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NADPH diaphorase histochemistry in rat gastric nitric oxide synthase neurons

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We use this protocol and it's working

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Abstract

NOS (nitric oxide synthase) is a NADPH oxidase, requiring NADPH as a proton donor for the conversion of arginine to citrulline and the production of nitric oxide (NO). Thus nitrergic neurons can be localised by nNOS immunoreactivity or histochemically, using the NADPH diaphorase reaction (Young et al. 1992) to enable investigation of their morphology.

Materials

Tris buffer, **Vendor, Cat#**
Triton X-100, **Vendor, Cat#**
β-NADPH, Cat# N1630, Sigma-Aldrich, Sydney, Australia
Nitro blue tetrazolium, Cat# N6876, Sigma-Aldrich, Sydney, Australia
Nicardipine, Cat# N7510, Sigma-Aldrich, Sydney, Australia
Dako fluorescence mounting medium, Agilent, Tullamarine, Australia

Primary antibodies:
Human anti-Hu C/D antibody, V.A. Lennon, Mayo Clinic, Rochester, Minnesota, USA **RRID:AB_2314657**
Sheep anti-nNOS antibody, P.C. Emson, Babraham Institute Cat# NOS, **RRID:AB_2314960**

Secondary antibodies:
Donkey anti-Human Alexa Fluor 594, Jackson ImmunoResearch Labs Cat# 709-585-149 **RRID:AB_2340572**
Donkey anti-Goat Alexa Fluor Plus 488, Thermo Fisher Scientific Cat# A32814, **RRID:AB_2762838**

Microscope:
Axio Imager (Zeiss, Sydney, Australia)
LMS800 confocal microscope (Zeiss, Sydney, Australia)
Mirax Digital Slide Scanner (Zeiss, Sydney, Australia)
Olympus SZH Drawing Tube (Camera Lucida)

Software:
CorelDRAW Graphics Suite, RRID:SCR_014235

1 *Tissue sources*

All procedures were approved by the University of Melbourne Animal Ethics Committee (approval 10272).Rats were supplied with food and water ad libitum prior to any experiments. Stomach samples were collected from female and male Sprague Dawley (SD) rats, 7-10 weeks old, 168-236 g for females and 308-382 g for males.

NADPH diaphorase histochemistry

2 NADPH diaphorase (NADPHd) histochemistry was based on a previously published procedure (Young et al. 1992, **doi.org/10.1007/BF00270041**). All procedures were at room temperature (RT) unless otherwise specified. Rats were deeply anesthetised by isoflurane inhalation followed by decapitation, and then stomachs were removed and placed into PBS (phosphate-buffered saline: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) containing nicardipine (1 µM; Sigma-Aldrich, Sydney, Australia) before being opened along the greater curvature and stretched and pinned to balsa board, mucosal side down, for fixation in 4% formaldehyde, in 0.1M phosphate buffer, pH 7.0, for 1 hour at 4°C. Fixative was removed by 3-6 × 10 min washes in PBS. Tissue samples were taken from the ventral fundus, corpus and antrum. Wholemounts of the longitudinal muscle and myenteric plexus were created by dissecting away the mucosa, submucosa and most of the circular muscle. Wholemounts of the submucosa were also prepared. Wholemounts were incubated with 0.3% Triton X-100 in PBS for 10 min followed by a 1 × 10 min PBS wash. NADPH was dissolved in 0.01M sodium hydroxide to create a 50mg/ml stock solution, which was then used to prepare the NADPHd solution (0.1M Tris buffer, pH 7.6, 0.5% Triton X-100, 0.25mg/mL nitroblue tetrazolium, 1mg/mL β-NADPH) (Sigma-Aldrich). Tissue was incubated in the NADPHd solution for 10-25 min at 37°C, while the colour reaction was monitored. Wholemounts were washed again with 3 x 10 min changes of PBS and mounted with Dako fluorescence mounting medium (Agilent, Tullamarine, Australia). Staining was assessed and individual cell drawings were completed using an Olympus camera lucida system and original pencil drawings were then scanned to pdfs and outlines were converted into Corel draw images (CorelDRAW Graphics Suite, RRID:SCR_014235). Samples were also imaged using a Mirax Digital Slide Scanner (Zeiss, Sydney, Australia).

Combined NADPH diaphorase and neuronal NOS staining

3 Wholemount preparations, that were fixed and dissected as above, were immunohistochemically stained with nNOS prior to NADPHd staining (Young et al. 1992). Wholemounts were incubated with a mixture of sheep anti-nNOS and human anti-Hu in PBS with 0.25% Triton X-100, overnight at RT. The preparations were then washed 3 × 10 min in PBS before a 2-hour incubation with fluorescently labelled secondary antibodies in PBS containing 0.25% triton. Samples received a further 3 × 10 min

washes in PBS, before being mounted with buffered glycerol (90% glycerol in PBS). Selected ganglia were imaged using an Axio Imager microscope (Zeiss, Sydney, Australia) or an LMS800 confocal microscope (Zeiss, Sydney, Australia). The coverslip was then removed and the samples histologically stained for NADPHd, as described above. The samples were mounted with Dako fluorescence mounting media and the cells that were identified initially, were re-imaged for NADPHd staining.

Protocol references

Young, H.M., Furness, J.B., Shuttleworth, C.W.R.*et al.* Co-localization of nitric oxide synthase immunoreactivity and NADPH diaphorase staining in neurons of the guinea-pig intestine.*Histochemistry* **97**, 375–378 (1992). https://doi.org/10.1007/BF00270041