Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism

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O’Malley D, Liston M, Hyland NP, Dinan TG, Cryan JF. Colonic soluble mediators from the maternal separation model of irritable bowel syndrome activate submucosal neurons via an interleukin-6-dependent mechanism. Am J Physiol Gastrointest Liver Physiol 300: G241–G252, 2011. First published November 25, 2010; doi:10.1152/ajpgi.00385.2010.—Irritable bowel syndrome (IBS) is characterized by episodic bouts of abdominal pain, bloating, and altered bowel habit. Accumulating evidence has linked immune activation with IBS, including reports of increases in circulating levels of the proinflammatory cytokine interleukin (IL)-6. However, it is unknown whether IL-6 contributes directly to disease manifestation. As enteric nervous activity mediates motility and secretory function, the aims of this study were to determine the effects of IL-6 on submucosal neurons and related gastrointestinal (GI) function. In these studies, we examined the colons of maternally separated (MS) rats, which exhibit elevated circulating levels of IL-6 in addition to GI dysfunction. To our knowledge, these studies are the first to provide evidence of the sensitivity of submucosal neurons to colonic secretions from MS rats (n = 50, P < 0.05), thus recapitulating clinical biopsy data. Moreover, we demonstrated that the excitatory action is IL-6 dependent. Thereafter, the impact of IL-6 on neuronal and glial activation and absorptive secretory function was pharmacologically characterized. Other proinflammatory cytokines including IL-8 (n = 30, P > 0.05), IL-1β (n = 56, P > 0.05), and TNF-α (n = 56, P > 0.05) excited fewer neurons. Both muscarinic and nicotinic cholinergic receptors participate in the effect and cause downstream activation of ERK, JAK-STAT, and NF-kB signaling cascades. Functionally, IL-6 increases transepithelial resistance and enhances neurally and cholinergically mediated ion transport. These data provide a role for IL-6 in colonic secretory functions and relate these effects to GI dysfunction in an animal model of IBS, thereby elucidating a potential relationship between circulating levels of IL-6 and aberrant GI function.

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functional effects of IL-6. Furthermore, the effects of IL-6 on rat submucosal neurons including the underlying cellular mechanisms and signaling cascades being activated by this cytokine are elucidated. Additionally, given that functional absorption and secretion are key aspects of IBS, the effects of IL-6 on neural activation and GI function are examined.

**MATERIALS AND METHODS**

**Ethical approval.** All experiments were in full accordance with the European Community Council Directive (86/609/EEC) and were approved by the local University College Cork animal ethical committee.

**Animals.** Sprague-Dawley rats (225–300 g) purchased from Harlan UK were group housed four to six per cage and maintained on a 12:12-h dark-light cycle (0800–2000) with a room temperature of 22 ± 1°C. For the maternal separation protocol, virgin female Sprague-Dawley rats (7–9 wk old) were used as the parental colony. As previously described (31, 48), the rat litter was separated from the dam for 3 h/day (0900–1200) between postnatal days 2 and 12. Each litter was placed in a clean cage over a heated blanket (30–33°C). Control, nonseparated (NS) groups were left undisturbed in the home cage with the dam. Male rats from both groups were weaned and allowed to grow to adulthood before experimentation.

**Supernatants.** As previously reported (5), 3-cm segments of full-thickness distal colon excised from nonanesthetized adult male MS and NS rats were bubbled in 2 ml of Hanks’ balanced salt solution (1 h, 37°C). Supernatants were pooled from five rats of each group and diluted in Krebs buffer (1:250 or 1:500). Supernatant concentrations of IL-6 were measured by an electrochemiluminescence system. The cytokine measurements were carried out in duplicate and were analyzed with the Mesoscale Discovery (MSD) Sector 2400 imager (Gaithersburg, MD), where antibodies labeled with Sulfo-tag reagents emitted light upon electrochemical stimulation. This ultrasensitive method has a detection limit of 0.3 pg/ml for IL-6.

IL-6 neutralization was conducted by incubating supernatants with anti-IL-6 antibody (affinity-purified goat antibody immunized with *Escherichia coli*-derived rrIL-6; R&D Systems, Abingdon, UK). The neutralization dose for this antibody was calculated at ~30–90 ng/ml in the presence of 0.6 ng/ml of rrIL-6. Neutralizing antibody was added in excess (0.5 mg/ml, 1 h at 37°C) to the pooled supernatants, which had an estimated IL-6 content of <40 pg/ml.

**Tissue preparation.** Distal segments of colon were excised from naïve Sprague-Dawley rats and maintained in bubbled (95% O₂–5% CO₂) Krebs saline consisting of (in mmol/l) 117 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, and 11 d-glucose, with 1 μM nifedipine to inhibit smooth muscle contractions. Longitudinal and circular muscle layers were removed to prepare a mucosa-submucosa preparation for Ussing chamber electrophysiology. For calcium imaging and immunohistochemical studies, the mucosal layer was removed to expose the submucosal plexus (SMP).

**Ussing chamber electrophysiology.** As previously described (22), mucosa-submucosa preparations were mounted in Ussing chambers (exposed area of 0.12 cm²) with 5 ml of Krebs solution (95% O₂–5% CO₂, 37°C) in the basolateral and luminal reservoirs. Tissues were voltage clamped at 0 mV with an automatic voltage clamp (EVC 4000, World Precision Instruments (WPI), Sarasota, FL), and the short-circuit current (Iₛₑ) required to maintain the 0-mV potential was monitored as a recording of the net active ion transport across the epithelium. Resistance was calculated by Ohm’s law. Experiments were carried out simultaneously in chambers connected to a PC equipped with DataTrax II software (WPI).

**Calcium imaging.** Tissue samples were loaded with fura-2 AM (7 μM, 1 h), and Cell R software (Olympus Soft Imaging Solutions, 1986–2009) was used to record excitation and emission wavelengths of 340/380 and 510 nm, respectively. Images were acquired at 3 Hz with a xenon/mercury arc burner (MT20 illumination system, Olympus America, Melville, NY), a charge-coupled device digital camera (F-view II, Soft Imaging System, Munster, Germany), and a ×40 water-immersion objective on a fixed-stage upright microscope (Olympus BX51W1). Enteric glial cells were identifiable on the basis of their size, shape, and position within the ganglia. Ganglionic neurons were identified on the basis of morphology and responsibility to 75 mM KCl. Depending on the size of the ganglia, between 5 and 17 cells were imaged. Neurons sensitive to IL-6 were defined as those with increases in intracellular calcium greater than 2 SD from baseline (calculated as the average ratio during the 150 s preceding stimulus application). Variation in kinetics and characteristics of IL-6- and supernatant-mediated responses were observed, but all responses fitting the selection criteria were included for analysis. Responses were considered blocked if they were reduced to below 2 SD from baseline. Responses were reported as a change in ratio. A perfusion system continuously superfused the colonic tissue with carboxen-bubbled Krebs-buffered saline, which was circulated at 1.5 ml/min. The lag time for reagents to reach the neurons was calculated at 1.9 (±0.13) min based on the time taken for 75 mM KCl to cause neuronal activation in 24 individual experiments. Reagents, including IL-6, were added to the superfusate, whereas supernatants diluted in 1 ml of Krebs solution were added directly to the dish because of low volume availability. This meant there was no lag time for neuronal exposure to the supernatants. The perfusion pump was stopped during incubation (3–5 min) and restarted for washout.

**Immunofluorescence.** For cross sections of colon, fixed (4% paraformaldehyde) distal colons were cryoprotected in 30% (wt/vol) sucrose and snap frozen (~80°C). The frozen tissue was cryostat (Leica, Nussloch, Germany; CM1900 UV) sectioned (10 μm) and mounted on glass slides. The sections were incubated with antibodies specific for anti-IL-6 antibody (affinity-purified goat antibody immunized with *E. coli*-derived rrIL-6, 1:300; R&D Systems) and anti-rrIL-6 receptor (IL-6R) antibody (affinity-purified rabbit polyclonal antibody mapping to the COOH terminus of IL-6Rα, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA). Fluorescent images were captured with an Olympus D71 upright fluorescence microscope and Cell F software (Soft Imaging Solutions).

Whole mount SMP preparations were fixed overnight in Zamboni’s fixative, blockaded with 1% donkey serum, and permeabilized with 0.1% Triton X-100. Colocalization studies were conducted by staining the submucosal ganglia with antibodies for IL-6 or IL-6Rs (1:300, as described above) with neuronal markers for calbindin (1:300, mouse; Swant, Bellinzona, Switzerland), calretinin (1:300, goat; Swant), neuronal nitric oxide synthase (nNOS, 1:300, goat; Abcam, Cambridge, UK), and the glial cell marker S100 (1:300, mouse; Sigma-Aldrich, St. Louis, MO). With the Cell F software, the numbers of cells positive for both the cell markers and IL-6 or IL-6Rs were counted. Phospho-p44/42 mitogen-activated protein kinase (MAPK) (1:400, rabbit) and phospho-signal transducer and activator of transcription (STAT)3 (1:100, mouse; Cell Signaling Technologies, Boston, MA) antibodies were used to assess the effects of IL-6 on these signaling cascades. Fluorescein isothiocyanate (FITC; anti-rabbit and anti-goat, 1:400, 2 h, 37°C) and rhodamine (anti-mouse, 1:400, 2 h, 37°C)-conjugated antibodies were used as fluorophores (Jackson Immunoresearch). Controls in the absence of the primary or secondary antibody and after preabsorption with IL-6 were negative. MultiGauge version 2.2 imaging software (Fujiﬁlm) was used to compare relative intensities of staining by measuring the quantitative light absorbance units minus background (QL-BG) for a defined area within the mucosal layer.

**Statistics.** The data are represented as mean ± SE values. Student’s t-test and one-way ANOVA with Newman-Keuls post hoc test were used where appropriate. P ≤ 0.05 was considered signiﬁcant. All experiments were conducted in at least three different animals.
RESULTS

Soluble mediators secreted from colons of MS rats activate submucosal neurons. Secretions from the distal colon of NS and MS rats were applied to naive SMP preparations. Both supernatants stimulated an increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)); however, the peak response evoked by MS supernatants was significantly greater than that evoked by NS secretions (1:250 dilution, \(n = 50, P < 0.05\); Fig. 1A). The lag from time of application of MS supernatants to calcium response was 2.2 ± 0.4 min (\(n = 9\) experiments). The MS-evoked response was attenuated by pretreatment with a neutralizing anti-IL-6 antibody (xIL-6, 0.5 mg/ml, \(n = 24, P < 0.05\); Fig. 1Ai). With a rat-specific IL-6 assay, the concentration of IL-6 in the pooled MS supernatant (31.5 ± 4.3 pg/ml or ~1.5 nM) was higher than the NS sample (3.4 ± 1.2 pg/ml or ~0.2 nM; \(n = 5\) samples pooled, 2 runs completed in duplicate).

Transverse sections of distal colon from NS and MS rats were immunolabeled for IL-6 and IL-6R expression. IL-6 was widely expressed in the mucosal epithelial cells, whereas IL-6R staining was more restricted to immunolabeled cells within the lamina propria and the neuronal plexi (Fig. 1B). Mucosally expressed IL-6 was higher in MS rats (\(n = 3\) sections from 4 different rats, \(P < 0.05\), Fig. 1B), whereas mucosal IL-6R expression was not significantly different between NS and MS rats. The remaining studies were conducted in naive whole mount SMP preparations. Stimulation of SMP samples with NS or MS supernatants (1:500 dilution, 1 h) did not affect IL-6 labeling (\(P > 0.05\); Fig. 1Ci) but caused a significant increase in IL-6R expression in MS stimulated tissues (\(n = 3\) ganglia from 6 animals, \(P < 0.05\); Fig. 1Cii).

IL-6-induced excitation of submucosal ganglia. To verify that IL-6 can mimic the effects of the colonic soluble mediators, recombinant IL-6 was applied to preparations of submucosal neurons from naive Sprague-Dawley rats. IL-6 concentrations of 0.01, 0.1, and 1 nM evoked increasing calcium responses (Fig. 2A). The remaining studies were conducted with 1 nM recombinant IL-6. Brief exposure to IL-6 (3 min) increased somatic calcium concentrations in 43.4% (\(n = 85/196\) neurons) of ganglionic neurons compared with control nonstimulated calcium levels (\(n = 15\) rats, \(P < 0.001\) in responders only). The response varied from slowly developing long-lasting rises to short peaks or oscillatory responses. A 17.5 ± 2.5% (\(n = 85\); Fig. 2B) increase in the peak amplitude of the [Ca\(^{2+}\)]\(_i\) response was observed compared with control. The specificity of the IL-6 effect was demonstrated by attenuating the calcium responses with a neutralizing IL-6 antibody (0.5 mg/ml, 1 h, \(n = 34, P < 0.01\); Fig. 2C). Also, prior incubation of the tissue with anti-IL-6R antibodies (1:100, 1 h, 37°C) resulted in IL-6-stimulated values (1.5 ± 0.06) being no different from basal values (1.55 ± 0.06, \(P > 0.05\), \(n = 18\)). As circulating levels of IL-6 are elevated in IBS patients, blood vessels feeding the basolateral side of the colon are a likely source of IL-6. Therefore, in functional Ussing chamber studies, recombinant IL-6 was added to the basolateral reservoir. When \(I_{sc}\) values were recorded 15 min after the addition of IL-6, a small but significant increase in basal current was observed (\(n = 26, P < 0.05\); Fig. 2D).

The IL-6-induced increase in [Ca\(^{2+}\)]\(_i\), displayed kinetics different from those of the supernatants. Excluding the time in the tubing (calculated as the mean time taken for 75 mM KCl to activate neurons), the onset latency to the first response was an average of 2.95 ± 0.37 min (\(n = 21\) experiments), a result that is not significantly different from the latency in response time for the MS supernatants (2.2 ± 0.4 min, \(n = 9\) experiments, \(P > 0.05\)). The lag time for IL-6-induced neuronal activation is suggestive of potential intermediary steps being required before neuronal activation. Thus potential candidates include the glial cells, and, indeed, in a subset of experiments, when activation times for ganglionic neurons and glial cells were compared the time to initial activation of glial cells (1.5 ± 0.36 min) was shorter than that for neuronal activation (3.05 ± 0.5 min, \(n = 6, P < 0.01\)).

Other cytokines such as IL-8 (11) and IL-1β and TNF-α (29) have also been reported to be altered in IBS. However, compared with IL-6, which activated 43.4% of neurons, reduced numbers were activated by IL-8 (\(n = 6/29\)), IL-1β (\(n = 2/46\)), and TNF-α (\(n = 4/46\); Table 1). Onset latencies for IL-8, IL-1β, and TNF-α were 1 ± 0.29 (\(n = 6\)), 1.2 ± 0.25 (\(n = 2\)), and 2.4 ± 0.9 (\(n = 5\)) min, respectively, which were not significantly different from the time lag for IL-6 (\(n = 21, P > 0.05\)).

Functional effects of IL-6. The sensitivity of the IL-6 effect to TTX (100 nM, \(P < 0.01\); Fig. 3A) suggests that signal transmission is necessary for neuronal activation. The importance of Na\(^+\) channel-facilitated neuronal activation was further documented in functional Ussing chamber studies, where prior addition of IL-6 (1 nM, 30 min) to the basolateral reservoir resulted in a significant enhancement of veratridine-induced current (\(n = 4, P < 0.05\); Fig. 3B). This is in contrast to the sensory nerve stimulant capsaicin (1 μM), which was unchanged in the presence of IL-6 (\(n = 5, P > 0.05\); Fig. 3C). Interestingly, the relative change in transepithelial resistance (TER) increased over the course of the experiment in tissues stimulated with IL-6 (19.3 ± 5.5 Ω/cm\(^2\)) relative to control preparations (−2.9 ± 4.1 Ω/cm\(^2\), \(n = 12, P < 0.01\)), indicating reduced epithelial barrier permeability.

Characterization of IL-6-mediated activation of submucosal neurons. To assess the importance of intracellular stores in the IL-6 effect, IL-6 was applied after removal of extracellular calcium (90 s). Under these conditions, IL-6 elicited a calcium peak that was short in duration (\(n = 15\)). When the neurons were reperfused with calcium-containing Krebs solution, IL-6 caused a longer, more sustained response (\(n = 19\); Fig. 4A), suggesting secondary calcium influx from extracellular sources. Indeed, incubation with the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor, thapsigargin (1 μM, 1 h), which itself caused a response in 23 of 33 neurons, did not prevent IL-6 from activating 42% (\(n = 14/33\)) of neurons (\(P < 0.001\) in IL-6-sensitive neurons; Fig. 4B).

Given the continuous presence of nifedipine (1 μM) in all experiments to reduce muscle-mediated movement, the IL-6-mediated response occurs independent of L-type voltage-gated calcium channels (VGCCs). However, application of the P- and Q-type VGCC blocker ω-agatoxin IVA (100 nM) inhibited the IL-6 response (\(n = 10, P < 0.05\); Fig. 4C). N-type calcium channels appeared to be the major contributor to calcium influx, as ω-conotoxin GVIA (100 nM) attenuated IL-6 response to a greater extent (\(n = 11, P < 0.01\); Fig. 4D). Calcium influx via glutamatergic N-methyl-D-aspartate (NMDA) receptors does not appear to be important, as control IL-6-induced increases in [Ca\(^{2+}\)]\(_i\) (0.075 ± 0.02), were unaffected by expo-
Fig. 1. Interleukin (IL)-6 in maternally separated (MS) rats. A, i: representative trace and histogram demonstrate the calcium responses from nonseparated (NS) and MS supernatants (spnt; 1:250 dilution, n = 50). ii: Prior incubation with anti-IL-6 (xIL-6) antibodies decreased the MS-stimulated response. B: histograms illustrate mean intensities (calculated as mean intensity/area) of mucosal IL-6 (red) and IL-6 receptor (IL-6R; green) staining in NS and MS colons (n = 10–12 sections from at least 3 rats, scale bar = 200 μm). Images illustrate staining in cross sections of colons (magnified regions are in insets). Arrows indicate strong IL-6/IL-6R colocalization in cells in the lamina propria, and arrowhead indicates higher mucosal expression of IL-6 in MS tissue. C: images and histograms show the effects of exposure to NS and MS supernatants (1:500, 1 h, scale bars = 500 μm) on IL-6 (i) and IL-6R (ii) expression. *P < 0.05.
sure to the inhibitor with d-2-amino-5-phosphonovalerate (d-APV, 10 μM, 0.05 ± 0.04, n = 11, P > 0.05).

Contribution of acetylcholine to IL-6 action. Given the importance of acetylcholine in secretion and GI motility, in addition to its contribution to IBS pathophysiology (10), pharmacological tools were used to assess its role in the effects of IL-6. Hexamethonium (1 μM), the nicotinic acetylcholine receptor (nAChR) inhibitor, attenuated most (10/12) IL-6-induced Ca\(^{2+}\) responses (n = 10, P < 0.01 in responders; Fig. 5A). The muscarinic acetylcholine receptor (mAChR) antagonist atropine (1 μM) was effective in blocking the IL-6 responses in 11 of 17 neurons tested (n = 11, P < 0.001 in responders; Fig. 5B). Furthermore, in Ussing chamber experiments, prior addition of IL-6 (1 nM, 30 min) augmented a bethanechol (10 μM)-stimulated secretory current in 7 of 12 samples (n = 7, P < 0.05 in responders; Fig. 5B). The contribution of neuronal transmission was demonstrated when TTX (100 nM, 15 min) blocked the capacity of IL-6 to enhance the bethanechol current such that control (120.7 ± 28.5) and IL-6 plus TTX (100.2 ± 24.5) bethanechol responses were similar (n = 6, P > 0.05).

Immunohistochemical characterization of IL-6- and IL-6R-positive ganglionic cells. Immunohistochemical techniques were utilized to characterize the ganglionic cell types expressing IL-6.

Table 1. Percentage of submucosal neurons activated by cytokines

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>IL-6</th>
<th>IL-8</th>
<th>TNF-α</th>
<th>IL-1β</th>
</tr>
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<tr>
<td>% Neurons activated</td>
<td>43.4</td>
<td>20.7</td>
<td>4.35</td>
<td>8.7</td>
</tr>
</tbody>
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Data are % of submucosal neurons sensitive to interleukin (IL)-6 (n = 85/196), IL-8 (n = 6/29), IL-1β (n = 2/46), and TNF-α (n = 4/46). Ca\(^{2+}\) responses (n = 10, P < 0.01 in responders; Fig. 5A). The muscarinic acetylcholine receptor (mAChR) antagonist atropine (1 μM) was effective in blocking the IL-6 responses in 11 of 17 neurons tested (n = 11, P < 0.001 in responders; Fig. 5B). Furthermore, in Ussing chamber experiments, prior addition of IL-6 (1 nM, 30 min) augmented a bethanechol (10 μM)-stimulated secretory current in 7 of 12 samples (n = 7, P < 0.05 in responders; Fig. 5B). The contribution of neuronal transmission was demonstrated when TTX (100 nM, 15 min) blocked the capacity of IL-6 to enhance the bethanechol current such that control (120.7 ± 28.5) and IL-6 plus TTX (100.2 ± 24.5) bethanechol responses were similar (n = 6, P > 0.05).
ligands and receptors. IL-6 immunostaining was diffuse, primarily cytosolic and colocalized with all S100-positive glial cells (n = 112) and 78% (n = 47/60) of calbindin-positive, 87% (n = 62/71) of calretinin-positive, and 83% (n = 34/41) of nNOS-positive neurons (Fig. 5Ci; n = 3 ganglia per tissue from 3 different rats). IL-6R labeling was punctate and most prominent on nerve fibers. Within ganglia, 61% (n = 54/89) of S100-positive glial cells and 73% (n = 27/37) of calbindin-positive, 72% (n = 54/72) of calretinin-positive, and 67% (n = 29/43) of nNOS-positive neurons were immunoreactive for IL-6Rs (Fig. 5Ci). Cytokines are known to stimulate increased receptor expression, and exposure to IL-6 (1 nM, 10 min) resulted in a significant increase in the intensity of ganglionic IL-6R immunostaining (including both glia and neurons, n = 5 ganglia each from 3 rats. P < 0.05; Fig. 5D).

**IL-6 activates STAT3, MAPK, and NF-κB signaling pathways.** Finally, we sought to clarify the role of intracellular signaling pathways in the IL-6-induced response. The role of the Janus tyrosine kinase (JAK)-STAT signaling pathway was investigated with WP1066 (1 μM), a STAT3 pathway inhibitor. WP1066 abolished the IL-6 response (n = 35, P < 0.001; Fig. 6Ai). MAPK or ERK1/2 activation is also linked with IL-6 signaling. Because of the long incubation period (2 h) required for UO126, the MAPK cascade inhibitor, the imaging protocol was adjusted. Control responses to IL-6 were analyzed in untreated tissue preparations and compared with IL-6 responses obtained in the presence of UO126 (10 μM). In the absence of UO126, 47% (n = 18/37) of neurons were activated by IL-6, whereas 23% (n = 7/30) were activated in the presence of UO126. Even those cells that did respond in the presence of UO126 generated significantly reduced responses (P < 0.05, n = 7; Fig. 6Bi). Nuclear factor-κB (NF-κB) is a ubiquitously expressed transcription factor and a major regulator of inflammatory genes including cytokines such as IL-6. Application of the inhibitor IκB kinase (IKK)2 (5 μM, 1-h incubation) to block NF-κB signaling (26) caused an independent increase in [Ca^{2+}]; however, control responses induced by IL-6 (0.97 ± 0.02) were decreased to 0.05 ± 0.01 (n = 13, P < 0.05; Fig. 6C) in the presence of this inhibitor. Phosphatidylinositol 3-kinase (PI3-kinase) has been proposed as an alternative signaling pathway for IL-6; however, incubation with wortmannin (10 μM) had no effect on the IL-6 response, such that the control response (0.1 ± 0.03) was not significantly different from the IL-6 response in the presence of wortmannin (0.08 ± 0.06, n = 15, P > 0.05).

When whole mount preparations were stimulated with IL-6 (1 nM, 10 min) the intensity of phosphorylated STAT3 staining was increased in cell bodies within the submucosal ganglia (n = 3 ganglia each from 4 different rats, P = 0.07; Fig. 6Aii). Additionally, IL-6 (1 nM, 10 min) increased phosphorylation of ganglionic p44/42 MAPK, which was most apparent in extraneuronal cells and nerve fibers (n = 3 ganglia per preparation from 4 different rats, P < 0.05; Fig. 6Bii).

**DISCUSSION**

To our knowledge, these studies are the first to provide evidence of the sensitivity of submucosal neurons to colonic secretions from an animal model of IBS, thus recapitulating
Moreover, we demonstrated that the excitatory action is, at least in part, dependent on IL-6. Thereafter, in an effort to understand the mechanisms underlying such effects, we pharmacologically characterized the impact of IL-6 on neuronal activation and absorptive/secretory function. As colonic function is primarily regulated by enteric neurons, these data offer a plausible explanation as to how the reported elevations in circulating IL-6 in IBS patients (10, 11) could feasibly increase activity of submucosal neurons and thereby contribute to the pathophysiological changes in gut function that underlie IBS symptomatology.

The MS rat provides a well-characterized model of IBS, exhibiting altered morphology and defection patterns (33) and visceral hypersensitivity (31). Mimicking the human condition, these animals also exhibit elevated circulating levels of IL-6 after stimulation (31). Because recent studies have shown activation of submucosal neurons with supernatants derived from IBS colonic biopsies (4) and have intimated the involvement of soluble mediators, such as histamine, serotonin, and proteases, we sought to investigate whether MS colonic secretions could activate rat submucosal neurons. Indeed, MS supernatants caused larger increases in $[\text{Ca}^{2+}]_i$ than those in NS controls, and this was found to be due, at least in part, to increased IL-6 secretion. Calcium increases to the supernatants were variable, in both the lag time to response and the amplitude of the peak stimulated. This may be due to different neuronal cell types within the ganglia being activated. Indeed, we have observed expression of the IL-6 ligand and receptor in all neuronal cell types.

Incomplete attenuation of the response by the IL-6 neutralizing antibody suggests that other factors in the milieu of...
colonic secretions could also contribute to the response. Previous reports (1, 4, 5) and our own observations of an inhibitory effect of the antihistamine, pyrilamine (O’Malley et al., unpublished observations) indicate an important role for factors released from mast cells. Interestingly, there is some suggestion that PAR2 and IL-6 signaling may converge (25). Indeed, the effects of IL-6 may lie downstream to the release of mast cell mediators, and this is worthy of further investigation.

The cellular source of secreted IL-6 is as yet unclear, but as the supernatants were produced by segments of whole colon, likely candidates could include epithelial cells, immune cells, glia, or enteric neurons. Furthermore, exposure of naive whole mount SMP preparations to MS supernatants caused an increase in IL-6R fluorescence intensity, despite no changes in IL-6R expression in MS mucosa being observed. It may be that submucosal neurons in MS animals are more sensitive to the neuromodulatory effects of IL-6. These findings may therefore be important in understanding alterations in neuronally regulated control of motility, secretion/absorption, blood flow, and immune function. The data suggest a possible mechanism by which cytokine-induced activation of submucosal neurons is associated with the dysregulation of GI function that is char-
characteristic of the IBS-like phenotype of the early-life stress model.

Over 40% of ganglionic submucosal neurons in the distal colon were activated by recombinant IL-6 at a concentration (1 nM) that is comparable to the concentration of the cytokine secreted from the MS colons (1.6 nM). Previous studies observed increased activation of enteric neurons by IL-1β (43, 49) and TNF-α (35); however, in our preparations, the neuroexcitatory effects of IL-8, TNF-α, and IL-1β were muted compared with IL-6.

The IL-6-induced increase in somatic calcium levels demonstrated slow kinetics, taking an average of ~3 min for
initiation. This time lag was similar to the onset latency evoked by the supernatants and not statistically different from activation times for IL-8, IL-1β, or TNF-α. Although these results contrast with electrophysiological studies that demonstrated that IL-6 could enhance the excitability of submucosal neurons within ~20 s (50), other studies displayed an onset latency of up to 2 min in myenteric neurons (23) following stimulation with IL-6 and IL-1β. Furthermore, the excitatory effects of IL-1 in hypothalamic neurons had a lag time ranging from 2 to 6 min (20). Possible reasons for the delay may be due to intermediary steps before neuronal activation. Potential candidates include the enteric glia, which are proposed to act as immunomodulatory cells in the enteric nervous system (ENS) (36) and were stained for the IL-6 ligand and receptor. Indeed, temporal studies of initial activation times demonstrated that submucosal glial cells responded to IL-6 stimulation more quickly than neurons. Our findings suggest a mechanism whereby IL-6 stimulates glial cell activation, which in turn stimulates TTX-sensitive transmission to neurons. In older studies conducted in guinea pig ileum, IL-6-induced cellular depolarization was unaffected by TTX (50). However, spike discharge in S-type motor neurons is TTX sensitive. Thus the calcium responses to IL-6, which are critically dependent on action potential discharge and show TTX sensitivity, suggest that spike discharge is blocked, causing a subsequent reduction in the calcium signal. We also observed nucleated immunelike cells in the lamina propria that were immunopositive for IL-6 and IL-6Rs and may contribute to neuronal activation. IL-6-mediated activation of immune cells may result in the release of cytokines and other factors capable of neuromodulation; however, in the submucosal preparation the numbers of immune cells are unlikely to be high. Ligand-induced receptor upregulation may also play a role in the slowly developing calcium response, as within the time frame of activation IL-6 also induced increases in IL-6R expression.

The IL-6-stimulated calcium influx was dependent on release from intracellular stores, and calcium influx was via N-type VGCCs and to a lesser extent P/Q-type channels. Both channel types are classed as high-voltage-activated calcium channels (12) and are highly expressed in presynaptic terminals and directly coupled to neurotransmission (46, 47), highlighting a potential mechanism by which IL-6 increases neuronal excitability.

Consistent with IL-6-mediated activation of submucosal neurons we observed an IL-6-stimulated increase in basal current in Ussing studies. Given the elevated levels of IL-6 in MS colonic mucosa, IL-6 may influence basal Isc in MS colons, a supposition that warrants further investigation. Additionally, we noted an IL-6-mediated increase in TER over the course of the experiment (1–1.5 h), which may be due to remodeling of gap junctions and a reduction in colonic permeability. This is consistent with IL-6 playing a protective role in the integrity of the intestinal epithelial layer (45). However, as other studies have demonstrated that longer incubation with IL-6 and other proinflammatory cytokines increases epithelial permeability (19, 30), chronic exposure to high levels of IL-6 could actually facilitate the movement of soluble mediators from the lumen into the colonic tissue, where immune cells may be activated causing IL-6 release and subsequent activation of submucosal ganglia. However, as is the case in IBS patients (10, 11), circulating IL-6 levels are elevated, providing access to submucosal neurons from the basolateral side of the colon.

IL-6 caused an enhancement of veratridine- and bethanochol-stimulated Isc values without affecting capsaicin-induced activation of sensory neurons. The effect on the bethanochol-induced secretory current was attenuated by prior incubation with TTX, further emphasizing the neuromodulatory actions of IL-6. This fits with our data demonstrating the inhibition of neuronal IL-6-induced calcium responses with a mACHR antagonist, suggesting that IL-6 modulates enteric neural control of basal ion transport and illustrating the importance of the cholinergic system to IL-6 secretion (10). This may indicate that IL-6-induced activation of secretomotor neurons contributes to neurogenic secretory diarrhea, as described in diarrhea-predominant IBS patients (27). Alternatively, others have shown that IL-6 can attenuate the presynaptic inhibition of norepinephrine release, thereby releasing the sympathetic brake and further contributing to a prosecretory state (50).

Several functional classes of neurons have been identified in the SMP, including intrinsic primary afferent neurons and secretomotor and vasomotor neurons (14). With distinct electrophysiological characteristics, these neurons have been classified as cholinergic AH-type sensory neurons and cholinergic or noncholinergic S-type motor neurons. Using immunohistochemical techniques we determined that IL-6 was present in most neuronal cell types and glial cells, which has implications for mucosal barrier integrity, intestinal homeostasis, and immunomodulation of enteric nerve-mediated GI function. When activated, membrane-bound IL-6Rs combine with the signal transducing component gp130 to activate intracellular signaling cascades. High proportions of calbindin-, calretinin-, and nNOS-positive neurons expressed IL-6Rs with a lower prevalence in glial cells. Our studies showed that IL-6R expression was predominant on neuronal fibers. These data illustrate the array of neuronal and glial cell types sensitive to IL-6 within the SMP. Additionally, alternative mechanisms of activation such as stimulation by IL-1β (36, 43) or binding of the soluble IL-6R to gp130 (40) may be important for IL-6 secretion.

Downstream of IL-6R/gp130 complex activation, we found that the JAK-STAT, MAPK/ERK, and NF-κB signaling cascades were important in IL-6-mediated activation of submucosal neurons. Activation of the JAK-STAT pathway is crucial for signal transduction leading to transcription but also for surface expression of receptors (18) and activation of ion channels (52). IL-6-induced activation of the serine/threonine-specific MAPK molecule has also been described to initiate transcription, cell survival, differentiation, and proliferation (see Ref. 17 for review) in addition to mediating the effects of other neuromodulators (32). NF-κB is an important transcription factor mainly involved in inflammatory and immune responses (3). Our studies demonstrate that the IL-6-induced increase in [Ca2+]i, in submucosal neurons utilizes MAPK, JAK-STAT, and NF-κB, but not PI3-kinase, signaling cascades. Immunohistochemical data also illustrate increased phosphorylation of MAPK and STAT3 in the whole ganglia. However, in the case of pSTAT3 the increase appears to be confined to the neurons, whereas the stimulated increase in MAPK phosphorylation appears to primarily occur in nonneuronal, possibly glial, cells, thus providing additional evidence for the importance of intermediary glial signaling in the actions of IL-6.
In the central nervous system, IL-6 can exert neurotrophic and neuroprotective effects, but depending on the cellular context IL-6 can also mediate inflammation, demyelination, and astroglialosis (28, 42, 44). The functional effects of IL-6 in the ENS have not been delineated so clearly. IL-6 has been shown to be central to intestinal inflammatory disorders such as Crohn’s disease (51), but as far as we are aware, these data are the first to demonstrate GI-specific changes in IL-6 expression and secretion and provide a tangible link between GI dysfunction and alterations in cytokine levels in a model of IBS. These data demonstrate that the MS rat model of IBS, which exhibits altered GI function and an exaggerated stress response, has increased expression of IL-6 and IL-6R in colonic epithelial and submucosal neurons, respectively. Furthermore, secretions from MS colons have increased levels of IL-6 compared with NS supernatants. Soluble mediators present in the supernatants from MS rats caused increased activation of submucosal neurons, and this is mediated, at least in part, by IL-6, although discrepancies between response profiles from the supernatants and recombinant IL-6 indicate the likelihood that other soluble mediators present in the supernatants may also contribute to this effect. Cholinergic receptors participate in the IL-6-induced effect and cause downstream activation of ERK, JAK-STAT, and NF-κB signaling cascades. At a functional level, IL-6 increases TER and enhances neurally and cholinergically mediated ion transport. Taken together, these data provide a role for IL-6 in colonic secretory function and relate these effects to GI dysfunction in an animal model of IBS. These studies elucidate a potential relationship between circulating IL-6 observed in IBS patients (10, 11, 38) and manifestation of IBS pathophysiology.

REFERENCES


