

**Gruppo Italiano per lo Studio della Neuromorfologia (G.I.S.N.)**  
*Sezione Sistema Nervoso Autonomo – Sistema Nervoso Enterico*



*G.I.S.N.*

**SIMPOSIO**

**IL FUTURO DEL SISTEMA  
NERVOSO ENTERICO:  
FRA NEUROBIOLOGIA E  
APPLICAZIONI CLINICHE**

*19 Novembre 2010*

**Accademia delle Scienze dell'Istituto di Bologna**  
**Via Zamboni, 31**  
*Bologna*



**Gruppo Italiano per lo Studio della Neuromorfologia (G.I.S.N.)**  
*Sezione Sistema Nervoso Autonomo – Sistema Nervoso Enterico*

**Accademia delle Scienze dell'Istituto di Bologna**  
Sala Ulisse

**Bologna, 19 Novembre 2010**

**Mattino**

10:00 - 10:10

Introduzione al simposio:

**“IL FUTURO DEL SISTEMA NERVOSO ENTERICO: FRA NEUROBIOLOGIA E  
APPLICAZIONI CLINICHE”**

Prof. Ruggero Bortolami, Università di Bologna

Dr. Roberto De Giorgio, Dipartimento di Medicina Clinica, Università di Bologna

10:10 - 11:10

Lettura Magistrale

**“ENDOCITOSI DEI RECETTORI OPIOIDI  $\mu$  NEI NEURONI ENTERICI:  
MECCANISMO DI REGOLAZIONE DELLA RISPOSTA CELLULARE”**

Prof.ssa Catia Sternini, UCLA School of Medicine, Los Angeles, CA, USA

**11:15 – 12:30**

**I SESSIONE**

- **ACTIVATION OF  $\mu$  OPIOID RECEPTORS AMELIORATES MUCOSAL INJURY INDUCED BY INTESTINAL ISCHEMIA AND REPERFUSION IN MICE**  
*Saccani F., Anselmi L., Jaramillo I., Bretoni S., Barocelli E., Sternini C.*
- **IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL CHANGES IN THE INTERSTITIAL CELLS OF CAJAL, SMOOTH MUSCLE CELLS AND NEURONS OF CAVEOLIN-1 KNOCKOUT MOUSE SMALL INTESTINE**  
*Vannucchi M.G., Cipriani G., Fausone-Pellegrini M.S.*
- **5-HYDOXYTRYPTAMINE RECEPTORS EXPRESSED IN THE GUINEA PIG COLON ACT HIERARCHICALLY IN THE CONTROL OF MOTILITY**  
*Balestra B., Cervio M., Colucci M., Dellabianca A., Tonini M., De Giorgio R.*
- **FUNCTIONAL AND NEUROCHEMICAL CHANGES IN THE INTESTINAL TRACT IN A RODENT MODEL OF PARKINSON'S DISEASE CENTRALLY TREATED WITH 6-OHDA**  
*Cervio M., Balestra B., Colucci M., Levandis G., Tassorelli C., Blandini F., Tonini M., De Giorgio R.*
- **EVALUATION OF ENTERIC IMMUNOREACTIVITY FOR CALCIUM-BINDING PROTEINS AND ALPHA-SYNUCLEIN IN RATS WITH 6-HYDROXYDOPAMINE INDUCED NIGROSTRIATAL LESION**

*Colucci M., Cervio M., Fragassi G., Levandis G., Balestra B., Tassorelli C., Tonini M., De Giorgio R., Blandini F.*

- **EXCITATORY EFFECTS INDUCED BY CIRCULATING NEURONAL AUTOANTIBODIES ON MYENTERIC NEURONS IN VITRO: A NEUROIMAGING ANALYSIS**

*De Giorgio R., Michel K., Li Q., Demir I.E., Ceyhan GO, Zeller F., Gibbons S.J., Linden D.R., Farrugia G., Chamberlain J., Lennon V.A., Schemann M.*

13:00 – Pausa pranzo

**Pomeriggio**

14:30 – 15:30

Lettura Magistrale:

**“LE CELLULE INTERSTIZIALI DI CAJAL IN CONDIZIONI NORMALI E PATOLOGICHE”**

Prof.ssa Maria Simonetta Fausone Pellegrini, Dipartimento di Anatomia, Istologia e Medicina Forense, Università di Firenze

**15:45 – 17:00**

**II SESSIONE**

- **ENTERIC NERVOUS SYSTEM IN ULCERATIVE COLITIS: STATE OF THE ART**

*Segnani C., Ippolito C., Colucci R., Blandizzi C., De Giorgio R., Fausone-Pellegrini M.S., Battolla B., Dolfi A., Bernardini N.*

- **DIFFERENTIAL REGULATION OF COLONIC EXCITATORY NERVE PATHWAYS BY CYCLOOXYGENASE ISOFORMS IN DIVERTICULAR DISEASE**

*Fornai M, Antonioli L, Colucci R, De Giorgio R, Bucciante P, Chiarugi M, Bernardini N, Blandizzi C.*

- **INTESTINAL SEROTONIN RELEASE, SENSORY NEURON ACTIVATION AND ABDOMINAL PAIN IN IRRITABLE BOWEL SYNDROME**

*Cremon C, Carini G, Bellicosa L, Dothel G, Vasina V, De Giorgio R, Stanghellini V, Grundy D, Tonini M, De Ponti F, Corinaldesi R, Barbara G.*

- **IDENTIFICATION OF NEUROLOGICAL DISORDERS IN PATIENTS WITH CHRONIC INTESTINAL PSEUDO-OBSTRUCTION**

*Serra M., Felicani C., Caputo C., Rinaldi R., Liguori R., Carelli V., Cogliandro R.F., Stanghellini V., Barbara G., Cremon C., De Giorgio R., Corinaldesi R.*

**Conclusioni del simposio**

Prof. Ruggero Bortolami, Dr. Roberto De Giorgio

## **DIFFERENTIAL LIGAND-INDUCED $\mu$ OPIOID RECEPTOR INTERNALIZATION IN ENTERIC NEURONS: A POSSIBLE MECHANISM REGULATING NEURONAL RESPONSIVENESS.**

**Catia STERNINI,**

Department of Medicine, Division of Digestive Diseases, UCLA School of Medicine, Los Angeles, CA, USA

$\mu$  opioid receptors ( $\mu$ ORs) are G protein coupled receptors widely expressed throughout the body, including the gastrointestinal (GI) tract, which mediate a variety of effects including analgesia, GI motility and secretion, stress response and immune processes. In the GI tract,  $\mu$ ORs are localized to functionally distinct populations of enteric neurons and immune cells.  $\mu$ OR activation initiates a cascade of events including phosphorylation, receptor endocytosis, intracellular sorting and recycling resulting in desensitization and resensitization, important regulatory processes that control signaling and cellular response. Receptor endocytosis contributes to the regulation of receptor mediated functions by removing receptors from the cell surface and participating to the attenuation and the recovery of cellular response.  $\mu$ OR endocytosis is of particular interest because it is differentially regulated by native opioids and opiate drugs. Opioids such as enkephalins and endomorphins as well as several opiates like etorphine and fentanyl induce rapid and pronounced  $\mu$ OR internalization in cell lines and in neurons, including enteric neurons via a clathrin-mediated mechanism. By contrast, morphine and heroin differ in their inefficiency to trigger receptor endocytosis in multiple cell types, though they activate  $\mu$ OR to induce analgesia, tolerance and constipation. The resistance of morphine-activated  $\mu$ ORs to undergo internalization has gained considerable attention because morphine is a drug of clinical relevance given its widespread use for pain control and following surgery and its higher propensity to induce opioid tolerance compared to other opiates highly efficient in triggering receptor internalization. Whether the ability of morphine to induce  $\mu$ OR endocytosis is affected by prolonged receptor activation is not known. Chronic stimulation of  $\mu$ OR induces a variety of intracellular adaptations including changes in the expression of proteins implicated in receptor trafficking in regions of the brain expressing  $\mu$ ORs. We compared the effects of morphine, a poor  $\mu$ OR internalizing opiate, and [D-Ala<sup>2</sup>, MePhe<sup>4</sup>, Gly<sup>-ol5</sup>] enkephalin (DAMGO), a potent  $\mu$ OR internalizing agonist, on  $\mu$ OR trafficking in enteric neurons and on the expression of dynamin and  $\beta$ -arrestin, proteins regulating receptor trafficking, in the ileum of guinea pigs rendered tolerant by chronic morphine treatment. Morphine induced strong  $\mu$ OR endocytosis in tolerant but not naïve neurons, whereas DAMGO induced pronounced and comparable  $\mu$ OR internalization in neurons from both tolerant and naïve animals. Morphine- or DAMGO-induced  $\mu$ OR endocytosis was due to direct ligand interaction with  $\mu$ OR since it was not affected by tetrodotoxin, a blocker of endogenous neurotransmitter release. Morphine-induced  $\mu$ OR internalization was inhibited by pretreatment with the dynamin inhibitor, dynasore. Chronic morphine treatment resulted in a significant increase and translocation of dynamin immunoreactivity from intracellular pool to plasma membrane, but did not affect  $\beta$  arrestin immunoreactivity. These findings suggest that chronic activation of  $\mu$ ORs increases morphine's efficacy to induce  $\mu$ OR endocytosis in enteric neurons, which might be dependent upon the level and cellular localization of dynamin, a regulatory protein that plays an important role in receptor-mediated signal transduction affecting cellular signaling.

## **INTERSTITIAL CELLS OF CAJAL IN HEALTH AND DISEASE**

**Maria-Simonetta FAUSSONE-PELLEGRINI**

*Department of Anatomy, Histology and forensic Medicine, University of Florence, ITALY*

The interstitial cells of Cajal (ICC) form networks within the gastrointestinal (GI) muscle coat, immunohistochemistry demonstrated these cells are c-kit-positive and electron microscopy showed their peculiar ultrastructural features. The ICC distributed throughout the entire gut at the level of the myenteric plexus area (ICC-MP) and those in the colon located at the submucosal border of the circular muscle layer (ICC-SM) generate slow waves responsible for gut pacemaker activity; the ICC located intramuscularly (ICC-IM and ICC-SEP) have a role in neurotransmission; the ICC located in between the two subdivisions of the circular muscle layer specific of the small intestine (ICC-DMP) exert mechanosensitivity as intestinal stretch receptor. Both ultrastructural and immunohistochemical studies are of high importance in view of the implication of these ICC subtypes in human GI motor diseases and results obtained in patients affected by achalasia, hypertensive sphincter, esophageal atresia, slow transit constipation, diabetic and idiopathic gastroparesis, gastroschisis, aganglionosis, ulcerative colitis and also by gastrointestinal stromal tumours, deserve to be compared and discussed. Furthermore, data from mouse muscular dystrophy, mouse and rat bowel inflammation, and rabbit colonic dysmotility showed ICC abnormalities and, in certain conditions, a possible recovery. The data so far collected are of high interest, although the majority of these results have been obtained with important technical limitations: in some cases the lack of c-kit-positivity was interpreted as an absence of ICC not always confirmed by electron microscopy examination; in the presence of a normal c-kit-positivity, electron microscopy has revealed that ICC might have abnormal or immature features; ICC were considered absent when actually these cells were not recognizable due to their significantly changed or too immature features. Before claiming that ICC are involved in the altered motility underlying various GI diseases, in the severity of this involvement and that can recover, it should be advisable to verify not only whether ICC maintain the c-kit-positivity, but also whether their ultrastructural phenotype is typical, immature, or abnormal.

## **ACTIVATION OF $\mu$ OPIOID RECEPTORS AMELIORATES MUCOSAL INJURY INDUCED BY INTESTINAL ISCHEMIA AND REPERFUSION IN MICE.**

**Saccani F.<sup>1,2</sup>, Anselmi L.<sup>1</sup>, Jaramillo I.<sup>1</sup>, Bertoni S.<sup>2</sup>, Barocelli E.<sup>2</sup>, Sternini C.<sup>1</sup>**

<sup>1</sup> Department of Medicine, Division of Digestive Diseases, David Geffen School of Medicine at University of California, Los Angeles, CA90095, USA;<sup>2</sup> Department of Pharmacological, Biological and Applied Chemical Sciences, University of Parma, via Usberti 27, 43100 Parma, Italy.

**BACKGROUND:** Intestinal ischemia is a clinical gastrointestinal emergency associated with high morbidity and mortality. This severe condition can occur after a variety of intestinal pathologies, including intestinal occlusion and bowel transplantation. The  $\mu$  opioid receptor ( $\mu$ OR), which is expressed in enteric neurons and lymphocytes in the gastrointestinal tract, has been shown to possess anti-inflammatory properties in experimental colitis.

**AIM:** The aim of this study was to determine if activation of  $\mu$ ORs protects from inflammatory injury induced by intestinal ischemia using a mouse model of ischemia and reperfusion.

**METHODS:** Intestinal ischemia was induced in female C57BL/6 mice by occlusion of the superior mesenteric artery for 45 min and was followed by 5 hr reperfusion (I/R). Sham Operated (SO) mice served as controls. All experiments were performed according to Guiding Principles in the Care and Use of Animals. 20 minutes before ischemia each group of mice received subcutaneously one of the following treatments: (1) saline solution; (2) the  $\mu$ OR selective agonist, [D-Ala<sup>2</sup>,N-Me-Phe<sup>4</sup>,Gly<sup>5</sup>-ol]-enkephalin (DAMGO) (0.01 mg kg<sup>-1</sup>); (3) DAMGO and the selective  $\mu$ OR antagonist [H-D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH<sub>2</sub>] (CTAP) (0.1 mg kg<sup>-1</sup>) or (4) CTAP alone. The intestinal damage was assessed histologically. The degree of intestinal inflammation was evaluated by measuring the myeloperoxidase activity (MPO index of tissue neutrophil accumulation) and the levels of the pro-inflammatory cytokine TNF- $\alpha$  mRNA in ileum tissue samples.

**RESULTS:** Histological examination revealed that intestinal I/R, induced neutrophil infiltration, edema, epithelial lifting, apoptotic cells in crypts and muscle thickening. Treatment with DAMGO reduced histological damage in I/R mice by 30%, which was reversed by the co-administration of CTAP.

Intestinal I/R induced a marked and significant increase in MPO activity compared to SO. DAMGO induced a significant reduction of the MPO levels (-65%) in I/R mice compared to saline; such effect was completely reversed by the  $\mu$ OR antagonist, indicating that this was a receptor-mediated effect. DAMGO had no significant effects on MPO levels in SO mice.

There was a two-fold increase of TNF- $\alpha$  mRNA levels in I/R compared to SO animals. DAMGO reduced TNF- $\alpha$  mRNA levels by about 50% in I/R mice, without affecting the TNF- $\alpha$  mRNA levels in SO mice, and this reduction was abolished by the  $\mu$ OR antagonist, CTAP.

**CONCLUSION:** These data provide evidence that exogenous activation of  $\mu$ ORs protects from the mucosal injury induced by ischemia and reperfusion, through a mechanism that appears to be in part mediated by TNF- $\alpha$ . This suggests that peripheral  $\mu$ OR agonists might be potential therapeutic approaches for intestinal I/R-induced inflammatory injury.

## **IMMUNOHISTOCHEMICAL AND ULTRASTRUCTURAL CHANGES IN THE INTERSTITIAL CELLS OF CAJAL, SMOOTH MUSCLE CELLS AND NEURONS OF CAVEOLIN-1 KNOCKOUT MOUSE SMALL INTESTINE**

***Vannucchi M.G., Cipriani G., Faussone-Pellegrini M.S.***

*Dept. Anatomy, Histology and forensic Medicine, Section of Histology. University of Florence*

Caveolae are plasma membrane invaginations of smooth muscle cells (SMC) and interstitial cells of Cajal (ICC) whose main component is an integral membrane protein called caveolin (Cav)-1. Caveolae play a crucial role in various signal transduction pathways and Cav-1 knockout mice show abnormalities in pacing and contractile activity of the small intestine. Presently, we verified, by immunohistochemistry and transmission electron microscopy (TEM), whether ICC, SMC and neurons of mouse small intestine are affected by the absence of Cav-1. ICC were labeled with two specific markers, Kit and Ano1 (also known as Dog1 or TMEM16A), excitatory neurons with Substance P (SP) antibody and SMC, ICC and neurons with antibodies against two of the SP receptors (NK1r and NK2r). Furthermore, the ultrastructural features of all these cell types were also investigated. Cav-1 knockout mice showed a complete loss of Ano1 labeling with

no changes in Kit expression, a significant decrease of SP-positive structures associated with an increase in NK1r and NK2r internalization and labeling intensity. Under TEM, ICC and SMC showed a marked reduction in caveolae and the few still present were larger and internalized. Moreover, both cell types contained a greater number of mitochondria. No ultrastructural change was appreciable in the neurons. The present results demonstrate that the absence of Cav-1 consistently affect ICC, SMC and neurons compromising their ability to express specific receptors such as those for neurokinins and those for Anol1 in the ICC. Interestingly, the loss of Anol1 and the maintenance of Kit indicate that the former should be mainly expressed in membrane portions rich in caveolae, in agreement with its functional properties its absence might be responsible for the altered contractile activity of the ileum of these mice. Since no ultrastructural changes in neurons were seen, the reduction of SP labeling likely represents an epiphenomenon consequent to the NK1r and NK2r changes.

## **5-HYDOXYTRYPTAMINE RECEPTORS EXPRESSED IN THE GUINEA PIG COLON ACT HIERARCHICALLY IN THE CONTROL OF MOTILITY**

***Balestra B.<sup>1</sup>, Cervio M.<sup>1</sup>, Colucci M.<sup>1</sup>, Dellabianca A.<sup>2</sup>, Tonini M.<sup>1</sup>, De Giorgio R.<sup>3</sup>***

<sup>1</sup>Dept. of Legal Medicine, Forensic Sciences & Pharmacology-Toxicology, University of Pavia, <sup>2</sup>Dept of Preventive, Occupational & Community Medicine, University of Pavia, <sup>3</sup>Dept. of Internal Medicine & Gastroenterology, University of Bologna, Italy

**Background.** Enterochromaffin cells of the intestinal mucosa are the main source of 5-hydroxytryptamine (5-HT) in the body and when stimulated by mechanical/chemical stimuli they release 5-HT in the lumen. Released 5-HT participate to the regulation of intestinal motility by activating at least five receptor types, 5-HT<sub>1,2,3,4,7</sub>.

**Aims.** To determine the presence of 5-HT<sub>2B</sub>, 5-HT<sub>3</sub>, 5-HT<sub>4</sub> and 5-HT<sub>7</sub> receptor mRNAs by classical RT-PCR, evaluate their relative expression by quantitative real time RT-PCR, define their role in propulsive activity and define the role of 5-HT<sub>7</sub> receptors in the gut wall accommodation in isolated distal colon segments.

**Methods:** Male albino guinea pigs were used. Total RNA was isolated by the RNeasy Protect Mini Kit. Real time RT-PCR experiments were performed by the FastStart SYBR Green Master kit. In functional studies, propulsion was elicited by distending intraluminally a rubber balloon (0.05-0.1 ml). The velocity of balloon propulsion (mm/s) was considered as the main parameter of peristaltic activity. Selective antagonists at 5-HT<sub>2B</sub> (SB 204741: 10-100 nM), 5-HT<sub>3</sub> (ondansetron: 1 µM), 5-HT<sub>4</sub> (RS-39604: 1 µM), and 5-HT<sub>7</sub> (SB-269970: 1-1000 nM) receptors were used to find out the involvement of these receptors in propulsive activity. The effect of SB-269970 (100 nM) on accommodation was evaluated in 1-cm long segments after slow intraluminal fluid infusion.

**Results:** PCR products corresponding to the four receptor mRNAs were all present in the colon and real time RT-PCR showed comparable levels of 5-HT<sub>2B</sub> (6.8 fg), 5-HT<sub>3</sub> (7.7 fg) and 5-HT<sub>7</sub> (8 fg) receptor mRNAs, which were higher in density compared to the 5-HT<sub>4</sub> (3.5 fg) mRNA. In functional studies, receptor blockade reduced propulsive activity by 40% (5-HT<sub>2B</sub>), 65% (5-HT<sub>3</sub>), 85% (5-HT<sub>4</sub>), and 53% (5-HT<sub>7</sub>). Peristaltic activity was invariably blocked by the simultaneous administration of the last three antagonists. Intraluminal infusion (1 min duration) of short colonic segments with 0.1-0.3 ml caused a slight increase of intraluminal pressure from 0 to 980 Pa. SB 269970 (100nM) failed to affect this effect.

**Conclusions:** 5-HT<sub>3</sub>, 5-HT<sub>4</sub> and to a lesser extent 5-HT<sub>2B</sub> and 5-HT<sub>7</sub> receptors participate in the regulation of colonic propulsive activity. 5-HT<sub>7</sub> receptors not mediate accommodation in the colon. Since 5-HT<sub>7</sub> receptors have been previously shown to mediate accommodation in the ileum, these receptors may emerge as a novel therapeutic target for the development of drugs affecting functional bowel disorders.

## **FUNCTIONAL AND NEUROCHEMICAL CHANGES IN THE INTESTINAL TRACT IN A RODENT MODEL OF PARKINSON'S DISEASE**

**Cervio M.<sup>1</sup>, Balestra B.<sup>1</sup>, Colucci M.<sup>1</sup>, Levandis G.<sup>2</sup>, Tassorelli C.<sup>2</sup>, Blandini F.<sup>2</sup>, Tonini M.<sup>1</sup>, De Giorgio R.<sup>3</sup>.**

<sup>1</sup>Department of Legal Medicine, Forensic Sciences and Pharmacology-Toxicology, University of Pavia, Pavia, Italy; <sup>2</sup>Interdepartmental Research Center for Parkinson's Disease (CRIMP), Neurological Institute C. Mondino, Pavia, Italy; <sup>3</sup>Department of Clinical Medicine, University of Bologna, Bologna, Italy.

In Parkinson's disease (PD), the impaired execution of voluntary movements caused by the degeneration of nigrostriatal dopaminergic neurons is often accompanied by several autonomic dysfunctions, including gut disorders. We have previously shown that rats with a unilateral nigrostriatal lesion, induced by stereotaxic injection of 6-hydroxydopamine (6-OHDA), develop marked constipation, combined with a reduction of neuronal nitrergic expression in the distal ileum and proximal colon. To further investigate the neurochemical changes in the enteric nervous system associated with the nigrostriatal damage, we analyzed the expression of the inhibitory enteric neurotransmitter VIP, its co-expression in nitrergic neurons, and the expression of the main excitatory enteric neurotransmitter acetylcholine (ACh) in the intestinal tract of 6-OHDA treated rats. The small bowel and colon of rats, with or without nigrostriatal lesion, were processed for whole mount immunohistochemistry; functional studies investigating peristalsis efficiency were also conducted.

Our data revealed an increase in the percentage of VIP-containing neurons and of neurons containing both VIP and nNOS in distal ileum and in proximal colon of 6-OHDA treated rats (respectively, +25.4% and +21.9% in distal ileum; +19.2% and +30.8% in proximal colon;  $p < 0.05$ ;  $n = 4$ ). Functional studies performed in isolated colonic segments showed a reduced peristalsis efficiency, including a peak pressure reduction (-19%,  $p < 0.05$ ;  $n = 8$ ) and a residual intraluminal pressure increase (+23%;  $p < 0.05$ ;  $n = 8$ ) in 6-OHDA treated rats.

In conclusion, our results show that a lesion of dopaminergic nigrostriatal neurons is associated with changes in the expression of enteric inhibitory neurotransmitters and impairment of peristalsis efficiency. These changes may reflect the enteric neuronal impairment underlying chronic constipation that frequently affects PD patients.

## **ALTERATIONS EVALUATION OF ENTERIC IMMUNOREACTIVITY FOR CALCIUM-BINDING PROTEINS IMMUNOREACTIVITY AND ALPHA-SYNUCLEIN EXPRESSION IN RATS CENTRALLY TREATED WITH 6-HYDROXYDOPAMINE INDUCED 6-OHDANIGROSTRIATAL LESION**

**Colucci M.<sup>1</sup>, Cervio M.<sup>1</sup>, Fragrassi G.<sup>3</sup>, Levandis G.<sup>2</sup>, Balestra B.<sup>1</sup>, Tassorelli C.<sup>2</sup>, Blandini F.<sup>2</sup>, Tonini M.<sup>1</sup>, De Giorgio R.<sup>3</sup>, Blandini F.<sup>2</sup>**

<sup>1</sup>Department of Legal Medicine, Forensic Sciences and Pharmacology-Toxicology, University of Pavia, Pavia, Italy; <sup>2</sup>Interdepartmental Research Center for Parkinson's Disease (CRIMP), Neurological Institute C. Mondino, Pavia, Italy; <sup>3</sup>Department of Clinical Medicine, University of Bologna, Bologna, Italy.

Calbindin and calretinin are two calcium-binding proteins (CBPs) largely expressed in the cholinergic neurons of the enteric nervous system (ENS), as well as also in various types of neurons within the central nervous system (CNS). In particular, these proteins are strongly expressed in surviving neurons of the substantia nigra pars compacta of patients with PD, where they may exert a putative neuroprotective role. In addition, alpha-synuclein, the main constituent of Lewy bodies, is constitutively expressed in both in the CNS and in the ENS; and recent evidences show that Lewy bodies have also been found in the ENS of PD patients, which are typically affected by alpha-synuclein inclusions are associated with the spread of neuropathology both in the enteric and in the CNS, and appear to be involved with the autonomic disorders responsible for the gastrointestinal (GI) symptoms of PD patients. We have

Our previously demonstrated that rats bearing a nigrostriatal lesion induced by 6-hydroxydopamine (6-OHDA) show rat model is a reliable model to reproduce the constipation-like symptoms. In the present study this context, to better understand the neurochemical remodelling underlying

the intestinal impairment of the parkinsonian rats, we investigated calbindin and calretinin expression and the normative expression of alpha-synuclein in the ileum and colon segments.

Our data revealed We found a marked increased expression of both of CBPs expression in both the proximal (calbindin: +13,5%,  $p < 0,05$ ; calretinin: +20,9%,  $p < 0,001$ ;  $n=4$ ) and distal ileum (calbindin: +16.5%,  $p < 0,005$ ; calretinin: +15,8%,  $p < 0,001$ ;  $n=4$ ) and a decrease of calbindin expression in the distal colon (-16%;  $p < 0,05$ ;  $n=4$ ) of rats treated with 6-OHDA. When we analyzed the neuronal subsets where the two proteins co-localize, we found increased These two proteins are co-expressed in a neuronal subset and the quantitative analysis showed a marked increase co-expression of calretinin also and calbindin immunopositive neurons in both the proximal (+15,1%,  $p < 0,005$ ;  $n=4$ ) and distal ileum (+19.5%,  $p < 0,005$ ;  $n=4$ ) of 6-OHDA treated rats. Alpha-synuclein immunoreactive neurons were detected in the ileum and colon (about 27% and 18% in the ileum and colon, respectively;  $n=2$ ), and but no changes in the expression were has been found in the 6-OHDA treated rats, compared to controls. Qualitative analysis showed that alpha-synuclein is largely co-expressed with calretinin immunoreactive neurons, especially in the ileum (respectively, 96% in the proximal and 99% in the distal ileum; 73% in the proximal and 88% in the distal colon;  $n=2$ ). A modest increase of alpha-synuclein/calretinin co-localization (+6.6%) was detected Only in the proximal ileum an increase of co-localization (+6,6%) was detected in the parkinsonian of 6-OHDA treated rats. compared to the controls while no differences were found in the other intestinal segments between the two groups of rats.

In conclusion, our results show that a selective, monolateral nigrostriatal lesion alters is associated with alterations of enteric CBPs expression, with regional specific patterns thereby suggesting a role for these proteins in the GI alterations associated with PD. A region-specific pattern seems to emerge, with . The ileum being seems to be more affected that by the central dopaminergic alteration as compared to the colon. As for Furthermore, alpha-synuclein, the protein tend protein tends to co-localize with immunoreactive neurons are co-expressed in particular with calretinin, but no relevant changes seem to be elicited by the central dopaminergic denervation. which in the CNS has been associated with neuroprotective mechanisms vs 6-OHDA treatment. This study provides the basis to understand the role of the enteric CBPs in pathological conditions.

## **EXCITATORY EFFECTS INDUCED BY CIRCULATING NEURONAL AUTOANTIBODIES ON MYENTERIC NEURONS IN VITRO: A NEUROIMAGING ANALYSIS**

***De Giorgio R.<sup>1</sup>, Michel K.<sup>2</sup>, Li Q.<sup>2</sup>, Demir I.E.<sup>3</sup>, Ceyhan G.O.<sup>3</sup>, Zeller F.<sup>4</sup>, Gibbons S.G.<sup>5</sup>, Linden D.R.<sup>5</sup>, Farrugia G.<sup>5</sup>, Chamberlain J.<sup>6</sup>, Lennon V.A.<sup>6</sup>, Schemann M.<sup>2</sup>***

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Humoral autoimmunity (i.e., IgG), either primary or secondary to paraneoplastic syndromes, may play a role in altering the enteric nervous system (ENS) function thereby eliciting gastrointestinal (GI) dysmotility and symptom generation. However, the mechanisms through which these autoantibodies impair neuronal function remains largely unexplained. Serum IgG prepared from patients with anti-neuronal nuclear autoantibody type 1 (ANNA-1 [or, based on the protein name, Hu],  $n=7$ ) or ganglionic nicotinic acetylcholine receptor ( $\alpha 3$ AChR,  $n=6$ ) autoantibody were tested on myenteric neurons in longitudinal muscle myenteric plexus (LMMP) preparations from the ileum of 38 guinea pigs. IgG prepared from healthy subjects or neurologic patients ( $n=7$ ) without GI symptoms served as controls. Action potential discharges in all neurons of a ganglion in response to acute application of IgG were monitored by fast neuroimaging analysis using a voltage-sensitive dye. Data were analysed as percentage of neurons per ganglion responding and frequency of action potential discharge. Only  $0.7 \pm 1.2\%$  of 708 neurons (26 ganglia) responded to 2 of 7 control IgG samples (mean action potential frequency  $0.5 \pm 0.9$ Hz). Six of 7 samples containing ANNA-1-IgG evoked a response in  $7.4 \pm 5.7\%$  of 1266 neurons (45 ganglia) ( $P < 0.05$ ) with a significantly higher action potential discharge ( $1.8 \pm 1.1$ Hz,  $P < 0.05$ ). We observed no correlation between ANNA-1 titer and the percentage of responding neurons or action potential frequency. Four of 6 samples containing  $\alpha 3$ AChR-IgG

evoked a response in a smaller number of neurons (6.5±9.9% of 612 neurons; 32 ganglia) with an action potential frequency of 1.9±1.5Hz.

In conclusion, our data indicate that IgG in serum of patients with autoimmune GI dysmotility elicits hyperexcitability in enteric neurons. IgGs targeting the ENS can contribute to gut dysmotility observed in the clinical setting.

## **ENTERIC NERVOUS SYSTEM IN ULCERATIVE COLITIS: STATE OF THE ART**

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**Background.** Ulcerative colitis (UC) is a chronic inflammatory bowel disorder associated with abnormal colonic motility and transit. These motor disturbances are suggestive of alterations of neuromuscular cellular components, including cells of the enteric nervous system (ENS, i.e. myenteric neurons and glial cells, interstitial cells of Cajal [ICC]) and circular and longitudinal smooth muscle cells. Although colonic dysmotility in UC patients has been well established, scarce attention has been paid to the anatomical structures which control gut neuromuscular activity in this pathological condition. Furthermore, previous efforts, made to obtain reliable quantitative estimations of ganglionic cells and ICC in UC, yielded hardly comparable, or even conflicting, results. Thus, in order to overcome the heterogeneity of current data, careful morphological examinations and development of standardized procedures are still particularly required in the field of gastrointestinal neuromuscular pathology.

**Aim.** To perform an accurate and standardized quantitative histopathologic analysis of the neural-glial components of myenteric ganglia and ICC population in UC patients.

**Patients and Methods.** Full-thickness archival samples of the left colon were collected from 10 patients with established, severe and pharmacologically unresponsive UC undergone bowel resection. The colonic neuromuscular compartment, with particular regard for myenteric ganglia, was evaluated histologically and immunohistochemically in paraffin cross-sections. The distribution and number of neurons, glial cells and ICC were assessed by anti-HuC/D, -S100 $\beta$  and -c-kit antibodies, respectively. Data were compared with findings on archival samples of normal left colon from 10 subjects undergone surgery for uncomplicated colon cancer.

**Results and Conclusions.** As compared to controls, the ENS of patients with UC showed: (a) reduced density of myenteric HuC/D-positive neurons and S100 $\beta$ -positive glial cells, with a loss over 50% and 34%, respectively; (b) increased glial cell/neuron ratio; (c) ICC decrease in the whole neuromuscular compartment, suggesting a role in the motor abnormalities reported in UC patients. Thus, the histopathological analysis of colonic neuromuscular tissue may represent a morphological basis for understanding changes in ENS morphology and the physiopathological mechanisms of colonic dysmotility.

## **DIFFERENTIAL REGULATION OF COLONIC EXCITATORY NERVE PATHWAYS BY CYCLOOXYGENASE ISOFORMS IN DIVERTICULAR DISEASE**

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**Introduction.** Prostanoids generated by cyclooxygenase isoforms (COX-1, COX-2) contribute to regulate intestinal motor functions. However, the role played by these pathways in human intestinal motility during inflammation is unknown. This study examines the effects of COX inhibitors on excitatory neuromuscular activity of colonic tissues dissected from patients with diverticular disease (DD).

Methods. Longitudinal muscle preparations were obtained from distal colon of patients undergoing surgery for DD (5 M, 3 F; age range: 33-68) or uncomplicated cancer (sex-age matched controls). Colonic strips were set up in organ baths and connected to isotonic transducers to determine the effects of indomethacin (IND, COX-1/COX-2 inhibitor, 1  $\mu$ M), SC-560 (COX-1 inhibitor, 0.1  $\mu$ M) or DFU (COX-2 inhibitor, 1  $\mu$ M) on contractions evoked by electrical field stimulation (EFS: 0.5 ms, 30 mA, 10 Hz, 100 pulses), in the presence of guanethidine (10  $\mu$ M) and N $\omega$ -nitro-L-arginine methylester (100  $\mu$ M). Atropine (1  $\mu$ M) or L-732,138 (NK1 receptor antagonist, 10  $\mu$ M) were used to record contractions driven by tachykinins or acetylcholine, respectively. The distribution pattern and expression of COX-1 and COX-2 in the neuromuscular compartment were assessed by immunofluorescence on paraffin cross-sections of full thickness colonic samples.

Results. In the presence of L-732,138, EFS of normal preparations evoked atropine-sensitive contractions. IND enhanced EFS-induced cholinergic responses (+103.9%). SC-560 or DFU mimicked the effect of IND, but were less effective (+67.1% and +53.9%, respectively). When incubated with atropine, normal colonic preparations responded to EFS with contractions antagonized by L-732,138. In this setting, IND, DFU and SC-560 blunted EFS-induced tachykininergic responses at different extents (-56.4%, -44.9% and -23.2%, respectively). In colonic tissues from DD patients, none of tested COX inhibitors affected the cholinergic contractions evoked by EFS. When preparations of diverticular colon were maintained in the presence of atropine, both IND and DFU decreased tachykininergic contractions elicited by EFS (-67.3% and -71.1%, respectively), while SC-560 was without effects. The pattern of COX expression was found to be changed in DD patients, mainly for the COX-1 isoform which was decreased in myenteric neurons and abolished in glial cells.

Conclusions. COX isoforms are involved in differential regulations of excitatory myenteric nerves in normal human colon: COX-1 and COX-2 down-regulate the contractile responses driven by cholinergic nerves, while both enzymes enhance tachykininergic pathways. In the presence of DD, these regulatory patterns are subjected to remodelling: there is a loss of modulation by both COX isoforms on cholinergic system, while COX-2 ensures an enhanced facilitatory control on tachykininergic motor activity. These functional changes may contribute to an enhanced colonic contractility and thereby painful stimuli in patients with DD.

## **INTESTINAL SEROTONIN RELEASE, SENSORY NEURON ACTIVATION AND ABDOMINAL PAIN IN IRRITABLE BOWEL SYNDROME**

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Introduction: Serotonin (5-hydroxytryptamine, 5-HT) metabolism may be altered in gut disorders including the irritable bowel syndrome (IBS). We assess in patients with IBS vs healthy controls (HC): the number of colonic 5-HT-positive cells; the amount of mucosal 5-HT release; the correlation between 5-HT metabolism and IBS symptoms; and the effects of mucosal 5-HT on electrophysiological responses in vitro. Methods: We enrolled 25 Rome II IBS patients and 12 HC. IBS symptom severity and frequency were graded 0-4. 5-HT-positive EC cells were assessed with quantitative immunohistochemistry on colonic biopsies. Mucosal 5-HT was assessed by high-performance liquid chromatography. The impact of mucosal 5-HT on electrophysiological activity of rat mesenteric afferent nerves was evaluated in vitro.

Results: Compared with HC, patients with IBS showed a significant increase in 5-HT+ve cell counts ( $0.37 \pm 0.16\%$  vs  $0.56 \pm 0.26\%$ ;  $P=0.039$ ), which was significantly greater in patients with IBS-D vs IBS-C ( $P=0.035$ ). Compared with HC, 5-HT release in patients with IBS was 10-fold significantly increased ( $P<0.001$ ), irrespective of bowel habit. A significant correlation was found between the mucosal 5-HT release and the severity of abdominal pain ( $r_s=0.582$ ,  $P=0.047$ ). The area under the curve, but not peak sensory afferent discharge evoked by IBS samples in rat jejunum was significantly inhibited by the 5-HT<sub>3</sub> receptor antagonist granisetron ( $P<0.005$ ).

Conclusions: In patients with IBS, 5-HT spontaneous release was significantly increased irrespective of bowel habit and correlated with the severity of abdominal pain. Our results support a role of mucosal 5-HT in abdominal pain in IBS.

## **IDENTIFICATION OF NEUROLOGICAL DISORDERS IN PATIENTS WITH CHRONIC INTESTINAL PSEUDO-OBSTRUCTION**

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**Background:** Chronic intestinal pseudo-obstruction (CIPO) is a failure of gut motility leading to recurrent episodes of intestinal sub-occlusion with no demonstrable mechanical reason. CIPO can be idiopathic in origin or secondary to a variety of conditions including neurological disorders.

**Objective:** To define the occurrence of neurological abnormalities in a cohort of prospectively identified CIPO patients.

**Methods:** 25 CIPO patients (10 F, age range: 16-85 yrs), diagnosed between 2006 and 2009 according to defined clinical, radiologic and manometric criteria (Stanghellini et al., Gut, 1987), entered the study. Each patient underwent a formal neurological assessment, electromyography and sympathetic skin response.

Based on the neurological examination and neurophysiological tests, CIPO patients were divided into three groups:

- 1) polyneuropathy;
- 2) suspected mitochondrial encephalomyopathy;
- 3) idiopathic CIPO;

Group 1 and 2 were examined by skeletal muscle or skin biopsy, microneurography, MR spectroscopy, genetic tests as well as serological and cerebrospinal fluid exams, to define the origin of their neurological impairment.

**Results:** A number of 8/25 (32%) CIPO showed neurological impairment: 5 patients had mitochondrial encephalomyopathies and 3 peripheral small fiber (including autonomic) neuropathy. Of the 5 encephalomyopathies, 4 had mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) with mutations in TP/ECGF1 gene and 1 had myoclonic epilepsy and ragged red fiber disease (MERRF) phenotype carrying the 3243A>G mtDNA MELAS mutation. Notably, these patients came to medical observation because of prominent and severe gastrointestinal symptoms present at the onset of their disease. Muscle biopsies showed COX negative fibers in the MNGIE and MERRF patients, whereas the MERRF patient also had red-ragged fibers. Increased SDH staining and myopathic features were seen in all biopsies. mtDNA analysis revealed a combination of multiple deletions and depletion in the MNGIE and MERRF patients. Ultrastructural analysis showed intramitochondrial paracrystalline inclusions. Of the 3 patients with small fibers polyneuropathy, 1 had chronic inflammatory demyelinating polyneuropathy, whereas 2 had an idiopathic disease. Notably, the first patient of this group improved dramatically with a high dose course of i.v. Ig therapy.

**Conclusions:** Accurate evaluation and tests unravelled a subgroup of CIPO patients with a neurological origin. Mitochondrial diseases and peripheral neuropathy can both contribute to severe gut dysmotility. Neurological identification is crucial for an appropriate, multidisciplinary management of these complicated cases.