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Quantitative analysis of enteric neurons containing choline acetyltransferase and nitric oxide synthase immunoreactivities in the submucosal and myenteric plexuses of the porcine colon

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ABSTRACT

The enteric nervous system (ENS) controls gastrointestinal functions. In large mammals' intestine, it comprises an inner (ISP) and outer (OSP) submucous plexus and a myenteric plexus (MP). This study quantifies enteric neurons in the ISP, OSP and MP of the pig ascending (AC) and descending colon (DC) using the HuC/D, choline acetyltransferase (ChAT) and neuronal nitric oxide synthase (nNOS) neuronal markers in wholemount preparations with multiple lbeling immunoflorescence. We established that the ISP contains the highest number of HuC/D neurons/mm2 , which were more abundant in AC vs. DC, followed by OSP and MP with similar density in AC and DC. In the ISP, the density of ChAT immunoreactive (IR) neurons was very similar in AC and DC (31% and 35%), nNOS-IR neurons were less abundant in AC than DC (15% vs. 42%, P< 0.001) and ChAT/nNOS-IR neurons were 5% and 10%, respectively. In the OSP, 39-44% of neurons were ChAT-IR in AC and DC, while 45% and 38% were nNOS-IR and 10- 15% were ChAT/nNOS-IR (AC vs. DC P< 0.05). In the MP, ChAT-IR neurons were 44% in AC and 54% in DC (P< 0.05), nNOS-IR neurons were 50% in both and ChAT/nNOS-IR neurons were 12 and 16%, respectively. The ENS architecture with multilayered submucosal plexuses and the distribution of functionally distinct groups of neurons in the pig colon are similar to humans, supporting the suitability of the pig as a model and providing the platform for investigating the mechanisms underlying human colonic diseases

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Enteric nervous system, excitatory motor neurons, inhibitory motor neurons, secretomotor neurons, interneurons

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MATERIALS TEXT

MATERIALS

Mo anti-nNOS **Santa Cruz Biotechnology Catalog #SC-5302**

Rb anti-nNOS **Abcam Catalog #ab15203**

Rb anti-ChAT **Contributed by users Catalog #P3YEB**

Mo anti-HuC/D **Contributed by users Catalog #A-21271**

Goat anti-mouse Alexa Fluor® 594 **Contributed by users Catalog #A11032**

Goat anti-rabbit Alexa Fluor® 488 **Thermo Fisher Scientific Catalog #A11008**

Goat anti-mouse Alexa Fluor® 488 **Thermo Fisher Scientific Catalog #A11029**

Goat anti mouse Alexa Fluor ®405 **Thermo Fisher Scientific Catalog #A31553**

1 Tissue Preparation

1.1 Animal care and procedures described in this study were carried out in strict accordance with the National Institutes of Health recommendations for the humane use of animals. The experimental procedures were approved by University of California, Los Angeles (UCLA), Chancellor's Animal Research Committee (ARC) (protocol 2018-074-01), and all efforts were made to avoid suffering. Specimens were obtained from 12 hours fasted 15 male castrated Yucatan minipigs (average weight 25-30 Kg of body weight). All animals were anaesthetized by intramuscular application of midazolam (1 mg/kg, cat # 067595, Covetrus, Dublin, OH), ketamine (15 mg/kg, cat # 068317, Covetrus, Dublin, OH) and meloxicam (0.3 mg/kg, #049755, Covetrus, Dublin, OH). These animals were first used for colonic motility analysis with manometry probes apposed on the serosa, then tissues were collected

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10-20 cm distally 5 hours post induction of anesthesia. The ascending colon (AC) in correspondence to the central flexure and descending colon (DC) (about 30 cm from the anus) were collected. We have compared these tissues to specimens collected 6 immediately after induction of anesthesia and we have not seen differences in the total density of enteric neurons and the localization of different neuronal markers, thus providing assurance that this procedure did not affect neuronal distribution and neurochemical expression. The samples were immersed in 0.01 M phosphate buffer saline (PBS, pH 7.0) containing the Ltype calcium channel blocker, nicardipine (20 mM) for 15-40 min. The tissues were then opened along the mesenteric border, vigorously flushed out with PBS and pinned tightly on balsa wood, mucosal surface facing down. Specimens were subsequently fixed in 2% paraformaldehyde containing 0.2% picric acid in PBS at 4 °C overnight, removed from the balsa wood, washed (3 x 10 min) in dimethyl-sulfoxide (DMSO, Sigma-Aldrich), followed by washing in PBS (3 x 10 min) and stored at 4 °C in PBS containing sodium azide (0.1%). Wholemount preparations of the MP were obtained by separating the longitudinal muscle layer with attached the MP from the submucosa and mucosa using a dissecting microscope. The mucosa was removed from the submucosa and the submucosal layer was separated into the inner (ISP) and outer (OSP) parts of the submucosal plexus.

Immunohistochemistry

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2.1 In order to establish the total number of neurons in each plexus and the distribution of subclasses of neurons, HuC/D, ChAT and nNOS primary antibodies were used (Table 1). Initial single labeling immunofluorescence experiments determined each individual antibody best dilution and incubation time. Wholemount preparations were incubated in 10% normal goat serum (NGS) in PBS containing 1% Triton-X100 and 1% BSA for 1 hour at room temperature (RT) to reduce nonspecific binding of the secondary antibodies and to permeabilize the tissue to the antisera. All primary antibodies were diluted in PBS containing 1% Triton-X100 and 3% NGS. All secondary antibodies were diluted in PBS containing 3% NGS. Tissues were then incubated at 4°C in a humid chamber for 2 days with the primary antibody (e.g. mouse anti-HuC/D), washed in PBS (3 x 10 min), and incubated for 3 hours at RT in a humid 7 chamber in a solution containing the secondary antibody (e.g. goat anti-mouse Alexa Fluor® 594 or 405) (Table 1). For double and triple-labeling experiments, we used the sequential staining procedure (Ho et al. 2003), which has been shown to produce the best staining/ background ratio and allow the use of two mouse monoclonal antibodies in the same preparation. For double labeling, tissue was first incubated with the first antibody (e.g. mouse anti-NOS or rabbit anti-ChAT), followed by goat anti-mouse or goat anti-rabbit Alexa Fluor® 488, then incubated with mouse anti-Hu C/D followed by goat anti-mouse Alexa Fluor® 594. For triple labeling, tissue was first incubated with the first antibody (e.g. rabbit anti-ChAT), followed by goat anti-rabbit Alexa Fluor® 488, then incubated with mouse anti-NOS, followed by goat anti-mouse Alexa Fluor® 594, finally incubated with mouse anti-Hu C/D, followed by goat anti-mouse Alexa Fluor® 405, then mounted on gelatin-coated slides with ProLongTM Gold antifade reagent (Invitrogen). Tissues were washed several times before each step.

Table 1. List of primary and secondary antibodies and their respective dilutions

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Marker Code	Code	Case product	Dilutions
Mo anti-nNOS	SC-5302	Santa Cruz	1:100
Rb anti-nNOS	ab15203	Abcam	1:100
Rb anti-ChAT	P3YEB	Prof. Schemann	1:800
Mo anti-HuC/D	A-21271	Thermofisher Scientific	1:100
Secondary antibody			
Goat anti-mouse Alexa Fluor® 594	A11032	Thermofisher Scientific	1:800
Goat anti-rabbit Alexa Fluor® 488	A11008	Thermofisher Scientific	1:2000
Goat anti-mouse Alexa Fluor® 488	A11029	Thermofisher Scientific	1:1000
Goat anti mouse Alexa Fluor ®405	A31553	Thermofisher Scientific	1:1000

Specificity of primary antibodies

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3.1 The specificity of the monoclonal antibodies to nNOS and HuC/D and of the polyclonal rabbit antiChAT antiserum has been previously tested (Murphy et al., 2007; Russo et al. 2013; Hens et al. 8 2000). The staining obtained with the rabbit anti-nNOS antiserum was superimposable to the one observed with the mouse monoclonal anti-nNOS antibody, supporting its specificity

Quantitative analysis of ISP, OSP and MP neurons

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4.1 Specimens were examined using Zeiss LSM 880 Fast-Airyscan confocal microscope and the Imaris software (Imaris for Neuroscientist) for quantification. In each specimen, we counted the total number of neurons immunoreactive (-IR) for the generalized neuronal marker Hu C/D and we expressed it as number of neurons per mm2 and number of neurons per ganglion. We then counted the number of

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ChAT-, nNOS- or ChAT/nNOS-IR neurons and expressed them as number per mm2 and percentage of the total number of HuC/D-IR neurons. Data were expressed as means ± standard error of the mean (SEM). One-way and two-way ANOVA followed by Bonferroni post-test for multiple comparisons were used for statistical analysis ($P < 0.05$ for significance). The statistical software package Prism 8.3.0 (GraphPad Software, San Diego, CA) was used for these analyses.

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