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. Therefore, 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-κB 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.

cholinergic receptor; mitogen-activated protein kinase; signal transducer and activator of transcription 3; supernatant

IRRITABLE BOWEL SYNDROME (IBS) is a functional gastrointestinal (GI) disorder characterized by episodic bouts of abdominal pain, bloating, and altered bowel habit. Although IBS is generally accepted as a brain-gut disorder, the underlying pathophysiology of IBS remains elusive. There is, however, growing support for the importance of immune activation in IBS symptomatology (34), as evidenced by increased expression of mucosal T cells, lymphocytes, and mast cells (6). Mucosal inflammation has been linked to altered sensory and motor function in the GI tract (8), and cytokine profiles indicative of a proinflammatory state have been described in IBS (29). Of particular interest are recent reports of elevated circulating levels of the proinflammatory cytokine interleukin (IL)-6 in IBS patients (7, 10, 11).

An additional consideration in understanding the pathophysiology of IBS is the associated sensitivity to stress found in patients (13), as low-grade inflammatory changes have been correlated with alterations in the hypothalamic-pituitary-adrenal (HPA) stress axis (2, 11). The impact of psychological stress on the immune system has been extensively studied, and immune cells are known to express receptors for several different stress-related peptides (39). Indeed, early-life stress in the form of maternal separation (MS) results in altered HPA axis function with increased stress-induced defecation (33) and reduced pain threshold to colorectal distension (CRD) (16), in addition to increased circulating IL-6 levels following an immunological challenge (31). Interestingly, early-life stress models of brain-gut axis dysfunction are also considered models of depression (9), which is not surprising given the comorbidity of stress-related psychiatric illnesses and functional bowel disorders (41).

In previous studies, evidence has been provided that factors secreted from IBS biopsies, including serotonin, histamine, and proteases, can activate submucosal neurons from healthy colons (4). Other research similarly implicated histamine and proteases in activation of rodent visceral afferent neurons and implicated mast cell release in this activation (1, 5). IL-6 can be secreted from mast cells (15) and has been shown to induce direct activation of submucosal secretomotor neurons, thereby modulating gut function (50). Cytokine-induced modulation of enteric neurons can subsequently alter GI motility, absorption, secretion, and blood flow. Moreover, mucosal ion transport and epithelial permeability, in addition to enhancement of cholinergically mediated neurotransmission, are altered by proinflammatory cytokines such as IL-6 and IL-1β (24, 30). Further support for IL-6 as a secretory cytokine is provided in its ability to suppress the inhibitory and antisercretory effects of norepinephrine by blocking its release from sympathetic fibers (37). The importance of cytokines in neuromuscular dysfunction in the inflamed intestine has been demonstrated (21, 37). Thus, with particular relevance to postinfective IBS, immunomodulation of enteric neurons by cytokines released from within the GI milieu may be important in the persistence of IBS symptomatology (36).

Proinflammatory cytokines have been linked to altered GI function in inflammatory disorders such as inflammatory bowel disorder, but there is a paucity of studies investigating their role in the context of IBS. In these studies, GI dysfunction in the MS model of IBS has been investigated in relation to the
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 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 with 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 IgG (affinity-purified goat antibody immunized with Escherichia coli-derived rIL-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 rIL-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 naive 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 (Isc) 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 BX51WI). 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 responsiveness 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 are reported as a change in ratio. A perfusion system continuously superfused the colonic tissue with carbogen-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-rat IL-6 antibody (affinity-purified goat antibody immunized with E. coli-derived rIL-6, 1:300; R&D Systems) and anti-rat IL-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, blocked 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-6R (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-6R 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\(^{2+}\)])

(74x243) 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-modulated neuronal activation was further demonstrated 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\(^{2+}\)] (0.075 ± 0.02), were unaffected by expo-
A. (i) Representative trace and histogram demonstrate the calcium responses from non-separated (NS) and MS supernatants (spnt; 1:250 dilution, n = 50). Prior incubation with anti-IL-6 (xIL-6) antibodies decreased the MS-stimulated response. (ii) 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 

Table 1. **Percentage of submucosal neurons activated by cytokines**

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<td>% Neurons activated</td>
<td>43.4</td>
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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).

**Fig. 2.** IL-6-mediated activation of submucosal neurons. A: trace and histogram show the change in calcium ratio (Δ ratio) to increasing concentrations of IL-6 (0.01, 0.1, and 1 nM, n = 17 neurons). B: sample trace and histogram demonstrating the IL-6 (1 nM)-induced increase in intracellular calcium concentration ([Ca^{2+}]_i; n = 196). C: prior neutralization of IL-6 (1 nM) with anti-IL-6 (200 nM) attenuated the IL-6 response (n = 34), as illustrated by the trace and histogram. D: representative traces and histogram show that 15-min incubation with IL-6 causes a small increase in short-circuit current (I_{sc}; n = 26). *P < 0.05, **P < 0.01, ***P < 0.001.

Ca^{2+} responses (n = 10, P < 0.01 in responders; Fig. 5Ai). 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. 5Aii). 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

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The presence of UO126 generated significantly reduced responses (P < 0.05, n = 7; Fig. 6B). 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+}]_{i}; 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 defecation 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 supernatants could activate rat submucosal neurons. Indeed, MS supernatants caused larger increases in \( \left[ Ca^{2+} \right]_{i} \) 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. Others have proposed the importance of proteinase-activated receptor 2 (PAR2) (4). 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-

Fig. 5. Cholinergic-mediated activation of submucosal neurons by IL-6. A: histograms illustrating the mean changes in IL-6-induced calcium influx in the presence of hexamethonium (Hex, i; 1 μM) and atropine (ii; 1 μM). B: trace and histogram demonstrating the effect of IL-6 on bethanechol (BCh, 10 μM)-induced increase in \( I_C \) (n = 5). C: i: dual-labeled images showing the diffuse distribution of IL-6 (green) in S100-positive glial cells, calbindin- and calretinin-positive cholinergic neurons, and neuronal nitric oxide synthase (nNOS)-positive nitrergic neurons (red; n = 5 ganglia from 3 rats). ii: Images dual-labeled with an antibody for IL-6Rs (green) and cellular markers for S100, calbindin, calretinin, and nNOS (red; n = 9 ganglia from 3 rats). D: histogram and images illustrate the increase in IL-6R fluorescence after incubation with IL-6 (1 nM, 10 min, n = 10–12 preparations from 3 rats). Scale bars = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001.
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 immune-like 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 classified 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 $I_{sc}$ 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 bethanechol-stimulated $I_{sc}$ values without affecting capsaicin-induced activation of sensory neurons. The effect on the bethanechol-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 calcium responses with a mAChR antagonist, suggesting that IL-6 modulates enteric neural control of basal ion transport and illustrating the importance of the cholineric 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 cholineric or noncholineric 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 activates 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 [Ca$^{2+}$]$_{i}$, 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 astrogliosis (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 relates 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.

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DISCLOSURES

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