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Mapping dichotomising colon and bladder sensory afferent neurons and terminals and if they undergo structural plasticity post-colitis. V.2

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SPARC

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This protocol is used to identify the sensory afferent neurons innervating the colon and bladder in dorsal root ganglia and nodose ganglia in order to map the distribution of dichotomising neurons (those innervating both organs) in the spinal and vagal sensory afferent pathways. This protocol was also used to identify how the central terminals of colon and bladder sensory afferent nerves are organised within the spinal cord and caudal medulla. This protocol was also used to identify if colon and/or bladder sensory afferent neurons or their central terminals under when structural plasticity in a mouse model of post-colitis chronic visceral hypersensitivity.

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protocol

Grundy, L et al. Chronic linaclotide treatment reduces colitis-induced neuroplasticity and reverses persistent bladder dysfunction JCI Insight. 2018;3(19):e121841. <https://doi.org/10.1172/jci.insight.121841>

colon, bladder, retrograde tracing, sensory afferent neurons, sensory afferent central terminals, colitis, chronic visceral hypersensitivity, nodose ganglia, dorsal root ganglia, spinal cord, dorsal vagal complex

_____ protocol ,

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2,4-Dinitrobenzenesulfonic acid hydrate (DNBS), MERCK Sigma-Aldrich catalogue number: 556971

KY Lubricating Jelly, Durex

Cholera Toxin Subunit-B conjugated to AlexaFluor (CTB-AF) 488 (500ug) ThermoFisher catalogue number: C22841

Cholera Toxin Subunit-B conjugated to AlexaFluor (CTB-AF) 594 (500ug) ThermoFisher catalogue number: C34777

30 GAUGE HUB RN Needle length 1 inch point style: 4 (10-12) Hamilton Company catalogue number: HAMC7803-07

Lethabarb (325 mg/ml pentobarbitone sodium solution) Virbac Australia

Paraformaldehyde Powder (95%) MERCK Sigma-Aldrich catalogue number: [158127](#)

Bis-acrylamide (19:1), MERCK Sigma-Aldrich catalogue number: A2917

VA-044 thermal initiator, FUJIFILM Wako Pure Chemical Corporation catalogue number: 27776-21-2

Sodium dodecyl sulfate, MERCK Sigma-Aldrich catalogue number: L3771

Boric acid, MERCK Sigma-Aldrich catalogue number: B6768

RapiClear 1.47 refractive index solution, SunJin Lab catalogue number: RC147001

Leica TCS SP8X, Leica Microsystems

Image J (NIH)

1 Induction of mouse model of post-colitis chronic visceral hypersensitivity.

<https://dx.doi.org/10.17504/protocols.io.14egn7mpqv5d/v1>

Colitis was induced by administration of Dinitrobenzene sulfonic acid (DNBS) via colorectal enema. Briefly, after an overnight fast with free access to a 5% glucose solution, 13-week-old mice anaesthetised via isoflurane inhalation (isoflurane 2-4% in oxygen) were administered a single intracolonic enema of 0.1 ml (6.5mg in 35% ethanol) DNBS via a polyethylene catheter. Healthy shams received an enema of sterile saline.

2 Cholera Toxin Subunit B (CTB) Retrograde tracing from the mouse colon and bladder wall.

4w

<https://dx.doi.org/10.17504/protocols.io.x54v9y391g3e/v1>

21-24 days after DNBS enema, mice underwent retrograde tracing from the colon and bladder wall using cholera toxin subunit b conjugated to Alexa Fluor 488 (bladder) and 594 (colon).

3 Perfuse fixation

4d

Four days after retrograde tracing surgery, to identify CTB labelled afferent cell bodies and seven days after retrograde tracing surgery to identify CTB labelled terminals mice underwent transcardial perfuse fixation as per *Harrington AM. et al.. Colonic afferent input and dorsal horn neuron activation differs between the thoracolumbar and lumbosacral spinal cord. Am J Physiol Gastrointest Liver Physiol. 317(3):G285-G303 (2019)*

<https://doi.org/10.1152/ajpgi.00013.2019>

Briefly, mice were given an overdose with Lethobarb (Virbac Australia, Milperra, NSW) via intraperitoneal injection and appropriate level of surgical anesthesia reached the chest cavity was opened and 0.5ml heparin-saline was injected into the left ventricle followed by insertion of a 22 gauge needle, attached to tubing and a peristaltic perfusion pump, into the left ventricle. The right atrium was snipped allowing for perfusate drainage. Warm saline (0.85 % physiological sterile saline) was perfused prior to ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer.

Following complete perfusion thoracic T9-T13 and lumbar L1-L6 and sacral S1 pairs of dorsal root ganglia (DRG) were collected with the lowest rib used as an anatomical marker of T13 DRG. Following this, the vagal nerve was used to located the nodose/jugular ganglia which was removed (both sides). To visualise terminals of traced spinal sensory nerves spinal cord was dissected at spinal levels T10-L1 and L5-S3. To visualise terminals of traced vagal sensory nerves the caudal medulla was excised.

4 CLARITY clearing.

Methods for CLARITY clearing were followed as per *Tomer, R. et al. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protoc 9,1682–1697 (2014).*

<https://doi.org/10.1038/nprot.2014.123>

. One exception being that animals were not perfused with 16% PFA-hydrogel solution (Hydrogel Matrix (HM) solution) as per the protocol as additional samples were collected that were not to undergo CLARITY clearing. After 4% paraformaldehyde perfusion, tissue was then placed in 16% PFA-HM solution (4% Bis-

acrylamide, 0.25% VA-044 in PBS). Samples were then kept at 4°C for a minimum of 24 hours. Residual oxygen was then removed from samples, as oxygen inhibits hydrogel polymerization, using a standard vacuum pump and desiccation chamber attached to a nitrogen gas supply. Samples were degassed in their tubes for 10 minutes before transfer to a 37°C water bath for 2 hours until hydrogel solution had uniformly polymerized. Tissue was removed from the hydrogel and underwent passive clearing in 8% SDS/200mM boric acid solution gently shaking at 37°C in a water bath. Buffer was changed every three days until samples were transparent at which point, they were washed with PBS-triton X-100 (0.2%) solution for 7 days at 37°C prior to placing individual ganglia into a 18-well glass slide. Wells with ganglia were filled with RapiClear 1.47 refractive index solution (SunJin Lab, Taiwan) and cover slipped 1 hour prior to confocal microscopy.

5 Confocal microscopy.

2w

Confocal laser scanning microscope (Leica TCS SP8X) was used to optically section cleared ganglia. Confocal images (1024 × 1024 pixels) were obtained with PL APO CS2 air 10X. Sequential scanning (5 line average) was performed with the following settings using a tunable white light laser and photomultiplier detectors: 495 nm-excitation and 503 / 538 nm-emission detection for AF488 and 561 nm-excitation and 570-625 nm-emission detection for AF594. Ganglia were optically sectioned (10µm thick) and z-projected images reconstructed for each ganglia (230-390µm).

6 Quantification of CTB labelled neurons

The number of colonic labeled (red), bladder labeled (green) or dual labeled (yellow) were obtained from projected images of CLARITY cleared ganglia using Image J (NIH) counting tool. Where it wasn't clear if overlapping neurons are counted, individual z-sections were used to confirm co-labelling. The mean number of neurons labelled from the colon, bladder or both in each ganglia was obtained and the mean number of labelled neurons / ganglia (± standard error of the mean) and the proportion of colon-, bladder- or dual- labelled neurons as a total of labelled neurons was used as the final representation and compared between the ganglia and between healthy and post-colitis mice.