RRID: SCR_017041) under a CC-BY 4.0 license.

Results

Macroscopic neuroanatomy of epicardial neurons

In mice, epicardial ganglia are found on the epicardial surface beneath a layer of connective tissue on the dorsal atrial surface near the pulmonary veins (Fig 1A). Approximately 4–7 large ganglia were identified following fixation and incubation with an antibody to the pan-neuronal protein PGP9.5 (Fig 1A). Counted ganglia (8 ganglia from 8 mice) contained 332 ± 142 neurons, with a mean ganglion area of $110 \pm 74 \text{ mm}^2$, and neuron area of $347 \pm 60 \text{ }\mu\text{m}^2$, giving a total neuronal density of 37 ± 21 neurons per $100 \text{ }\mu\text{m}^2$. From this data, we estimate the population of mouse ICNs to be between 1000 and 2400 neurons. This is the first quantification of ganglion cell numbers using whole isolated ganglia and three-dimensional imaging with confocal microscopy. Representative quantified ganglia are shown in Figure 1A. For single cell electrophysiology and morphology, we focused on neurons within the ganglion located at the merger of the superior and inferior vena cava, as indicated in Figure 1A.

In adult pig and human cardiac tissues, epicardial ganglia were more challenging to visualize given a thick layer of enveloping subepicardial fat. We focused on clusters of epicardial ganglia referred to commonly as the RAGP. The localization of the RAGP in pig and human hearts is shown in Figures 1B and 1C. Neurons from these ganglia primarily project to the right atrial myocardium and sinoatrial node (Bluemel *et al.*, 1990; Randall *et al.*, 1998; Hanna *et al.*, 2021a). Macroscopically, in pig and human, the RAGP appeared as an interconnecting web of nerve bundles with interspersed ganglia of varying size, consistent with the nomenclature of a 'ganglionated plexus' (Fig 1B₃ & C₂). This neuroanatomy, while conserved between animals, showed significant interspecies, and more subtly, inter-animal variability. The areas of individual ganglia, $168 \pm 200 \text{ mm}^2$ in pig (30 ganglia from 14 pigs) and $188 \pm 207 \text{ mm}^2$ in human (26 ganglia from 11 humans), were similar between pig and human; however, the cross-sectional area of the neuronal soma ($1140 \pm 207 \text{ }\mu\text{m}^2$ vs $1689 \pm$

242 μm^2) were smaller in pigs (298 ICNs in 14 ganglia from 9 pigs) compared to humans (521 ICNs in 7 ganglia from 7 humans). The density of neurons in pig (6 ganglia from 6 pigs) and human (7 ganglia from 7 humans) ganglia were similar (6.7 ± 1.2 neurons/100 μm^2 vs 3.0 ± 1.5 neurons/100 μm^2). While ganglion size was not significantly different between all three species, the number of ICNs per unit area was greater in mouse compared to pig ($P = 0.0034$) or human ($P = 0.0054$).

Immunohistochemical observations of neurotransmitter diversity

Cellular neurochemical expression was probed with rigorously validated antibodies (Table 2), in both ganglion sections and whole mounts. In all three species, intense punctate labeling of the vesicular acetylcholine transporter (VACHT) was observed surrounding most neuronal perikarya, supporting a ubiquitous preganglionic cholinergic innervation to these neurons. Intracellular VACHT+ labeling was observed in most, but not all neurons (Fig 2), indicative of a large population of post-ganglionic cholinergic neurons in epicardial ganglia from mice, pigs and humans.

Immunolabeling with an antibody to tyrosine hydroxylase (TH), a marker for noradrenergic neurons, revealed moderate to strong staining of the neuronal cytoplasm for some ICNs (Fig 2). While cell bodies were strongly positive for TH in each species, only within human ganglia did we clearly observe TH-immunoreactive (IR) nerve terminals encircling the postganglionic neurons (Fig 2).

In addition to adrenergic and cholinergic neurons, nitrergic neurons have also been identified within intracardiac ganglia (Parsons & Dun, 2004). Using an antibody to the neuronal isoform of nitric oxide synthase (nNOS) (Table 2), sparse cytoplasmic immunolabeling of nNOS was observed in several neurons from mouse and pig, but not human (Fig 2). No intraganglionic nNOS-IR fibers were observed in sections or whole mounts of cardiac ganglia from mice, pigs or humans.

Peptidergic neuromodulators are also present in peripheral ganglia of multiple species, including human (Hoover *et al.*, 2009a). Immunostaining for the sensory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) exposed striking differences across species. In mice, SP and CGRP are colocalized within nerve fibers surrounding neurons as well as within intracardiac nerve bundles; however, no CGRP, or SP-IR cell bodies were identified (Fig 3). Pig intracardiac ganglia showed little to no staining for CGRP or SP containing perineuronal fibers or cell bodies. We only identified a few dually positive fibers within the intracardiac nerve bundles. Human intracardiac ganglia showed colocalization of CGRP and SP, like mouse, and displayed a dense meshwork of perineuronal fibers, also as observed in mice. Two human ICNs out of approximately 100 appeared to exhibit immunoreactivity (IR) to these peptides, though a definitive confirmation of sensory neurons within intracardiac ganglia is lacking.

Neuropeptide Y (NPY) and vasoactive intestinal polypeptide (VIP) are two peptidergic co-transmitters commonly identified within sympathetic and parasympathetic neurons, respectively (Dalsgaard *et al.*, 1986; Pardini *et al.*, 1992; Richardson *et al.*, 2003). In the mouse, NPY-IR was observed in cell bodies, a few fibers, and was often colocalized with

TH in nerve fibers surrounding blood vessels (Fig 3). In the pig, few NPY-IR nerve fibers were identified. NPY was principally localized to cell bodies and some co-labeling with TH was observed (Fig 3). In human, a greater abundance of NPY-IR nerve fibers surrounding postganglionic neuronal somata was identified. Perineuronal VIP-IR nerve fibers were also significantly more abundant in human ganglia compared to pig or mouse. Within pig RAGP, only a few VIP-IR cell bodies were identified. In mouse, a few varicose VIP-IR fibers were observed.

Importantly, immunoreactivity for the peptides showed variability across ganglia. The above observations were inferred from imaging of 18 sections and 2 whole mounts from 3 mice, 27 sections and 2 whole mounts from 5 pigs, and 32 sections and 2 whole mounts from 4 humans.

Passive and active membrane properties

Intracellular microelectrode recordings from intact ganglion preparations were used to assess basic membrane properties of ICNs from each species (Fig 4). Recordings were obtained in identical extracellular solutions made at 34 ± 2 °C. The ganglion preparations rested for at least 1 hr in PSS before commencement of recording. Representative action potentials from each species are overlaid in Fig 4D and show similar initial rates of depolarization and repolarization. The mouse neurons had low resting membrane potentials (-51 ± 7 mV, $n = 31$), relative to pig (-57 ± 7 mV, $n = 43$) or human (-56 ± 5 mV, $n = 19$)(Fig 4E). 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). Action potential half-widths were similar between groups (Fig 4G). The overlaid after-hyperpolarizing (AHP) potentials illustrate the markedly larger AHP of human ICNs, which differed in amplitude but not duration between species (Fig 4H,I,J). The Human AHP amplitudes (-14.2 ± 4.0 mV, $n = 22$) were greater than those of mouse (-9.9 ± 2.7 mV, $n = 28$; $P < 0.0001$) or pig (-11.4 ± 2.6 , $n = 33$; $P = 0.0034$). The smaller mouse ICNs had higher whole cell input resistance (166 ± 88 M Ω , $n = 31$), in comparison to the larger pig (90 ± 58 M Ω , $n = 44$) and human (87 ± 35 M Ω , $n = 20$) neurons (Fig 4K). The threshold current for action potential general (rheobase) was less in mouse (60 ± 26 pA, $n = 20$ ICNs from 9 mice) compared to human (179 ± 70 pA, 14 ICNs from 10 humans; $P = 0.0124$) or pig (435 ± 161 pA, 29 ICNs from 10 pigs; $P < 0.0001$) neurons. The pig neurons required significantly more current to elicit an action potential compared to human ($P < 0.0001$).

Excitability of the post-synaptic membrane was assessed in response to long duration, depolarizing current steps (+50 to +800 pA, 500ms). Autonomic neurons are characterized based on the number of action potentials elicited by depolarizing stimuli(Cassell *et al.*, 1986). Phasic neurons fire short bursts of action potentials in response to long depolarizing current steps, while tonic neurons fire steadily throughout the depolarization. In mice, both phasic and tonic neurons were identified (Fig 5A). In pig, no tonic neurons were observed (0/40), and in the human, mostly tonic neurons (19/20) were observed (Fig 5B,C). Tonic human ICNs reached a peak firing frequency of 23 spikes/500ms (46 Hz) while mouse neurons peaked at twice that frequency (45 spikes/500ms; 90Hz). Excitability curves

showing the number of action potentials elicited by each depolarizing current step are shown for phasic and tonic neurons in each species (Fig 5D,E,F).

The membrane time constant, tau, was calculated from -100pA hyperpolarizing steps. Mouse neurons had the shortest charge time ($2.6 \pm 1.2\text{ ms}$, $n = 34$ ICNs from 12 mice) relative to human ($15.6 \pm 11.7\text{ ms}$, $n = 19$ ICNs from 10 humans; $P < 0.0001$) or pig ($9.7 \pm 8\text{ ms}$, 33 ICNs from 11 pigs; $P = 0.0011$). Despite being similar in size (based on neuron volume), pig neurons charged faster than human neurons in response to -100pA steps ($P = 0.0121$). Larger amplitude, long duration (500ms), hyperpolarizing steps (-100 to -600 pA) were given to assess responsiveness to membrane hyperpolarization. A difference between the instantaneous and steady-state voltage response (Fig 5D–F) indicated the presence of a hyperpolarization-activated non-selective cationic current, known to be mediated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels (Cuevas *et al.*, 1997), which opposes the hyperpolarization. This change in the hyperpolarization is commonly termed ‘sag’. A ‘sag’ or difference potential of greater than 2 mV was observed in 75% human ICNs, 33% of porcine ICNs, and 85% of mouse ICNs.

Both the A-type voltage-gated potassium channels and small conductance Ca^{2+} -activated potassium (SK) channels have been shown to affect intrinsic excitability of mammalian ICNs (Xi-Moy & Dun, 1995; Rimmer & Harper, 2006). 4-AP, an inhibitor of A-type channels, increased excitability of pig but not human or mouse ICNs (Fig 6A,B,C). 4-AP (1 mM), slowed repolarization of the action potential which was measured as an increased action potential half-width in mouse and human but not pig ICNs (Fig 6A,B,C). Apamin, an inhibitor of SK channels, significantly increased the number of action potentials elicited with depolarizing current steps in all three species (Fig 6D,E,F). In mouse and pig, phasic neurons were converted to tonically firing ones. After 5 mins, apamin (100 nM) significantly reduced the duration of the AHP in human ($130 \pm 34\text{ ms}$ before, $51 \pm 21\text{ ms}$ after, $P = 0.03$, $n = 3$ ICNs from 3 humans, paired t test) and mouse ($81 \pm 20\text{ ms}$ before, $12 \pm 2\text{ ms}$ after; $P = 0.02$, $n = 3$ ICNs from 3 mice, paired t test), but not pig ICNs ($62 \pm 8\text{ ms}$ before, $53 \pm 12\text{ ms}$ after; $P = 0.1$, $n = 3$ ICNs from 3 pigs, paired t test).

Nerve evoked potentials at intracardiac neurons

Brief (100 μs) stimulation of interganglionic nerves elicited orthodromic and antidromic potentials at ICNs from mice, pigs, and humans (Fig 7). Single stimuli elicited fast, excitatory potentials which, at mouse ICNs, were always suprathreshold for the generation of an action potential. These neurons exhibited a large excitatory post synaptic potential (EPSP) (Fig 7A). At pig and human ICNs, both subthreshold and suprathreshold EPSPs were observed (Fig 7B,C). At all inputs tested with the ganglion nicotinic blocker hexamethonium (500 μM), the potentials were blocked, almost completely, confirming activation of ganglion nicotinic receptors with acetylcholine as the principal mechanism for fast excitatory neurotransmission (data not shown). Antidromic potentials could be discerned from synaptic potentials in that they were all-or-none and were unaffected by hexamethonium.

To determine if the ganglionic neurons received multiple inputs, we used either graded stimuli at a single input, or, where possible, multiple ganglionic inputs were stimulated with independent extracellular electrodes. At pig ICNs, stimulation of ganglionic nerves evoked

fast synaptic potentials. Most of the nerve evoked responses (32/35) were orthodromic, with only three antidromic spikes. 8 of 28 synapses tested received two discrete inputs based on latency alone. As an example, the pig stimulus recruitment curve shown in figure 7B shows a short latency input in response to a 400 μ A current and a long latency input was revealed with a 100 μ A pulse. 9 of 15 neurons tested for dual inputs with multiple electrodes had them. At human ICNs, nerve evoked potentials were obtained in 8/20 ganglion preparations. 5/7 of the potentials were identified as antidromic. In 4/8 preparations, the synaptically mediated EPSPs were graded in nature, showed variability in synaptic latency, and were sensitive to inhibition by hexamethonium (500 μ M). Similar to mouse, a large suprathreshold EPSP was observed, which occasionally, due to the excitability of the post-synaptic membrane, elicited multiple spikes. In one cell, two synaptically-evoked responses were observed after stimulation of a single nerve (Fig 7C), with unique latency values that were both inhibited by hexamethonium, supporting the concept of convergent innervation, as observed for pig but not mouse ICNs. The frequency distributions of latency values are shown in the bottom panels of figure 7 A, B and C. Mouse latencies were tightly distributed around a mean value of 2.1 ± 0.7 ms, while pig and human latencies were more broadly distributed around means of 5.1 ± 2.2 ms and 24.5 ± 23.76 ms respectively. Upon stimulation of interganglionic nerves, latency values exceeding 20ms were observed in 3 human ganglion preparations indicating the possibility of multisynaptic pathways within the ganglion.

In addition to single stimuli, trains of stimuli (5, 10, and 20 Hz) were given to test for presence of slow potentials and to measure synaptic efficacy. At pig ICNs, of 18 neurons tested for slow potentials, 8/18 exhibited a slow hyperpolarization. No slow depolarizations were observed out of the 18 neurons tested. Synaptic efficacy tended to be very low at porcine ICNs, with fewer than 40% of the evoked potentials triggering spikes. In contrast, at mouse ICNs, the evoked potentials elicited spikes nearly 100% of the time, and at human ICNs multiple spikes were occasionally observed giving greater than 100% synaptic efficacy (Fig 7D).

Spontaneous EPSPs (sEPSPs) were observed in mouse, pig and human ICNs. sEPSPs are mediated by spontaneous vesicle fusion, release of transmitter, and quantal-like activation of post-synaptic receptors. Representative sEPSPs from mouse, pig and human ICNs are shown in Figure 7E. The sEPSPs observed at pig ICNs (2.4 ± 0.6 mV, $n = 8$) were smaller in amplitude than mouse (4.5 ± 1.1 mV, $n = 8$; $P < 0.001$) but were comparable to human sEPSPs (3.3 ± 1.1 mV, $n = 7$; $P = 0.16$)(Fig 7F). The durations, measured from 90% to 10% peak amplitude, of mouse sEPSPs (16.2 ± 3.4 ms, $n = 8$) were shorter than those from pig (38.3 ± 15.3 ms, $n = 8$) or human (64.3 ± 25.6 ms, $n = 7$), and the human sEPSPs lasted longer than pig (Fig 7G).

Morphologic properties of single ICNs

The segmented cellular morphologies of representative neurons from each species are shown in Figure 8. Mouse neurons (15 ICNs from 10 mice) were unique from pig or human ICNs in that few or no dendritic projections were observed with light microscopy. In only 2/15 neurons were any small dendrites observed. The small diameter neuronal cell bodies

projected a single axon which typically, though not always, exited the ganglion (Fig 8A₁₋₄). In contrast, the manually segmented neurons from pig (13 ICNs from 8 pigs) and human (11 ICNs from 9 humans) exhibited elaborate dendritic arbors consisting of multiple branches projecting from the soma (Fig 8B,C). Typically, these projections extended from the inner (epicardial facing) membrane. A few neurons located deeper within the ganglion had projections extending from both inner and outer membranes. Ganglion depth varied from 1 to 2 layers of neurons to as many as 7 layers of neurons with the greatest neuronal mass concentrated in the ganglion center. Axons were identified based on larger cross-sectional diameter of the initial segment, and as the longest projections which exited the ganglion to join a nerve bundle. These projections are labeled 'Ax' on the shown reconstructions (Fig 8). Axons appeared to originate near dendrites on the soma. Given that the ganglia were isolated from the whole heart, the eventual targets of the axons could not be determined. Some pig and human ICN axons showed small bifurcations near the soma, giving rise to one or two short collateral neurite projections (Fig 8B,C). The 3D confocal images and 3D cellular reconstructions are available for download (see Data Availability statement).

The measured morphological properties are presented in Figure 9. The height (Y axis), width (X axis), and depth (Z axis) of the dendritic arbors, including cell somas, was similar between pig and humans ICNs (Fig 9A,B,C). Both dendrite area and ICN soma volume were significantly greater in pig and human than mouse (Fig 9A,B,C,D). 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). The number of dendrite stems extending from the human ICNs was greater than for pig ICNs (Fig 9E). In mice, only 2 of 15 manually segmented ICNs had dendrites, each with three dendrite stems and a total dendrite length of less than 100 μm . The number of bifurcations and branches of human ICNs were greater than pig ICNs, but only the number of dendrite tips was significantly different from the pig neurons. Total dendrite length and were similar between the pig and human ICNs, both greater than mice. Despite the similar dendritic arbor size, human ICNs had more dendrite length close to the cell body, as shown with Sholl analysis of pig and human dendrite length (Fig 9k).

Across the population of manually segmented mouse, pig and human ICNs, some variability in neuronal morphology was observed. In mouse, we found that not all axons were fully matured and showed growth cones at the distal end of the axon (Fig 8A₁). From most neurons we observed only a single axon according to the classification given above. In pig, a couple of neurons appeared to lack any axon (Fig 8B₂), and there was one human cell in which an axon could not be identified. In human, two neurons lacked long dendritic projections and had only short dendritic projections constrained within an envelope around the soma (Fig 8C₂). Every effort was made to completely delineate neuronal morphology, although, truncation due to either incomplete filling or imaging cannot be excluded. Whether these unique morphologies relate to a difference in function have not been determined. Also, whether the long intraganglionic projections were definitively dendrites or might function as collateral axons cannot be determined at this time.

ICN ultrastructure

Ultrastructural organization of the intracardiac ganglia increased with complexity from mouse to human. The differences observed exceed a simple increase in cell size but include a reorganization of synaptic contact and a greater partitioning of the membrane in human and pig epicardial neurons. At mouse ICNs, synaptic specializations formed upon the somatic membrane forming axosomatic connections (Fig 10B). The synaptic boutons were filled with small clear vesicles (SCVs) and mitochondria (Fig 10B1,B2). However, large dense core vesicles (LDCVs) were also observed among the SCVs (Fig 10B1). The mouse did not show any dendritic projections from the ICN somata, validating the results from light microscopy. In stark contrast, at pig ICNs, no axosomatic specializations were identified out of 19 ICNs inspected with TEM (Fig 10C). Instead, multiple protrusions of the somatic cytoplasm into dendritic processes were observed (Fig 10E). Synaptic densities were challenging to locate but could be found at appositions between axonal and dendritic segments in areas adjacent to the neuronal cell bodies (Fig 10D,D1,F). These terminals were filled with mitochondria, SCVs, and a few LDCVs.

TEM of the somata of human ICNs revealed a nucleus with a prominent nucleolus and surrounding cytoplasm filled with varied quantities of vacuoles and lipofuscin granules (Fig 11). In total 54 neurons were examined. All images available on the online database. Some of the somata showed dendritic processes (Fig 11B1,D). As in pig, axosomatic synapses were not encountered in the human ICN samples. Instead, axodendritic connections were present (Fig 11E2). These axon terminals contained mitochondria, SCVs, and some LDCVs; and showed synaptic specialization with an electron dense active zone on the pre-terminal side of the synaptic cleft (Fig 11 E2), similar to axodendritic terminals in pigs.

The cross-sectional areas of the SCVs in ICNs of mice, pigs, and humans were quantified from high resolution TEM images. All tissues had been processed for ultrastructural studies using identical fixation and staining protocols. The mean area of mouse SCVs ($1437 \pm 89 \text{ nm}^2$, $n = 3$ terminals on 3 ICNs) was significantly smaller than SCVs at minipig ($2174 \pm 320 \text{ nm}^2$, $n = 3$ terminals on 3 ICNs) and human ICNs ($2136 \pm 115 \text{ nm}^2$, $n = 3$ terminals on 3 ICNs) (Fig 11 F). There was no difference in the size of minipig or human SCVs.

Discussion

We identified structural and functional specification of human ICNs relative to two commonly used laboratory mammals, mice and pigs. Mice offer many advantages given available molecular tools and genetic manipulation, while pigs offer a preclinical model with potential for cardiac xenotransplantation. Across species we identify: 1) a scaling of somatic innervation density inversely proportional to dendritic arborization, 2) unique species dependent profiles of ganglionic neuropeptide expression, and 3) unique regulation of intrinsic membrane excitability. These properties shape intracardiac neuronal processing which by extrapolation would be distinct among these species. Human ICNs share structural similarity with the larger ICNs from pigs but exhibit membrane properties and neuropeptide expression patterns more common to mouse. The human neurons showed hyperexcitable membranes, perineuronal sympathetic fibers, presence of VIP, the largest AHP amplitudes, greater proximal dendrite density, and large spontaneous synaptic potentials.

Changes in cell size and synaptic complexity among species has been shown for sympathetic(Purves & Lichtman, 1985) and parasympathetic neurons(Snider, 1987). Here we identify consistency with these scaling paradigms which now include data from human ICNs. The compact and densely organized ICNs of mice, have few if any dendrites observed with both light and electron microscopy. Accordingly, the density of axosomatic synapses increased. A reduced convergence of axonal inputs was also identified. This is consistent with the finding of greater somatic innervation at some sympathetic neurons which have reduced dendritic area(Forehand, 1985). The larger ICNs of pigs and humans grow elaborate dendritic arbors where convergence of axonal input was observed. We cannot say for certain that all long neurite projections were in fact dendrites or whether these may function as collateral axons to nearby neurons. Interestingly both 'dendrites' and axons appeared to originate from adjacent membrane locations. We also observed that axons from pigs and humans ICNs often bifurcated near the soma giving rise to short axonal collaterals. We cannot yet determine if these are functional connections to adjacent neurons. Given the unique morphology among large mammals, factors governing dendrite growth and maturation should prove informative to understanding factors regulating synaptic integration within the human heart.

Three classes of ICNs based on neurotransmitter contents are classically identified including cholinergic, adrenergic, and nitrergic neurons (*for review see* (Parsons & Dun, 2004)). The most abundant cell type in each species is cholinergic neurons, identified in this study by the presence of the vesicular acetylcholine transporter (VAChT). Neurons with immunoreactivity for TH are also identified, yet, from previous observations, not all TH+ neurons are believed to be functionally adrenergic, particularly in mice, due to the absence of proteins required for further synthesis and release of norepinephrine(Hoard *et al.*, 2008). TH-IR varicosities have not been identified surrounding mouse ICNs, indicating a limited convergence of sympathetic terminals onto intracardiac ganglia in this species(Bizanti *et al.*, 2023). Based on previous immunohistochemical studies, functionally adrenergic neurons are thought to exist within human intracardiac ganglia(Weihe *et al.*, 2005). We clearly identify TH-IR varicosities surrounding human ICNs, indicating the likely convergence of sympathetic nerves onto postganglionic neurons within human epicardial ganglia. We identified very few nitrergic neurons or fibers within ICNs of mice, pigs, or humans, though they were previously observed in human RAGP removed during bypass surgery(Hoover *et al.*, 2009a), and in guinea pigs(Conlon & Kidd, 1999; Calupca *et al.*, 2000). A recent study of human ICNs isolated during coronary artery bypass surgery from patients with and without atrial fibrillation (AF), identified an increased proportion of TH-IR ICNs with AF.(Ashton *et al.*, 2024) Remodeling of neurochemical expression in cardiac disease requires further study. These new data support unique functional roles of these classical fast neurotransmitters across species.

The expression and localization of neuropeptides was also unique between mice, pigs, and humans. The sensory neuropeptides SP and CGRP are found in some afferent neurons of DRG and nodose ganglia. SP causes slow depolarization at ICNs of rat, rabbit, and guinea pigs(Jiang *et al.*, 1982; Hardwick *et al.*, 1995, 1997; Tompkins *et al.*, 1999; Chang *et al.*, 2000). Interganglionic SP/CGRP fibers were identified in all three species, though perineuronal varicosities were only observed in mouse and human ganglia. The apparent

density of SP/CGRP fibers was also noticeably lacking in pig intracardiac ganglia. The peptides NPY and VIP are commonly identified within sympathetic(Herring *et al.*, 2012; Sharma *et al.*, 2023) and parasympathetic(Horackova *et al.*, 1999; Richardson *et al.*, 2003) neurons, respectively, and can modulate ICN activity(Pardini *et al.*, 1992; Cuevas & Adams, 1996; Kennedy *et al.*, 1998; Liu *et al.*, 2000; Hoover *et al.*, 2009b). A network of nerve fibers immunoreactive for NPY were abundant in human ganglia, but lesser so in ganglia from mice or pigs. NPY+ ICNs were identified in all three species suggesting that NPY might be co-released from intracardiac neurons within the heart. Ganglion fibers immunoreactive for VIP were also much more prevalent within human ganglia, with little to none observed in pigs and only a few VIP+ fibers observed in mice. Importantly, the presence of the peptide does not, in and of itself, demonstrate a functional role of these molecules. Additional work is necessary to confirm the unique expression profiles, localization, and activities of these peptides within human epicardial ganglia.

Like all neurons, the excitability of ICNs is dictated by the expression, localization, and activity of ion channels in the plasma membrane. Much work has identified roles for ionic conductances in the regulation of intrinsic excitability of ICNs from mice(Harper & Adams, 2021), rats(Seabrook *et al.*, 1990; Selyanko, 1992; Selyanko & Skok, 1992a, 1992b; Xu & Adams, 1992a, 1992b; Xi-Moy & Dun, 1995; Cuevas *et al.*, 1997), guinea pigs(Hardwick *et al.*, 1995; Merriam *et al.*, 2004; Tompkins *et al.*, 2006), pigs(Smith *et al.*, 1992; Smith, 1999; Hanna *et al.*, 2021a; Tompkins *et al.*, 2022b), and dogs(Xi-Moy *et al.*, 1993; Xi *et al.*, 1994; Smith *et al.*, 2001b, 2001a; Scornik *et al.*, 2006); however, there has been no data identifying the conductances regulating human ICN excitability. Neuronal excitability is regulated in part by the non-inactivating voltage-dependent K^+ current I_M , the hyperpolarization-activated cationic conductance I_h , and the transient outward K^+ current I_A (Xi-Moy & Dun, 1995). In all three species, membrane hyperpolarization evoked the characteristic ‘sag’ in membrane potential indicative of activation of I_h . This first identification of H-current in human ICNs is clinically relevant given a prescribed use of the H-current blocker ivabradine for heart failure(MEERS *et al.*, 2016), where it inhibits I_h , also known as I_f , at cardiac myocytes(Thollon *et al.*, 1994). Ivabradine would likely have an effect at human ICNs. Additional work is necessary to identify the HCN channel subtypes expressed. Inhibition of A-current with 4-AP markedly prolonged action potential half-width in mouse and human ICNs. In pig, conversely, 4-AP significantly increased AHP amplitude. A-current inhibition had little effect on human ICN excitability, moderately increased pig ICN excitability, and produced mixed effects on the mouse neurons with both increases and decreases in excitability observed. Inhibition of SK channels with apamin greatly increased the number of spikes triggered by depolarization in all three species, identifying an important functional role for the small-conductance Ca^{2+} -activated K^+ current in regulation of membrane excitability. Sk-channel inhibition had a significant impact on the duration of the AHP at mouse and human neurons, but less so at pig neurons. These results identify greater similarity in the underlying A- and SK-current of human and mouse neurons compared to pig ICNs. Indecently, after inhibition of SK-channels, mouse neurons reached a maximum firing rate of 60hz while pig and human neurons peaked at 20Hz and 26Hz, respectively. This suggests, that while the general ionic conductances are conserved, the kinetics of these channels may be uniquely tuned within each species, with mouse ICNs

capable of the highest frequency of nerve activity. This higher frequency of nerve activity is likely associated with the higher intrinsic heart rate and greater need for high frequency vagal firing in mice.

Electron microscopy identified both similarities and differences in the ultrastructural organization of ICNs between species. Synaptic terminals on ICNs of mouse, pig, and human contain SCVs and, similar to previous reports based on TEM studies in these species (Ellison & Hibbs, 1976; Armour *et al.*, 1997; Pauziene & Pauza, 2003; Hanna *et al.*, 2021a). However, in mice, the synaptic terminals are situated on the ICN somata, whereas in pigs and humans synapses are in apposition to dendrites. Similar axodendritic synapses have previously been reported in human ICNs (Armour *et al.*, 1997). A close relationship was also demonstrated across the studied species between the ICNs and glial processes. Similar to prior studies, satellite cell nuclei were readily identified around ICN somata and their glial processes extending to form near apposition with the ICN cell membranes (Armour *et al.*, 1997; Hanna *et al.*, 2021a). Similar glial processes were also in close proximity to axodendritic synaptic contacts in pigs and humans. Although the synaptic terminals in ICNs shared many ultrastructural features between species, studies here of SCV size showed significantly larger SCVs in pigs and humans compared to mice, indicating species differences in transmitter quantities or packaging. Functionally, the amplitude of spontaneous postsynaptic potentials (i.e. quantal-like events) were larger but briefer in duration in mouse, whereas in pig a human, where synaptic contact is made on high-resistance dendrites, the events had smaller amplitude and longer duration.

It is important to consider the impact of cardiac disease on the functional and structural properties of human ICNs reported here. Cardiac disease can impact ICN function. For example, several studies report that myocardial infarction and heart failure cause remodeling of ICNs and ganglionic neurotransmission. (Bibevski & Dunlap, 1999, 2004; Hardwick *et al.*, 2008, 2012, 2014; Tompkins *et al.*, 2022b) Human ICNs in this report came from donor hearts denied for transplant. Hearts may be excluded from transplant due to multiple factors including presence of cardiac disease, drug use, or congenital abnormality (Table 1). Given the diversity in cardiac disease pathology and the relatively small number of cells studied, we have not attempted to make comparison between diseased and non-diseased ICNs. A recent study by Ashton *et al.* studied both morphology and physiology of ICNs removed from human patients, both with and without atrial fibrillation, during coronary artery bypass surgery. (Ashton *et al.*, 2024) The ICNs of this study ($n = 8$) had fewer primary dendrites (aka stems), fewer branches and shorter total neurite length compared to the human ICNs reported here. Membrane physiology appears mostly consistent between the studies, though Ashton *et al.* report shorter AP and AHP amplitude, and higher input resistances. Some differences between these studies may be attributable to recording methodology. Whole-cell patch-clamp can washout molecules associated with ion channel function (e.g. I_h , I_M). It appears consistent that most if not all human ICNs are tonically firing, though disease (i.e. atrial fibrillation) may influence spike frequency. All data from the current study are publicly available to facilitate future comparison to data from diseased and non-diseased ICNs.

In summary, the importance of the intrinsic cardiac nervous system and its cellular components is well recognized and is increasingly being targeted in the management of human heart disease including atrial fibrillation, ventricular arrhythmias, and heart failure (Scherlag *et al.*, 2006, 2009; Vaseghi & Shivkumar, 2008; Florea & Cohn, 2014; Shen & Zipes, 2014; Shivkumar *et al.*, 2016; Wu & Vaseghi, 2020; Hanna *et al.*, 2021b). Post-synaptic autonomic receptors at the heart have been the mainstay of cardiovascular pharmacology for the last half century. Increasingly more targeted approaches to manipulate the upstream neural circuitry, including the ICNs (Tsuboi *et al.*, 2002; Steinberg & Romanov, 2022), preganglionic fibers (Hadaya *et al.*, 2023), or afferent fibers (Yoshie *et al.*, 2019), are in clinical and preclinical testing. The use of human tissues is important to ensure that the findings obtained in research animals can be generalizable to human systems. The identification of ICN specialization across species described herein identifies areas for greater need regarding the understanding of dendrite and synapse formation, the ionic currents regulating membrane excitability, and the actions of cardiac neuropeptides unique to humans. While we are just beginning to scratch the surface of the many subtleties at the cellular level of human ICNs, we have made a significant step forward in the understanding of the uniqueness of these neurons among species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ICNs	Intrinsic Cardiac Neurons
VIP	Vasoactive Intestinal Polypeptide
SP	Substance P
CGRP	Calcitonin Gene-Related Peptide
SAN	Sinoatrial node
AV	Atrioventricular

RAGP	Right Atrial Ganglionated Plexus
SPARC	Stimulating Peripheral Activity to Relieve Conditions
PSS	Physiologic Salt Solution
SVC	Superior Vena Cava
IVC	Inferior Vena Cava
IHC	Immunohistochemistry
sEPSPs	Spontaneous Excitatory Post-Synaptic Potentials
PBS	Phosphate Buffered Saline
VACHT	Vesicular Acetylcholine Transporter
TH	Tyrosine Hydroxylase
IR	Immunoreactive
VMAT2	Vesicular Monoamine Transporter 2
nNOS	Neuronal Isoform of Nitric Oxide Synthase
NPY	Neuropeptide Y
AHP	After Hyper-polarization
EPSP	Excitatory Post-Synaptic Potentials
SCVs	Small Clear Vesicles
LDCVs	Large Dense Core Vesicles
DRG	Dorsal Root Ganglia
ICG	Intrinsic Cardiac Ganglia

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Key Points

- Intrinsic cardiac neurons (ICNs) are essential to the regulation of cardiac function.
- We investigated the neurochemistry, morphology, ultrastructure, membrane physiology and synaptic transmission of ICNs from donated human hearts in parallel with identical studies of ICNs from mice and pigs to create a publicly accessible cellular atlas detailing the structure and function of these neurons across species.
- In addition to presenting foundational data on human ICNs, this comparative study identifies both conserved and derived attributes of these neurons within mammals.
- The findings have significant implications for understanding the regulation of cardiac autonomic function in humans and may greatly influence strategies for neuromodulation in conditions such as atrial fibrillation and heart failure.

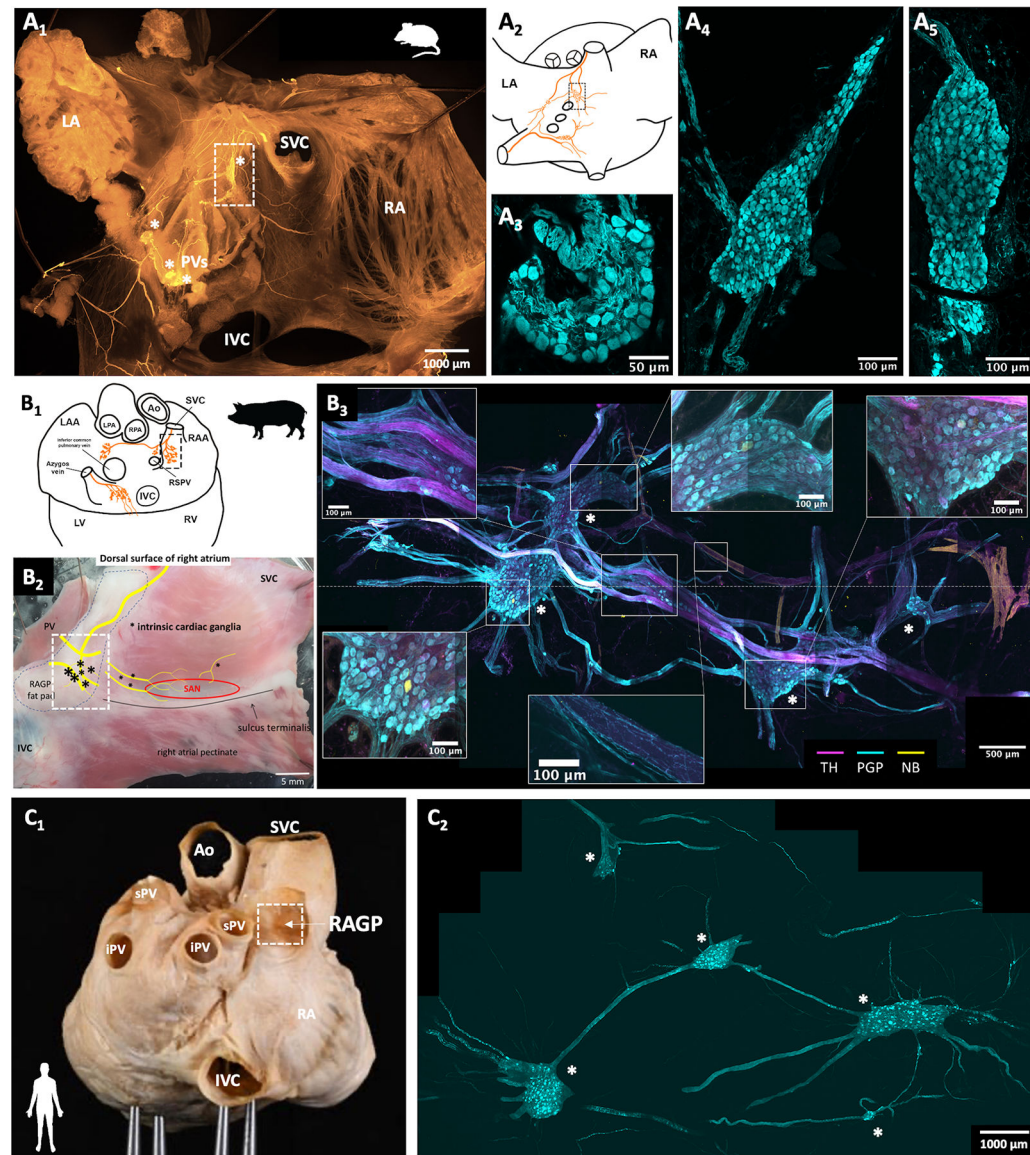


Figure 1. The right atrial ganglionated plexus (RAGP) of mice, pigs and humans.

(A₁) Fluorescent immunolabeling of PGP9.5 in fixed tissue shows intrinsic and extrinsic nerve fibers, and epicardial ganglia (*) on the dorsal surface of the mouse atria. Abbreviations: left atrium (LA); right atrium (RA); inferior vena cava (IVC); superior vena cava (SVC); pulmonary veins (PVs). (A₂) Ganglion locations illustrated on mouse heart drawing. Boxed region shows location of mouse RAGP. (A₃₋₅) Three mouse RAGPs, isolated from boxed regions identified in A₁&A₂, showing immunofluorescence for PGP9.5. (B₁) Cartoon illustrates location of epicardial ganglia on minipig dorsal atria. (B₂) Photo of right atrial dorsal epicardium indicating location of RAGP identified by boxed regions shown in B₁&B₂. Approximate location of ganglia (*), nerves (yellow lines), and the sinoatrial node (red oval) are shown. (B₃) An RAGP isolated from the area shown in B₂. Immunofluorescence to PGP9.5, tyrosine hydroxylase (TH), and injected neurobiotin (NB) are shown. Insets show greater magnification of identified areas. (C₁) Photo of a human

heart after perfusion fixation identifies location of human RAGP isolation. Abbreviations: superior vena cava (SVC); inferior vena cava (IVC); inferior pulmonary vein (iPV); superior pulmonary vein (sPV); right atrium (RA); aorta (Ao). (C₂) Photomicrograph of an RAGP isolated from a human heart showing immunofluorescence to PGP9.5. Five epicardial ganglia (*) connected by interganglionic nerves are shown.

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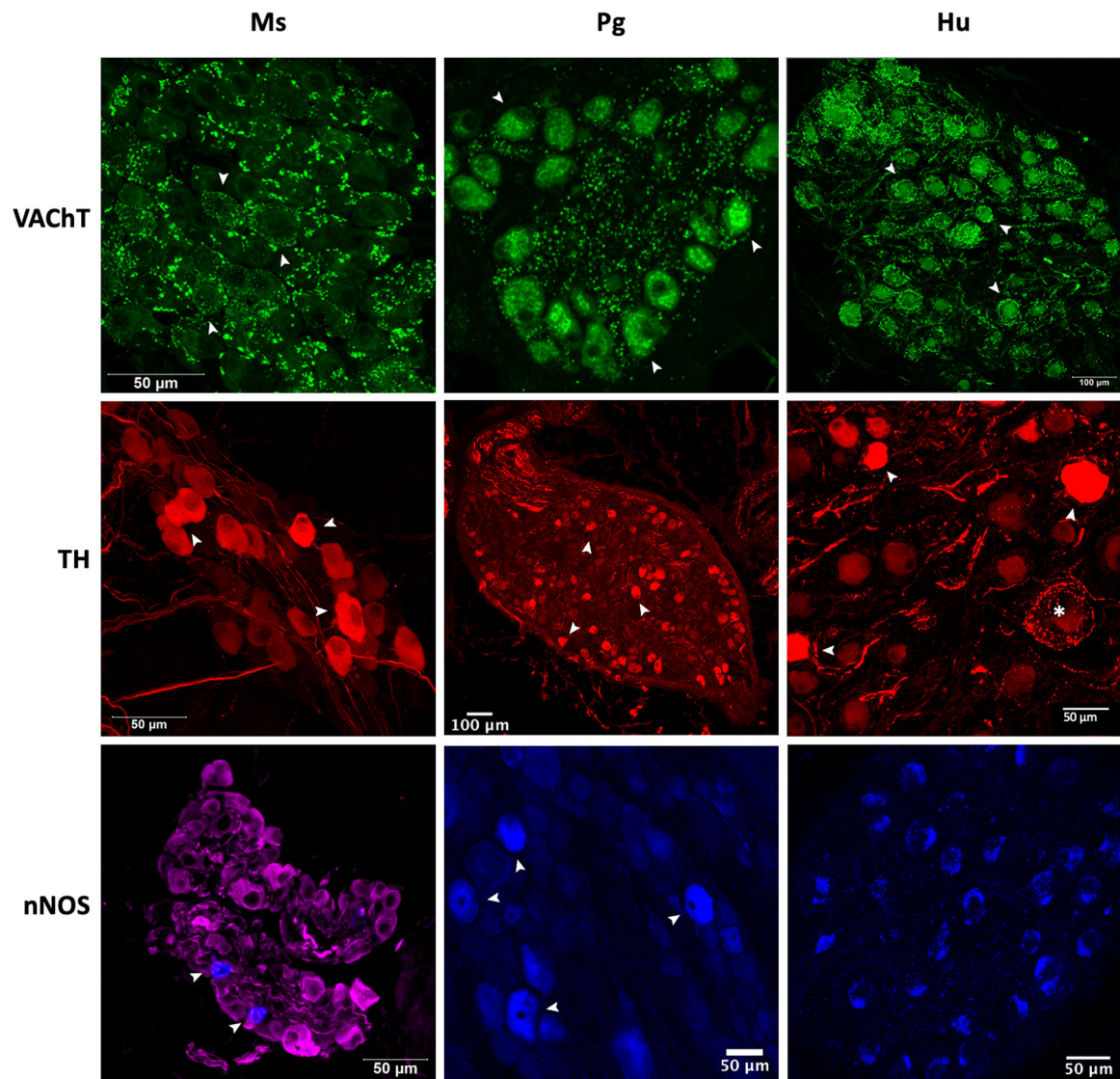


Figure 2. Fluorescently immunolabeled ganglia from mice, pigs and humans showing presence and localization of cholinergic, adrenergic and nitrergic proteins.

Confocal photomicrographs of sectioned and whole-mount ganglia show representative stainings for the indicated proteins (vesicular acetylcholine transporter (VAcHT); tyrosine hydroxylase (TH); neuronal nitric oxide synthase (nNOS)). Top panel arrows indicate pericellular, cholinergic varicosities and VAcHT+ cell bodies in each species. Middle panel arrows indicate TH+ neurons. The asterisk in the human RAGP section indicates a cell with pericellular localization of TH+ nerves. Arrows in bottom panels indicate nNOS+ neurons. Note that the staining observed in the human tissue was autofluorescence from lipofuscin. No nNOS+ neurons were identified in human ganglia.

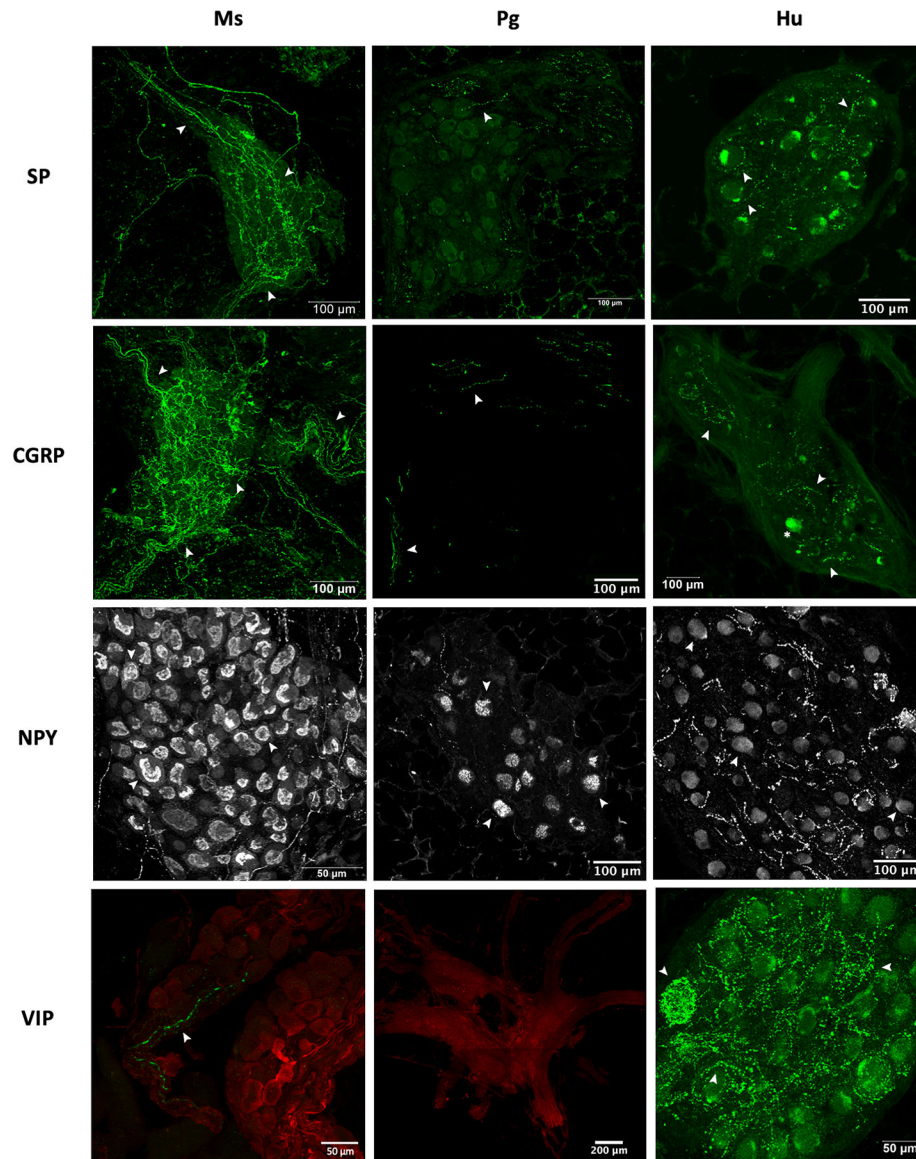


Figure 3. Immunolabeled ganglia from mice, pigs and humans show presence and localization of the indicated neuropeptides.

Confocal photomicrographs of sectioned and whole-mount ganglia show representative stainings for the indicated proteins (substance P (SP); calcitonin gene related peptide (CGRP); neuropeptide Y (NPY); vasoactive intestinal polypeptide (VIP)). White arrows in top two row indicated representative SP+ or CGRP+ nerve fibers. The asterisks in human ganglion section of second row indicates a putative CGRP+ neuron. Note apparent staining in cytoplasm of human neurons (top row) was autofluorescence from lipofuscin. Arrows in third row show NPY+ neuronal cell bodies. Arrows in bottom row indicate VIP+ intraganglionic nerve fibers.

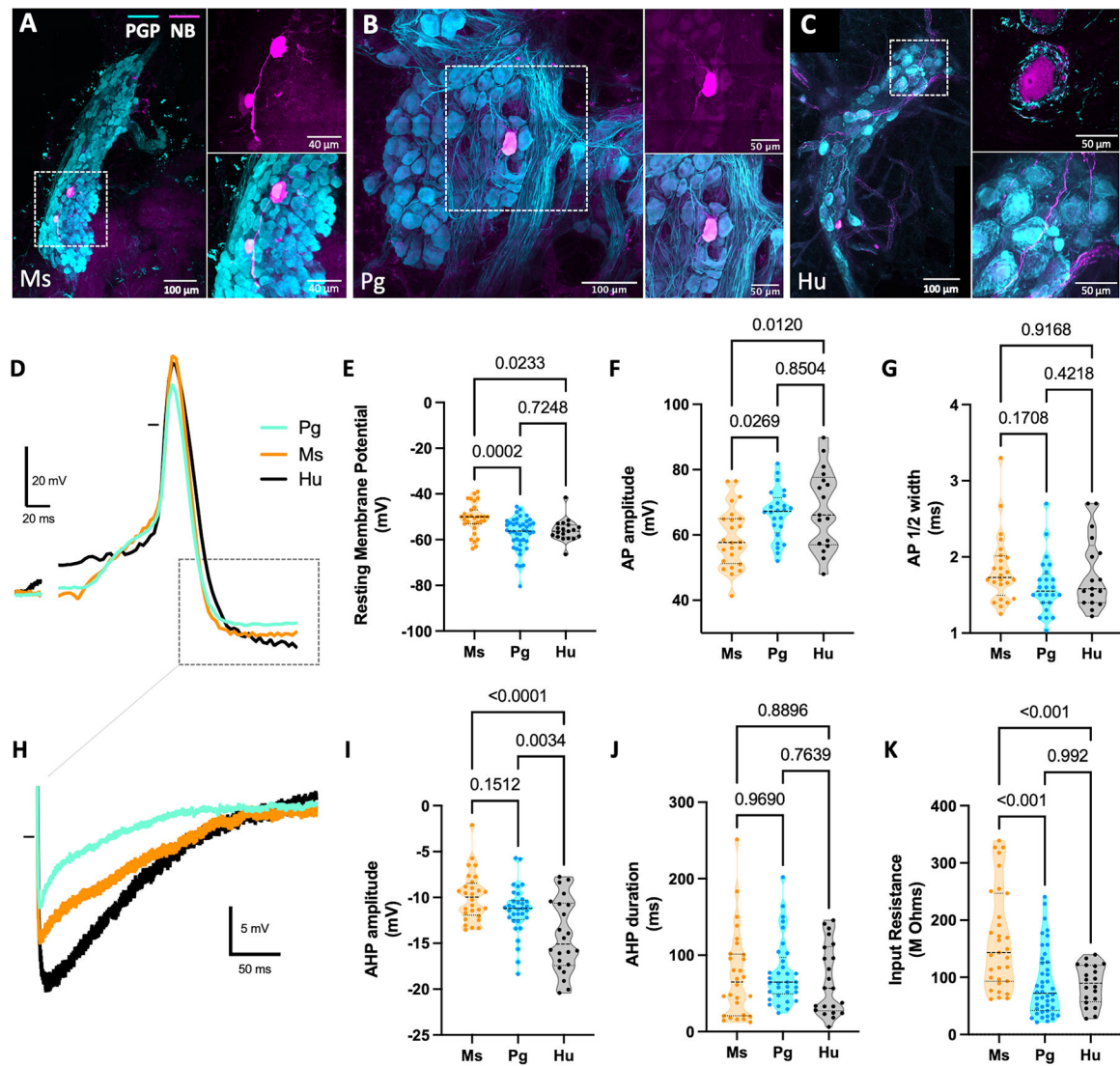


Figure 4. Passive and active membrane properties of ICNs recorded with intracellular microelectrodes.

(A-C) Confocal images of right atrial ganglia isolated from a mouse (A), a minipig (B), and a human (C). ICNs were visualized with fluorescently labeled antibodies to PGP9.5 (cyan) and intracellularly injected neurobiotin (magenta). White boxed area in left panels show at higher magnification to right. (D) Overlaid action potentials from a mouse (orange), a pig (cyan), and a human (black) ICN. (E) Resting potentials of mouse neurons ($n = 32$) were less negative than pig ($n = 33$) or human ($n = 14$) ICNs. (F) Action potential amplitudes were greater in pig ($n = 21$) and human ($n = 13$) ICNs compared to mice ($n = 21$). (G) The durations of these action potential were similar between species. (H) After-hyperpolarizations (AHPs) following the action potentials in (D) are shown. (I) The AHPs recorded from human ICNs ($n = 15$) were greater in amplitude than either mouse ($n = 28$) or pig ($n = 23$) AHPs. (J) The durations of these AHPs varied greatly across species, with both short and long-lasting AHPs observed. There was not difference in mean amplitude between species. (K) The smaller mouse ICNs ($n = 31$) had higher input resistance than pig ($n = 33$)

or human (n = 14) ICNs. Measurements were made in the indicated number of neurons (n) in 12 mice, 11 pigs and 10 humans. P values computed by Tukey post-hoc analysis after ANOVA.

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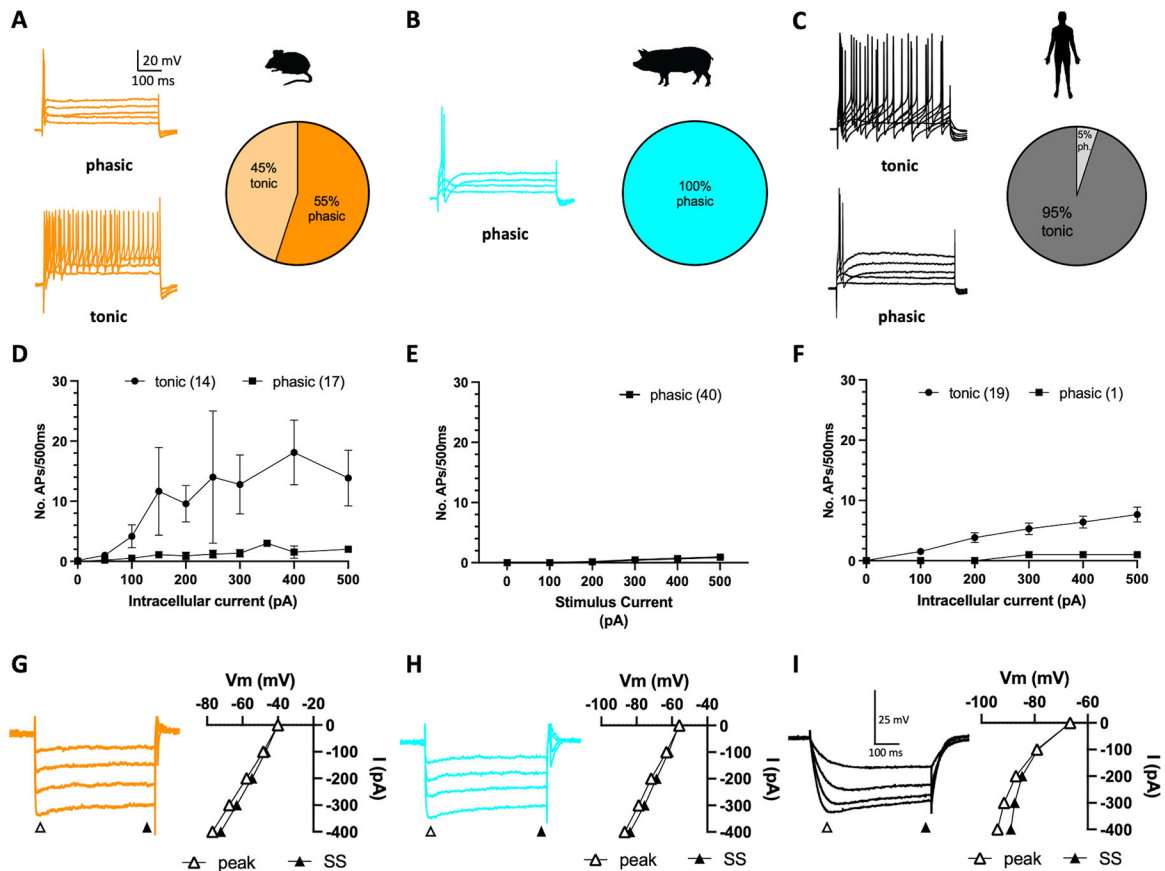


Figure 5. Regulation of membrane excitability varies across species.

(A) In mice, phasic and tonic neurons were identified from 31 neurons sampled in 14 mice). (B) In pig, no tonic neurons were observed out of 40 ICNs sampled from 15 animals. (C) In humans, all but one cell was tonically firing out of 20 ICNs sampled from 13 donor hearts. (D-F) Membrane excitability curves show number of action potentials elicited by depolarizing current steps of phasic and tonic neurons in each species. (G-I) Representative membrane potential responses to hyperpolarizing current (-100 to -400 pA) are shown. The peak (open triangle) and steady-state (closed triangle) change in membrane potential is plotted at the right. Triangles indicate where the peak and steady-state (SS) membrane potential changes were measured. Sag in the hyperpolarization, likely caused by activation of H-current, was observed in all three species.

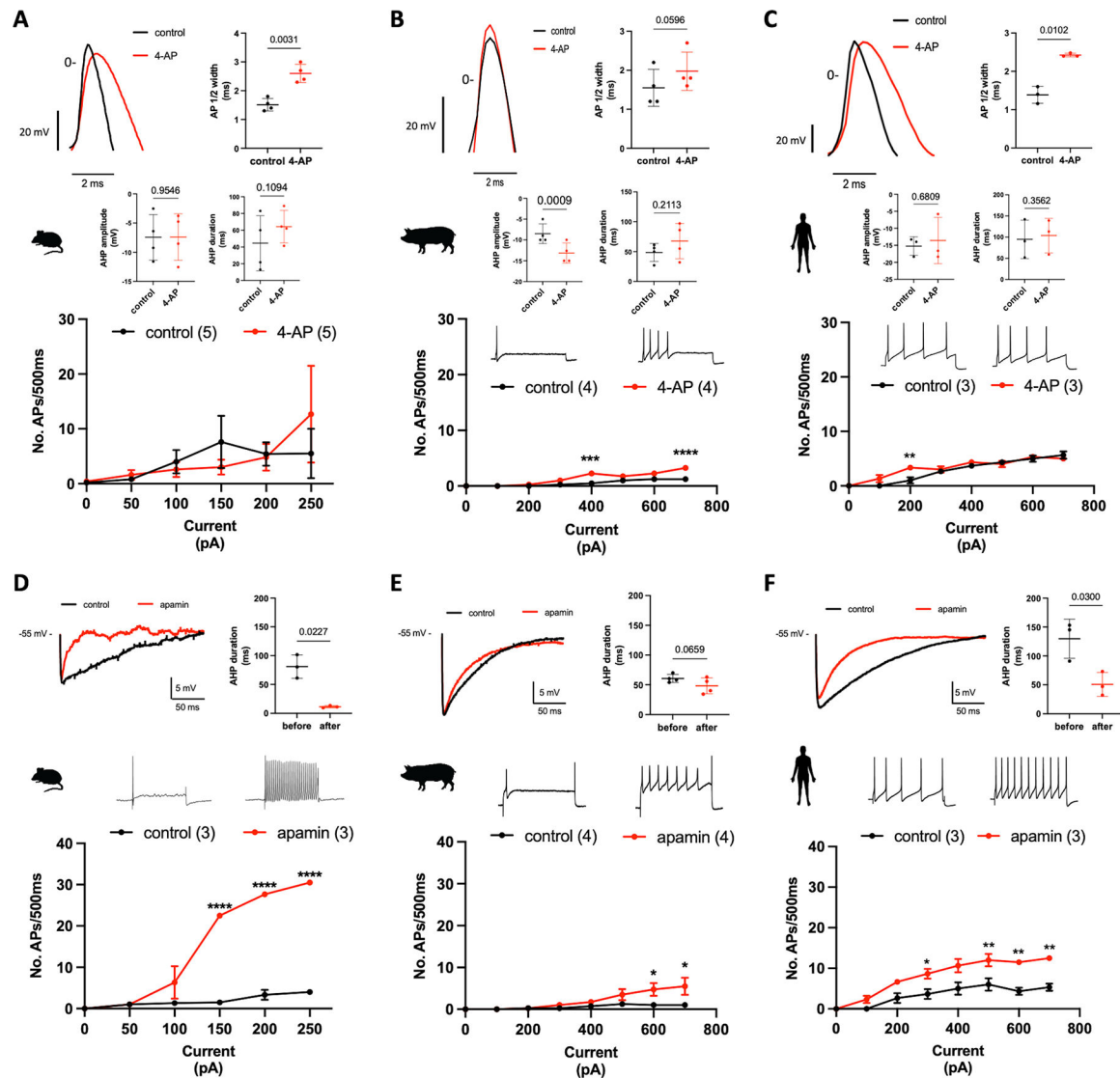


Figure 6. Pharmacological antagonism of A- and SK-currents produce differential effects in mouse, pig or human ICNs.

The effects of two K^+ -channel inhibitors were tested on the mammalian ICNs. (A) In mice, 1mM 4-AP, an inhibitor of A-current, prolonged action potential half-width but did not affect AHP amplitude or duration. Both increases and decreases in membrane excitability were observed in 4 neurons tested from 4 mice. (B) In pigs, 4-AP had no effect on action potential half-width, but increased AHP amplitude and membrane excitability. (4 neurons from 4 pigs) (C) At human ICNs, 4-AP increased action potential half-width, but did not affect the AHP or membrane excitability. (3 neurons from 3 humans) (D) 100nM apamin consistently decreased AHP duration in 3 neurons from 3 separate mice. A robust change in membrane excitability was also observed, as evidenced by the increased slope of the excitability curve five minutes after apamin application (red lines). (E) At pig ICNs, apamin shortened the AHP, although just short of statistical significance. Excitability was increased in 4 neurons from 4 animals tested. (F) At human ICNs, apamin significantly decreased AHP duration and increased the number of spikes elicited with depolarizing stimuli (3

neurons from 3 humans). For paired analyses, P values were computed by student's t test. For excitability curves, P values were computed by Šídák post-hoc analysis after Two-Way ANOVA. Asterisks indicate * $P < 0.05$, ** $P < 0.0332$, *** $P < 0.0021$, **** $P < 0.0001$.

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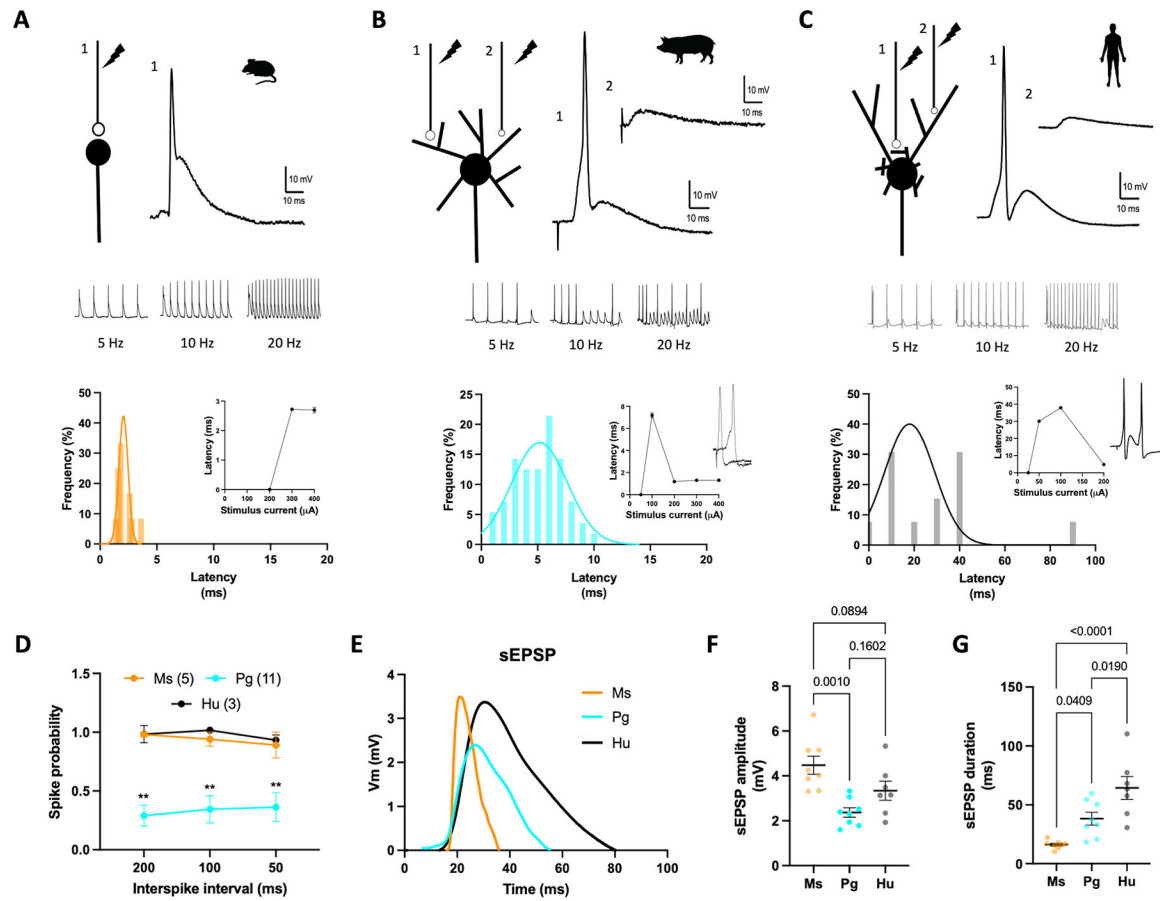


Figure 7. Assessment of ganglionic neurotransmission between species.

Stimulation of preganglionic nerves evoked fast excitatory synaptic potentials (EPSPs) at mouse (A), pig (B) and human (C) ICNs. Representative responses are shown adjacent to a schematic illustration of synaptic connectivity as determined by light and electron microscopy. In mouse, single stimuli evoked suprathreshold EPSPs, while at pig and human ICNs, graded stimulation evoked both supra- and subthreshold EPSPs, providing evidence for convergence of cholinergic inputs. Synaptic potentials were blocked by the ganglion nicotinic antagonist hexamethonium (500 μ M, data not shown). Shown below these traces are representative trains of stimuli, tested at multiple frequencies. Below these traces, latency times for each species are shown as a frequency histogram for all neurons with nerve evoked synaptic potentials, as well as a representative stimulus recruitment curve in each species. At pig and human ICNs, titration of the extracellular stimulus current revealed inputs with unique latency values, supporting, in part, innervation by multiple inputs. The distribution of latency values increased greatly in the large mammals, with some nerve evoked action potentials observed 90ms after the preganglionic stimulation. (D) The probability of a stimulation evoked action potential was measured for each interspike interval. Multiple failures for spike generation were observed at pig ICNs resulting in low synaptic efficacy at these synapses. Measurements were made from 9 mouse ICNs in 5 mice, 11 ICNs in 7 pigs, and 3 ICNs in 3 human hearts. P values computed by Tukey post-hoc analysis after Two-Way ANOVA. Asterisks indicate ** $P < 0.0332$. (E) Representative

tracings for an average sized spontaneous EPSPs (sEPSPs) from a mouse, a pig and a human ICN. Note that pig sEPSPs were significantly smaller than mouse potentials (F), while mouse sEPSPs had shortest duration and the spontaneous mini potentials at human ICNs had the longest duration. Sample size includes mean sEPSP amplitudes from 8 ICNs of 8 mice, 8 ICNs of 7 pigs, 7 ICNs of 7 human hearts. P values computed by Tukey post-hoc analysis after ANOVA, mixed-effects model.

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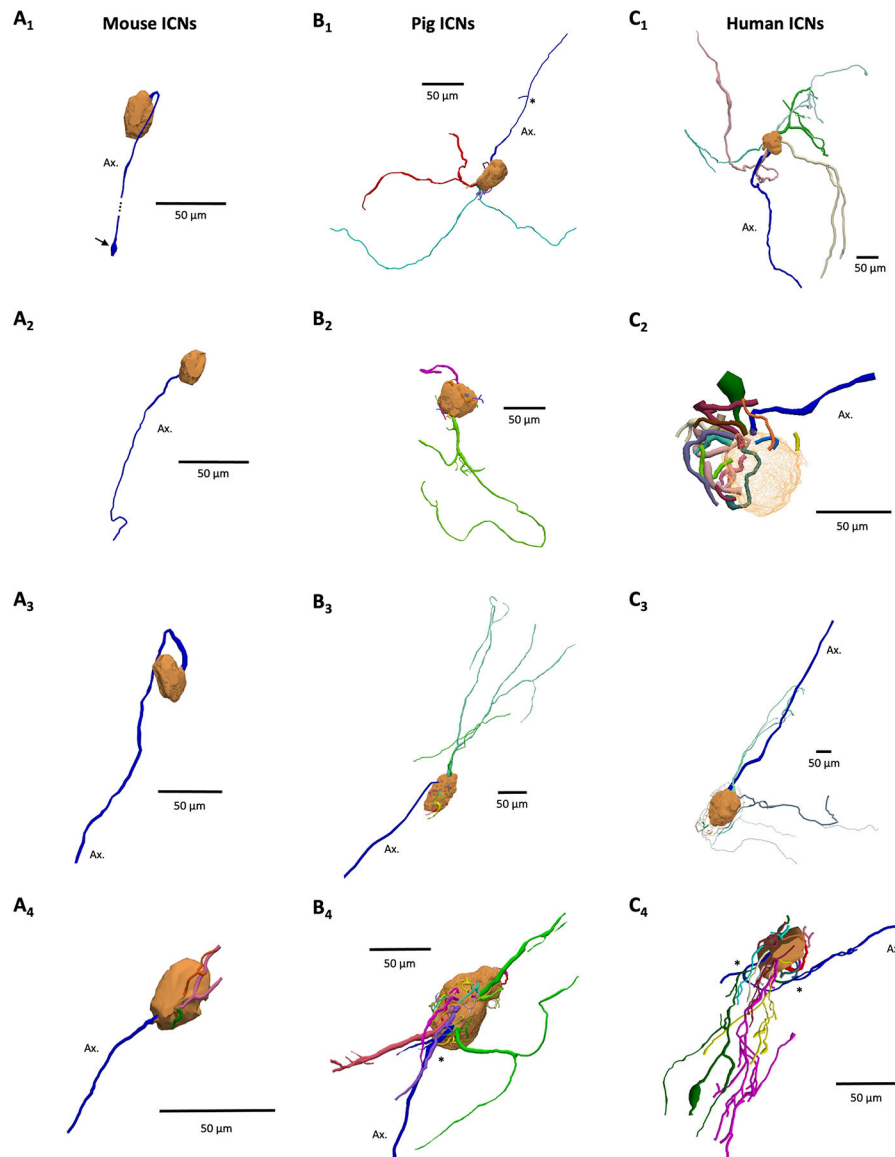


Figure 8. Morphologies of mouse, pig and human ICNs.

Segmentation of neurobiotin filled neurons reveals diversity of cellular morphologies among species. (A₁₋₄) Mouse ICNs have a single axon (Ax.) and most neurons have no dendrites. Arrow in A₁ indicates presumed axon growth cone. Pig (B₁₋₄) and human (C₁₋₄) ICNs have complex dendritic arbors and typically a single axon. The neuron in B₂ did not appear to have an axon. Dendritic branches for human cell in C₂ stayed very close to cell body. All axons are shown in blue. Cell bodies are light orange. Each dendritic stem and all its branches match in color. Axon bifurcations (*) at pig and human ICNs gave rise to short collateral axon branches.

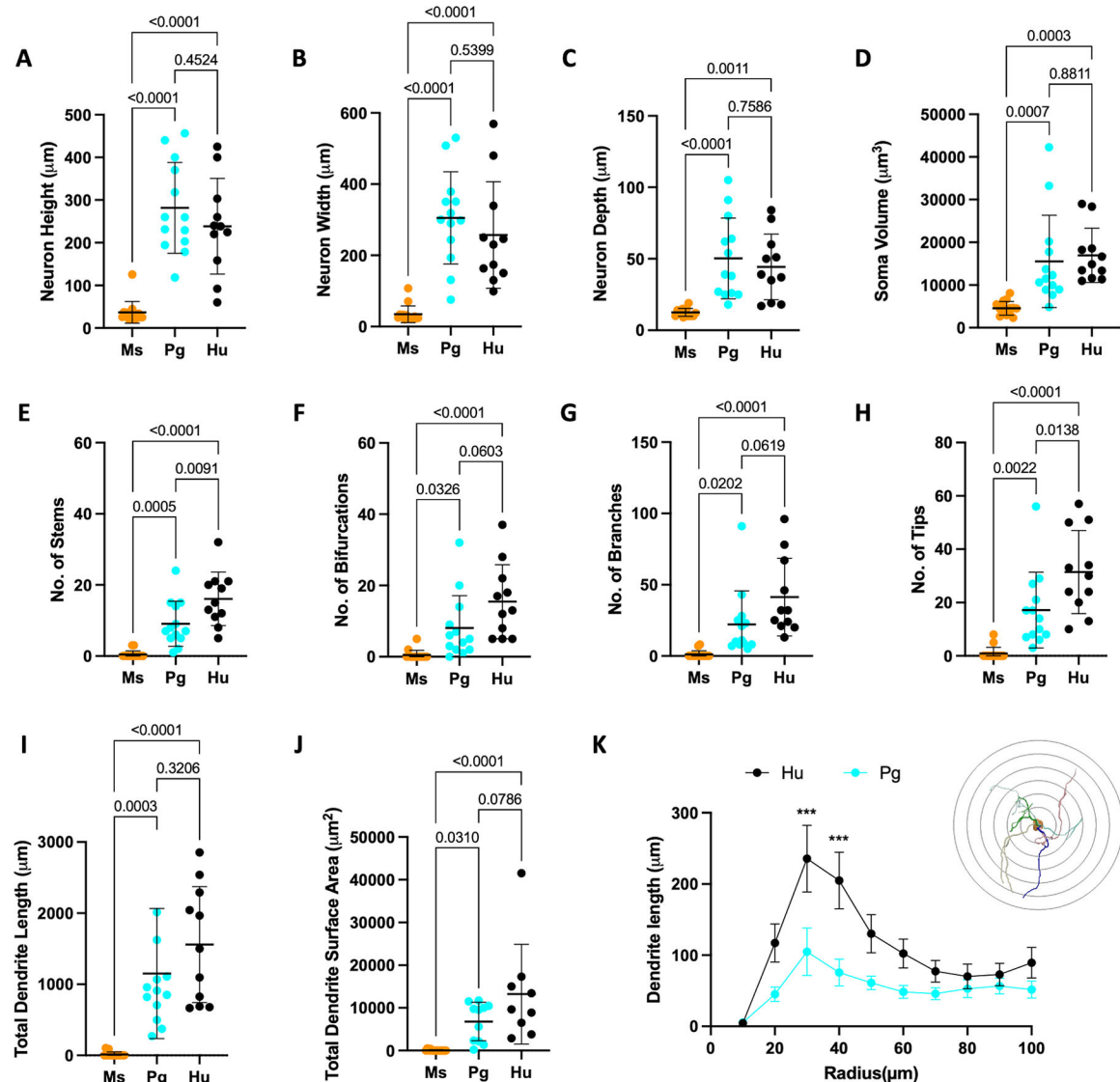


Figure 9. Quantification of morphological properties of mouse, pig and human ICNs.

(A) Height, (B) width and (C) depth of neuron soma and dendrites. Mouse ICNs have smaller cell bodies and only 1/7 had dendrites. (D) The volume of the mouse ICN soma was significantly less than pig and human neurons, which were similar in size. The number of dendritic stems (i.e. originating branch segments)(E), dendrite bifurcations (F), dendrite branches (G), and number of dendrite tips(H) were similar for pig and human neurons, which were both greater than mouse ICNs. Human neuronal dendrites showed greatest total length (I) and total dendrite surface area (J). Sample size for each measurement are 7 neurons from 7 mice, 9 neurons from 6 pigs, and 6 neurons from 6 humans. P values computed by Tukey post-hoc analysis after ANOVA. (K) Sholl analysis identifies greater dendritic length proximal to the cell body in human ICNs compared to pig. Measurements from 9 neurons in 6 pigs and 6 neurons in 6 humans. Means at each radius compared with an unpaired t test. Asterisks indicate $P < 0.05$.

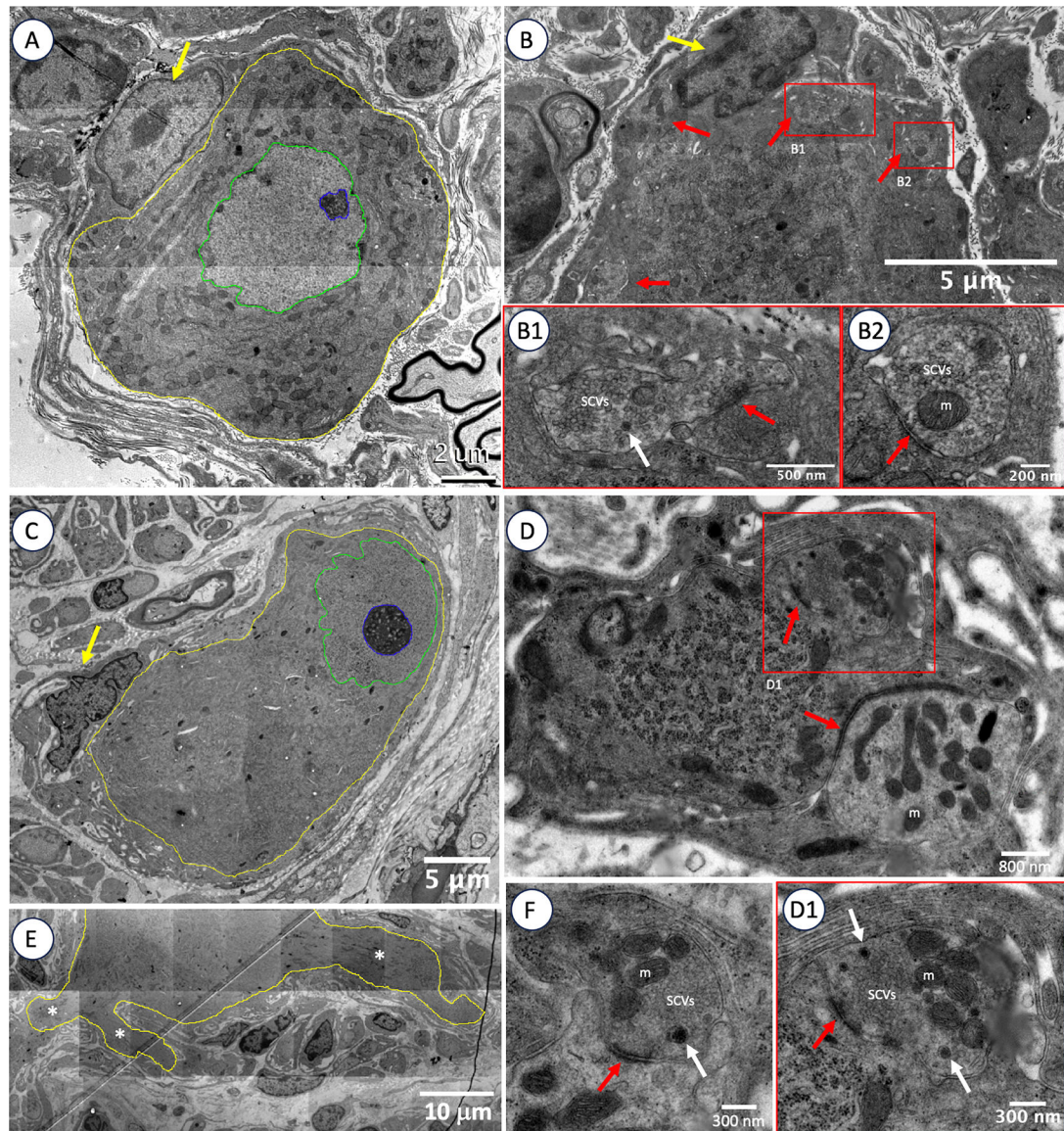


Figure 10. Ultrastructure of mouse and pig ICNs.

TEM photomicrographs show ultrastructural features of mouse and pig ICNs. (A) TEM image of a ganglionic neuron from a mouse intracardiac ganglion. Yellow arrow indicates a satellite cell nuclei. Neuronal membrane outlined in yellow. Nuclear envelope outlined in green. Nucleolus outlined in blue. (B) Another mouse ICN TEM image shows a satellite cell nucleus (*yellow arrow*) and multiple synaptic specializations on the somatic membrane (*red arrows*). Red boxed areas are expanded in B1 and B2, which show ultrastructure of the axon terminal including small clear vesicles (SCVs) and mitochondria (m). Red arrow indicates synaptic specialization. (C) TEM image of a ganglionic neuron from a pig intracardiac ganglion. Yellow arrows indicates satellite cell nuclei. Neuronal membrane, nuclear envelope and nucleolus traced in yellow, green and blue, respectively. (D) Synapse specialization forming on a dendrite in an area adjacent to the pig ICN soma. Synaptic specialization indicated with red arrows. Red boxed region expanded in D1. (E) Region of

pig ICN membrane showing neurites (*white asterisks*) extending from the soma. (F, D1)
Higher magnification images of axon terminals containing mitochondria and SCVs. Red
arrows indicate synaptic specialization. White arrows indicate large dense core vesicles.

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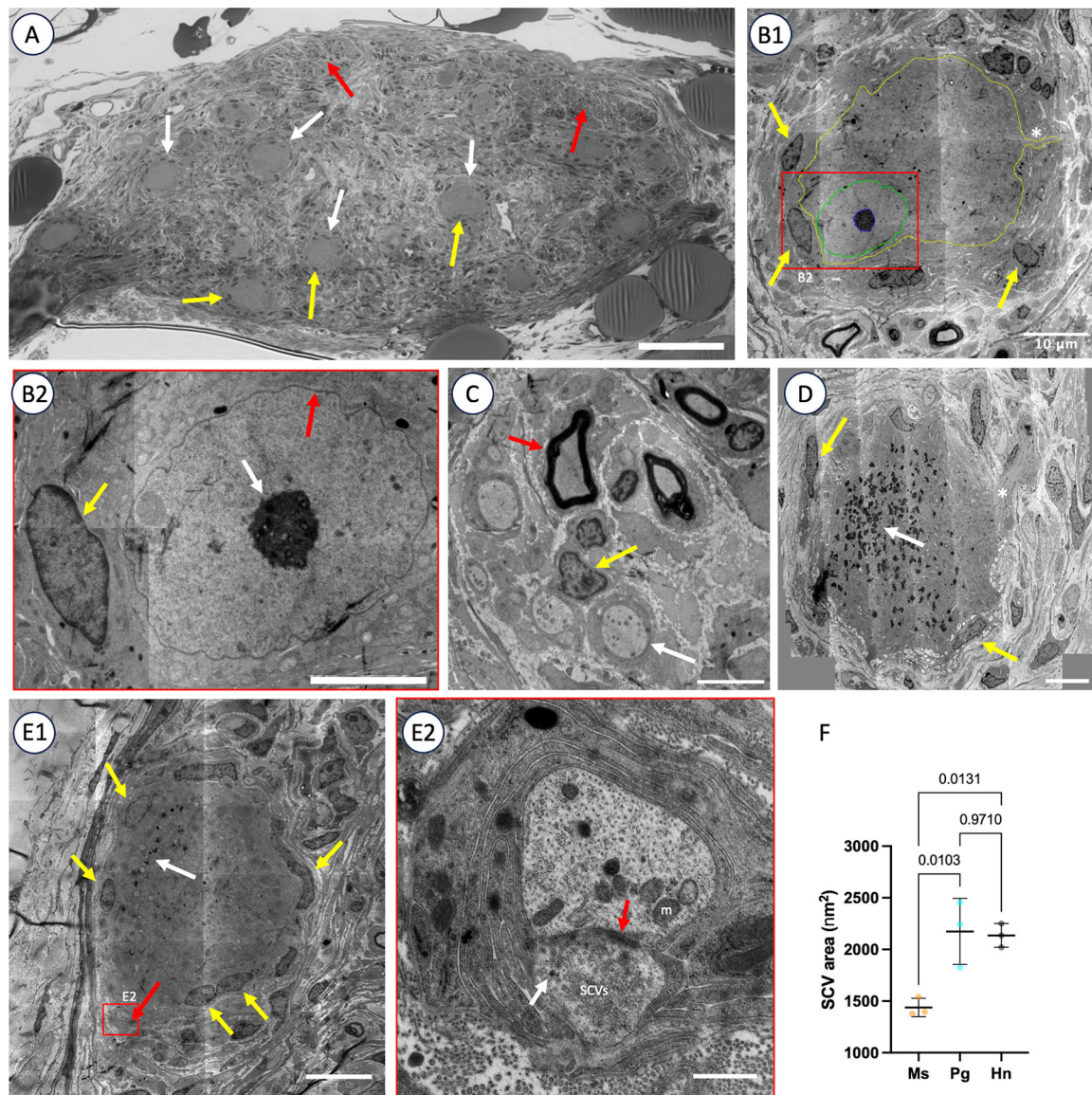


Figure 11. Ultrastructure of human ICNs.

(A) Plastic embedded and toluidine blue section of human intracardiac ganglion. White arrows indicate neuronal somata. Yellow arrows indicate satellite cell nuclei. Red arrows indicate neuropil areas at the periphery of the ganglion with myelinated and unmyelinated nerve fibers. Scale bar 80 μm . (B1) TEM image of ganglionic neuron from human intracardiac ganglion. Neuronal membrane outlined in yellow. Nuclear envelope outlined in green. Nucleolus outlined in blue. White asterisk indicates neurite extending from soma. Yellow arrows indicate satellite cell nuclei. Red boxed area expanded in B2. (B2) Yellow arrow indicates satellite cell nucleus, red arrow indicates nuclear membrane, white arrow indicates nucleolus. Scale bar 5 μm . (C) TEM image of neuropil area with red, yellow, and white arrows indicating a myelinated axon, Schwann cell nucleus, and unmyelinated axon. Scale bar 4 μm . (D) TEM image of somata of human ICN with yellow, white, and red arrows indicating satellite cell nuclei, lipofuscin, and a dendritic process, respectively.

White asterisk indicates neurite extending from soma. Scale bar 5 μm . (E1) TEM image of human ganglionic neuron with yellow, white, and red arrows indicating satellite cell nuclei, vacuoles and lipofuscin, and axodendritic synapse, respectively. Red boxed region expanded in E2. Scale bar 5 μm . (E2) Larger magnification of axodendritic synapse with SCVs, a LDCV (white arrow), and a synaptic cleft with a pre-terminal active zone (red arrow). The axon terminal is indicated with a red arrow. Scale bar 500 nm. (F) The area of SCVs was larger in pig (3 synapses from 3 neurons) and human (3 synapses from 3 neurons) terminals compared to mouse ((3 synapses from 3 neurons). Porcine and human SCVs were similar in size. P values computed by Tukey post-hoc analysis after ANOVA.

Table 1.

Organ donor demographic, transplant time and ECG data

Age	Race	Sex	Transplant time (hrs) *	ECG Notes
52	Caucasian	F	5	Sinus tachycardia, low voltage QRS, cannot rule out anterior infarct, abnormal ECG
57	Caucasian	M	6.5	NA
44	Hispanic	M	4	Normal Sinus rhythm, ST & T wave abnormality, consider inferolateral ischemia, prolonged QT, abnormal ECG
48	Hispanic	M	2	Sinus tachycardia, ventricular premature complex, old inferior infarct
72	Caucasian	M	2.5	Atrial fibrillation with rapid ventricular response. Moderate ST depression. Abnormal rhythm ECG.
57	Hispanic	M	2	Normal heart rhythm, normal ECG
64	Asian	M	1.75	Normal sinus rhythm, T wave abnormality, consider anterior ischemia, abnormal ECG
62	African American	F	2.5	Normal sinus rhythm, prolonged QT, abnormal ECG, low voltage QRS, nonspecific T wave abnormality
63	Hispanic	M	3.5	Sinus rhythm with marked sinus arrhythmia, left anterior fascicular block, anterolateral infarct, age undetermined, abnormal ECG
40	Caucasian	M	4	Sinus rhythm, low voltage, precordial leads, abnormal T, consider ischemia, lateral leads, abnormal ECG
52	Hispanic	M	3.5	Sinus tachycardia, Moderate T-wave abnormality, consider later and inferior ischemia, abnormal ECG
66	Hispanic	F	1	Atrial fibrillation and slow ventricular response, abnormal ECG
64	Hispanic	M	4	Sinus tachycardia, T wave abnormality, consider inferior ischemia. Abnormal ECG
66	Hispanic	F	2.5	Sinus tachycardia, abnormal R-wave progression, early transition. Borderline T wave abnormality
47	Hispanic	M	3.5	NA
57	African American	M	2	Sinus rhythm with first degree AV block, Low QRS voltage, prolonged QT interval, abnormal ECG
44	African American	M	5.5	Normal sinus rhythm, possible left atrial enlargement, left axis deviation, septal infarct, age undetermined, abnormal ECG
48	Caucasian	F	2.5	Sinus rhythm, long QT, abnormal ECG
53	African American	M	3	Sinus rhythm with 1st degree AV Block. Possible left atrial enlargement, junctional ST Depression, prolonged QT
50	Caucasian	F	6	Sinus rhythm with shorter PR interval, nonspecific T-Wave abnormality
31	Caucasian	M	2	Normal sinus rhythm, incomplete right bundle branch block

* elapsed time between aortic cross-clamp and tissue arrival

Table 2.

List of antibodies, sources, and concentrations used

Antibody	Concentration	Source
rabbit anti-PGP9.5	1:500	Abcam Cat# ab108986, RRID:AB_10891773
sheep anti-TH	1:200	Millipore Cat# AB1542, RRID:AB_90755
rabbit anti-VACHT	1:500	Synaptic Systems Cat# 139 103, RRID:AB_887864
goat-Anti-nNOS	1:1000	Abcam Cat# ab1376, RRID:AB_300614
rabbit anti-SP	1:1000	ImmunoStar Cat# 20064, RRID:AB_572266
mouse anti-CGRP	1:1000	Abcam Cat# ab81887, RRID:AB_1658411
rabbit anti-NPY	1:1000	ImmunoStar Cat# 22940, RRID:AB_2307354
rabbit anti-VIP	1:1000	ImmunoStar Cat# 20077, RRID:AB_572270
donkey anti-mouse Alexa Fluor 594	1:200	Jackson ImmunoResearch Labs Cat# 715-585-150, RRID:AB_2340854
donkey anti-rabbit Cy3	1:200	Jackson ImmunoResearch Labs Cat# 711-165-152, RRID:AB_2307443
donkey anti-sheep 488	1:200	Jackson ImmunoResearch Labs Cat# 713-545-147, RRID:AB_2340745
donkey anti-mouse Cy3	1:200	Jackson ImmunoResearch Labs Cat# 715-005-150, RRID:AB_2340758
streptavidin conjugated ATTO-647N	1:500	Sigma-Aldrich Cat#94149