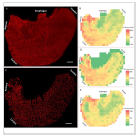

Jun 01, 2022

Topographical Organization, Morphology, and Density Analysis of Substance P (SP)-IR axons in the Whole Mouse Stomach

Duyen Nguyen[1], Anas Mistareehi[1], Jichao Ma[1], Jazune Madas[1], Andrew M. Kwiat[1], Kohlton Bendowski[1], Jin Chen[1], De-Pei Li[2], John Furness[3,4], Terry Powley[5], Zixi Jack Cheng[1]

[1]University of Central Florida; [2]University of Missouri; [3]The University of Melbourne; [4]The Florey Institute of Neuroscience and Mental Health; [5]Department of Psychological Sciences, Purdue University

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Tech. support email: info@neuinfo.org

Duyen Nguyen
University of Central Florida

This protocol describes the process of mapping the topographical organization of substance P (SP) immuno-reactive axons and terminals in the mouse stomach. Stomachs were removed from the abdominal cavities, layers of the organ were separated and gone under immunohistochemistry as whole mounts, then scanned using confocal and/or Zeiss microscopes. The stomach was reconstructed into a 2D maximum projection image and was evaluated for density of SP-IR axons. This analysis method was performed on the stomach as a whole as well as on separated layers (myenteric plexus, circular and longitudinal muscles).

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substance P, nociceptive afferent, myenteric plexus, longitudinal and circular muscles, stomach

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Animals

1

Mouse cohorts for feeding **Jackson**

Male **Laboratory Catalog #C57BL/6J** (age range: 12-16 weeks) were used. All animals were housed in a room in which light/dark cycles were set to 12h/12h (6:00 AM to 6:00 PM light cycle) and provided food and water ad libitum.

All procedures were approved by the University of Central Florida Animal Care and Use Committee and strictly followed the guidelines established by NIH. All experiments conformed to the University of Central Florida guidelines on the ethical use of animals.

Tissue preparation

2

Mice were deeply anesthetized 4% Isoflurane **Covetrus Catalog #029404** . Absence of the hind paw pinch withdrawal reflex was used as an indicator to ensure sufficient depth of anesthesia.

The inferior vena cava was cut to drain the blood and perfusate.

Mice were perfused through the left ventricle with 38-40 °C phosphate-buffered saline (0.1 M PBS, pH = 7.4) for 5 minutes, then fixed by perfusion with 4 °C Zamboni's fixative (15% picric acid and 2% paraformaldehyde in PBS, pH = 7.4).

The whole stomach with a small portion of distal esophagus and proximal duodenum were removed.
The stomach was cut open along the lesser and greater curvatures into ventral and dorsal halves, the contents within the stomach were removed and the stomach was rinsed with distilled water, followed by a 24h post-fixation in the same fixative.

After post-fixation, the mucosal and submucosal layers of the ventral and dorsal halves were peeled off of the muscular wall of the stomach. The longitudinal muscle, circular muscle and myenteric plexus comprise the flat-mounts (or the whole-mounts) of the entire stomach muscular wall.

Immunohistochemistry (IHC)

3

Single and double labeling of mouse stomach to reveal the morphology and distribution of SP-IR axons and terminals, as well as the origin of this neuropeptide.

3.1 *Single labeling. S*imilar to the protocol that we previously used for the heart (Li et al. 2014), the flat-mounts of the whole stomach muscular walls (ventral n = 3, dorsal n = 3), brainstems (n = 2) and celiac ganglia (n = 3) were processed with primary antibody
☒ Guinea pig polyclonal anti-SP antibody **Abcam Catalog #10353** .

All IHC procedures were performed in 24-well plates on a shaker **120 rpm, Room temperature**

3.2 The stomach walls were washed six times, 5 minutes each in 0.01 M PBS pH = 7.4 to remove any remaining fixative.

Tissues were then blocked for 4 days in a blocking mixture containing 2% bovine serum albumin, 10% normal goat and donkey serum, 2% Triton X-100, and 0.08% NaN3 in 0.01 M PBS, pH = 7.4, followed by a primary antibody incubation for 4 days (12:1000) in a solution comprising 2% bovine serum albumin, 4% normal goat and donkey serum, 0.5% Triton X-100, and 0.08% NaN3 in 0.01 M PBS, pH = 7.4).

3.3 The stomachs were thoroughly washed six times, 5 minutes each in PBS-T (0.5% Triton X-100 in 0.01 M PBS) to remove unbound primary antibodies.

The stomachs were kept in the dark and incubated in a fluorescent secondary antibody
☒ Alexa Fluor 594 goat anti guinea pig **Invitrogen Catalog #A11076** (24:1000 in PBS-T solution) for 2 days.

3.4 Unbound secondary antibodies were removed by washing the stomachs six times, 5 minutes each in PBS-T.

Negative controls were performed by omitting either primary or secondary antibodies and did not show any SP-IR axons or terminals.

3.5 The ventral and dorsal stomach flat-mounts were then mounted on glass slides, flattened with lead weights (total: 6.75 kg) for 4 hours, and air-dried under a fume hood for one hour.

The slides were then dehydrated using four ascending concentrations of ethanol (75%, 95%, 100%, and 100%) each for 4 minutes followed by a clearing step using xylene for 20 minutes.

Cover slips were used to cover the tissue after applying
☒ DEPEX mounting medium **Electron Microscopy Sciences Catalog #50-980-371** (Product # 13514).

The tissue and the slides were air dried overnight in the fume hood.

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3.6 *Double-labeling:* To investigate whether SP-IR axons in the stomach originated from sympathetic (catecholaminergic) or enteric ganglia, whole muscular walls (ventral n=3, dorsal n=3) were double labeled for SP with tyrosine hydroxylase (TH, a sympathetic marker) or Vesicular Acetylcholine Transporter (VAChT, a marker of enteric excitatory muscle motor neurons, vagal pre-enteric neurons and possibly gastric interneurons). The samples were processed using the same protocol as single labeling with the addition of either TH or VAChT sequentially to the stomach samples.

The primary antibodies were:
☒Rabbit anti-substance P antibody; AB_572266 **Immunostar Catalog #20064** polyclonal,
☒Sheep anti-tyrosine hydroxylase antibody **Merck Millipore Catalog #AB1542** polyclonal,
☒Goat anti-Vesicular Acetylcholine Transporter (VAChT) antibody **Millipore Sigma Catalog #ABN100** polyclonal.

The secondary antibodies were:
☒Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody Alexa Fluor 594 **Thermo Fisher Scientific Catalog #A-21207**

☒Donkey anti-Sheep IgG (H L) Cross-Adsorbed Secondary Antibody Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A-11015**

☒Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 **Thermo Fisher Scientific Catalog #A11055**
(RRID:AB_2534102)

We used Rabbit anti-SP primary antibody rather than guinea pig anti-SP primary antibody that we used for single labeling above. This was because we aimed to reduce any possibility that the secondary antibody (Anti-Guinea pig; host goat) against the Guinea pig SP primary antibody could react with the secondary antibody (Anti-goat, host donkey) against the Goat anti-VAChT primary antibody that we used for double labeling. Before the double labeling experiment, Rabbit anti-SP primary antibody was used to label stomach walls (n=5) which yielded a similar distribution pattern and morphology of the SP-IR axons (not shown) that had been seen using Guinea pig SP primary antibody in the single labeling experiment above.

A	B	C	D	E
Antibody	Host	Concentration	Emission	Company
Anti-SP (1)	Guinea pig	12:1000	n/a	Abcam
Anti-SP (1)	Rabbit	12:1000	n/a	Immunostar
Anti-TH (1)	Sheep	12:1000	n/a	Millipore Sigma
Anti-VAChT (1)	Goat	12:1000	n/a	Millipore Sigma
Alexa Fluor Anti-Guinea pig (2)	Goat	24:1000	594 nm	Invitrogen
Alexa Fluor Anti-rabbit (2)	Donkey	24:1000	594 nm	Thermo Fisher Scientific
Alexa Fluor Anti-sheep (2)	Donkey	24:1000	488 nm	Thermo Fisher Scientific
Alexa Fluor Anti-goat (2)	Donkey	24:1000	488 nm	Thermo Fisher Scientific

1: primary antibody, 2: secondary antibody

Tracer injection into the stomach wall

4 To verify whether the SP-IR axons in the stomach originated from the dorsal root ganglia (DRG) and vagal nodose-petrosal ganglia complex (VNG), mice (n=5) were anesthetized with 2-3% ☒Isoflurane **Covetrus Catalog #029404** . The depth of anesthesia was determined by absence of the hind paw pinch withdrawal reflex.

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Tracer

FAST DiI™ oil; DiIΔ9,12-C18(3), ClO4 (1,1'-Dilinoleyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate) **Thermo Fisher Catalog #D3899**

was injected into the ventral or dorsal stomach walls using a

NANOFIL-100 sub-microliter injection system **World Precision Instruments** in the regions of the fundus, corpus, and antrum-pylorus to cover the whole surface of the ventral or dorsal walls. Each region received 3-4 injections (2 µl/each, 9-12 injections per surface).

Following injections, the incisions were closed and the animals were returned to their cages.

14-16 days after injection, animals were perfused, and the left and right DRGs (T1-12), as well as the left and right VNG, were removed for IHC labeling of SP.

Fluoro-Gold (FG) counterstaining

5

To counterstain neurons in the myenteric plexus and determine whether vagal motor neurons in the dorsal motor nucleus of the vagus (DmnX) in the brainstem which project to the stomach contain SP-IR, 3 additional animals were used.

Fluorogold **Fluorochrome** (0.3 ml of 3 mg/ml per mouse) was injected (i.p.) to counterstain neurons in the peripheral ganglia and the DmnX (Ai et al. 2007, Cheng et al. 1997b, Cheng et al. 2004, Li et al. 2010, Lin et al. 2008).

Animals in these groups were perfused 3–5 days after FG injections.

The stomachs (n = 3) were removed and peeled as described above for SP IHC labeling.

The brainstems (n = 2) were also removed and used for possible SP labeling of vagal motor neurons in the DmnX.

The brainstems were cross-sectioned (100 µm) relative to the obex (-1000 to + 1000 µm) using

Cryostat
Leica **Cryostat**

which included the nucleus of the solitary tract (NTS) and DmnX as shown previously (Cheng et al. 2002, Yan et al. 2009).

Data acquisition and analysis

6

Eclipse 80i
Fluorescent Microscope
Nikon **M318E**

(Lens: 20X and 40X) was first used to survey the quality of SP labeling in flat-mounts of stomach walls. Then,

TCS SP5 Laser Scanning
Confocal Microscope
Leica **TCSSP5**

was used to acquire images and put together image montages of whole ventral and dorsal stomach walls.

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The helium-neon (HeNe) laser (excitation 543 nm) was used to detect SP-IR axons and an argon–krypton (ArKr) laser (excitation 488 nm) was used to detect background autofluorescence of the tissues (e.g., smooth muscles and myenteric ganglion cells).

In addition, the 405 nm laser (excitation) was used for detecting and verifying FG-labeled myenteric ganglionic neurons and brainstem DmnX neurons.

Each whole ventral or dorsal stomach was first scanned using a 20X oil immersion objective lens (Z-step: 1.5 µm), which led to over 600 frames of confocal image stacks per montage. The confocal projection images of these stacks were used to assemble montages of whole ventral or dorsal stomachs using

ImageJ/Fiji 1.8.0
Windows 7
source by National Institutes of Health

(https://imagej.net/, RRID: SCR_003070) and

Adobe Photoshop 2020
by Adobe

(RRID: SCR_014199). The generated montages showed the whole stomach with the resolution of single cell/axon scale.

To better show the details of varicose SP-IR axons and connections with their targets, regions of interest were scanned at high magnification (40x oil immersion objective lens, zoom: 1.5 X, Z-step: 1 µm). An additional whole flat-mount stomach labeled with FG was imaged using

Axio Imager M2
Microscope
Zeiss AI2

with a 350 nm excitation wavelength. Modifications, including brightness and contrast adjustments, and scale bar additions were conducted utilizing Photoshop , ImageJ, or Zen (for Zeiss M2) software.

Regional density analysis of SP-IR axons in the whole stomach

7 *Overall regional density:* The overall regional density is the SP-IR axon density within the whole stomach with all layers, including the muscular layers (longitudinal and circular) and myenteric plexus.

Overall regional density analysis of SP-IR axons in the whole ventral and dorsal stomachs started by importing their respective confocal montages into Photoshop, each montage including over 600 frames of all-in-focus confocal projection images (30-40 optical sections/each frame, Z-step: 1.5 µm).

Then, a sampling grid with a window size of 1067x1067 pixels was used to cover each montage completely. The individual windows were accurately labeled according to their positions and exported as .png files. Using

Icy
Windows/MacOS/Linux
source by BioImage Analysis Lab (Institut Pasteur)

which is a free open-source bio-image analysis program (RRID:SCR_010587), two pixel numbers were calculated and exported per window into an Excel file: all signals and axon signals.

The “all signals” in a window referring to the areas where any signals (e.g., muscles, ganglia, axons, and noises) may exist. The “axon signals” referred to the areas where only axon signals existed.

For "all signals", we used a lower threshold to exclude any dark/black areas where no signal existed in the window and obtained the surface area where all signals were included. For "axon signals", we used a higher threshold to detect pure axon fibers only.

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An equation was applied to calculate the axon density in each window of the grid in Excel:

axon density of the window = nerve signal pixels/surface area pixels

Finally, an overall density montage was reconstructed from all windows of the sampling grid. Conditional formatting (Low density = green; High density = red) was used on the density montage to create a heatmap so that areas of high axon density could be easily seen.

Due to the minimal background in the entire stomach, the SP-IR axon signals to noise ratio was very high. Thus, we did not have any problems in obtaining such heatmaps.

Laminar regional density in the myenteric plexus and muscular layers: For the SP-IR axon innervation of the myenteric plexus, the overall montage was imported into Photoshop, and then the areas outside the myenteric plexus were cut out. Since the shape of SP-IR axons in myenteric ganglia could be easily identified in the montage, there were no problems in removing these areas outside the myenteric plexus.

After this myenteric plexus was isolated, we were able to use a similar process as above to create a heatmap for it.

The heat-map for muscular layers was obtained by subtracting the myenteric plexus heat-map from the overall heat-map.

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