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## Changes in Nitrenergic and Tachykininergic Pathways in Rat Proximal Colon In Response to Chronic Treatment With Otilonium Bromide

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### Abstract

**Background**—Otilonium bromide (OB) is used as a spasmolytic in the treatment of the functional bowel disorder irritable bowel syndrome. Although its acute effects on colonic relaxation are well-characterized, little is known about the effects of chronic administration of OB on enteric neurons, neuromuscular transmission, and interstitial cells of Cajal (ICC), key regulators of the gut function.

**Methods**—Adult Sprague-Dawley rats were treated with OB in drinking water at a dose of 2 mg/kg for 30 days. The colons of OB-treated and age-matched control rats were studied by confocal immunohistochemistry to detect immunoreactivity (IR) in myenteric plexus neurons for nitrenergic and tachykininergic markers, and also by microelectrode electrophysiology.

**Results**—Using immunohistochemistry, chronic OB administration did not change total neuron number, assessed by anti-Hu IR, but resulted in a significant increase in NK1 receptor positive neurons, a decrease in neuronal nitric oxide synthase (nNOS) expressing neurons, and a reduction in volume of substance P in nerve fibers in the myenteric plexus. Chronic OB administration potentiated inhibitory and excitatory junction potentials evoked by repetitive electrical field

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#### CONFLICT OF INTEREST

Dr. Stefano Evangelista is an employee of Menarini Ricerche. No other conflicts

#### AUTHOR CONTRIBUTIONS

Research design: SJG, JHS, SE, MSFP, MG, and GF

Performed experiments: GC and SSA

Data analysis/interpretation: All authors

Provided reagents/materials/analysis tools: SE, JHS, and GF

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stimulation. The various types of colonic ICC, detected by Kit IR, were not altered nor were slow waves or smooth muscle membrane potential.

**Conclusions and Inferences**—Chronic treatment with OB caused significant changes in the nitrergic and tachykinergic components of the myenteric plexus and in both inhibitory and excitatory neurotransmission in the rat colon.

### Keywords

Enteric nervous system; anti-spasmodic; excitability; confocal microscopy; interstitial cells of Cajal; junction potential; slow wave; nerve-evoked activity; myenteric plexus

## INTRODUCTION

Irritable bowel syndrome (IBS), a heterogeneous sensory and motility disorder of the gastrointestinal tract, affects approximately 15% of the population with significant negative impact on quality of life.<sup>1, 2</sup> Its heterogeneous and highly variable combination of symptoms include altered bowel habits, abdominal pain, discomfort, bloating, excessive flatulence, stool urgency, and a feeling of incomplete bowel evacuation. These symptoms often manifest to varying degrees reflecting the complex and multifaceted nature of this incompletely understood medical condition.

Otilonium bromide (OB) – also known as octylonium bromide and by its IUPAC chemical name N,N-diethyl-N-methyl-2-(4-[2-(octyloxy)benzamido]benzoyloxy)ethanaminium bromide - is prescribed in Europe, Asia, and South America as an antispasmodic for the treatment of IBS. In support of its clinical utility, clinical trials have studied the efficacy of OB in IBS (reviewed in<sup>3-5</sup>). For example, Clave et al.<sup>6</sup> reported superior efficacy of OB-treatment over placebo in IBS patients, specifically noting reductions in the frequency of abdominal pain and severity of abdominal bloating. OB contains a charged quaternary ammonium favoring OB accumulation within the gastrointestinal wall and poor systematic absorption.<sup>7, 8</sup> As such, the primary site of action for OB is considered to be within the gut wall, especially in the colon.<sup>9</sup>

Within the intestinal musculature, interactions among neurons, interstitial cells of Cajal (ICC), and smooth muscle cells determine the function of this tissue especially contractility.<sup>10-12</sup> Several neurotransmitters – including tachykinins and nitric oxide that, respectively, contribute to excitatory and inhibitory neurotransmission to the smooth muscle - provide key regulation of the gut musculature. Substance P, a member of the tachykinin family of neuropeptides, with high affinity for NK1 and lesser for NK2 and NK3 receptors, has been implicated in efferent transmission of the enteric nervous system and also in afferent gut perception.<sup>13-16</sup> The subtype of the NK receptor activated and its localization on smooth muscle cells and/or neurons determines the overall response in the gut. Indeed, although stimulation of motility is the prevailing response to SP via activation of smooth muscle NK1/2 receptors, SP can also inhibit intestinal motor activity via a neural site of action mediated by NK1/3 receptors.<sup>13, 16</sup> Nitric oxide (NO) is the major nonadrenergic, noncholinergic (NANC) inhibitory neurotransmitter in the gastrointestinal tract. NO acts directly on the intestinal smooth muscle cells causing relaxation and on enteric neurons or

ICC modifying neurotransmission and attenuating release of excitatory neurotransmitters such as SP and acetylcholine.<sup>17, 18</sup> SP, NK1/2 receptors, and NO have been implicated in IBS or experimental models of this disorder,<sup>19–23</sup> and they are potential targets for OB.<sup>24</sup>

The contractility of the gastrointestinal musculature is determined by the balance of the net excitatory and inhibitory signals to smooth muscle cells and the postsynaptic responses generated. Experimentally, electrical field stimulation (EFS) of excitatory and inhibitory enteric nerves evokes, respectively, excitatory (EJP) and inhibitory (IJP) junction potentials.<sup>25, 26</sup> Specific experimental conditions have been established for the study of effects of pharmacological agents, including OB, on EJPs and IJPs. Indeed, acute exposure to OB caused a biphasic effect on excitatory neurotransmission in the rat colon.<sup>27</sup> At high concentrations (> 3  $\mu$ M), OB caused inhibition of EFS-induced cholinergic EJP consistent with its antimuscarinic pharmacology, whereas a low concentration (100 nM) augmented EJP via an unknown mechanism. IJPs were not affected by acute administration of OB in rat or human colon.<sup>27, 28</sup>

Acting directly on smooth muscle, acute application of OB has been shown to attenuate calcium fluxes from extracellular and intracellular pools in the intestine acting as a mixed antagonist of muscarinic and NK1/2 receptors,<sup>28–31</sup> and an inhibitor of L-type and T-type  $\text{Ca}^{2+}$  channels as demonstrated by electrophysiological characterization.<sup>29, 30, 32, 33</sup> Reduced internalization of NK2 receptors has been also found for acute OB either when applied alone or with a tachykinergic agonist.<sup>34</sup> Additional effects attributed to acute OB, identified in non-gastrointestinal cells, include inhibition of nicotinic acetylcholine receptors<sup>35</sup> and interaction with platelet activating factor (PAF) receptors;<sup>31</sup> however, the relevance of these properties in the gut remains unclear. In contrast, chronic exposure to OB up to 30 days - translationally relevant to human drug treatment - caused redistribution of NK1 receptors and L-type  $\text{Ca}^{2+}$  channels, morphological alterations in smooth muscle cells as well as enhanced contractile responses to the selective NK1 agonist [Sar<sup>9</sup>,Met(O<sub>2</sub>)<sup>11</sup>]-SP and the muscarinic agonist metacholine.<sup>36, 37</sup>

*Much less is known about the effects of OB on interstitial cells of Cajal (ICC).* In the colon of several species including rodents and humans, ICC are distributed at the level of myenteric plexus (ICC-MY), at the submucosal border of the circular muscle layer (ICC-SM), and within the musculature (ICC-IM and septal ICC).<sup>12, 38, 39</sup> Electrical recordings from gastrointestinal musculature show spontaneous rhythmic oscillations in the membrane potential, which are called “slow waves”, generation of which depends on ICC.<sup>39–41</sup> Given the proposed role of T-type  $\text{Ca}^{2+}$  channels in slow waves and the inhibitory effect of OB on these  $\text{Ca}^{2+}$  channels,<sup>42–44</sup> OB chronic treatment may affect both slow waves and interstitial cells of Cajal although a preliminary study found ICC-SM to be unaltered by chronic OB.<sup>36</sup> In comparison, acute OB application at a very high concentration of 100  $\mu$ M abolished L-type  $\text{Ca}^{2+}$  channel-mediated spontaneous electrical activity (not slow waves) and induced depolarization in the rat colon.<sup>45</sup>

Although acute effects of OB are well-established,<sup>29, 30, 32–34, 45</sup> they likely contribute only partially to the clinical efficacy of OB. Clinical trials demonstrate superiority of OB dosing over placebo occurring a number of weeks into treatment,<sup>6, 46, 47</sup> in some cases reporting an

increase in gastrointestinal transit,<sup>8</sup> and relatively long-lasting beneficial effects after cessation of the treatment.<sup>6</sup> All of these observations suggest an additional effect attributed to chronic OB exposure. In this study, we aimed to elucidate how *chronic treatment of OB - at a dose relevant to human pharmacotherapy in IBS- modifies the enteric nervous system excitatory and inhibitory components in the colon by employing immunohistochemistry with specific neural markers and functional assessments of IJPs and EJPs in the rat colon. We also investigated if chronic treatment with OB alters various types of ICC and slow waves. The studies presented herein demonstrate a novel finding that chronic OB treatment alters the distribution of excitatory and inhibitory signaling markers and neurotransmission to the smooth muscle in the rat colon.*

## MATERIALS AND METHODS

### Animals

Experiments were performed in male Sprague Dawley (Indianapolis, IN, USA) of 170–190 g at the beginning of the treatments. Animals were randomly divided into two groups: vehicle (control) - and OB-treated. OB was added in the drinking water for 30 days and adjusted every 2 days for changes (usually increases) in body weight and water intake to maintain the dose of 2 mg/kg per day. Vehicle control rats received only water. At the end of treatment animals were sacrificed by CO<sub>2</sub> inhalation, and samples of the proximal colon (around 5 cm) were quickly removed. The first part, approximately 1 cm below the ileocecal junction, was used for electrophysiological experiments (1.5 cm) while the remaining (3 cm) for confocal immunohistochemistry as described previously.<sup>38</sup> The Institutional Animal Care and Use Committee at Mayo Clinic approved all animal-handling procedures described in this manuscript.

### Compounds and Drugs

The following drugs were used: otilonium bromide (Menarini Ricerche SpA, Florence, Italy), nicardipine (Sigma-Aldrich, St Louis, MO, USA), N'-nitro-L-arginine (L-NNA) (Tocris Bioscience, Ellisville, MO, USA) and (1R,2S)-4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonoxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS-2500) (Tocris Bioscience). Drugs were dissolved in ethanol (for nicardipine), in distilled water (for MRS 2500) or in the Krebs solution (for L-NNA).

### Immunohistochemistry

*The tissue samples used for immunohistochemistry were opened along the mesenteric border and pinned flat in a Sylgard-coated petri dish (Sylgard 184, Dow Corning, Midland, MI, USA) with the mucosa facing up. After cleaning carefully with Krebs solution the tissue was fixed overnight in PFA fixative (pH 7.4) at 4°C. After fixation, the proximal colon tissue was rinsed (3 times for 10 min each) in PBS, and then the mucosa was scraped off. The submucosa was then carefully removed using fine forceps in order to maintain intact ICC-SM attached to the circular muscle layer. Proximal colon whole mount preparations were rinsed (3 times for 15 min each) in PBS. After a further rinsing (2 times for 5 min each) in PBS, tissues were incubated for 4 h at 4 °C with BSA (5%) in PBS containing 0.3% Triton X-100 as a blocking solution to prevent the non-specific binding before being placed in*

primary antibody. Tissues were then incubated with a combination of primary antibodies (Table 1), as described, diluted in the blocking solution. After the primary antibody treatment, tissues were rinsed (3 times for 15 min each) in PBS and incubated for 24 h at room temperature with secondary antibodies (Table 1) diluted in PBS containing 0.3% Triton X-100. After removing the secondary antibody with PBS, tissues were rinsed (3 times for 10 min each). Tissues were maintained in 1 mL of 300 nM of DAPI in water for 45 min and washed 5 times for 5 min. Specimens were examined using a laser scanning confocal microscope (Olympus FV1000, Center Valley, PA, USA). Stacks of optical sections were collected and 3-D reconstruction images were made using Analyze™ (Mayo Foundation, Rochester, MN, USA) computer software to determine SP-IR fibers as previously reported.<sup>48</sup> Only cells with identifiable DAPI staining, to ensure a nucleus was present, were counted in analyses.

### Electrophysiology

Electrical recordings were made in the proximal colon of controls and OB-treated animals using two different preparations. In order to record electrical slow wave activity, in the first preparation the mucosa was carefully removed to preserve intact ICC-SM network on the mucosal side of the muscle layer. In the second preparation, ICC-SM were removed during the peeling process. The two types of muscle strip preparations were pinned with the circular muscle layer facing upward. Strips were allowed to equilibrate for 1–2 hrs with continuously perfused oxygenated Krebs solution (in mM: NaCl 120.3, KCl 5.9, MgCl<sub>2</sub> 1.2, NaHCO<sub>3</sub> 15.5, NaH<sub>2</sub>PO<sub>4</sub> 1.2, glucose 11.5, CaCl<sub>2</sub> 2.5; the pH was 7.3–7.4 when bubbled with 97% O<sub>2</sub>/3% CO<sub>2</sub>) at 36.5 ± 0.5 °C. Sharp microelectrode recording were made at the same temperature using glass electrodes filled with 3 M KCl (70–90 MΩ) in the presence of 2 μM nifedipine. Transmembrane potentials were measured with an Axoclamp 2B amplifier and a Digidata 1440A acquisition system, and stored in a computer running Axoscope 10.0 software (Axon Instruments/Molecular Devices Corp.). EFS was applied with increasing voltage steps (40 to 100V, pulse duration 0.5 msec) or by train stimulation at 90 V for 300 msec at 20 Hz. IJPs were measured without blockers while EJPs were measured in the presence of 1 mM N<sup>G</sup>-nitro-L-arginine (L-NNA), an inhibitor of NOS, and 1 μM MRS2500, a blocker of P2Y<sub>1</sub> receptors. Data were analyzed as maximum IJP or EJP peak amplitudes in relation to pre-EFS stable baseline or duration responses, which were measured from its beginning marked by EFS artifact upon which the events superimposed (unless clearly differentiated) to the end when the response recovered back to pre-stimulation level in Clampfit ver. 10 using appropriate tool functions. The nitroergic and purinergic components of IJPs, and cholinergic and tachykininergic components of EJPs were not pharmacologically isolated.

### Statistics

Data are expressed as means ± SEM. Statistical significance was determined by GraphPad Prism using either ANOVA or Student's t-test (two-tailed) as described. P values of less than 0.05 were taken as statistically significant. The 'n' value refers to the number of separate cells recorded from tissue strips, and the 'N' value identifies the animal count. In confocal experiments to detect nNOS, NK1 receptor, or SP IR, 4–10 different optical fields were analyzed and averaged to obtain a single value for each individual animal (OB-

treatment or control). In Kit experiments, images were scored for staining intensity on a visual analog scale by marking the score on a 10 cm line. The investigators were blinded to the treatment variable and had extensive prior expertise in immunohistochemistry. The values from different animals were then analyzed and reported as means  $\pm$  SEM (N).

## RESULTS

### Chronic OB treatment does not change total number of enteric neurons

In the first set of experiments, we investigated whether chronic treatment with OB affects the number of total neurons within the myenteric plexus using an anti-Hu antibody. As summarized in Fig. 1 and 2, the total number of neurons was not different in OB-treated rats. Within the myenteric plexus of the colon, there were  $50.0 \pm 2.2$  (N=6) and  $51.6 \pm 2.3$  cells/ganglion (N=6,  $P > 0.05$ , t-test) in OB and saline-treated (controls) rats, respectively.

### Chronic OB treatment reduces the number of neurons expressing nNOS

Simultaneous co-labelling with anti-Hu and nNOS antibodies showed a significant decrease in the nNOS-IR neurons in the OB-treated animals (Fig. 1). The number of nNOS-IR neurons per ganglion in the chronically treated OB rats was  $14.6 \pm 2.9$  (N=6), and in the controls it was  $19.7 \pm 1.8$  neuron per ganglion (N=6,  $P < 0.05$ , t-test).

### Chronic OB treatment alters the expression of tachykinergic signaling at the level of myenteric plexus with an increase in NK1 receptor expressing neurons and a decrease in Substance P immunoreactivity

Co-labelling with anti-Hu and NK1 receptor antibodies demonstrated a significant increase in NK1 receptor positive neurons in the chronic OB treated rats compared to controls (Fig. 2). The total number of these neurons per ganglion was  $9.2 \pm 3.8$  (N=6) and  $4.4 \pm 2.1$  (N=6,  $P < 0.05$ , t-test) in OB-treated and control rats, respectively. SP is an endogenous agonist for the NK1 receptors, and therefore we determined its expression in response to chronic OB treatment, both at the level of myenteric plexus and in the circular muscle layer. SP-IR in the nerve fibers was quantified by three dimensional reconstruction of the digital confocal images and volume rendering using Analyze<sup>TM</sup> software. The data are summarized in Fig. 3 and demonstrate a decrease of 30% in the SP expression at the level of myenteric plexus (OB:  $555.00 \pm 61.00$  voxels/field versus control:  $794.00 \pm 96.00$  voxels/field; N=6 each,  $P < 0.05$ , t-test) and no change in the circular muscle muscular layer (OB:  $270.00 \pm 24.16$  voxels/field versus control:  $266.54 \pm 22.36$  voxels/field,  $P > 0.05$ , t-test).

### Chronic OB treatment enhances inhibitory junction potentials evoked by repeated pulses and excitatory junction potentials by both single and repeated pulses in the circular muscle layer

To assess the functional consequence of chronic OB treatment on innervation to the smooth muscle cells of the colon, intracellular sharp microelectrode recordings were made of the electrical responses evoked by EFS in muscle preparation devoid of ICC-SM and slow wave activity. These experiments were conducted in the presence of nicardipine, an L-type  $Ca^{2+}$  channel blocker, to limit tissue contractility and to aid in maintaining stable recordings. Using a protocol in which the voltage of stimulation intensity was increased from 40 V to

100 V using a single pulse duration stimulation, IJPs of increasing amplitudes were measured from OB-treated and control rats. There was no difference ( $P>0.05$ , ANOVA) in the treatment variable (saline vs. OB) under this experimental condition (Fig. 4). IJP duration changes approached but did not reach significance. For 90 V pulse, IJP duration intervals were  $2.86\pm 0.21$  s ( $n=25$ ,  $N=4$ ) and  $2.22\pm 0.17$  s ( $n=13$ ,  $N=3$ ;  $P=0.058$ , t-test) for OB-treated and control rats, respectively, indicating a strong tendency for an increase. EFS-mediated responses were measured in the presence of L-NNA (1 mM) and MRS2500 (1  $\mu$ M) to block IJPs and to optimally measure EJPs. Increasing voltages caused stimulus-dependent enhancement in the peak amplitude of EJPs (Fig. 4B). When comparing the responses from OB-treated and control rats, there was a significant effect of the treatment variable ( $P<0.0001$ , ANOVA). For example, at 90 V the EJP amplitudes for the OB-treated and control groups were  $2.67\pm 0.52$  mV ( $n=21$ ,  $N=4$ ) and  $0.78\pm 0.30$  mV ( $n=8$ ,  $N=3$ ), respectively.

In additional series of experiments depicted in Fig. 5, EFS was altered from a single pulse to a train of stimulations (at 90 V, for 300 ms at 20 Hz). In contrast to the results obtained by a single pulse, the IJP amplitude (OB-treated:  $15.36\pm 1.02$  mV,  $n=36$ ;  $N=4$ ; control:  $10.43\pm 1.48$  mV,  $n=16$ ,  $N=3$ ;  $P<0.05$ , t-test) and duration (OB-treated:  $3.96\pm 0.19$  ms,  $n=36$ ;  $N=4$ ; control:  $3.32\pm 0.18$ ,  $n=16$ ,  $N=3$ ;  $P<0.05$ , t-test) were increased by the chronic treatment. EJP amplitude (measured in the presence of L-NNA and MRS2500) also increased (OB-treated:  $4.15\pm 0.54$  mV,  $n=20$ ,  $N=4$ ; control:  $2.11\pm 0.48$  mV,  $n=13$ ,  $N=3$ ;  $P<0.05$ , t-test). Due to low amplitudes of EJPs under the control experimental conditions of this study, we were unable to reliably measure the duration.

### **Chronic OB treatment does not alter interstitial cells of Cajal networks and electrical slow wave activity**

In intact muscle preparations carefully prepared to preserve ICC-SM, three different Kit positive layers of ICC were detected associated with submucosal plexus, myenteric plexus and within the circular muscle layer (Fig. 6). There were no effects on Kit labelling in any of the three layers. When visually quantified for Kit staining, there was no difference between OB-treated ( $5.3\pm 1.5$ ,  $N=3$ ) and control rats ( $4.2\pm 1.9$ ,  $N=3$ ;  $P>0.05$ , t-test).

In preparations with an intact ICC-SM layer, electrical slow wave activity was not significantly different between the OB-treated and control groups (Fig. 6B-E) with respect to membrane potential (Fig. 6C; OB-treated:  $-45.40\pm 1.65$  mV,  $n=15$ ,  $N=7$ ; control:  $-48.67\pm 1.56$  mV,  $n=18$ ,  $N=7$ ;  $P>0.05$ , t-test), slow wave amplitude (Fig. 6D; OB-treated:  $12.48\pm 1.25$  mV,  $n=15$ ,  $N=7$ ; control:  $13.30\pm 1.46$  mV,  $n=18$ ;  $N=7$ ;  $P>0.05$ , t-test), or slow wave frequency (Fig. 6E; OB-treated:  $8.47\pm 0.20$  cpm,  $n=15$ ,  $N=7$ ; control:  $8.26\pm 0.11$  cpm,  $n=18$ ,  $N=7$ ;  $P>0.05$ , t-test). These experiments indicate that chronic treatment with OB has no effect on ICC or slow wave activity.

## **DISCUSSION**

The mechanism underlying OB actions remains incompletely understood, especially in chronic treatment. In this study, we examined if and how chronic OB treatment alters the excitatory and inhibitory neural components, and ICC expression and function in the rat



colon. We show the novel findings that chronic OB treatment altered the enteric nervous system at the level of the myenteric plexus. Specifically it caused an increase in the number of neurons expressing NK1 receptors, a decrease in SP-containing nerve fibers, and a reduction in neurons expressing nNOS in the absence of any change in the total number of neurons. Furthermore, chronic OB enhanced nerve-evoked IJPs and EJPs induced by repetitive pulse stimulation, and it did not affect ICC or slow wave activity.

This current paper and two recent reports<sup>36, 37</sup> are the only studies describing the effects of chronic administration of OB in the colon. Collectively, the results of these three studies suggest that chronic OB treatment exerts complex effects. Herein, changes in the expression of NK1 receptor and nNOS in neurons of the myenteric plexus were detected using co-labelling of the two markers with the specific neuronal marker anti-Hu antibody, which reliably labels all neurons in the intestine.<sup>49</sup> Chronic administration of OB increased the number of NK1 receptor expressing neurons and decreased the number of nNOS positive neurons while the total number of neurons remained unaltered by the treatment. No previous study has examined the expression of nNOS and NK1 receptors in the enteric nervous system by confocal microscopy following chronic OB administration. We are unable to determine whether the same subset of neurons gains NK1 receptor expression while at the same time loses nNOS in response to OB. Consistent with a previous study,<sup>36</sup> we found a decrease in the density of SP-positive fibers in the myenteric plexus after OB chronic administration. In contrast, we did not find a significant decrease in SP-positive fibers in the circular muscle layer. This may reflect a methodological difference. We used a 3-D reconstruction method to quantify the actual volume of SP-positive fibers whereas the previous study<sup>36</sup> analyzed overall pixel intensity and number. It is known that SP exerts complex effects in the gut causing a direct excitation of smooth muscle cells and an indirect inhibition of the enteric nervous system via nNOS.<sup>13–16</sup> Our data suggest a primary effect of chronic OB on neuron to neuron communication rather than a post-synaptic effect on smooth muscle cells. The reduced expression of myenteric SP likely affects both sensory perception and motility of the colon. Since both afferent signaling and contractility have been implicated in the action of OB,<sup>5, 24, 50</sup> our observations further expand the current knowledge by identifying potential mechanisms involved at the level of myenteric plexus.

Previous studies on the rat colon following chronic administration of OB at 2 mg/kg or 20 mg/kg reported NOS up-regulation in smooth muscle cells, alteration of NK1 receptor expression pattern in the musculature, and redistribution of NK1 receptors and L-type Ca<sup>2+</sup> channels receptors within smooth muscle cells.<sup>37</sup> Specific descriptions of changes in smooth muscle and their consequences are described in detail in the original papers of Traini et al<sup>36, 37</sup> and were beyond the scope of the current study. OB applied acutely attenuates smooth muscle excitability and contractility via various mechanisms including mixed antagonism of muscarinic and NK1/2 receptors,<sup>28–31</sup> and blockade of L-type and T-type Ca<sup>2+</sup> channels.<sup>29, 30, 32, 33</sup> In general the smooth muscle effects of OB are thought to determine its antispasmodic pharmacology. Ultra-structural changes in smooth muscle cells were detected by electron microscopy in the chronic OB group and showed increases in the number of smooth endoplasmic cisternae and number of caveolae.<sup>36</sup>

Under our experimental conditions, chronic OB treatment enhanced EJPs under both single and repeated pulse stimulation and IJPs only with repeated pulses in the rat colon. The differential effect of stimulation might be explained by greater release of inhibitory neurotransmitters by repeated stimulation due to presynaptic or postsynaptic membrane potential summation resulting in higher neurotransmitter release at the neuromuscular junction or enhanced smooth muscle excitability. The repetitive stimulation also more optimally mimics ongoing activity in vivo than the single pulse EFS. Indeed, we observed IJP amplitudes of ~10 mV and ~18 mV and durations of ~2.2 s and 3.3 s, respectively, for single and repetitive train stimulations. The greater IJP amplitude obtained with repetitive stimulation is most likely caused by an enhancement in purinergic neurotransmission since the peak amplitude IJP response is primarily due to the purinergic component of the IJP.<sup>51</sup> In contrast, nitrgergic component of IJP contributes predominately to its duration with slower kinetics.<sup>51</sup> The observation that chronic OB enhanced IJP duration also supports modulation of nitrgergic neuromuscular transmission by the treatment. Moreover, our results on IJP and nNOS expression are not directly intuitive. The nNOS positive neurons within the myenteric plexus are afferent (sensory) efferent (motor), or interneurons, and therefore differentially control colonic function.<sup>52, 53</sup> Since we did not observe any attenuation of IJP response, our data suggest that the reduction in the number of myenteric nNOS by chronic OB may preferentially affect afferent neurons or interneurons, likely involved in sensory perception or processing, rather than motor neurons although we have no direct evidence for this.

Previously, only the effects of acute application of OB were examined on EJPs or IJPs in the colon. Acute OB at 0.1  $\mu$ M failed to alter IJPs and enhanced EJPs in guinea pig colon.<sup>27</sup> In both guinea pigs and rats, higher concentrations of OB (above 1  $\mu$ M) blocked EJPs.<sup>27, 28</sup> Further, acute OB blocked depolarization in smooth muscle cells evoked by muscarinic and tachykininergic agonists.<sup>27, 28</sup> Acute OB also effectively blocked nerve-evoked contractions in guinea pig, rat and human colon.<sup>27, 28, 54</sup> These data provide potential mechanisms by which OB exerts its antispasmodic activity. Our observation of an increase in IJP response by chronic OB – likely by altering both purinergic and nitrgergic components as supported by increases in the primary fast peak amplitude and trend for an increase in overall duration – is consistent with the antispasmodic pharmacology. Of note, the potentiation in IJPs occurred despite a decrease in the proportion of nNOS expressing myenteric plexus neurons in relation to NK1 expressing neurons. This suggests that expression of myenteric plexus nNOS alone may not be sufficient to drive overall inhibitory neuromuscular transmission. On the other hand, the increase in the proportion of neurons expressing NK1 receptors and enhancement in EJPs may promote increased excitation-contraction coupling. The increase in EJP following chronic OB was associated with a decrease in SP volume within the myenteric plexus. In our analysis, we used a threshold detection rendering method, which does not quantify the overall SP content or density. We are thus unable to definitely link the neuronal myenteric plexus SP volume with EJPs, and specifically the tachykininergic component. Collectively, OB exerts complex pharmacological profile involving both acute and chronic effects involving smooth muscle cells and neurons.

Chronic in vivo treatment of rats at 20 mg/kg in drinking water – a ten-fold higher concentration than the dose used in the present study for structural and functional studies - did not have an overall significant effect on nerve-evoked relaxation or contraction.<sup>36, 37</sup>

However, a tendency toward enhancement was observed, and the authors stated that “contractile responses obtained in preparations from treated animals have a tendency to be higher“. The differences between electrophysiological and mechanical assessments to detect enhancement in function by chronic OB were most likely due to experimental approaches (sharp microelectrode electrophysiology vs. contractions), stimulation parameters (voltage vs pulse duration, and single versus train stimulations), dosing (2 vs 20 mg/kg), or tissue preparations (with and without intact submucosal plexus). Thus, additional studies are still needed to fully access the relationships among chronic OB, nerve-evoked electrical activities, and nerve-induced contractions or relaxations using the same treatment regimen, preparations, electrical stimulation parameters, and sufficient statistical power. Future studies are needed to determine how chronic OB alters the components of IJPs (purinergic and nitrergic) and EJPs (cholinergic and tachykinergic), especially employing pharmacological means, and how they correlate with the changes in the nitrergic and tachykininergic markers identified in this study.

Enteric nerves and ICC are often found in close proximity to each other and their interactions regulate normal function in the gastrointestinal tract.<sup>17, 41, 55</sup> The possibility that chronic OB administration might alter ICC was tested using whole mount immunohistochemistry and electrophysiology experiments. In the colon of rats, Kit positive cells are found at the level of the myenteric plexus (ICC-MY), the submucosal border of the circular muscle (ICC-SM), and within both muscle layers (ICC-IM).<sup>12, 38, 39</sup> All were unaffected by chronic OB administration consistent with a recent study.<sup>36</sup> We further demonstrated that the slow wave activity also remained unchanged under chronic OB administration.

In summary, we show that chronic OB administration in rats, administered at a dose similar to that currently used clinically for IBS treatment in humans, affects the myenteric plexus in the colon increasing the ratio of NK1 receptor to nNOS expressing neurons with no effect on the total neuronal population. Chronic OB decreases the SP fiber volume, and enhances inhibitory and excitatory neurotransmission to smooth muscle. Chronic OB treatment does not affect various types of ICC or the pacemaker slow wave activity. We propose that the enteric nervous system of the colon is a key target for chronic OB treatment that interplays together with multiple and complex acute effects of the drug on the smooth muscle cells and neurons. The changes in the expression of key neurotransmitters may contribute to the long acting effects of OB, even after cessation of drug administration.

## Acknowledgments

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## ABBREVIATIONS

|      |                               |
|------|-------------------------------|
| DAPI | 4',6-diamidino-2-phenylindole |
| EFS  | electrical field stimulation  |

|               |                                |
|---------------|--------------------------------|
| <b>EJP</b>    | excitatory junction potential  |
| <b>IBS</b>    | irritable bowel syndrome       |
| <b>ICC</b>    | interstitial cells of Cajal    |
| <b>ICC-MY</b> | myenteric plexus ICC           |
| <b>ICC-SM</b> | submucosal ICC                 |
| <b>ICC-IM</b> | intramuscular ICC              |
| <b>IJP</b>    | inhibitory junction potential  |
| <b>IR</b>     | immunoreactivity               |
| <b>OB</b>     | otilonium bromide              |
| <b>NANC</b>   | non-adrenergic non-cholinergic |
| <b>NK</b>     | neurokinin                     |
| <b>NO</b>     | nitric oxide                   |
| <b>nNOS</b>   | neuronal nitric oxide synthase |
| <b>SP</b>     | substance P                    |

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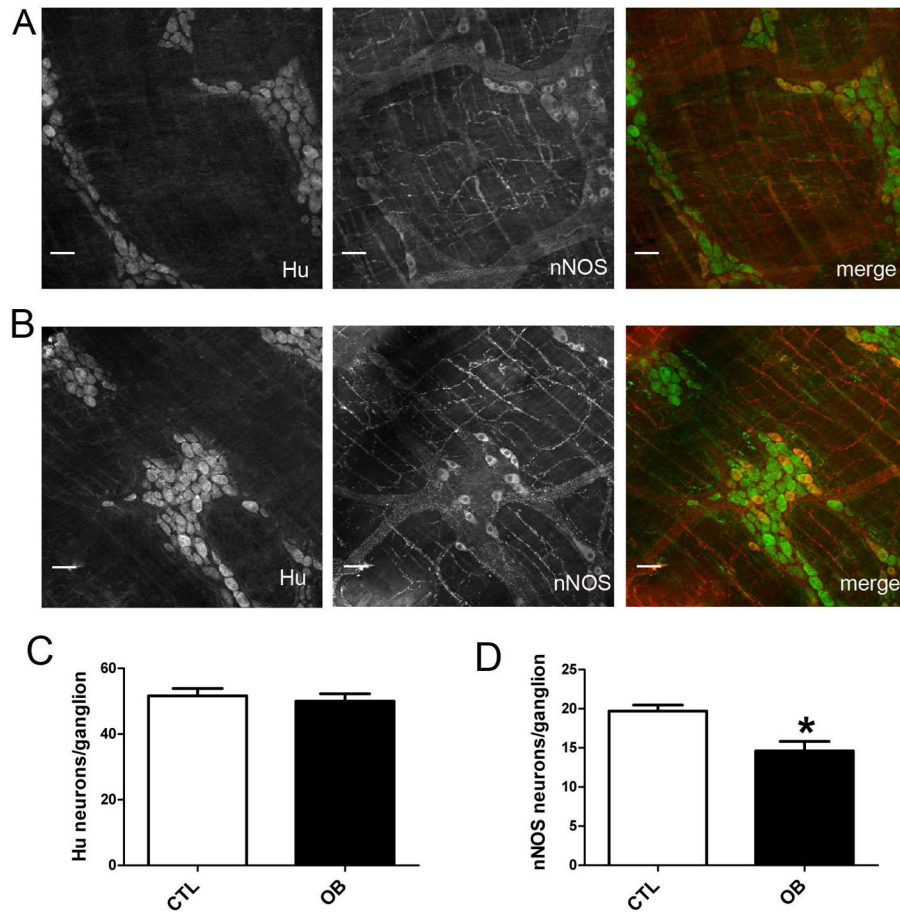
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**KEY MESSAGES**

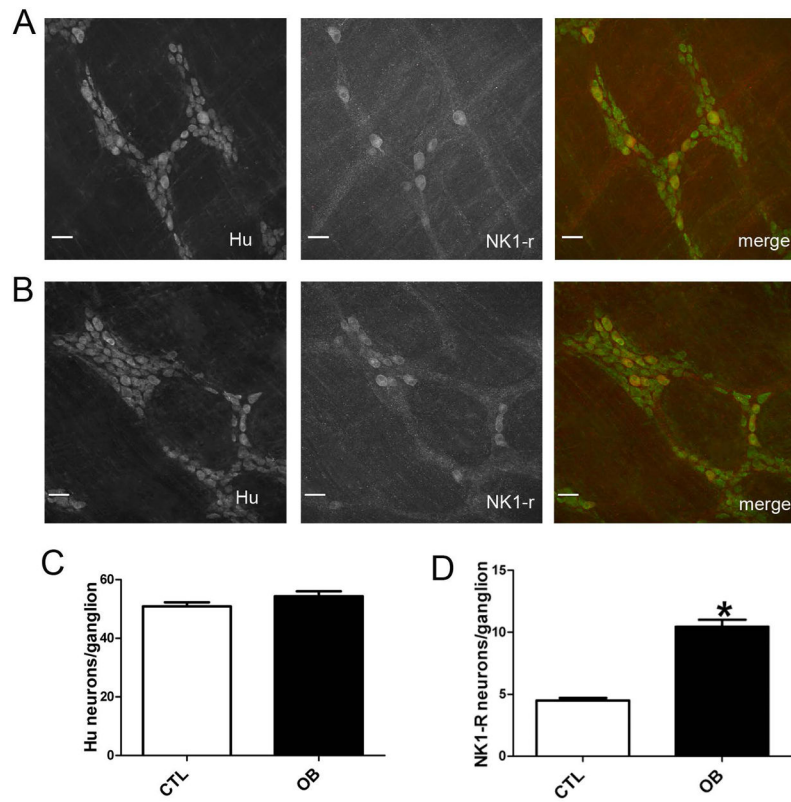
- Chronic administration of otilonium bromide (OB) provides relief from irritable bowel syndrome (IBS) in humans; yet the underlying mechanisms remain to be fully elucidated.
- In rats, chronic OB administered at a dose relevant to human pharmacotherapy altered colonic enteric nervous system markers at the level of myenteric plexus (neurokinin1 receptor and substance P, and neuronal nitric oxide synthase) and neuromuscular transmission.
- Chronic OB treatment did not affect interstitial cells of Cajal or slow wave activity in the colon.
- A novel mechanism for the effects of chronic OB administration involving the myenteric plexus is revealed that, together with the established inhibitory effects of OB acting directly on smooth muscle cells, modulates colonic function.





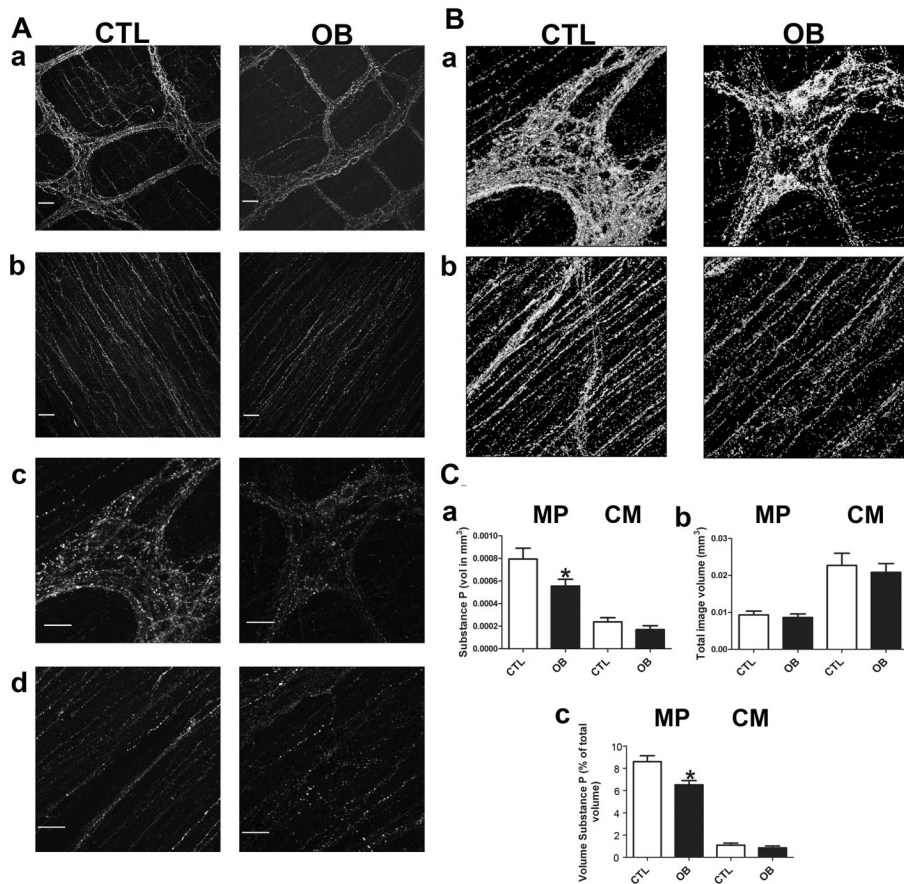
**Figure 1. Chronic OB-induced decrease in nNOS expressing neurons of the myenteric plexus of proximal colon**

Representative images depicting labelling of neurons with anti-Hu antibody and of nNOS positive neurons in control (A) and chronic OB-treated (B) rats. Bars in (A, B) = 40 μm. Summary bar-graphs showing the total neuron number (C) and the number of nNOS positive neurons (D) in controls (CTL) and chronically OB-treated rats. Each data-point in (C) and (D) is N=6 and normalized per individual ganglion; \*P < 0.05 (t-test).



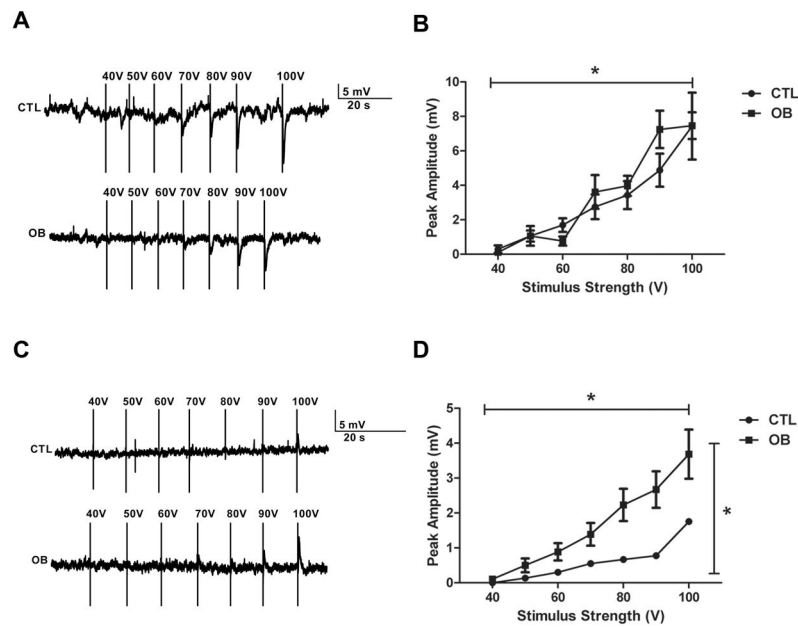
**Figure 2. OB-induced increase in NK1 receptor-expressing neurons in the myenteric plexus of proximal colon**

Representative images of all neurons labeled with anti-Hu and anti-NK1 receptor (NK1-r) antibodies and their co-localization (merged) in control (A) and chronic OB-treated (B) rats. Bars in (A,B) = 40 μm. Summary bar-graphs showing the total neuron number (C) and the number of NK1-r positive neurons (D) in controls (CTL) and chronically OB-treated rats. Each data-point in (C) and (D) is N=6 and normalized per individual ganglion; \*P < 0.05 (t-test).

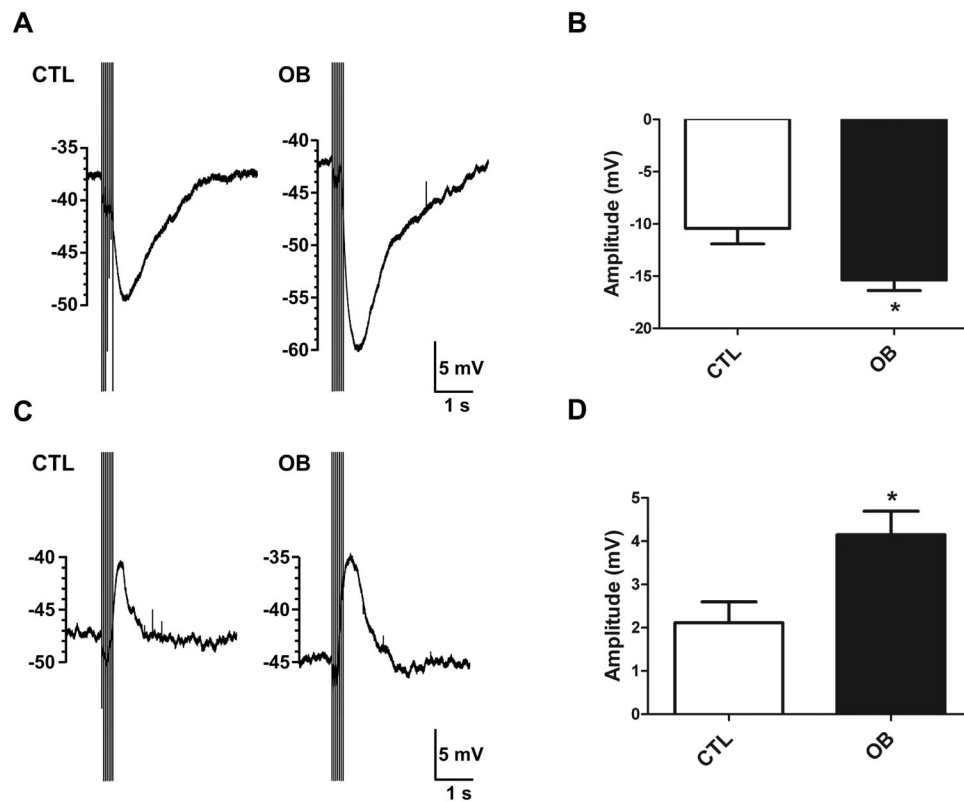


**Figure 3. Chronic OB-induced decrease in Substance P expression in the myenteric plexus but not in the circular muscle of proximal colon**

Panel (A) depicts images of SP-immunoreactive (SP-IR) fibers located in the myenteric plexus (a, low magnification; c, high magnification) and intramuscularly (b, low magnification; d, high magnification) in controls and OB-treated rats. Bars in (a) and (b) = 40  $\mu\text{m}$ , and (c) and (d) = 20  $\mu\text{m}$ . Panel (B) shows SP-IR reconstruction images using threshold analysis of controls and OB-treated rats within the myenteric plexus (a) and intramuscularly (b) for high magnification images. Panel (C) summarizes quantifications of Substance P positive structures (a), total volume (b) and percentage (%) of SP volume versus total volume in the myenteric plexus (MP) and circular muscle layer (CM) regions (c). Each data-point in (C, a-c) is N=6; \*P < 0.05 (t-test).

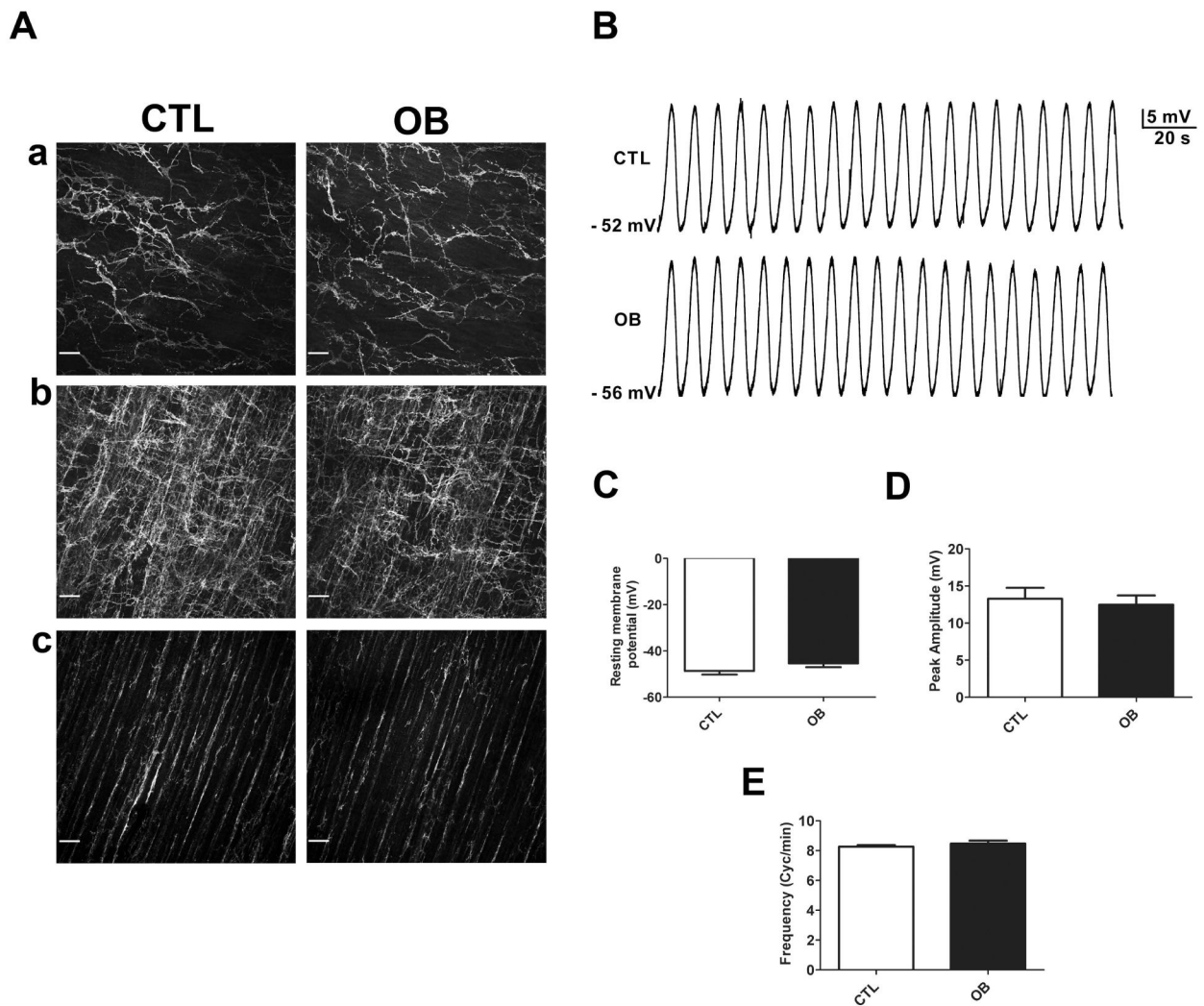


**Figure 4. Chronic OB treatment enhanced excitatory junction potentials but not inhibitory junction potentials evoked by single pulse stimulation in colonic circular muscle**  
 Shown are representative traces for inhibitory (A) and excitatory (C) junction potentials measured in controls and OB-treated rats. The panels in (B) and (D) describe, respectively, the mean amplitude responses for inhibitory and excitatory junction potentials in controls (n=8–13, N=3) and chronically OB-treated rats (n=21–27, N=4). In (B), statistical difference was noted for the stimulus strength ( $P < 0.0001$ , ANOVA) and in (D) for both the stimulus strength ( $P < 0.0001$ , ANOVA) and in-between treatment groups ( $P < 0.05$ , ANOVA). Recordings were made in the presence of nicardipine ( $2 \mu\text{M}$ ), an L-type  $\text{Ca}^{2+}$  channel blocker. Excitatory junction potentials were measured in the presence of L-NNA ( $1 \text{ mM}$ ) and MRS2500 ( $1 \mu\text{M}$ ) to block NOS and purinergic receptors, respectively.



**Figure 5. Chronic OB treatment increased excitatory and inhibitory junction potentials evoked by repeated pulse stimulation in colonic circular muscle**

Shown are representative traces for inhibitory (A) and excitatory (C) junction potentials measured in controls and OB-treated rats. The panels in (B) and (D) describe the mean amplitude responses for inhibitory and excitatory junction potentials, respectively, in controls (n=13 or 16, N=3) and chronically OB-treated rats (n=20 or 36, N=4) evoked by train stimulation (90 V, 300 msec, 20 Hz). Statistical differences were noted for both types of junction potentials (\*P < 0.05, t-test). Recordings were made in the presence of nifedipine (2  $\mu$ M), an L-type Ca<sup>2+</sup> channel blocker. Excitatory junction potentials were measured in the presence of L-NNA (1 mM) and MRS2500 (1  $\mu$ M) to block NOS and purinergic receptors, respectively.



**Figure 6. Chronic OB treatment does not alter interstitial cells of Cajal or slow wave activity in colonic circular smooth muscle**

(A) Kit immunoreactivity showing ICC at the submucosal border of the circular muscle (a), at the myenteric plexus (b), and within the muscle layers (c) in control and OB-treated rats; bar: a-c=40  $\mu$ m. (B) Shown are representative traces recorded in control and OB-treated rats. (C-E) Summary data for the membrane potential, peak amplitude, and frequency in control and OB-treated rats; each data-point in (C-E) is n=15 or 18, N=7 (all P>0.05).

**Table 1**

Sources of commercial antibodies used in immunohistochemistry experiments.

| Primary Antibody           | Supplier               | Concentr-ation        | Host        | Clonality        | Cat. number        |
|----------------------------|------------------------|-----------------------|-------------|------------------|--------------------|
| Anti-Hu                    | Millipore              | 1 µg/mL               | Mouse       | Monoclonal       | MAB377             |
| NK1-r                      | Millipore              | 0.33 µg/mL            | Rabbit      | Polyclonal       | AB5060             |
| nNOS                       | Millipore              | 0.33 µg/mL            | Rabbit      | Polyclonal       | AP3580             |
| Substance P                | Abcam                  | 0.5 µg/mL             | Guinea Pig  | Polyclonal       | ab10353            |
| Kit                        | R&D                    | 0.2 µg/mL             | Goat        | Polyclonal       | AF1356             |
| <b>Secondary Antibody</b>  | <b>Supplier</b>        | <b>Concentr-ation</b> | <b>Host</b> | <b>Clonality</b> | <b>Cat. number</b> |
| Cy3 anti-goat              | Jackson ImmunoResearch | 1.75 µg/mL            | Donkey      | Polyclonal       | 705-165-147        |
| Cy3 anti-guinea pig        | Jackson ImmunoResearch | 1.75 µg/mL            | Donkey      | Polyclonal       | 706-165-148        |
| Alexa Fluor 488 anti-mouse | Jackson ImmunoResearch | 2.33 µg/mL            | Donkey      | Polyclonal       | 715-545-151        |
| Cy3 anti-rabbit            | Jackson ImmunoResearch | 1.75 µg/mL            | Donkey      | Polyclonal       | 711-165-152        |