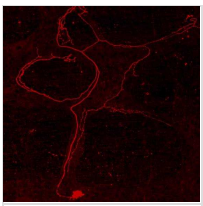

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Intracellular recordings and post hoc immunofluorescence

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SPARC
Tech. support email: **info@neuinfo.org**

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The following protocol was submitted on behalf of the authors from the Bornstein lab by the SPARC project.

This protocol describes methods for standard intracellular recording from mouse myenteric neurons impaled with intracellular electrodes containing biocytin, followed by processing for immunohistochemistry to map the projections of the neurites of enteric neurons. Methods described in this protocol are adapted from decades of studies on guinea-pig enteric neurons and more recent analyses of mouse duodenal myenteric neurons and colonic submucosal neurons. The immunofluorescence is used to reveal either calretinin or neuronal nitric oxide synthase (nNOS), as well as the enteric neurons in the proximal colon of the mouse. The methods can be generalized to whole mount preparations from any gut region in any species.

Mice were sacrificed by cervical dislocation, a procedure approved by the University Melbourne Animal Experimentation Ethics committee. The experimental procedures should incorporate all local requirements for standards of animal experimentation.

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protocol ,

Nov 23, 2021

Apr 01, 2022

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Electrophysiology:

- nicardipine - Sigma Aldrich, NSW, Australia
- hyoscine - Sigma Aldrich, NSW, Australia
- biocytin - Sigma Aldrich, NSW, Australia
- silicone elastomer - Sylgard 184, Dow Corning, NSW, Australia
- dissection forceps, ultrafine - Dumont #5 INOX, sourced from Fine Science Tools, Canada
- inverted microscope - Zeiss Axiovert 200
- Axoprobe 1A microelectrode amplifier - Axon Instruments, USA
- PowerLab/4SP acquisition system - ADInstruments, NSW, Australia
- Chart5 for Windows - ADInstruments, NSW, Australia
- Master 8 pulse generator - A.M.P. Instruments, Jerusalem, Israel
- ISO-Flex stimulus isolation unit - A.M.P. Instruments, Jerusalem, Israel

Pharmacology:

- hyoscine (1μM) - Sigma Aldrich, NSW, Australia
- hexamethonium (200μM) - Sigma Aldrich, NSW, Australia
- nicardipine (2.5μM) - Sigma Aldrich, NSW, Australia
- NOLA (100μM) - Sigma Aldrich, NSW, Australia
- TNP-ATP triethylammonium salt, *Tocris,* (5μM) - sourced from In Vitro Technologies Pty Ltd. Vic, Australia)
- ondansetron (3μM) - Glaxo Research Group, Greenford, UK

Immunohistochemistry:

- CAS-Block - Invitrogen, CA, USA
- Triton X-100 - ProSci Tech, QLD, Australia
- glass slides - Livingstone International, NSW, Australia
- Dako mounting medium - Agilent Technologies

Primary antibodies:

- Goat anti-calretinin - Swant, Bellinzona, Switzerland, Catalog #CG1
- Sheep neuronal nitric oxide synthase (nNOS) - P.C. Emson, The Babraham Institute, Cambridge, UK, Catalog #K205

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A	B	C	D	E	F	G
Primary Antiserum	**Specific Immunogen**	**RRID**	**Host Species**	**Dilution**	**Supplier**	**Catalog**
Calretinin	Human recombinant calretinin	AB_10000342	Goat	1:1000	Swant, Bellinzona, Switzerland	CG1
Nitric oxide synthase (NOS)	Neuronal NOS (nNOS)	AB_2895154	Sheep	1:1000	P.C. Emson - The Babraham Institute, Cambridge, UK	K205

Secondary Antibodies:

A	B	C	D	E	F	G
Secondary Antiserum	**Fluorophore**	**RRID**	**Host Species**	**Dilution**	**Supplier**	**Catalog**
Sheep	Alexa 488	AB_141362	Donkey	1:200	Molecular Probes, Mt Waverley, VIC, Australia	A11015
Sheep	Alexa 647	AB_10374882	Donkey	1:200	Molecular Probes, Mt Waverley, VIC, Australia	A21448
	Alexa 594 Streptavidin Conjugates			1:200	Molecular Probes, Mt Waverley, VIC, Australia	S32356 Lot 55981A

Confocal Microscopy:

- confocal microscope - Zeiss LSM 880, Biological Optical Microscopy Platform, University of Melbourne

Electrophysiology

1 Sacrifice mice by cervical dislocation.

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2 Open the abdominal cavity using a midline incision and remove the colon.

3 To minimize the contractions of the muscle layers during intracellular recordings place the colon in oxygenated [95% O_2, 5% CO_2] physiological saline [composition in mM: NaCl 118, KCl 4.6, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 1, $NaHCO_3$ 25, d-glucose 11] containing nicardipine (1.25 µM) (Sigma Aldrich, NSW, Australia) and hyoscine (1 µM) (Sigma Aldrich, NSW, Australia).

4 Open 1cm^2 segment of the proximal colon along its mesenteric border. Pin it flat in a petri dish lined with a silicone elastomer (Sylgard 184, Dow Corning, NSW, Australia). Remove mucosa and submucosal plexus (SMP) together in a sheet.

5 To allow access to the longitudinal muscle layer turn the segment over and repin it.

6 Remove the longitudinal muscle layer in fine stripes in the longitudinal direction using ultrafine dissection forceps (Dumont #5 INOX, sourced from Fine Science Tools, Canada).

7 Transfer the preparation to a recording bath (volume **1-2 mL**) and superfuse with warmed physiological saline (**35 °C**) at 5mL/min.

8 After 1 hour equilibration period, visualize myenteric ganglia using an inverted microscope (Zeiss Axiovert 200).

9 Impale myenteric neurons using glass microelectrodes (100 – 200 MΩ resistance, pulled on a Sutter P-97 microelectrode puller) containing 1M KCl with 2% biocytin (Sigma Aldrich, NSW, Australia).

10 To obtain and acquire voltage recordings, use an Axoprobe 1A microelectrode amplifier (Axon Insturments, USA), a personal computer connected to a PowerLab/4SP acquisition system, and Chart5 for Windows (ADInstruments, NSW, Australia).

11 Apply single electrical stimuli or trains of stimuli (3, 5 or 15 P at 20 Hz) to an interganglionic fibre tract entering the impaled ganglion using a unipolar stimulating electrode (100 µM stainless steel insulated with 15 µM Teflon, 0.5 – 3.0 mA, duration 0.5 ms) driven by a Master 8 pulse generator via an ISO-Flex stimulus isolation unit (A.M.P. Instruments, Jerusalem,

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Israel).

Record fast excitatory postsynaptic potentials (EPSP) at hyperpolarized membrane potentials using hyperpolarizing current injected into the neuron, -90 mV.

Record slow EPSPs and inhibitory postsynaptic potentials (IPSP) at resting membrane potential (RMP) or close to RMP (-50 to -60 mV) using hyperpolarizing current.

12 Examine the excitability of neurons by injecting depolarizing current pulses (500 ms duration) in 50 pA increments over the range 50 – 350 pA while the membrane potential (MP) is held at -55 mV.

Examine the number of action potentials (AP) and the duration of AP firing for each current pulse amplitude. Measure the duration of AP firing from the start of the first AP to the end of the last AP triggered by the pulse.

To determine the input resistance (IR) apply hyperpolarizing current pulses (50 pA - 300 pA) and plot against the associated voltage changes.

Pharmacology

13 Make up concentrated stock solutions of antagonists in distilled water.

14 On the day of the experiment dilute the stock solutions to working concentrations.

Drugs used in the experiment included:

- hyoscine (1μM) - Sigma Aldrich, NSW, Australia
- hexamethonium (200μM) - Sigma Aldrich, NSW, Australia
- nicardipine (2.5μM) - Sigma Aldrich, NSW, Australia
- NOLA (100μM) - Sigma Aldrich, NSW, Australia
- TNP-ATP triethylammonium salt (5μM) - Tocris, sourced from In Vitro Technologies Pty Ltd. Vic, Australia)
- ondansetron (3μM) - Glaxo Research Group, Greenford, UK

15 Record a minimum of 3 synaptic responses before adding antagonist to the perfusing solution.

16 Record at least 3 responses between 5 and 20 minutes with antagonists present, before washing the drugs from the bath (20-30 minutes).

17 Where possible, record responses after the washout period to determine if any effect seen with an antagonist present is reversible.

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Immunofluorescence

18 Following electrophysiological recordings:

18.1 Remove the proximal colon from the recording bath.

18.2 Repin it into a petri dish lined with silicone elastomer.

18.3 Fix it overnight in 4% formaldehyde solution (40% formaldehyde diluted with 0.2M phosphate buffer, pH 7.0).

18.4 Remove fixative with 3 x 10 minutes washes in phosphate buffered saline (PBS).

18.5 Soak the proximal colon in 10% CAS block plus 0.1% Triton X-100 for 20 minutes.

19 Apply primary antibodies (AB) against calretinin or nitric oxide synthase (NOS) and incubate for 2-3 nights in humidified containers at **4 °C** .

19.1 Wash excess primary AB with 3 washes in PBS.

20 Add secondary ABs to visualize calretinin or NOS labelling with streptavidin Alexa-Fluor 594 (high binding affinity for biotin injected into neurons during recordings) to allow identification of impaled neurons

20.1 Incubate for 3 hours in dark humid containers.

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20.2 Remove secondary ABs by washing 3 x in PBS.

21 Mount the preparations on glass slides (Livingstone premium grade, thickness 1.0-1.2 mm) in Dako mounting medium (Carpinteria, CA, USA).

Confocal Microscopy

22 To visualize impaled neurons and either calretinin or NOS labeling capture Z-stack images (.czi files) using two channels of a confocal laser scanning microscope.

22.1 Set the step distance so that adjacent planes overlap.

22.2 Take high power (63x or 40x oil) images of neuron cell bodies.

22.3 Trace and image neuron projections using 10 or 20x air objectives ensuring overlap between consecutive image stacks.

22.4 Project Z-stacks into single images (.tif files) using ImageJ.

22.5 To produce detailed black and white images (also .tif files) further process images in Corel Photo-Paint.

22.6 Stitch neuron projection images (.tif files) together in Corel Draw to produce a "map" for each neuron and export as a jpeg file.

23 Each neuron map is linked into the excel spreadsheet containing the electrophysiological data obtained from that neuron.

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