OXIDIZED PHOSPHOLIPIDS AFFECT SMALL INTESTINE NEUROMUSCULAR TRANSMISSION AND SEROTONERGIC PATHWAYS IN JUVENILE MICE

Journal:	*Neurogastroenterology and Motility*
Manuscript ID	NMO-00271-2020.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	14-Oct-2020
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Key Words:	oxidized phospholipids, enteric nervous system, small intestine neuromuscular contractility, Toll-like receptors, serotonine

OXIDIZED PHOSPHOLIPIDS AFFECT SMALL INTESTINE NEUROMUSCULAR TRANSMISSION AND SEROTONERGIC PATHWAYS IN JUVENILE MICE

Running title: Oxidized phospholipids alters gut function

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Abbreviations: ENS, enteric nervous system; 5-HT, serotonin; IBS, irritable bowel syndrome; TLRs, toll-like receptors; OxPLs, oxidized phospholipids; carbachol, CCh; EFS electric field stimulation

KEY POINTS

- OxPAPC alters the structure of the enteric neuroglial network.
- OxPAPC causes dysmotility of the juvenile small intestine associated with altered cholinergic and nitrergic neurotransmission.
- OxPAPC determines higher functional response to 5-HT, together with a marked decrease of 5-HT levels, shifting tryptophan metabolism towards kynurenine production.

ABSTRACT

Background. Oxidized phospholipid derivatives (OxPAPCs) act as bacterial lipopolysaccharide (LPS)-like damage-associated molecular patterns. OxPAPCs dose-dependently exert pro- or anti-inflammatory effects by interacting with several cellular receptors, mainly Toll-like receptor 2 and 4. It is currently unknown whether OxPAPCs may affect enteric nervous system (ENS) functional and structural integrity.

Methods. Juvenile (3 weeks old) male C57Bl/6 mice were treated intraperitoneally with OxPAPCs, twice daily for three days. Changes in small intestinal contractility were evaluated by isometric neuromuscular responses to receptor and non-receptor-mediated stimuli. Alterations in ENS integrity and serotonergic pathways were assessed by real-time PCR and confocal immunofluorescence microscopy in longitudinal muscle-myenteric plexus whole-mount preparations (LMMPs). Tissue levels of serotonin (5-HT), tryptophan and kynurenine were measured by HPLC coupled to UV/fluorescent detection.

Key Results. OxPAPCs treatment induced enteric gliosis, loss of myenteric plexus neurons, excitatory hypercontractility and reduced nitrergic neurotransmission with no changes in $nNOS^+$ neurons. Interestingly, these changes were associated with a higher functional response to 5-HT, altered immunoreactivity of 5-HT receptors and serotonin transporter (SERT) together with a marked decrease of 5-HT levels, shifting tryptophan metabolism

towards kynurenine production.

Conclusions & Inferences. OxPAPCs treatment disrupted structural and functional integrity of the ENS, affecting serotoninergic tone and 5-HT tissue levels towards a higher kynurenine content during adolescence, suggesting that changes in intestinal lipid metabolism toward oxidation can affect serotoninergic pathways, potentially increasing the risk of developing functional gastrointestinal disorders during critical stages of development.

Keywords: oxidized phospholipids, Toll-like receptor, serotonin, enteric nervous system, small intestine neuromuscular contractility, confocal microscopy

1. INTRODUCTION

Irritable bowel syndrome (IBS) is a multifactorial functional gastrointestinal disorder affecting about 10–44% of EU citizens.[1,2] IBS is diagnosed by a characteristic symptom profile (abdominal pain/discomfort, bloating/distension, alterations in defecatory function) in the absence of a definite organic disease of the gastrointestinal system.[3] Several preclinical and clinical studies have proposed a variety of genetic, environmental, and psychological factors, including gut dysbiosis, obesity, food intolerances, sleep habits, stress, inflammation and surgery, as possible triggers of IBS.[4] Higher levels of circulating pro-inflammatory polyunsaturated fatty acids[5] and cytokines[6,7] have been reported in IBS patients together with a low-grade immune activation.[8,9] However, these altered immune responses, observed in IBS patients, still lack of clear mechanistic insights.

During oxidative stress, endogenous phospholipids (PLs), such as 1-palmitoyl-2-arachidonoyl-*sn*-glycerol-3-phosphatidylcholine (PAPC), are easily peroxidized to a mixture of reaction products, termed as OxPAPCs, and include oxidized chain-shortened phospholipids and oxygenated phospholipids. In physiological conditions, submicromolar to micromolar levels of OxPAPCs are circulating in human and rodent plasma, potentially exerting anti-LPS activity.[10] At low concentrations OxPAPCs block both Toll-like receptors (TLRs) 2 and 4 signaling by competitively interfering with extracellular accessory proteins such as CD14, LPS-binding protein (LBP), and MD2.[11,12] Thus, oxidized phospholipids (OxPLs) might be useful tools for studying the role of TLRs signaling during homeostasis.[13]

TLRs are recognized as first-line sentinels of innate and adaptive immunity that sense a distinctive repertoire of diverse molecules, released by microbes, referred to as microbial-associated molecular patterns (PAMPs), or derived from mammal cells, termed as danger-associated molecular patterns (DAMPs).[14] In the gastrointestinal tract, TLRs are expressed on immune cells, and epithelial cells as well as in neurons, glia and smooth muscle cells,[15]

highlighting their key role in ensuring gut homeostasis, including enteric nervous system (ENS) integrity.[15,16] Juvenile mice deficient for TLR2 show morpho-functional alterations of the ENS,[17] similar to that observed in mice following antibiotic-induced dysbiosis of gut microbiota.[18] TLR4, the most studied pathogen-recognition receptor, participates in the control of nitrergic/purinergic pathways within enteric neuronal-glial networks, influencing gut motility.[15,19] Germ-free, antibiotic-treated mice and TLR4 deficient mice display similar gastrointestinal dysmotility and fewer $nNOS^+$ neurons.[15,18,19] Recent reports have highlighted that changes in TLR2 and/or TLR4 signaling in mouse small intestine influence the pattern of expression of serotonin (5-HT) receptors as well as motor responses to 5-HT, thus suggesting an interactive communication between TLRs and the intestinal serotonergic system.[15,20-22] Alteration of 5-HT homeostasis is a major feature in IBS patients and may account for dysmotility. Indeed, changes in the whole serotonergic neurotransmitter machinery, including synthesis, reuptake mechanisms and receptors have been observed in IBS patients.[23] Administration of $5\text{-}HT_3$ receptor antagonists to IBS patients has been shown to ameliorate motor dysfunction and visceral hypersensitivity.[23,24] In the gut, 5-HT is produced from the metabolism of the essential amino acid tryptophan (TRP) which may give origin to several other compounds, such as kynurenines (KYN), tryptamine and indolic compounds, participating to the microbiota-gut-brain communication in health and disease states.[25-27] Under normal conditions, approximately 3% of the assumed TRP is metabolized into 5-HT, whereas about 90% is catabolized into KYN, through the KYN pathway, and the remaining is degraded by the gut microbiota in order to produce indole and its derivatives.[28] Decreased 5-HT and increased KYN plasma concentrations as well as an elevation in the KYN/TRP ratio, possibly involving TLR activation, have been shown in IBS patients.[6,23] These findings highlight the potential regulatory activity for TRP and its metabolites on the secretory, motor and sensory gut functions in health and disease conditions. The effects of TRP metabolites

are, however, not only confined to the regulation of cell homeostasis within the enteric microenvironment, but may also extend to the CNS, influencing mood and cognitive functions. [23,29,30] This latter observation lends itself to the possibility of studying the involvement of tryptophan metabolites in the generation of microbiota-gut-brain axis signaling underlying psychiatric disturbances associated with IBS.[23,30] Based on this evidence, we aimed to evaluate the impact of OxPAPCs on functional and structural integrity of the ENS as well as on enteric serotonergic neurotransmission and tryptophan metabolism in male juvenile mice.

2. MATERIALS AND METHODS

2.1 Animals and in vivo treatment

All animal care and experimental procedures were approved by the Animal Care and Use Ethics Committee of the University of Padova and by the Italian Ministry of Health (authorization number: 1142/2015-PR) and were performed in accordance with national and EU guidelines for the handling and use of experimental animals. Animal studies are reported in compliance with the ARRIVE guidelines.[31,32]

Juvenile male C57BL/6J mice (3±1 weeks old, body weight 18±1 g; Charles River Laboratories, Italy) were housed in individually ventilated cages (four animals per cage) at the conventional animal facility of the Department of Pharmaceutical and Pharmacological Sciences, University of Padova under controlled environmental conditions (temperature 21±1°C; relative humidity 60–70%) with access to food and water *ad libitum* and maintained at a regular 12/12 h light/dark cycle. Mice were randomized and subjected to intraperitoneal administration of OxPAPCs (Invivogen; 1.5 μg/g in 0.9% saline; OxPAPC-treated mice)[33] or saline solution (control (CNTR) mice) for 3 days, twice a day. At the end of procedures, animals were killed by cervical dislocation. All the subsequent experimental procedures were conducted blindly.

2.2 Immunohistochemistry on ileal whole mount preparations

Distal ileum segments (10 cm) were filled with fixative solution 4% PFA in PBS for 1 hour at room temperature. Using a dissecting microscope, whole-mount preparations of longitudinal muscle with attached the myenteric plexus (LMMPs) were prepared as previously described.[18,19] LMMPs from CNTR and OxPAPC mice were gently pinned down on a wax support and washed by gentle shaking in PBT (PBS with 0.2% Triton X-100) for 45 minutes. After blocking non-specific-binding sites with 2% bovine serum albumin (BSA) in PBT for 1 hour at room temperature, LMMPs were incubated with primary antibodies (**Supplementary**

Table 1) diluted in PBT/2% BSA overnight at room temperature. LMMPs were then washed in PBT for 45 minutes and incubated at room temperature for 2 hours with the secondary antibodies (**Supplementary Table 1**) diluted in PBT and BSA 2%.[34,35] After 15-minutes washes with PBT, LMMPs were mounted on glass slides using a Mowiol Mounting Medium. Negative controls were obtained by incubating sections with isotype-matched control antibodies at the same concentration as primary antibody and/or pre-incubating each antibody with the corresponding control peptide (final concentration as indicated by manufacturer's instructions).

2.2.1 Imaging acquisition and analysis

Images were acquired with the Zeiss LSM 800 confocal imaging system (Oberkoken, Germany) equipped with an oil-immersion 63× objectives (NA 1.4). Z-series images (25 planes) of 1024×1024 pixels were captured and processed as maximum intensity projections. All microscope settings were set to collect images below saturation and were kept constant for all images. The number of HuC/D^+ or $nNOS^+$ neurons gathered was normalized to the total myenteric ganglia area as previously described.[18,19] Changes in the immunoreactivity for GFAP, S100β, 5-HT_{2A}, 5-HT_3, 5-HT_4, SERT and ChAT were determined by evaluation the density index of labelling per myenteric ganglia area and was reported as mean ± SEM.

2.3 In vitro contractility studies

Distal ileum segments (1 cm) were isolated and mounted along the longitudinal axis in organ baths containing 10 ml of oxygenated and heated (37°C) Krebs solution. In vitro contractility experiments were performed as previously described.[18,19,36] Changes in ileum mechanical activity were recorded by isometric transducers (World Precision Instruments, Berlin, Germany) connected to a quad bridge amplifier and PowerLab 4/30 data acquisition system using LabChart 6 software (ADInstruments, Besozzo, VA, Italy). After 45 min equilibration, ileal segments were stretched passively to an initial tension of 0.5 g and brought to their

optimal point of length-tension relationship using 1 μM CCh.[37] Ileal segments were either treated with CCh (0.001-100 μM) to study cholinergic-mediated responses or subjected to electrical field stimulation (EFS, 0-40 Hz; 1-ms pulse duration; 10-s pulse-trains, 40 V) using platinum electrodes connected to an S88 stimulator (Grass Instrument, Quincy, MA, USA) to evaluate neuronally-mediated contractions. To determine the neuronal influence on serotonergic response, cumulative EFS stimulation was performed in presence or absence of ondansetron (0.1 μM, 5-HT_3 receptor antagonist) or GR113808 (0.1 μM, 5-HT_4 receptor antagonist). 10 Hz-EFS-mediated NANC responses, obtained by adding 1 μM guanethidine and 1 μM atropine to Krebs solutions, were recorded in absence or presence of non-selective NOS inhibitor L-NAME, 100 μM (preincubation time=20min) or 30 μM 5-HT in absence or presence of ondansetron (0.1 μM) or GR113808 (0.1 μM). Concentration-response curves to 5-HT were constructed in a non-cumulative fashion (0.3–30 μM) on basal tone.[21] The effect of 30 μM 5-HT was observed also in in the presence of ketanserin (1 μM, 5-HT_{2A} receptor antagonist), or ondansetron (0.1 μM). The antagonist concentrations used were based on the pKi described in literature and all antagonists were allowed to equilibrate for 20 min before concentration-response curves were repeated.[38,39] Contractile responses were expressed as gram tension/gram dry tissue weight of ileal segments and ileal relaxation was calculated as AUC and normalized per g dry tissue weight.[18]

2.4 RNA isolation and real-time PCR

The relative abundance of SERT and 5-HT receptors (2A, 3 and 4) mRNA in distal ileum, was evaluated by real-time PCR as previously described.[21] RNA extractions from LMMPs were carried out with the RNeasy mini kit (Qiagen, Hilden, Germany) and the cDNA was synthesized using the Affinity Script Multiple Temperature cDNA synthesis kit (Stratagene, La Jolla, CA, USA) according to the supplier's protocol. cDNAs obtained were used to measure SERT and 5-HT receptors' mRNA expression levels by SYBR Green and specific

primers (**Supplementary Table 2**). Reactions were run using the StepOne Plus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The reaction mixture (10 µL) comprised 4.5 µL FastStart Universal SYBR Green Master (Roche, Mannheim, Germany), 0.5 µL of each primer 30 µM, 2.5 µL of sterile distilled water, and 2 µL of cDNA template (200 ng). Each sample was run in triplicate, and the mean Ct was determined from the three runs. Relative SERT and 5-HT receptors mRNA expression in each group of animals (CNTR or OxPAPC-treated) was expressed as $\Delta Ct = Ct_{5\text{-}HTreceptor} - Ct_{calibrator}$. GAPDH and HPRT housekeeping genes' expressions were used as calibrators after verification of their stability under our experimental conditions. Relative 5-HT receptors' mRNA expression was then calculated as $\Delta\Delta Ct = \Delta Ct_{OxPAPC} - \Delta Ct_{CNTR}$. Finally, the relative gene expression levels were converted and expressed as fold difference ($= 2^{-\Delta\Delta Ct}$).

2.5 HPLC analysis of tryptophan metabolites

TRP metabolites were analyzed on ileal homogenates by high-performance liquid chromatography (HPLC) as previously described.[40-42]Briefly, freshly isolated ileal segments were immersed in liquid nitrogen, and pulverized in a cooled stainless mortar containing 1N $HClO_4$ (0.5 mL). The homogenates were then sonicated with Elmasonic S30 sonicator (Elma, Singer, Germany). After centrifugation (13,000 *g* for 30 minutes at 4°C), the supernatants were stored at −80°C until HPLC analysis whereas the samples pellets were dissolved in 1N NaOH and boiled for 20 minutes at 60°C, and then centrifugate at 15,000 *g* for 10 minutes at 4°C. The isolated supernatants were used for protein determination.[37,43] The supernatants were brought to about pH 4-5 with 1 N NaOH and analyzed using a HPLC system (Shimadzu LC-10AD, Kyoto, Japan) equipped with a fluorometric detector (Shimadzu RF-10AXL) set at the excitation and emission wavelengths of 285 and 345 nm, respectively. Briefly, chromatographic separation of tryptophan metabolites was performed using an analytical Apollo EPS C18 100A column (5 µm; 250 mm × 4.6 mm; Grace, Deerfield, IL, USA) and an

Alltech guard column with stationary phase RP-8 (25-40 μm Lichroprep, Merck Darmstadt, Germany). Kynurenine analysis was carried out on an analytical Grace Smart RP-18 column (5 μm; 250 mm × 4.6 mm; Grace) using a UV-VIS (ultraviolet-visible) detector (SPD-10A, Shimadzu), set at 360 nm. The mobile phases were as follows: Phase A, 95% acetonitrile – 5% water, and Phase B, 90% water – 5% methanol (pH 3.8). The analytes elution was performed with an isocratic gradient (5% Phase A and 95% Phase B, v/v) at 1 ml/min flow rate. The concentration of TRP and its metabolites was extrapolated from calibration curves.

2.6 Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations on experimental design and analysis in pharmacology.[32] Animals were randomly allocated into the different experimental groups. All the experiments were analyzed by investigators blinded to the treatments. All data are expressed as mean ± SEM. Statistical significance was calculated with the paired or unpaired Student's *t*-test for two-sample comparisons, two-way ANOVA followed by Bonferroni's *post hoc* test for multiple comparison, using GraphPad Prism software version 8.0 (San Diego, CA, USA). The differences between groups were considered significant when $P<0.05$; 'N' values indicate the number of animals. *Post hoc* tests were run only if F achieved $P<0.05$ and there was no significant variance inhomogeneity.

2.7 Materials

Unless otherwise specified, chemicals were analytical grade and purchased from Sigma Aldrich (Milan, Italy). Paraformaldehyde (PFA) was purchased from Electron Microscopy Sciences - Società Italiana Chimici (Rome, Italy), and Triton-X-100 was from Applichem (Milan, Italy). All drugs for in vitro experiments were dissolved in Krebs solution, with the exception of ketanserin, which was dissolved in DMSO. Final concentration of DMSO never exceeded 0.01%, and controls were conclusive that this concentration had no effect on in vitro contractility studies.

OxPAPCs (Invivogen; San Diego, CA) was prepared as indicated by manufacturer's instructions. Briefly, suspended in 500 µL chloroform (final concentration 1 mg/mL) and carefully vortexed to obtain a homogeneous solution that was then aliquoted and evaporated under a gentle stream of nitrogen gas. On the day of experiment, saline was added to produce the desired concentration. Since at higher concentrations can induce inflammation,[10] OxPAPCs dosage was chosen based on previous published findings[33] as well as considering the recommended concentration reported on Invivogen datasheet (i.e. ≤30 µg/mL) and controlling animals' general health parameters during the treatment.

3. RESULTS

3.1 In vivo administration of OxPAPCs alters the architecture of juvenile mice myenteric plexus

Considering that gut dysbiosis as well as changes in TLRs signaling affects ENS integrity,[15,17-19,21] we sought to determine the impact of OxPAPC treatment on ENS architecture by confocal immunofluorescence. In the myenteric plexus of OxPAPC-treated mice, the total number of HuC/D$^+$ neurons was significantly lower than in CNTR mice (Figure 1A, B). This change was accompanied by a marked increase of the immunoreactivity for the glial markers GFAP (40%; Figure 1A, C) and S100β (17%; Figure 1D, E). Since OxPAPC-induced neuroglia plasticity may be linked to changes in the enteric motor function, ileal excitatory neuromuscular contractility was assessed in isolated small intestine preparation from control and OxPAPC-treated mice. This latter investigation was all the more stringent since mice deficient for TLR2 or TLR4 showed altered gastrointestinal motility.[15,17,19,21]

3.2 In vivo treatment with OxPAPCs increases excitatory neuromuscular contractility

In vitro neuromuscular responses have been evaluated after in vivo treatment with OxPAPCs, by measuring tension changes in isolated ileal preparations following cumulative addition of the non-selective cholinergic agonist, CCh. Ileal segments from OxPAPC-treated mice showed a significant upward shift of the concentration–response curve to CCh and a consequent increase in maximum response, Emax rising about 50% from the CNTR value (Figure 2A). Since cholinergic response was found to be modified after treatment, we sought to further test the neuromuscular function by analyzing frequency-response curves to EFS. Altered neurotransmission in OxPAPC-treated ileal segments was reflected by increased EFS-elicited contractions (by 80% at 10 Hz; Figure 2B). We previously confirmed that in mouse ileum, EFS-mediated responses to frequencies up to 10 Hz are of neuronal cholinergic origin,

being sensitive to both tetrodotoxin and atropine.[17,36,37] However, no changes in ChAT immunoreactivity was found in ileal whole mount preparations of OxPAPC mice (Figure 2C, D). A higher excitatory neuromuscular response in the absence of significant changes in ChAT immunostaining after OxPAPCs treatment, may depend upon the impairment of the inhibitory neurotransmission.

3.3 OxPAPCs in vivo administration affects inhibitory neurotransmission

To evaluate the contribution of inhibitory neurotransmission in the control of intestinal motility in our model, and considering that the increased excitatory contractions could be due, at least in part, to a reduction of the inhibitory component, we tested NO-mediated relaxation responses in the presence of guanethidine and atropine. In OxPAPC mice, EFS NANC-relaxations at 10 Hz were significantly reduced (by -45%) compared to those obtained in CNTR animals (Figure 3A). Pretreatment with L-NAME induced an EFS-mediated relaxation comparable between the two groups. To better characterize the impact of treatment with OxPAPCs on the nitrergic neurotransmission, we analyzed the distribution of nitrergic neurons in the myenteric plexus. However, the number of $nNOS^+$ neurons in the ileal myenteric plexus of both groups was not significantly different (Figure 3B, C). Rearrangements in the cholinergic and nitrergic neurotransmissions as well as in the neuroglia network following OxPAPCs suggest that blockade of TLR2 and TLR4 may bear important consequences on the morphology and function of the ENS which we decided to further investigate by evaluating the influence of OxPAPCs on enteric serotonergic pathways, which appear to be modulated by TLR2 and TLR4 signaling. [21,44]

3.4 In vivo exposure to OxPAPCs influences ileal SERT and 5-HT receptors expression

In order to elucidate if OxPAPCs can impact the enteric serotonergic system, which plays an important role in stimulating ENS and gut function[45], we analyzed SERT expression in the mouse ileum by evaluating the transporter immunoreactivity and mRNA levels in LMMPs

preparations. In LMMPs obtained from OxPAPC-treated animals SERT immunoreactivity was higher than in CNTR, which was reflected by a 1.3-fold increase in mRNA expression (Figure 4A-C).

Secondly, considering that the actions of 5-HT on gastrointestinal motility are transduced by a large family of 5-HT receptor subtypes, real-time PCR and confocal immunofluorescence were performed to characterize the involvement of 5-HT_{2A}, 5-HT_3, 5-HT_4 receptors in OxPAPC-mediated hypercontractility (Figure 5). In OxPAPC-treated LMMPs, the mRNA levels of 5-HT_{2A} and 5-HT_4 receptors increased by 3.4- and 1.8-fold, respectively, with no changes in 5-HT_3 receptor mRNA levels, when compared to CNTR preparations (Figure 4C, F, I). The enhanced expression levels of 5-HT_{2A} receptors by 85% was then confirmed by confocal immunofluorescence and density index analysis in whole mount preparation of LMMPs (Figure 5A-B), together with a significant increase of density index for 5-HT_3 receptor by 62% (Figure 5D, E). Considering that OxPAPCs treatment alters both ileal immunoreactivity and mRNA levels of SERT serotonergic receptors, we have evaluated whether these alterations could affect serotonergic neuromuscular response.

3.5 OxPAPCs in vivo administration modifies serotonergic neurotransmission

Since 5-HT is described to activate both intrinsic excitatory and inhibitory enteric motor neurons, we analyzed the influence of the *in vivo* treatment with OxPAPC on the contractile response evoked by the non-cumulative addition of exogenous 5-HT in isolated ileal segments. 5-HT evoked a concentration-dependent contractile response that was significantly higher in OxPAPC-treated mice compared to CNTR animals (by 40% Emax; Figure 6A). In both experimental groups, 5-HT-mediated ileal contractions were prevalently of neuronal origin since they were reduced by about 60% after addition of tetrodotoxin (data not shown). To further investigate which 5-HT receptor subtype is responsible for OxPAPC-induced neuromuscular hypercontractility to 5-HT, we first evaluated the effect of ondansetron

(antagonist of 5-HT_3 receptors) and GR113808 (antagonist of 5-HT_4 receptors) on frequency-response curves to EFS. In CNTR mice both antagonists caused a significantly increase of EFS-induced contractions (+53% at 10 Hz; +65% at 40 Hz; Figure 6B). In OxPAPC mice EFS-mediated enhancement of the contractile response was obtained only at 40 Hz (+38; Figure 6C). In a successive set of experiments, the inhibition of either 5-HT_{2A} receptors with ketanserin or 5-HT_3 receptors with ondansetron was evaluated on the submaximal contractile response elicited by 30 μM 5-HT. Both in presence and absence of OxPAPC treatment, the incubation with 0.1 μM ondansentron determined a significant reduction of the contractile response to 5-HT (Figure 6D-F), by about 75% and 85%, respectively, confirming the involvement of 5-HT_3 receptor in 5-HT-mediated ileal contraction. In ileal segments from both experimental groups, ketanserin increased the contractile response to 5-HT, by about 70% and 73%, respectively, indicating that 5-HT_{2A} receptor are involved in a relaxation response in the mouse ileum[46] (Figure 6E-F). Furthermore, the influence of 30 μM 5-HT was investigated on 10 Hz-EFS NANC-mediated relaxation in presence of 5-HT_3 or 5-HT_4 receptor antagonists. As shown in Figure 6G, 30 μM 5-HT caused a significant reduction of the inhibitory NANC response in CNTR and OxPAPC-treated mice (-32%, -45%, respectively). In ileal segment from both experimental groups, after incubation with 5-HT_3 or 5-HT_4 receptors antagonists, the inhibitory effect of 30 μM 5-HT was slightly, but not significantly, reduced with respect to control NANC conditions, highlighting the role of these receptors in the 5-HT-mediated neuromuscular response (Figure 6G). Since serotonergic neurotransmission as well as 5-HT receptors mRNA levels and immunoreactivity resulted modified by OxPAPC treatment, it is conceivable that tryptophan metabolism might be affected. Thus, we further tested the impact of OxPAPCs in vivo exposure on TRP metabolism in small intestine.

3.6 OxPAPCs in vivo administration impairs tryptophan metabolism

After OxPAPC treatment, TRP levels in ileal tissue were found to be comparable to CNTR specimens (Figure 7A). However, along the 5-HT pathway of TRP metabolism, after OxPAPC treatment the levels of the metabolite 5-hydroxytryptophan (5-HTP) significantly increased (Figure 7B) and were associated with a reduction of 5-HT levels (by 56%; Figure 7C). Interestingly, we found a significant increase of kynurenine (KYN) levels in ileal tissue of OxPAPC-treated mice (+50%; Figure 7D), suggesting that acute exposure to OxPAPC may shift TRP metabolism from the serotoninergic pathway to the physiologically relevant KYN arm.[46]

4. DISCUSSION

Phospholipids are the building blocks of all mammalian membranes, ensuring a permeable barrier as well as endogenous substrates in multiple enzymatic pathways usually involved in the formation of essential signaling mediators (e.g. platelet-activating factor, sphingosine-derived phospholipids, eicosanoids and many others).[47] However, several pathological conditions, including lung injury, autoimmune diseases, and sepsis, promote oxidative stress with the consequent formation of fatty acid hydroperoxides and further accumulation of full-length and fragmented OxPLs in cell membranes and circulating lipoproteins.[48] OxPLs elicit a multiplicity of bioactivities by interacting with different pattern-recognition receptors of the innate immune system cellular receptors, including TLRs and scavenger receptors.[48,49] In contrast to these proinflammatory effects, OxPLs have also been shown to mediate anti-inflammatory responses by negatively influencing the activation of TLRs following exposure to microbial ligands.[11,33,50,51] OxPAPCs are produced by the spontaneous oxidation of phosphorylcholine-containing lipids that are present in the plasma membrane of cells and are considered LPS-like DAMPs.[52,53]

Here, for the first time, we show the consequences of a 3-day treatment with OxPAPCs on the morphology and function of juvenile ENS and provide novel insights into the affected pathways. In particular, this study demonstrates that an acute increase of OxPLs during early adulthood has the following outcomes: (i) altered structure of the enteric glial network as well as alterations in cholinergic and nitrergic neurochemical coding; (ii) dysmotility of the small intestine associated with impaired cholinergic and nitrergic neurotransmission; (iii) increased expression of 5-HT_{2A}, 5-HT_3, 5-HT_4 receptors and SERT, further evidenced by altered immunofluorescence density in the neuromuscular layers; (iv) reduced 5-HT levels and higher KYN content together with increased 5-HT-mediated motor response via 5-HT_3 and 5-HT_4 receptors.

The structural evaluation by means of whole mount immunohistochemistry, performed on ileal LMMPs preparations, indicated anomalies in myenteric ganglia, denoted by a lower number of HuC/D$^+$ neurons in OxPAPC-treated mice, associated with a higher immunofluorescence of the glial proteins GFAP and S100β. The analysis of neuroplasticity is critical for investigating enteric morphological and functional changes that may occur during health and disease.[19,54-56] Reduced number in HuC/D$^+$ neurons together with an increased staining of the glial proteins GFAP and S100β, indicative of reactive gliosis, have been shown during hypoxia, impaired mitochondrial respiration, mechanical nerve injury[55] and absence of TLR4 signaling.[19] As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating mature astrocyte motility and shape by providing structural stability to astrocytic processes.[19,57] A rapid synthesis of GFAP has been shown in inflamed area of biopsies from patients with both ulcerative colitis and Crohn's disease,[57,58] although this cell marker is reduced during necrotizing enterocolitis and in the non-inflamed mucosa of patients with inflammatory bowel disease.[57,58] Aberrant distribution and release of the glial functional protein S100β is also associated with the gut inflammatory status, impaired TLR4 signaling and enteric dysbiosis.[15,18,19,59,60] We here add further findings indicating that OxPAPCs treatment may induce anomalies in both structural and regulatory enteric glial cell proteins, affecting glial homeostasis in the ENS. Okamoto et al. (2014) have shown that colonic serotonergic neurons of the myenteric plexus project their fibers to EGCs.[61] In particular, direct application of exogenous 5-HT to an isolated human EGC induced the onset of a Ca^{2+} wave, to underline the modulatory role of the serotonergic system in neuron-to-glial communication.[62] The morphological abnormalities observed in the ENS of OxPAPC-treated mice were associated with impaired gut motor function. In juvenile mice exposed to OxPAPCs, the receptor-mediated response to CCh as well as EFS-elicited neuromuscular contractions significantly increased. However, no changes in ChAT immunoreactivity were

detected, indicating that alterations of the EFS-induced contractions do not depend upon major changes in the cholinergic innervation. However, we cannot exclude the occurrence of changes of acetylcholine and/or of other excitatory co-transmitters, such as tachykinins or glutamate, synaptic turnover.[35,63] Evidences on smooth muscle cells (SMCs) have shown that OxPAPCs can induce a SMC inflammatory phenotype by accumulating within lipid-rich atherosclerotic lesions.[64] However, these effects more likely appear to locally and progressively develop at OxPLs tissue deposition sites. The low OxPAPC concentration used in the present study, mostly exerts an anti-LPS function, protecting from excessive systemic response to TLR4 ligands.[10,33]

Another hypothesis, which can be put forward, is that the enhancement of the cholinergic contractile response may homeostatically counterbalance changes in the inhibitory relaxation in order to sustain peristalsis after OxPAPCs treatment. However, a reduction of EFS-induced NANC relaxations at 10 Hz, prevalently of nitrergic origin,[19,65,66] was observed in ileal preparations of OxPAPC-treated mice. Derangement of the relaxation response was not associated with significant changes in $nNOS^+$ neurons in myenteric ganglia. Overall, these data showing neuronal loss, altered neuromuscular function, along with glial activation, strongly suggest that OxPAPCs treatment favors the development of a neuroplastic phenotype in the ENS similar to some neuroplastic changes observed both in the central and peripheral nervous system.[67] This observation is all the more interesting considering that an increasing amount of data are now available to sustain the existence of gut−brain disorders underlying the pathogenesis of peripheral and brain diseases.[68]

From a mechanistic view point, although the correlation between the observed alterations in the glial proteins S100β and GFAP and neuromuscular dysfunction are still to be elucidated, along with their pathophysiologic relevance, these changes clearly indicate that OxPAPCs

treatment favors development of a gut neuropathy, reminiscent of the structural and functional alterations found in TLR2-deficient mice.[17]

Recently, Latorre and colleagues[20] have demonstrated that TLR2 could be a negative repressor of SERT and the double knockout mice for TLR2 and 4 receptors (TLR2/4 DKO) express alterations in the pattern of expression of 5-HT receptors as well as their involvement in the modulation of enteric motor responses.[21,44] Thus, in juvenile mice we evaluated the effect of the acute pharmacological treatment with OxPAPCs on enteric serotonergic pathways. 5-HT is considered a neuromodulator of gastrointestinal motility, secretion and visceral sensitivity by acting on specific receptors, expressed on enteric neurons, although the function of the serotoninergic system in the control gut homeostasis has not yet been clear-cut defined.[26,46,69] As regards the motor function, for example, it is important to note that depletion of all endogenous 5-HT does not block peristalsis in the large intestine of vertebrates, nor inhibit transit.[70] In accordance with previous studies,[21,44] we found that inhibition of both TLR2 and TLR4 signaling affected the pattern of myenteric distribution of 5-HT_{2A} and 5-HT_3 receptors and SERT, while 5-HT_4 receptor distribution and expression was not largely influenced. In a recent study, both the human microbiota and conventional mouse microbiota were found to modulate 5-HT_3-mediated response in germ-free animals, suggesting the involvement of a common effect of commensal microbiota regardless of host species,[71] potentially through TLRs signaling. In the ileum of OxPAPC-treated mice, 5-HT_3-evoked contraction significantly increased, suggesting the presence of an interactive dialogue between TLR2 and TLR4 receptors and the intestinal serotonergic neurotransmission, since both receptors are expressed in a variety of cells within the enteric microenvironment.[17,72-74] The 5-$HT_{2,3,4,7}$ receptors are involved in the modulation of intestinal motor function.[45,46] In our study, in the isolated ileum of both OxPAPC-treated and CNTR mice the excitatory effect of a submaximal dose of 5-HT was significantly reduced by preincubation with ondansetron,

suggesting the involvement of 5-HT_3 receptors. Interestingly, in IBS patients, $5HT_3$ receptor expression is altered and administration of 5-HT_3 receptor antagonists may slow colonic transit, enhance small intestinal absorption and reduce visceral pain by activation of gut-brain pathways.[23,24] In a recent meta-analysis review of the literature different 5-HT_3 receptor antagonists have emerged as potential valid therapeutic tools to treat IBS with few associated adverse effects.[75] In the ileum of OxPAPC-treated mice, 5-HT-evoked a contractile response in presence of the 5-HT_{2A} receptor antagonist, significantly higher to that obtained from control preparations, indicating the involvement of 5-HT_{2A} receptors in the relaxation response.[76] Both preclinical and clinical studies point to $5HT_{2A}$ receptors as pathogenetically relevant to IBS and as potential targets for treating abdominal pain and discomfort in IBS.[77] Intriguingly, isolated small intestine of OxPAPC-treated mice showed higher and comparable excitatory responses following EFS in presence of ondansetron or GR113808, suggesting the involvement of 5-HT_3 and 5-HT_4 receptors. These findings were further corroborated by the reduced inhibitory responses of OxPAPC preparations following inhibition of 5-HT_3 and 5-HT_4 receptors. 5-HT_4 receptor agonists are implicated in the regulation of propulsive motility for alleviating constipation as well as relieving pain in IBS. They are known to modulate the release of nitric oxide from inhibitory nitrergic neurons, to counteract contraction, and the production of acetylcholine from myenteric excitatory cholinergic neurons, to ensure smooth muscle contraction, sustaining GI motility.[46] In the gut, 5-HT is produced from the metabolism of the essential amino acid TRP, which under direct or indirect control of the microbiota, may give origin to several other compounds, such as KYN, tryptamine and indolic compounds, participating to the microbiota-gut-brain communication in health and disease states.[25-27] OxPAPCs treatment induced a significant increase of 5-HTP and a reduction of 5-HT ileum tissue levels, suggesting that blockade of TLR2 and TLR4 signaling affects TRP metabolism. Under normal conditions, TRP is principally catabolized into KYN,

which therefore represents the main TRP degradation pathway, leading to the formation of a large number of metabolically active compounds.[28] Multiple enzymes are involved in the KYN pathway, some of which are tightly regulated by inflammatory mediators.[78] The main enzyme that catalyzes TRP conversion into KYN is indoelamine 2,3-dyoxigenase (IDO), expressed in the intestine and whose activity can be enhanced by inflammatory mediators, such as IFN-α and IFN-γ, TNF-α and LPS.[78] In the juvenile mouse ileum, OxPAPC treatment induced a significant increase of KYN levels, suggesting that from a metabolomic viewpoint TLRs may favor a diversion of TRP metabolism from the 5-HT to the KYN pathway. Two of the downstream metabolites of KYN, quinolinic and kynurenic acid are of particular interest for neurogastroenterology due to their excitotoxic and neuroprotective role in the CNS, respectively. Although much research is still needed to elucidate their role in the modulation of the gut homeostasis, especially kynurenic acid appears to be involved in immunoregulation and in regulation of enteric neuron excitability under inflammatory conditions.[63,79] Interestingly, studies focusing on the role of TRP in IBS, indicate that the severity of the disease positively correlates with enhanced activation of the KYN arm of TRP metabolism, suggesting that this metabolic pathway may represent a key mediator of the altered immune and neuronal responses associated with the disease.[6,23,25,28,30] Lipoproteins, the major carrier of OxPLs are implicated in clearing MAMPs (e.g. LPS and lipoteichoic acid), or preventing TLRs signaling and the subsequent release of proinflammatory cytokines.[80] From a translation point of you, our findings highlight that acute, but not chronic pro-inflammatory, changes in the levels of OxPLs affect the integrity of juvenile ENS in terms of structure, function and TRP metabolism, suggesting a key role of a healthy diet[81] in modelling a dynamic balance between the luminal environment and the physiological response of intestinal neural network to maintain gut homeostasis and health. Furthermore, OxPLs exert pleiotropic biological effects that are dependent on their structure (e.g. full-length OxPLs are barrier protective;

truncated OxPLs are barrier disruptive), tissue concentrations (e.g. at low levels full-length OxPLs are protective), and cellular context.[48] In this respect, OxPAPCs have been shown to be anti-inflammatory regulators involved in the negative control of the non-canonical inflammasome caspases both in humans and mice, suggesting their possible therapeutic potential in targeting non-canonical inflammasomes during Gram-negative bacterial infection.[51] This is all the more important since during adolescence enteric neurons undergo neuroplastic changes in response to genetic and environmental signals, and any insult undermining a normal physiologic neurodevelopment may contribute to the onset of functional intestinal diseases, such as IBS.

ACKNOWLEDGMENTS

We thank Francesca Patrese, DMV and Ludovico Scenna, DMV for veterinary assistance, Mauro Berto, Massimo Rizza for technical assistance in animal handling, Dr. Carla Argentini and Dr. Federico Cusinato for technical support in experimental procedures, and Dr. Andrea Pagetta for technical assistance on confocal microscopy.

This work was supported by grants from University of Padova (UNIPD-Assegno di Ricerca 2016, UNIPD-DSF-PRID2017, UNIPD-DOR-2018 and 2019 funds) and from San Camillo Hospital, Treviso (Italy) to MCG. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

IM: performed the research, analyzed the data, wrote the original draft and revised the manuscript; **VC**: performed the research, analyzed the data, wrote the original draft; **EL**: performed the research, analyzed the data, wrote the original draft; **SC**: performed the research, analyzed the data, wrote the original draft and revised the manuscript; **AP**: performed the research, analyzed the data; **AIA**: designed the research study, analyzed the data; **JEM**: analyzed the data, contributed essential reagents or tools; **SOM**: designed the research study, analyzed the data; **AB**: designed the research study, analyzed the data, wrote the original draft, contributed essential reagents or tools; **CG**: designed the research study, analyzed the data, wrote the original draft and revised the manuscript, contributed essential reagents or tools; **MCG**: designed the research study, analyzed the data, wrote the original draft and revised the manuscript, contributed essential reagents or tools, supervised & administered the project. All authors approved the final version of the manuscript.

COMPETING INTERESTS: The authors have no competing interests.

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Figure Legends

Figure 1. OxPAPC treatment alters the architecture of the ileal myenteric plexus. (**A**, **D**) Representative confocal microphotographs showing the distribution of GFAP (green; **A**), S100β (green; **D**) and HuC/D (red; **A**, **D**) in LMMP preparations from CNTR and OxPAPC-treated mice (bars = 22 μm). (**B**) Number of HuC/D$^+$ neurons per myenteric ganglia area. Relative analysis of GFAP (**C**) and S100β (**E**) density index. Data are reported as mean ± SEM (N = 5-6 mice/group). **P < 0.01 vs. CNTR.

Figure 2. Effect of OxPAPC on ileal neuromuscular contractility and cholinergic neurochemical coding. (**A**) Concentration-response curve to CCh in isolated ileal segments from CNTR and OxPAPC-treated mice. (**B**) Excitatory response to electric field stimulation (EFS) in isolated ileal segments from CNTR and OxPAPC-treated mice. Representative confocal microphotographs showing the distribution of ChAT (red; **C**) in CNTR and OxPAPC-treated LMMP preparations (bars = 22 μm) and relative analysis of ChAT (**D**) density index. Data are reported as mean ± SEM (N = 5-6 mice/group). ** P < 0.01 vs. CNTR.

Figure 3. OxPAPCs administration affects nitrergic neurotransmission. (**A**) 10-Hz-EFS induced NANC relaxation responses in presence or absence of L-NAME in ileal preparations from CNTR and OxPAPC-treated mice. (**B**) Representative confocal photomicrographs showing the distribution of nNOS (green) and HuC/D (red). (**C**) Number of nNOS$^+$ neurons per myenteric ganglia area in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice (bar = 22 μm). Data are reported as mean ± SEM (N = 5-6 mice/ group). *P < 0.05 vs. CNTR; #P < 0.05 vs. respective control in absence of L-NAME.

Figure 4. OxPAPC treatment alters serotonin transporter (SERT) expression. (**A**) Representative confocal microphotographs showing SERT (green) and HuC/D (red) distribution in LMMP preparations from CNTR and OxPAPC-treated mice (bars = 22 μm).

(**B**) Analysis of SERT density index in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice. (**C**) Real-time PCR analysis of SERT mRNA levels in ileal LMMPs from CNTR and OxPAPC-treated mice. Data are reported as mean ± SEM (N = 5-6 mice/group). **P < 0.01 vs. CNTR.

Figure 5. OxPAPC treatment affects serotonergic neurochemical coding. Representative confocal microphotographs showing 5-HT_{2A} (green; **A**), 5-HT_3 (green; **D**) and 5-HT_4 (green; **G**) receptors and HuC/D (red) distribution in CNTR and OxPAPC-treated LMMP preparations (bars = 22 μm). Analysis of 5-HT_{2A} (**B**), 5-HT_3 (**E**) and 5-HT_4 (**H**) receptors density index in ileal LMMP whole-mount preparations of CNTR and OxPAPC-treated mice. Real-time PCR analysis of 5-HT_{2A} (**C**), 5-HT_3 (**F**) and 5-HT_4 (**I**) receptors mRNA expression levels in ileum LMMPs from CNTR and OxPAPC-treated mice. Data are reported as mean ± SEM (N = 5-6 mice/group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. CNTR.

Figure 6. OxPAPC treatment alters serotonergic response. (**A**) Concentration-response curves to 5-HT (0.3 – 30 μM; in isolated ileal preparations from CNTR and OxPAPC-treated mice. (**B** and **C**) Excitatory response to EFS in absence or presence of 0.1 μM ondansetron or 0.1 μM GR113808 in isolated ileal segments from CNTR and OxPAPC-treated mice. **(D)** Representative tracings of responses induced by 30 μM 5-HT in CNTR and OxPAPC segments in absence or presence of 0.1 μM ondansetron or 1 μM ketanserin. **(E** and **F)** Contractile responses induced by 30 μM 5-HT in absence or presence of 0.1 μM ondansetron or 1 μM ketanserin in ileal preparations from CNTR and OxPAPC-treated mice. (**G**) 10-Hz-EFS induced NANC relaxation responses in absence or presence of 30 μM 5-HT and 0.1 μM ondansetron or 0.1 μM GR113808 in ileal preparations from CNTR and OxPAPC-treated mice. Data are reported as mean ± SEM (N = 5-6 mice/group). °°P<0.01 vs. CNTR ; *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective control in absence of antagonists.

Figure 7. OxPAPC treatment affects tryptophan (TRP) metabolism. TRP levels (**A**), 5-hydroxytryptophan (5-HTP) levels (**B**), serotonin (5-HT) levels (**C**), kynurenine (KYN) levels (**D**) and indoleamine 2,3-dioxygenase (IDO) activity (**E**) in ileal tissue measured by HPLC analysis in ileal specimens from CNTR and OxPAPC-treated mice. Data are reported as mean ± SEM (N = 6-8 mice/group). IDO activity was measured by assessing the ratio (Kyn/Trp)x10^3. *P < 0.05 vs. CNTR.

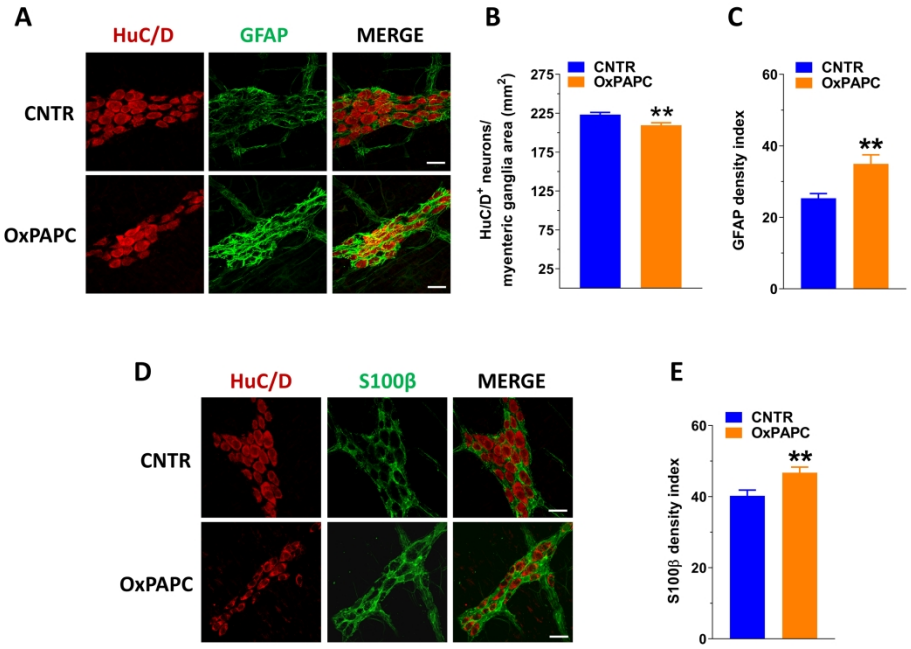

Figure 1

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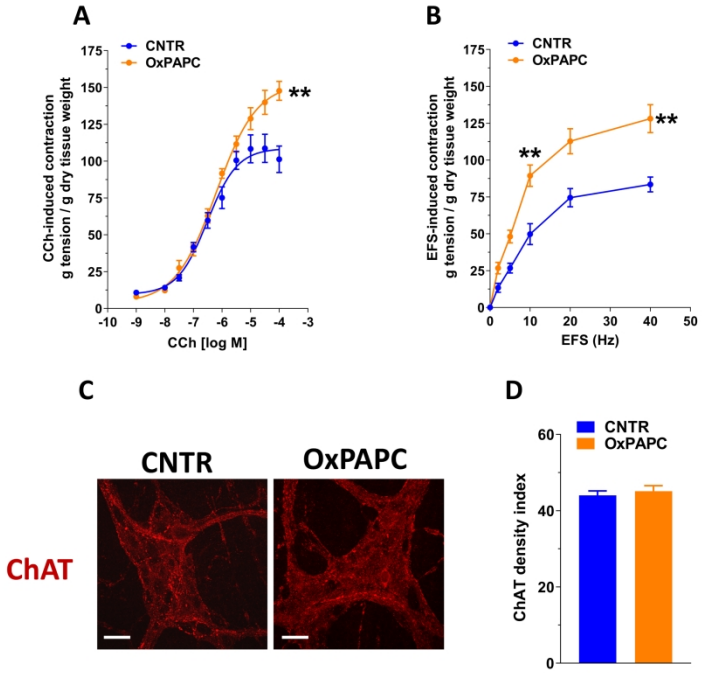

Figure 2

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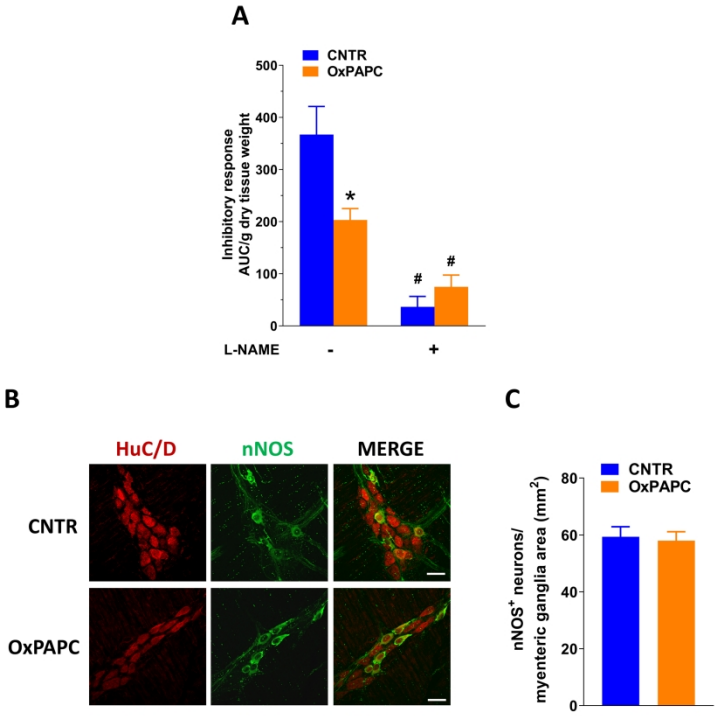

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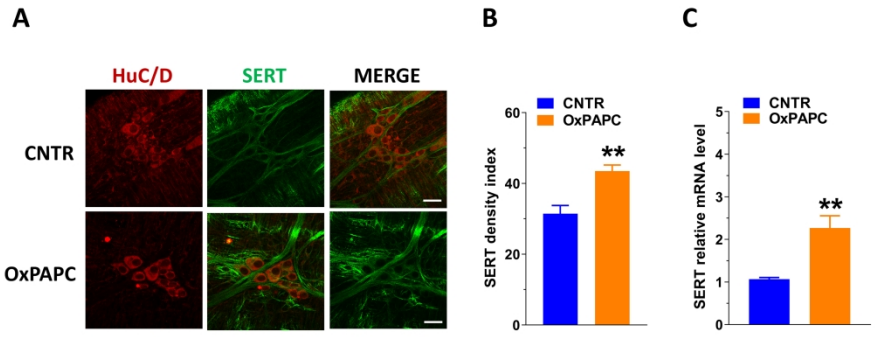

Figure 4

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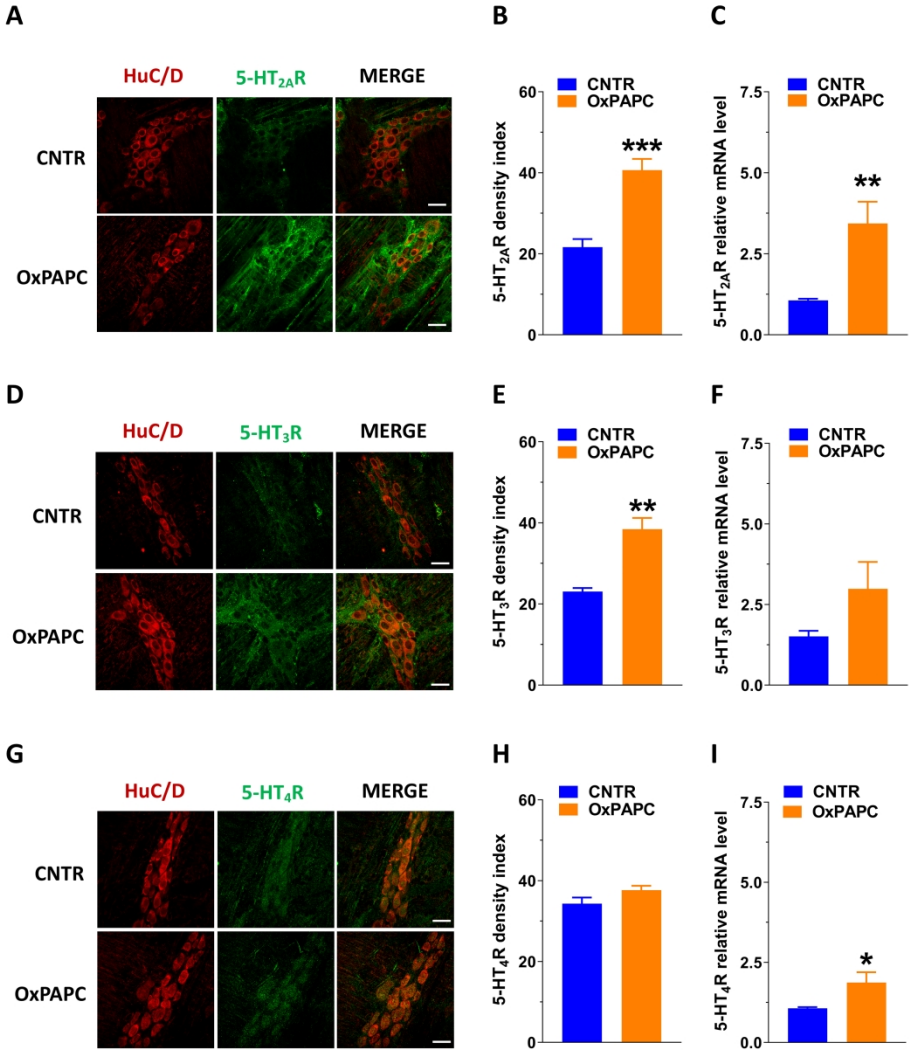

Figure 5

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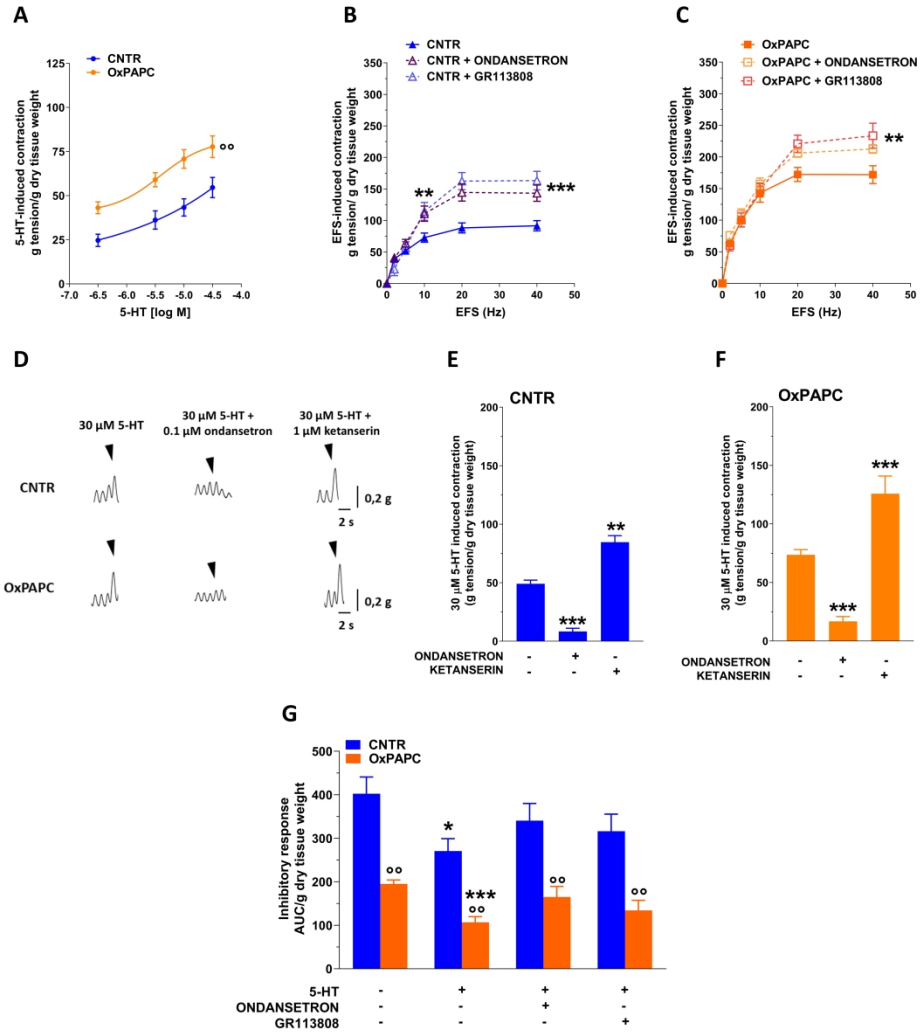

Figure 6

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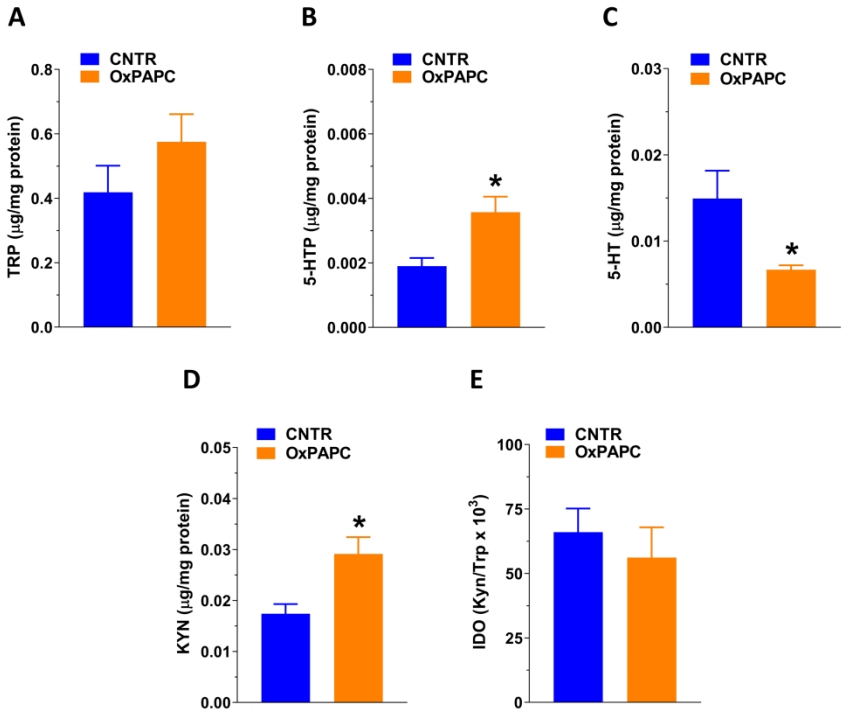

Figure 7

599x500mm (150 x 150 DPI)

Antibody	Host Species	Dilution	Catalog Number	Source
Primary Antisera (Clone)				
HuC/D (16A11)	Mouse biotin-conjugated	1:100	A-21272	Thermo Fisher Scientific (Monza, Italy)
nNOS (polyclonal)	Rabbit	1:100	61-700	Thermo Fisher Scientific
GFAP (polyclonal)	Rabbit	1:200	AB5804	Sigma-Aldrich (Milan, Italy)
S100β (EP1576Y)	Rabbit	1:100	04-1054	Sigma-Aldrich
ChAT (polyclonal)	Goat	1:50	AB144P	Sigma-Aldrich
SERT (polyclonal)	Rabbit	1:50	AMT-004	Alomone Labs (Jerusalem, Israel)
5-HT_{2A} (polyclonal)	Rabbit	1:50	ASR-033	Alomone Labs
5-HT_3 (polyclonal)	Rabbit	1:50	ASR-031	Alomone Labs
5-HT_4 (AG15226)	Rabbit	1:50	21165-1-AP	Proteintech (Manchester, UK)
Secondary Antisera				
Goat anti-rabbit IgG Alexa 488-conjugated	-	1:1000	A-11008	Thermo Fisher Scientific
Donkey anti-goat IgY Alexa 555-conjugated	-	1:500	A-21432	Thermo Fisher Scientific
Streptavidin Alexa 555-conjugated	-	1:1000	S21381	Thermo Fisher Scientific

Supplementary Table 1: Primary and secondary antibodies and their respective dilutions used for immunohistochemistry on ileal whole-mount preparations.

Gene	*Sequence 5'-3'*	*Lenght*
SERT	GGCAACATCTGGCGTTTTCC	138
	ATTTCGGTGGTACTGGCCCA	
5-HT$_{2A}$	TGCCGTCTGGATTTACCTGGATGT	169
	TACGGATATGGCAGTCCACACCAT	
5-HT$_3$	TCTTGCTGCCCAGTATCTTCCTCA	248
	TTATGCACCAGCCGCACAATGAAG	
5-HT$_4$	AATGCAAGGCTGGAACAACATCGG	210
	TGTATCTGCTGGGCATGCTCCTTA	
HPRT	CTGGTGAAAAGGACCTCTCGAA	110
	CTGAAGTACTCATTATAGTCAAGGGCAT	
GAPDH	AACGACCCCTTCATTGAC	191
	TCCACGACATACTCAGCAC	

Supplementary Table 2: Sequence of primers used for the real-time PCR analysis of 5-HT receptors in mouse intestine and relative length of the amplification products.