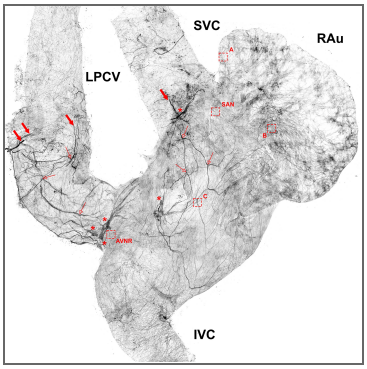


DEC 12, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.3byl4qd38vo5/v1

External link:
https://doi.org/10.1016/j.autneu.2023.103127

Protocol Citation: Jin Chen, Kohlton T Bendowski, Ariege Bizanti, Yuanyuan Zhang, Jichao Ma, Donald Hoover, David Gozal, Kalyanam Shivkumar, Zixi (Jack) Cheng 2023. Distribution and Morphology of Calcitonin Gene-Related Peptide (CGRP) Innervation in Flat Mounts of Whole Rat Atria and Ventricles. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl4qd38vo5/v1

Distribution and Morphology of Calcitonin Gene-Related Peptide (CGRP) Innervation in Flat Mounts of Whole Rat Atria and Ventricles

Jin Chen[1], Kohlton T Bendowski[1], Ariege Bizanti[1], Yuanyuan Zhang[1], Jichao Ma[1], Donald Hoover[2], David Gozal[3], Kalyanam Shivkumar[4], Zixi (Jack) Cheng[1]

[1]University of Central Florida; [2]East Tennessee State University; [3]Marshall University; [4]University of California Los Angeles

Kohlton T Bendowski
University of Central Florida

ABSTRACT

Calcitonin gene-related peptide (CGRP) is widely used as a marker for nociceptive afferent axons. However, the distribution of CGRP-IR axons has not been fully determined in the whole rat heart. Immunohistochemically labeled flat-mounts of the right and left atria and ventricles, and the interventricular septum in rats for CGRP were assessed with a Zeiss imager to generate complete montages of the entire atria, ventricles, and septum, and a confocal microscope was used to acquire detailed images of selected regions. We found that 1) CGRP-IR axons extensively innervated all regions of the atrial walls and the walls of the great vessels including the sinoatrial node region, auricles, atrioventricular node region, superior/inferior vena cava, left pre-caval vein, and pulmonary veins. 2) CGRP-IR axons formed varicose terminals around individual neurons in some cardiac ganglia but passed through other ganglia without making appositions with cardiac neurons. 3) Varicose CGRP-IR axons innervated the walls of blood vessels. 4) CGRP-IR axons extensively innervated the right/left ventricular walls and interventricular septum. Our data shows the rather ubiquitous distribution of CGRP-IR axons in the whole rat heart at single-cell/axon/varicosity resolution for the first time. This study lays the foundation for future studies to quantify the differences in CGRP-IR axon innervation between sexes, disease models, and species.

MANUSCRIPT CITATION:
J. Chen, K.T. Bendowski, A. Bizanti, et al., Distribution and morphology of calcitonin gene-related peptide (CGRP) innervation in flat mounts of whole rat atria and ventricles, Autonomic Neuroscience: Basic and Clinical (2023). https://doi.org/10.1016/j.autneu.2023.103127

Protocol status: Working
We use this protocol and it's working

Created: Nov 21, 2023

Last Modified: Dec 12, 2023

PROTOCOL integer ID: 91289

Keywords: Calcitonin gene-related peptide, Heart, Vasculature, Intrinsic cardiac ganglia, Atria, Ventricles

Funders Acknowledgement:
NIH
Grant ID: 1 U01 NS113867-01
NIH
Grant ID: R15HL137143-01A1

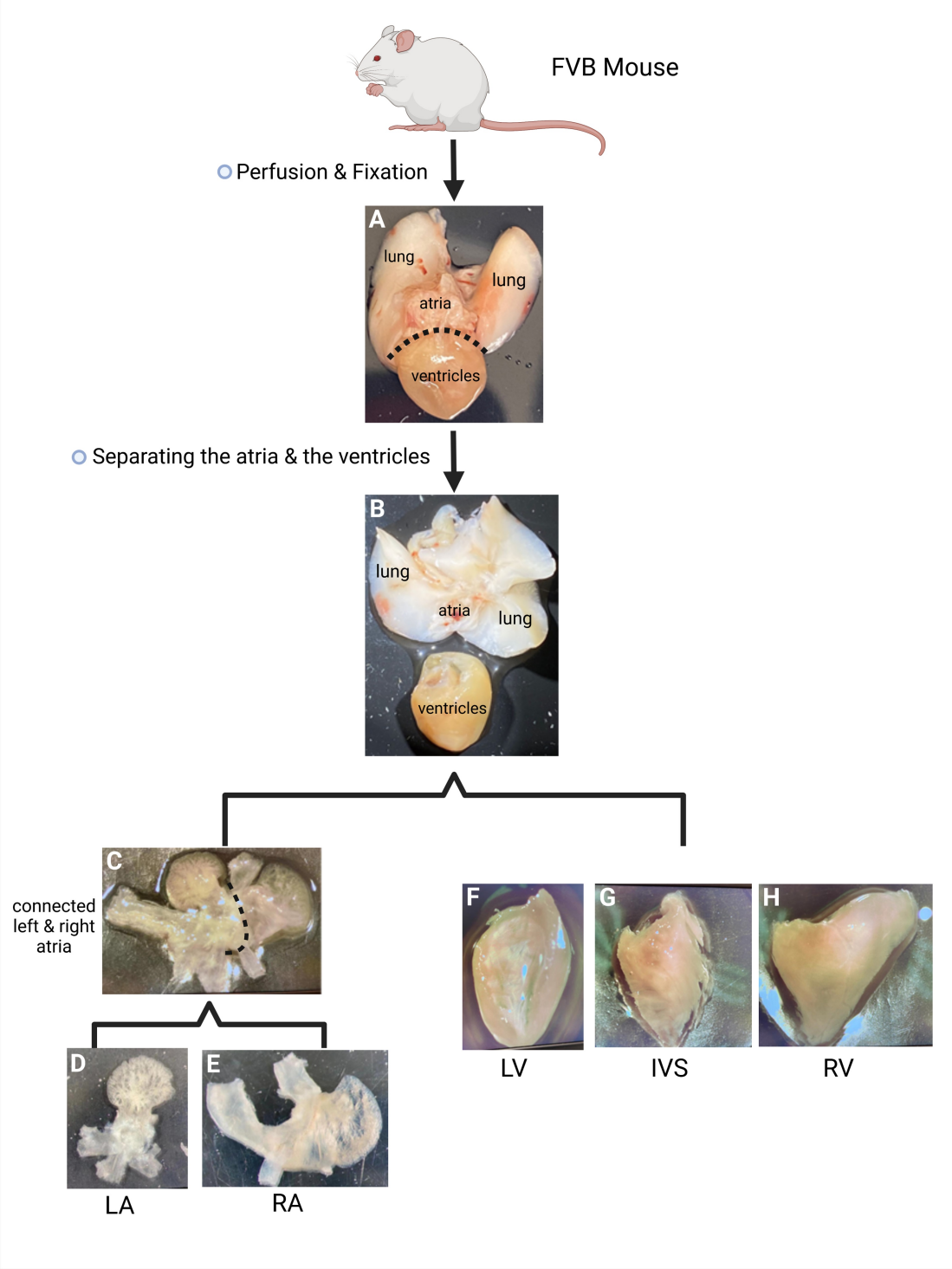
MATERIALS

- Healthy male Sprague-Dawley rats (RRID: RGD_737903; Envigo RMS, LLC, Indianapolis, IN) (n=6; 2-3 months; weighing: 200-350 g)
- Isoflurane (5%; Cat #: 029405, Covetrus North America)
- Heparin (0.2 mL; 1000 units/mL, Cat #: 25021-400-10, Sagent Pharmaceuticals)
- Paraformaldehyde (Cat #: 416780010, Acros Organics)
- Bovine serum albumin (Cat #: A7906, Sigma-Aldrich)
- Normal donkey serum (Cat #: 017-000-121, Jackson Immunoresearch)
- Triton X-100 (Cat #: T8787, Sigma-Aldrich)
- NaN_3 (Cat #: S2002, Sigma Aldrich)
- Anti-CGRP primary antibody (Rabbit; Cell Signaling, Danvers, MA; Cat # 14959, RRID: AB_2798662; 1:200)
- Alexa Fluor 488 secondary antibody (Donkey; Thermo Fisher, Waltham, MA, Cat# A21202, RRID:AB_141607; 1:100)
- Methanol (Cat #34860: , Sigma-Aldrich)
- DCM (dichloromethane) (Cat #: 270997, Sigma-Aldrich)
- Ethanol (Cat #: E7023, Sigma-Aldrich)
- Depex mounting medium (Electron Microscopy Sciences #13514)
- Zeiss Axio Imager M2 (Item no.: 430004-0000-711)
- Leica Confocal Microscope (Type: TCS SP5)
- Adobe Photoshop (Version: 23.3.1; Link: https://www.adobe.com/products/photoshop.html)
- FIJI ImageJ (Version 2.14.0; Link: https://imagej.net/software/fiji/)

Whole heart dissection and flat-mount preparation

1 Rats were first deeply anesthetized with isoflurane (5%; 5-10 minutes), with an oxygen flow rate of 1 liter per minute. When the animals were not responsive to the hind-toe pinch withdrawal reflex, the chest was opened and an injection of heparin (100 units) was made in the apex of the left ventricle. After the inferior vena cava was cut to drain the blood and perfusate, a needle was inserted into the left ventricle and the perfusion began. The rats were first perfused with 500 ml of 37-40 °C phosphate-buffered saline (0.1 M PBS, pH = 7.4) followed by 300 ml paraformaldehyde (4%; 4 °C).

2 The tissues were separated in the same manner that is shown in Figure 1 of Bizanti et al., 2023 [1] (see the figure below). **Note:** The following figure uses an FVB mouse as an example, however the current protocol uses Sprague-Dawley rats. The heart, lungs, and trachea were removed immediately after perfusion and stored in 4 % paraformaldehyde for at least 24 hours at 4 ºC. The atria and ventricles were separated at the atrial-ventricular groove, and the left and right atria and ventricles were separated. The left atrium was prepared with the entrance portions of all pulmonary veins (PVs) attached and the right atrium was prepared with the superior vena cava (SVC), inferior vena cava (IVC) and left precaval vein (LPCV) attached.



Preparation of flat-mounts of a mouse heart. (A) A heart with attached lungs, trachea, and

esophagus was collected after perfusion. (B) The heart was separated from other structures and followed by the separation of the atria and ventricles. (C) The brown adipose tissue and aortic arch were removed to expose the connected right atrium (RA) and left atrium (LA). The LA (D) along with the pulmonary veins (PV) was separated from the RA (E) with the superior vena cava (SVC), left precaval vein (LPCV), and inferior vena cava (IVC). The left ventricle (LV) (F) and right ventricle (RV) (H) were separated by cutting along the interventricular septum (IVS) (G).

Immunohistochemistry

3 Dissected tissues were washed 3 times for 15 min in 0.01 M PBS (pH = 7.4) in a 6-well plate on an orbital shaker to remove the remaining fixative.

4 To prevent non-specific binding and enhance antibody penetration, the atria were fully submerged in a blocking solution (2% bovine serum albumin, 10% normal donkey serum, 2% Triton X-100, 0.08% NaN3 (in 0.1 M PBS, pH = 7.4)) for 5 days (atria) at 4 ºC.

5 Anti-CGRP primary antibody (Cell Signaling, Danvers, MA; Cat # 14959, RRID: AB_2798662; 1:200) was mixed with the primary solution (2% bovine serum albumin, 4% normal donkey serum, 0.5% Triton X-100, 0.08% NaN3 in 0.1 M PBS, pH = 7.4) and incubated with the tissues for 5 days (for atria) or 7 days (for ventricles) at 4 ºC.

6 Tissues were thoroughly washed 6 times for 10 min in PBST (phosphate buffered saline with Triton X-100; 0.5% Triton X-100 in 0.01 M PBS) to remove unbound primary antibodies.

7 Tissues were then kept in the dark and incubated in a fluorescent secondary antibody solution (Alexa Fluor 488; Thermo Fisher, Waltham, MA, Cat# A21202, RRID:AB_141607; 1:100) overnight at room temperature. Unbound secondary antibodies were removed by washing 6 times for 10 minutes in PBS at room temperature.

7.1 Rat ventricles were pretreated with methanol (see the following sub-steps) and the immunolabeling procedures outlined in [2] **Note:** the clearing step was omitted entirely from the procedures outlined in the referenced publication.

7.2 Tissues were dehydrated in methanol/H_2O in the following series of concentrations: 20%, 40%, 60%, 80%, 100% for 1 hour each.

7.3 Tissues were further washed in 100% methanol for 1 hour and then chilled in 4^{o} C.

7.4 Tissues were incubated overnight on a shaker in 66% DCM/33% methanol at room temperature.

7.5 Tissues were washed twice in 100% methanol at room temperature and then they were chilled at 4^{o} C.

7.6 Tissues were bleached in chilled fresh 5% H_2O_2 in methanol (1 volume 30% H_2O_2 to 5 volumes methanol) overnight at 4^{o} C.

7.7 Tissues were rehydrated in the following series of methanol/H_2O solutions: 80%, 60%, 40%, 20%, PBS for 1 hour each at room temperature.

7.8 Tissues were washed twice in room temperature PBST (PTx.2) for 1 hour at room temperature.

8 Tissues were then mounted onto glass slides and flattened with lead weights for either 2 days (20 lbs for atria) or 3 weeks (30 lbs for ventricles) at 4 ºC.

9 Slides were dehydrated in four ascending concentrations of ethanol (75%, 95%, 100%, and 100%) for 2 min in each concentration, followed by 2 × 10 min washes in 100% xylene.

10 Coverslips were then attached using DEPEX mounting medium (Electron Microscopy Sciences #13514) and allowed to dry overnight.

11 Negative controls, which excluded primary antibodies, showed no immunoreactivity, confirming that nonspecific binding of secondary antibodies did not occur. Antibody specificity was tested by Cell Signaling Technology using Western Blot and immunofluorescence (https://www.cellsignal.com/products/primary-antibodies/cgrp-d5r8f-rabbitmab/14959). Western Blot analysis shows a single band at the correct molecular weight of CGRP in TT cells but not in HeLa cells. Moreover, confocal immunofluorescent analysis of TT and HeLa cells confirms that CGRP were in TT cells but not in HeLa cells.

12

	A	B	C	D	E	F	G
	Antibody	Concentration	Host	Company	Cat #	Emission	RRID
	Anti-CGRP	1:200	Rabbit	Cell Signaling, Danvers, MA	14959	n/a	AB_2798662
	Donkey Anti-Rabbit	1:100	Donkey	Thermo Fisher, Waltham, MA	A21202	488	AB_141607

Table 1: Antibodies sources and concentration

Image acquisition

13 Hundreds of overlapping maximum projection images from stacks of optical sections (z-step: 1.5 µm for atria and 2 µm for the ventricles) from each tissue were captured using a Zeiss M2 Imager (20x lens; NA 0.8) and stitched back together seamlessly to yield full photo montages of the right and left atria and ventricles. An LED light source with a 488 nm wavelength was used to visualize the CGRP-IR axons in the tissues.

14 A Leica TCS SP5 laser-scanning confocal microscope (40x oil immersion lens; NA 1.25; z-step: 1 µm) was used to capture detailed images of CGRP-IR axons and their targets in selected locations of the heart. An argon–krypton laser (488 nm) was used to visualize CGRP-IR axons in the tissue, and a helium-neon laser (543 nm) was used to detect the autofluorescence of the tissues in the background (e.g., cardiac muscles, ganglionic cells, and blood vessels).

15 Modifications, including brightness and contrast adjustments, and scale bar additions, were performed using Photoshop or FIJI ImageJ software [3]. Of the 6 rat hearts that were used in this study, we showed

the representative cases from each tissue in our figures (RA, LA, RV, LV, and IVS).