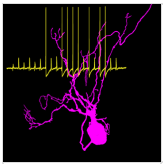

Version 2

May 06, 2021

Protocol for characterization of morphology, electrophysiology, and synaptology of mouse stellate ganglion neurons V.2

Amit Tsanhani[1], Youngjin Cho[2], Aidan Sullivan[3], Susan Tappan[3], John D Tompkins[1]

[1]Cardiac Arrhythmia Center, Neurocardiology Research Program of Excellence, Department of Medicine - Cardiology, University of California, Los Angeles, CA, USA;

[2]Seoul National University Bundang Hospital, Department of Inernal Medicine, Seongnam, Republic of Korea;

[3]MBF Bioscience, 185 Allen Brook Lane, Suite 101, Williston, VT, USA

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John Tompkins
Cardiac Arrhythmia Center, Neurocardiology Research Program ...

ABSTRACT

The stellate is a thoracic sympathetic ganglion supplying autonomic innervation to cells, tissues and organs of the upper thorax; including, but not limited to, the brachial sweat glands, mammary glands, cardiomyocytes, and sinoatrial and atrioventricular nodes. We sampled individual cells of the mouse right stellate ganglion with intracellular microelectrodes to create a topographic atlas of ganglion neuron structure and function. Membrane potential recordings, during stimulation of presynaptic nerves, were paired with confocal imaging of single cells, backfilled with fluorescent dyes, to quantify cell morphology, membrane physiology, and synaptology of the individual cells. The cellular database, including localization and projection of each cell, will be shared publicly through a link in the manuscript, which will be appended to this protocol once published. In the accompanying manuscript, we describe unique populations of cardiac-projecting stellate neurons and detail the heterogenous population of neurons comprising this ganglion which is vital to sympathetic control of the heart.The methods used in this study are detailed here.

EXTERNAL LINK

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KEYWORDS

stellate, ganglion, autonomic, sympathetic, neuron, cardiac, heart

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GUIDELINES

Euthanasia protocols are approved by the UCLA Animal Care and Use Committee, and conform to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (2011).

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Stellate Isolation

1 Adult mice (M/F; 12±2 weeks of age) are sacrificed under deep isoflurane (5%) anesthesia by cervical dislocation and exsanguination.

2 The thorax is 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.

3 Stellate ganglia are isolated from paravertebral chain at the origin of the 1st rib, and the overlying connective tissue is pinned to the SylGard (Dow Corning) floor of a glass bottom petri dish.

4 The dish is secured to a custom XY linear stage and ganglia are observed using an upright microscope (AxioExaminer, Zeiss) equipped with a dry 5X and a 40X water-immersion objective, differential interference contrast optics, and monochrome camera (Axiocam, Zeiss).

5 Concentric bipolar stimulation electrodes (FHC) are placed on either the grey rami communicantes, the inferior cardiac nerve, the ventral ansa, or the thoracic sympathetic trunk.

6 Images are captured at low magnification (5X) to document placement of the electrodes.

7 Distance from the stimulation electrode to the impaled cell is measured to determine nerve fiber conduction velocity.

Intracellular Microelectrode Recording

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8 Isolated ganglia are continuously superfused (6–7 ml/min) with PSS maintained at 32–35°C with a thermostatically controlled heater.

9 Neurons are identified visually and impaled with borosilicate-glass microelectrodes filled with either 2M KCl (60–120 MΩ) or 2M KCl + 2% Neurobiotin (80-160 MΩ; Vector Labs).

10 Membrane voltage is recorded using a Multiclamp 700B amplifier connected to a Digidata 1550B data acquisition system.

11 pCLAMP 10 software (Molecular Devices, CA) is used for acquisition and analysis of membrane potential data.

12 Intracellular current injected through the recording electrode is used to characterize membrane physiology.

13 Depolarizing current steps (0.1–0.5 nA, Δ100 pA, 500 ms duration) are used to assess neuronal excitability.

14 Cells are classified as either phasic (1) or non-phasic (≥2) based on the maximum number of action potentials elicited by the depolarizing current.

15 Hyperpolarizing current steps (500 ms) of decreasing amplitude (-0.4 to -0.1 nA, Δ100 pA) are used to test for rectification in the current-induced hyperpolarization, occurring when hyperpolarization-activated currents are initiated, and to measure whole-cell input resistance.

16 The amplitude and duration of the action potential are measured from either a spontaneous or a nerve evoked spike.

17 After-hyperpolarization amplitude and duration are measured from brief intracellular current pulses (0.1-0.8 nA,Δ100 pA, 5 ms) or spontaneous action potentials.

18 Inclusion criteria for analysis include a resting membrane potential less than or equal to -45mV, a holding current of greater than or equal to -100pA , and the cell must be excitable (cells with no action potential are excluded).

19 Graded stimulus shocks (100 µs) are delivered from the concentric bipolar electrodes in 50-100 µA steps, from 0 to 800 µA, to generate stimulus recruitment curves (Master 8 and IsoFlex optical Isolation unit, AMPI).

20 Five to 20 stimuli are delivered at each stimulus intensity, with an interval of 3 seconds between stimuli.

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21 Analysis of synaptic events focuses on latency of the excitatory post-synaptic potential (EPSP), measured from the start of the stimulus trigger to the beginning of the EPSP (V≥2xRMS),and jitter (SD of latency) as indices of conduction, path and release.

Retrograde Labeling of Cardiac-Projecting Stellate Neurons

22 Mice are given carprofen (5 mg kg^{-1}, s.c.) and buprenorphine (0.05 mg kg^{-1}, s.c.) 1 hour before surgery.

23 Animals are anesthetized with isoflurane (induction at 5%, maintenance at 1–3%, inhalation), intubated, and mechanically ventilated.

24 Core body temperature is measured and maintained at 37 °C.

25 The surgical incision site is cleaned 3 times with 10% povidone iodine and 70% ethanol in H_2O (vol/vol).

26 A left lateral thoracotomy is performed at the fourth intercostal space, the pericardium opened, and the heart is exposed.

27 Ten microliters of cholera toxin B conjugated to Alexa Fluor 488 (0.1% in 0.01 M PBS (vol/vol), ThermoFischer Scientific) is injected subepicardially with a 31-gauge needle.

28 The surgical wounds are closed with 6–0 suture.

29 Buprenorphine (0.05 mg kg^{-1}, s.c.) is administered once daily for up to 2 days after surgery.

30 Animals are sacrificed 6 days later for tissue collection.

Neurobiotin Backfilling of Inferior Cardiac Nerve-Projecting Neurons

31 Axons of the inferior cardiac nerve are filled retrogradely with neurobiotin by aspirating the iCN into a suction electrode filled with 2% neurobiotin in 1M KCl.

32 After 4 hours, ganglia are stained using immunohistochemical protocols described below.

Immunohistochemistry

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33 After dye injection and microelectrode recording, isolated ganglia are fixed in 4% paraformaldehyde, overnight.

34 Fixed tissue is rinsed (3 x 1 hr wash) in 0.01 M phosphate-buffered saline (PBS), and stored in 0.01 M PBS with 0.02% sodium azide.

35 The stellate ganglion whole mounts are blocked in 0.01 M PBS, 0.02% sodium azide, and 0.1% Triton X-100,and horse serum for 4 hours at room temperature with agitation.

36 The following primary antibodies are used: rabbit anti-PGP9.5 (Abcam, ab108986, 1:500), sheep anti-TH (Millipore Sigma, AB1542, 1:200) in a solution of 0.01 M PBS, 0.02% sodium azide, and 0.1% Triton X-100 with agitation for 2 nights.

37 Tissues are washed with a solution of 0.01 M PBS, 0.02% sodium azide every hour for 3 hours.

38 Tissue is then incubated in secondary antibodies diluted in 0.01 M PBS with 0.1% Triton X-100 and 0.02% sodium azide for 2 nights at room temperature with agitation.

39 The following secondary antibodies are used: donkey anti-rabbit Cy3 (Jackson ImmunoResearch, 711-165-152, 1:200),donkey anti-sheep 488 (Jackson ImmunoResearch, 713-545-147, 1:200).

40 Secondary staining with streptavidin conjugated ATTO-647N is used to visualize neurobiotin filling (1:500).

41 Stained tissue is rinsed in 0.01 M PBS, 0.02% sodium azide every hour for 3 hours, wiped dry, and mounted on glass slides with Citifluor (Electron Microscopy Sciences) and coverslipped.

Confocal Imaging

42 Tiled scans of whole ganglion neuroanatomy were acquired on confocal microscopes (Zess LSM 880 or Leica SP5) in X, Y, and Z planes using a dry 10X objective. Regions of interest were tile scanned using either 40x (1.3NA; Leica) or 63x (1.4NA; Zeiss) oil-immersion objectives. Z-stacks were taken at Nyquist sampling criteria given the NA of the respective lenses.

43 Image data was quantified offline using Zen (Zeiss), ImageJ, or Neurolucida360 (MBF) software. Quantified data includes: cell cross-sectional areas (measured at nucleus cross-sections), total cell counts per ganglia, numbers of back-labeled cells, numbers of axons, numbers of dendrites, length of dendrites.

44 Camera lucida drawings were made using a digital tablet and stylus. Cell structures were classified as either multipolar, adendritic or anaxonic based the presence of axons and dendrites.

45 Image Z-stacks were analyzed using Neurolucida360 to trace and measure all projections from the neuronal soma.

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Dendrite number and length were quantified from the skeletonized structures. Traced axons are used to measure conduction velocity.

Neurolucida360

46 Individual neurobiotin filled cells are segmented using Neurolucida360 (MBF Biosciences).

47 Within Neurolucida360, segmented neurons are analyzed by orthogonal view to determine maximum Z travel of longest dendrite (term name??).

48 Quantatiatve measurements of segmented neurons are computed within Neurolucida Explorer. Measurements for dendrite length and number of principal dendrites (attach to cell soma) in computed under the 'Analysis' window under 'Branched Structure Analysis'.

49 Scoll analysis of each neuron is computed in Neurolucida Explorer within 'Spatial Analysis." Scoll analysis was used to calculate the maximum extension of dendrites in XY plane from center of neuron soma, defined as 'maximum dendrite radius.'

50 Within Neurolucida Explorer under "orientation", polar histograms were computed to quantify directionality of dendritic extension. HOW REPORTED!!!

51 Many more analysis options are available within the Neurolucida software package from MBF Biosciences. We have chose to only focus on the above analysis options to define our neural population at this time but may append addtitional analysis over time as needed.

Analysis

52 The membrane properties are summarized: resting membrane potential, input resistance, variation of resistance according to the current (or rectification?)(defined as residual sum of squares in the linear regression between the current and voltage), AHP amplitude, 2/3 of AHP duration, excitability (phasic or non-phasic), amplitude of slow EPSP after train stimulation, and tau.

53 The morphological characteristics are summarized: axonal projection (cardiac or non-cardiac), number of principal dendrites, number of nodes in dendrites, total length of dendrites, maximal distance of the dendrite tip from the cell body, and cross-sectional cell area.

54 Correlation between parameters are screened using Pearson’s correlation test.

55 The membrane properties and morphological characteristics are compared between (1) male vs female mice, (2) phasic versus non-phasic cells, and (3) iCN versus non-iCN projecting cells. Student’s t-test is used for continuous variables, and chi-square test for categorical variables.

56 Linear regression analysis is used to study the relationship betweenrectification of input resistanceand amplitude of slow EPSP.

57 Nerve evoked potentials are evaluated: amplitude of AP (or EPSP), latency of EPSP, and recruitment at 5, 10, and 20Hz train stimulation.

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58 Modified latency, defined as latency divided by distance from the stimulating electrode, is calculated when available.

59 Latency (or modified latency) values are plotted for an individual cell. If the plot seems to form multiple clusters, the criteria of >2SD difference in the mean of latency is used to confirm separate clusters.

60 Linear regression analysis is used to study the relationship between latency of EPSP and its jitter.

61

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