Role of enteric glia in intestinal physiology: effects of the gliotoxin fluorocitrate on motor and secretory function


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The enteric nervous system (ENS) is an integrated neuronal network located in the wall of the gastrointestinal tract and is unique in its capacity to mediate reflex activity independent of the central nervous system (CNS) (14). The ENS is divided into two ganglionated plexuses, the myenteric and the submucosal plexus, which together coordinate the activity of the gut musculature, epithelium, and vasculature to regulate physiological functions such as digestion and absorption (14, 17).

Enteric ganglia are composed of neurons and glia (15). Enteric glia support and sustain enteric neurons and may regulate the extracellular environment (5, 9, 20, 44). As in the CNS, glia outnumber neurons and envelop both nerve cell bodies and interganglionic neuronal processes (9, 15, 44). Although the neurochemical classes and projections of enteric neurons have been relatively well characterized (8, 10), the functional role of enteric glia in gastrointestinal physiology remains largely unexplored. We examined the actions of the gliotoxin fluorocitrate (FC) on intestinal motility, secretion, and inflammation after assessing its efficacy and specificity in vitro. FC (100 μM) caused a significant decrease in the phosphorylation of the glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diaz-4-yl)amino]-2-deoxyglucose in enteric glial cultures and a reduction in glial uptake of the fluorescent dipeptide Ala-Lys-7-amino-4-methyl-coumarin-3-acetic acid in both the ileum and colon. Dipeptide uptake by resident murine macrophages or guinea pig myenteric neurons was unaffected by FC. Incubation of isolated guinea pig ileal segments with FC caused a specific and significant increase in glial expression of the phosphorylated form of ERK-1/2. Disruption of enteric glial function with FC in mice reduced small intestinal motility in vitro, including a significant decrease in basal tone and the amplitude of contractility in response to electrical field stimulation. Mice treated with 10 or 20 μmol/kg FC twice daily for 7 days demonstrated a concentration-dependent decrease in small intestinal transit. In contrast, no changes in colonic transit or ion transport in vitro were observed. There were no changes in glial or neuronal morphology, any signs of inflammation in the FC-treated mice, or any change in the number of myenteric nitric oxide synthase-expressing neurons. We conclude that FC treatment causes enteric glial dysfunction, without causing intestinal inflammation. Our data suggest that enteric glia are involved in the modulation of enteric neural circuits underlying the regulation of intestinal motility.

enteric glia; myenteric plexus; colonic ion transport

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We hypothesized that FC was a specific gliotoxin in the ENS to establish whether it is a selective gliotoxin in the enteric nervous system (18, 58). The effects of FC on enteric glia are unknown. Including metabolism (13, 22, 50) and neurotransmission (13, 26, 18, 58), the levels and the uptake and metabolism of neurotransmitters are dynamic functions such as the maintenance of extracellular K+ (47). Briefly, the jejunum from adult rats (5–8 wk, Charles River Laboratories), male Albino or BFA guinea pigs (350–600 g, Charles River Laboratories), and male Sprague-Dawley rats (180–200 g, Charles River Laboratories) were used for these studies. Animals were housed in plastic sawdust floor cages with free access to laboratory chow and tap water before animal use. Animal use in these studies in Calgary was approved by the University of Calgary Animal Care Committee, and all studies were carried out in accordance with the guidelines of the Canadian Council on Animal Care. Studies in Munich were conducted according to the German guidelines for animal care and approved by the Institutional Review Board and Animal Care Committee.

Preparation of Cultured Enteric Glia

Enteric glial cell cultures were generated as previously described (47). Briefly, the jejenum from adult rats (n = 2) was removed under sterile conditions, divided into ~4- to 6-cm segments, opened along the mesentry, and pinned with the serosal side up (47). The longitudinal muscle-myenteric plexus (LMMP) layer was peeled away from the circular muscle with dissection forceps, cut into ~5-mm pieces, and placed in DMEM (Sigma, Deisenhofen, Germany) containing dispase (6 U/ml at 37°C for ~30–60 min). Dissociated cells were then plated into T-75 flasks and sustained in DMEM containing 10% fetal bovine serum (GIBCO), 1% antibiotic-antimycotic solution, and 10−5 M of the antimitotic agent Ara-C (Sigma). After 2 wk, Ara-C was replaced with brain pituitary extract (500 µg/ml, Linaris, Wertheim, Germany) and forskolin (1 µM, Sigma) (47). After ~3–4 wk, cells had grown to near confluence and were dislodged from their substrate with 0.125% trypsin (Sigma). Contaminating fibroblasts were eliminated by antibody-mediated complement lysis employing the monoclonal mouse anti-rat Thy-1.1 antibody (Serotec, 1:100 dilution at 37°C for 30 min) (47). The remaining cells were then washed in HBSS (Sigma) and replated into T-75 flasks. Purified enteric glial cell cultures were morphologically characterized by phase-contrast microscopy and immunohistochemically by using GFAP and S-100β expression as well as by excluding the expression of markers from potentially contaminating cells such as smooth muscle actin and Thy-1.1.

Preparation of FC Solution

FC was prepared as described by Paulsen et al. (42). Briefly, barium FC (Sigma, St. Louis, MO) was dissolved in half of the final volume of distilled water and sonicated until a clear solution was obtained. A slight excess of sodium sulfate was added to the clear solution to precipitate the barium from the solution by forming barium sulfate. This solution was then filtered through a 0.2-µm filter, and distilled water was added to make up the final volume. NaCl (Sigma) was added to make the solution isotonic before use. A sodium sulfate vehicle solution similarly prepared, except for the omission of FC, was used in all studies.

2-NBDG Phosphorylation Assay

To assess whether FC inhibited glial metabolism, we studied the phosphorylation of a fluorescent glucose substrate, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amin]-2-deoxyglucose (2-NBDG; Molecular Probes no. N13195), a glucose analog that has recently been used to study glucose uptake and metabolism in astrocytes and neurons (26, 35). Phosphorylation of 2-NBDG by hexokinase yields a fluorescent dipeptide, whereas nonphosphorylated 2-NBDG is not fluorescent (26). All incubations were carried out in a humidified incubator (37°C, 95% O2:5% CO2). Cultured enteric glia were pretreated for 30 min with HBSS containing 100 µM FC or vehicle. After this initial incubation, 2 mM glucose was added to the flasks for a further 15 min. Cells were then incubated with 500 µM of 2-NBDG for another 15 min. Subsequently, the reaction mixture was replaced with a fresh HBSS solution containing 2 mM glucose and FC or vehicle for another 10 min to allow the removal of any nonphosphorylated 2-NBDG from the cells (26). Cells were washed in this solution, and fluorescence was visualized by use of an inverted microscope (Olympus IX 50) with a ×40 oil-immersion objective lens (numerical aperture 1.35), a Xe arc lamp, a charge-coupled device camera (Redshirt Imaging), and Neuroplex 7.01 imaging software (Redshirt Imaging). Excitation and emission wavelengths were set at 475 and 535 nm, respectively. Fluorescence intensity was calibrated using an empty culture dish containing 50 and 500 nM 2-NBDG in HBSS. Fluorescence intensity (FI) was assessed in 210 FC-treated and 266 vehicle-treated glial cells from 16 different cultures treated independently on 4 experimental days and expressed as arbitrary FI units.

Dipeptide Uptake

To directly assess whether FC alters glial function, we studied the cellular uptake of the fluorescent dipeptide Ala-Lys-7-amino-4-methylcoumarin-3-acetic acid (Ala-Lys-AMCA) in isolated tissues from the guinea pig proximal colon as well as from isolated tissues from the mouse ileum and distal colon. Ala-Lys-AMCA uptake has recently been described as a vital marker of enteric glia (45). In this study, we have used both isolated tissues from guinea pigs and mice because Ala-Lys-AMCA is not exclusively taken up by enteric glia but also by tissue macrophages. Furthermore, in the guinea pig, Ala-Lys-AMCA is also taken up by a class of cholinergic enteric neurons that are also immunoreactive for neuronal nuclei and/or calbindin, likely intrinsic primary afferent neurons (45). The procedure to assess Ala-Lys-AMCA uptake into enteric ganglia has been described in detail elsewhere (45). Briefly, guinea pigs (n = 3) were killed by concussion and exsanguination from their cervical vessels, and mice (n = 3) were killed by cervical dislocation. The gastrointestinal tract was quickly removed and immediately placed in sterile, ice-cold, oxygenated (95% O2:5% CO2; pH 7.4) Krebs buffer. When used to prepare mouse tissues, this buffer also contained 1 µM nifedipine (Sigma) and 1 µM atropine (Sigma) to immobilize the tissue segments during dissection (45). The relevant intestinal segments were separated, opened along the mesenteric border, washed, and pinned mucosal side up in sterile Sylgard-coated petri dishes. The mucosa was removed and the remaining tissue was covered with DMEM-Ham’s F-12 medium (Sigma) containing 10% heat-inactivated horse serum (ecPro, Neustadt, Germany). Samples were placed onto a horizontal shaker in a humidified incubator. After 15 min, 100 µM FC or vehicle were added to the dishes for a further 2-h incubation period. The fluorescent dipeptide Ala-Lys-AMCA (Biotrend, Cologne, Germany) was subsequently added to the incubation medium (final concentration of 2.5 µM), and the tissues were maintained in the incubator for a further 1.5 h. Tissues were then washed with ice-cold PBS (pH 7.4) and fixed overnight at 4°C in freshly prepared Zamboni’s fixative (picric acid-paraformaldehyde mixture; picric acid, Sigma; and paraformaldehyde,
Merlin). The next day, tissues were rinsed in PBS. The submucosal and circular muscle layers were removed to obtain a whole mount LMMP preparation (45, 49). Whole mount preparations were immediately mounted and assessed for AMCA fluorescence by use of an Olympus BX61 W1 fluorescence microscope (Olympus, Hamburg, Germany).

**Enteric Glial Activation**

To investigate whether FC selectively activates stress or other metabolic pathways in enteric glia, we employed an in vitro stimulation protocol that would allow us to see whether neurons, glia, and/or smooth muscle were activated in response to FC (52). Guinea pigs (n = 5) were fasted 24 h before the experiment. Animals were anesthetized with halothane (4% in oxygen), and a 30-cm segment of the distal ileum was removed and placed in ice-cold oxygenated (95% O2-5% CO2; pH 7.4) Krebs buffer consisting of (in mM) 117 NaCl, 4.8 KCl, 25 NaHCO3, 1.2 NaH2PO4, 1.2 MgCl2, 11 glucose, and 2.5 CaCl2 and containing 1 μM nifedipine (Sigma). Animals were killed by exsanguination. Tissues were minimally handled to decrease mechanical stimulation.

After 5 min, the ends of the tissue were trimmed, the tissue was cut into 5-cm segments, and four of these segments were transferred to tubes containing oxygenated Krebs solution at 37°C, whereas the fifth segment was immediately opened along the mesenteric border, pinned flat with the mucosa facing up, and fixed overnight in Zamboni’s fixative. The remaining four tissue segments were incubated for 3 × 20 min, to ensure that the tissues were fully exposed to fresh drug, with Krebs containing vehicle only (for 100 μM FC) or FC (30, 100, or 300 μM). Tissues were subsequently placed in ice-cold Krebs solution, opened along the mesenteric border, pinned flat, fixed in Zamboni’s fixative, and processed for immunohistochemistry as described below.

**Effects of FC In Vitro**

**Contractility studies.** Male C57Bl/6 mice were given intraperitoneal injections of FC (20 μmol/kg body wt) or vehicle at 90, 60, and 30 min before the start of the experiment. Mice were killed by cervical dislocation, and segments of the distal ileum were removed from both the vehicle and FC-treated mice and submerged in ice-cold oxygenated Krebs solution. Two segments, 1.5 cm each, from each animal were then ligated at each end with 6-0 silk thread, leaving an opening through the lumen. Each segment was mounted longitudinally in a 25-ml tissue bath inside an electrode sleeve and attached to an 8811A, Mississauga, Ontario, Canada), and recorded on a four-channel chart recorder (Hewlett-Packard, model 7754B) calibrated at 1 g of tension.

The tissue was stretched until an increase in muscle tension was observed. Tissues were allowed to equilibrate for 90 min and rinsed. The length of the muscle was measured and further stretched by 50% of the initial measurement. Tissues were allowed to stabilize for a further 30 min and subsequently rinsed. Maximal muscle contractions were obtained by adding 10−5 M bethanecol (Sigma) to the bath. After being rinsed and stabilized, the tissues were subjected to electrical field stimulation (EFS; Grass Instruments, model S88 stimulator, Quincy, MA) for 30 s at supramaximal voltage, delivered as 1-ms pulses at 0.5, 1, 2, 4, and 8 Hz. Tissues were allowed to stabilize for 2 min between each stimulating frequency. After EFS, the tissue response to 10−5 M bethanecol was again tested, and the average response was calculated. At the end of the experiment, the silk was removed, the lumen was opened, and the mucosa was scraped off. The muscle tissue was weighed to obtain a wet weight and then dried and reweighed to obtain the dry weight.

**Ion transport studies.** The same mice treated with FC or vehicle also had 4-cm segments of the colon removed immediately distal to the ileocecal junction and gently flushed with cold (4°C) glucose-free Krebs buffer. Segments were not stripped of external muscle layers and were mounted in Ussing-type diffusion chambers (0.4 cm2 exposed area) and bathed on the mucosal and serosal sides with oxygenated Krebs buffer containing 10 mM glucose and FC (30, 100, or 300 μM, n = 6–7) or an equivalent volume of vehicle (n = 7) at 37°C. Two pairs of Ag-AgCl electrodes were placed in contact with the buffer on either side of the tissue. One pair of electrodes was used to measure potential difference (PD), and the other was used to deliver short-circuit current (Isc). The PD generated by the epithelium was measured and clamped to zero with a voltage-clamp apparatus (VCC MC8, Physiologic Instruments, San Diego, CA). The Isc required to clamp the epithelial PD was measured as an indicator of net ionic transport. Tissues were allowed to equilibrate for 15–20 min to establish a stable baseline Isc before EFS or the addition of drugs. Tissues were treated with 2.5-, 5-, and 10-Hz EFS (stimulus intensity 50 V, pulse duration 0.5 ms, total stimulation duration 5 s), followed by a sequential serosal application of forskolin (10 μM, Sigma) and carbachol (10 μM, Sigma). Isc was allowed to return to baseline between EFS or drug treatments.

**Effects of FC In Vivo**

Male C57Bl/6 mice were given intraperitoneal injections of FC (10, 20, 30, or 40 μmol/kg body wt) or vehicle (n = 23–24) twice per day (9 AM and 6 PM) for 7 days. Animals were carefully monitored for signs of drug toxicity. We observed signs of toxicity and mortality in two animals treated with higher doses of FC; one animal treated with 30 μmol/kg died and one animal treated with 40 μmol/kg was emaciated and displayed piloerection and a decrease in normal exploratory activity. Subsequently, high-dose FC treatment was discontinued and only animals given the 10 (n = 10–12) and 20 μmol/kg doses (n = 14–16) were used in subsequent experiments. No signs of FC toxicity were observed at these lower doses. Before euthanasia, mice were fasted overnight but were given free access to drinking water. Animals were then used for intestinal transit studies and immunohistochemical and histological studies as described below.

**Assessment of inflammation.** Because ablation of enteric glia in two lines of transgenic mice led to severe intestinal inflammation (8, 10), we assessed inflammation after FC treatment in several ways: a change in body weight, a macroscopic damage score, and a microscopic or histological damage score as well as a tissue myeloperoxidase (MPO) assay. During the 7 days of FC or vehicle administration, mice were weighed daily. Subsequently, mice were killed by cervical dislocation, and the ileum and colon were removed. Macroscopic assessment of inflammation was performed by using previously established criteria (37) and included parameters such as the maximal bowel wall thickness and the presence of diarrhea as well as the presence and degree of ulceration, adhesions, and hyperemia. Macroscopic damage was assessed by an investigator blinded to the treatment. Tissue samples were then fixed in Zamboni’s fixative overnight as described above and processed for the histological examination of microscopic colitis as well as immunohistochemistry as described below. In addition, tissue samples from both the ileum and colon were used for MPO assays.

**Assessment of microscopic colitis.** After fixation, circumferential segments of both the ileum and colon were washed and transferred to a PBS solution containing 20% sucrose at 4°C overnight. Tissue segments were then embedded in OCT compound (Miles, Elkhardt, IN), cut on a cryostat (10 μm), and mounted onto poly-D-lysine-
coated slides. Tissue sections were subsequently stained with hematoxylin and eosin and scored by two blinded investigators. The criteria for the assessment of microscopic colitis have been previously described (37) and grade parameters such as the damage to normal mucosal architecture, the presence of crypt damage, the presence and extent of cellular infiltrate, and the amount of muscle thickening as well as the level of goblet cell destruction (37). Each of these criteria was added to reach a maximum possible score of 11.

**MPO assay.** MPO is an enzyme found in the granules of neutrophils and eosinophils and has been extensively used to determine cellular infiltration into gastrointestinal tissues (37). Samples of the full-thickness ileum and colon from mice treated with FC or vehicle were weighed, frozen on dry ice, and stored at −80°C for 48 h. Tissue MPO activity was assessed by using a previously described assay (37).

**Gastrointestinal transit.** An oral bolus of 0.25 ml of a solution containing 5% Evans blue (50 mg/ml, Sigma) in 5% gum arabic (50 mg/ml, Sigma) was administered to mice under light metophane anesthesia by gavage. Fifteen minutes later, animals were killed by overdose of anesthesia and both the dye front and the resting length of the small intestine were measured. The small intestine was also carefully inspected for any macroscopic signs of inflammation or mucosal damage, as described above. Segments of the ileum were then placed in PBS containing 1 μM nifedipine (Sigma). Tissues were opened along the mesenteric border, pinned flat with the mucosal side up, fixed in Zamboni’s fixative, and subsequently stored at 4°C in PBS. The LMMP and submucosal plexus were dissected as described above, whereas preparations of the submucosal plexus were dissected by removing the mucosa from the submucosa and any residual circular muscle from the submucosal plexus layer. Tissues were then processed for immunohistochemistry as described below (n = 3–4 per group).

**Colonic transit.** Colonic transit was assessed by using the expulsion of a small plastic bead the size and shape of a fecal pellet, as previously described (38). Bead expulsion latency has been previously used as an indicator of colonic motility in vivo (38). After being overnight fasted, mice were lightly anesthetized with metophane and a plastic bead (2 mm diameter) was inserted through the anus and gently pushed 2 cm aborally by a customized steel cannula covered with silicon tubing. The cannula was carefully withdrawn and the bead expulsion latency was measured. Animals were killed by an overdose of anesthetic, and segments of the colon were then placed in PBS containing 1 μM nifedipine. Tissues were opened along the mesenteric border, pinned flat with the mucosal side up, fixed in Zamboni’s fixative, and subsequently stored at 4°C in PBS. The LMMP and submucosal plexus were dissected as described above and processed for immunohistochemistry as described below (n = 3–4 per group).

**Immunohistochemistry.** In addition to the in vitro experiments described above, segments of the ileum and colon were removed from mice treated with FC. Tissues were incubated with FC (100 μM, n = 3) or an equivalent volume of vehicle (n = 3) for 1 h in a water bath containing Krebs solution maintained at a temperature of 37°C and oxygenated with 95% O2-5% CO2. Tissues were then cut into pieces and placed in PBS containing 1 μM nifedipine, pinned flat with the mucosal side up, fixed in Zamboni’s fixative, and subsequently stored at 4°C in PBS. The LMMP and submucosal plexus were dissected as described above and processed for immunohistochemistry as described below (n = 3–4 per group).

**Quantification of NOS-immunoreactive neurons.** To assess the number of NOS-immunoreactive neurons per ganglion, myenteric plexus preparations of both the ileum and colon from in vivo FC-treated (20 μmol/kg; n = 4) and vehicle-treated (n = 3) mice were labeled with antibodies directed toward NOS, as described above. NOS-positive cells were counted in at least 20 randomly chosen ganglia throughout the tissue preparation. Data were expressed as the mean numbers of cells per ganglion from each animal, and the number of these values are shown for the ileum and colon in the two groups.

**Statistics.** Data were analyzed using a Mann-Whitney rank-sum test for the 2-NBDG phosphorylation assay and the macroscopic and microscopic damage scores; a Kruskal-Wallis test followed by a Dunn’s multiple comparison test for the in vitro muscle contractility data; a one-way ANOVA for the Ussing chamber data; a one-way ANOVA followed by a Tukey’s multiple-comparison test for the pERK1/2 pixel intensity; a Students’ t-test for the forskolin and carbobach Ussing chamber data, the muscle thickness, body weight, and MPO data as well as the number of NOS-immunoreactive neurons. A P value of <0.05 was considered statistically significant in all cases.

**RESULTS**

**FC Alters Enteric Glial Function in In Vitro Systems**

To elucidate whether FC alters energy-dependent processes in enteric glia as it does in astrocytes, we first tested the effects of FC on isolated enteric glia. As FC decreases astrocyte glucose metabolism in culture (22), we investigated whether the same was true in cultured enteric glia. Cultured rat enteric glia were pretreated with 100 μM FC or vehicle and subsequently incubated with 2-NBDG. This dose was based on previous work in brain slices (18) and on our in vitro studies (see Effects of FC on Enteric Glial Activation). Average FI was significantly decreased in cultured enteric glial cells that were pretreated with 100 μM FC (Fig. 1; Mann-Whitney rank-sum
isolated guinea pig proximal colon segments with 100 preparations from both guinea pigs and mice. Pretreatment of porter PEPT2 (45), was altered in the presence of FC in ex vivo fluorescent dipeptide Ala-Lys-AMCA, by the dipeptide trans-signal significantly reduced in the presence of FC.

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P_{\text{test}, P < 0.001}, \text{indicating that glucose metabolism was significantly reduced in the presence of FC.}
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We next investigated whether enteric glial uptake of the fluorescent dipeptide Ala-Lys-AMCA, by the dipeptide transporter PEPT2 (45), was altered in the presence of FC in ex vivo preparations from both guinea pigs and mice. Pretreatment of isolated guinea pig proximal colon segments with 100 μM FC resulted in a marked decrease in AMCA fluorescence in the myenteric plexus (Fig. 2, C and D) compared with the robust fluorescence in enteric glial cells from preparations pretreated with the vehicle alone (Fig. 2, A and B). We utilized this preparation because a class of cholinergic enteric neurons (neuronal neurons and/or calbindin-immunoreactive intrinsic primary afferent neurons) also take up the dipeptide (45), allowing us to determine whether FC specifically inhibited dipeptide uptake in enteric glia. In FC-treated preparations, we did not observe any differences in neuronal dipeptide uptake compared with vehicle-treated controls (data not shown).

In the isolated mouse ileum preparations, robust AMCA fluorescence was observed in enteric glia of the myenteric plexus from tissues treated with vehicle alone (Fig. 3A). In contrast, pretreatment of tissues with 100 μM FC resulted in the disappearance of AMCA fluorescence in enteric glia (Fig. 3B). Uptake of the dipeptide by resident tissue macrophages was unchanged by FC pretreatment. In the mouse colon, widespread AMCA fluorescence was apparent in both enteric glia in myenteric ganglia as well as in resident tissue macrophages (Fig. 3C); pretreatment with 100 μM FC again caused a decrease in AMCA fluorescence in enteric glia but had no effect on dipeptide uptake by tissue macrophages (Fig. 3D).

**Effects of FC on Enteric Glial Activation**

Because FC selectively depletes ATP in astrocytes and so is a stressor to these cells, we investigated whether in vitro incubation of FC in isolated guinea pig ileal segments could alter the enteric glial expression of pSAPK and pERK1/2. Treatment of ileal segments with 100 μM FC resulted in a marked increase in the intensity of pERK1/2 immunofluorescence (Fig. 4Aii) compared with tissues treated with 30 μM FC (Fig. 4Aii), 300 μM FC (Fig. 4Aiv), or vehicle alone (Fig. 4Ai). Image analysis of the pERK1/2 pixel intensity demonstrated a maximum and significant increase in pERK1/2 immunoreactivity in ileal segments treated with 100 μM FC compared with vehicle (Fig. 4B; vehicle pixel intensity: 100%, 30 μM FC pixel intensity: 115.6 ± 5.04%, 100 μM FC pixel intensity: 131.6 ± 11.6%, 300 μM FC pixel intensity: 104.5 ± 4.8%; Kruskal-Wallis test, \( P = 0.04 \) between vehicle- and 100 μM FC-treated tissues, \( n = 5 \) for each group). The slight baseline pERK1/2 immunoreactivity in vehicle-treated tissues is likely a result of mechanical stimulation during the assay, as tissue segments that were immediately fixed displayed minimal glial pERK1/2 immunofluorescence (data not shown). FC selectively stimulated pERK1/2 immunoreactivity in enteric glia, as the brightly stained cells double labeled with the glial specific marker S-100β (Fig. 4C). pERK1/2 immunoreactivity was not observed in smooth muscle or neurons after FC treatment in these preparations. We also noted an increase in pSAPK immunoreactivity in enteric glia with FC treatment, but image analysis did not reveal any significant changes in pixel intensity between vehicle- and FC-treated tissues (data not shown).

**Effects of FC on Intestinal Function In Vitro**

To determine whether enteric glia are involved in the modulation of neurotransmission in intestinal motility and secretion, we tested whether FC altered glial and neuronal morphology as well as contractility and secretion in vitro.

Incubation of isolated segments of the mouse ileum and colon with FC did not cause any evident macroscopic damage. The cytoarchitecture of neurons and enteric glia in the myenteric (Fig. 5) as well as in the submucosal plexus (data not shown), appeared qualitatively well preserved. We did not observe any overt changes in enteric glial cell morphology, as visualized with GFAP and S-100β (Fig. 5) visualized with GFAP and S-100β (Fig. 5). Neuronal morphology was assessed with the neuronal marker PGP 9.5. Again, no differences were observed between vehicle-treated (Fig. 5, C and D) and FC-treated (Fig. 5, G and H) tissues. No obvious changes in the distribution or the intensity of staining of any of these markers were noted. In addition, we did not observe any obvious changes in the length or thickness of GFAP-immunoreactive glial processes when comparing FC-treated and vehicle-treated tissues.

We next examined whether selective impairment of enteric glial function had any effects on the neuronal circuitry involved in muscle contractility. No signs of inflammation or macroscopic damage were observed in either the ileum or colon of FC-treated mice. We assessed the contractile response...
such that tension was no different in tissues treated with 30 μM FC than in vehicle-treated tissues, but in tissues treated with 100 and 300 μM FC, the EFS-generated tension was lower than in vehicle-treated tissues (Fig. 6A). The amplitude of EFS-generated tension was significantly different in tissues treated with 100 and 300 μM FC than in tissues treated with vehicle alone (2-way ANOVA, P = 0.0015), as was the baseline tension (baseline tension: 3.4 ± 0.9% bethanechol response/mg dry tissue wt for vehicle-treated tissue; baseline tension: 3.7 ± 0.8% bethanechol response/mg dry tissue wt for 30 μM FC-treated tissue; baseline tension: 2.7 ± 0.7% bethanechol response/mg dry tissue wt for 100 μM FC-treated tissue; baseline tension: 1.3 ± 1.4% bethanechol response/mg dry tissue wt for 300 μM FC-treated tissue, linear regression P = 0.001; Fig. 6A). However, the slopes of the lines were not significantly different between the groups (slope: 1.23 ± 0.22% for vehicle-treated tissues, 1.40 ± 0.20% for 30 μM FC-treated tissues, 0.97 ± 0.18% for 100 μM FC-treated tissues, and 0.94 ± 0.33% for 300 μM FC-treated tissues, P = 0.52; Fig. 6A). No differences in the response to bethanechol were observed in vehicle- or FC-treated tissues (data not shown).

We also examined the effects of FC on ion transport. Segments of the proximal colon from the same mice treated with FC or vehicle as above were mounted in Ussing chambers. There were no differences in tissue conductance between tissues treated with FC or vehicle (data not shown). EFS elicited a frequency-dependent increase in Isc, but no differences in Isc were observed between vehicle and tissues treated with 100 μM FC (Fig. 7). No dose-dependent effect of FC was observed on EFS-elicited Isc (data not shown). Serosal administration of the cholinergic agonist carbachol or of the adenylate cyclase activator forskolin caused changes in Isc. No differences in the carbachol or forskolin responses were observed in tissues treated with 100 μM FC compared with vehicle-treated controls (Fig. 7). No dose-dependent effect of FC was observed on the carbachol- or forskolin-induced responses (data not shown).

Effects of FC on Intestinal Function In Vivo

Given the effects of FC in vitro, we next examined whether FC altered intestinal physiology in vivo. Again, to determine whether any effects of FC were secondary to intestinal damage or inflammation, we first examined whether FC had any effect on glial and neuronal morphology. Mice were treated with twice daily injections of FC or vehicle for 1 wk. No signs of FC toxicity were observed, and the behavior of the animals was normal. Animals treated with both FC and vehicle lost weight over the 7-day treatment period, but no significant differences in weight loss were observed between the two groups (mean decrease in body weight: vehicle-treated animals: −0.42 ± 0.64 g, n = 5; 20 μmol/kg FC-treated animals: −1.5 ± 0.23 g, n = 6; P = 0.11, Student’s t-test). In addition, we could find no evidence of macroscopic or microscopic inflammation in either the ileum or colon (Fig. 8). In both regions, neither the macroscopic (macroscopic damage score for vehicle-treated animals: 0.34 ± 0.01, n = 5; FC-treated animals: 0.33 ± 0.01, n = 6) nor the microscopic damage was significantly different.
Fig. 3. Dipeptide uptake is suppressed in mouse ileal and colonic enteric glia treated with FC but preserved in resident tissue macrophages. Robust uptake of the fluorescent dipeptide Ala-Lys-AMCA was observed in both enteric glia (indicated with the arrow) and resident tissue macrophages (indicated with the chevron) of vehicle-treated mouse preparations in both the ileum (A) and the colon (C). In contrast, pretreatment of tissues with 100 μM FC resulted in a marked reduction of AMCA fluorescence in enteric glia in both regions, whereas dipeptide uptake in resident macrophages was preserved (B: mouse ileum; a macrophage is indicated with the chevron; D: mouse colon; an enteric glial cell in the ganglion is indicated with the arrow and a macrophage in the muscular layer is indicated with the chevron). Scale bars = 100 μm.

between vehicle-treated animals and animals treated with 20 μmol/kg FC (Fig. 8A; ileum, n = 5 for vehicle and n = 5 for FC; colon, n = 5 for vehicle and n = 6 for FC). Hematoxylin and eosin-stained cross sections from the ileum (Fig. 8Bi) and the colon (Fig. 8Bii) of FC-treated animals appeared similar to samples taken from the ileum (Fig. 8Bii) or the colon (Fig. 8Biii) of vehicle-treated mice and no differences in gross morphology, goblet cell number, or inflammatory cell infiltrate were apparent. Furthermore, no differences in muscle thickness (Fig. 8C; ileum: n = 5 for vehicle and n = 5 for FC; colon: n = 5 for vehicle and n = 6 for FC) or in tissue MPO activity (Fig. 8D; ileum and colon: n = 5 for vehicle and n = 6 for FC) were observed between the two groups.

Again, we qualitatively examined glial and neuronal morphology in both the myenteric and submucosal plexuses (data not shown) of the ileum and colon. No discernable changes in enteric glial architecture, as assessed with GFAP and S-100β, were observed in the myenteric plexus of the ileum in mice treated with 10 μmol/kg FC (Fig. 9, D and E) or 20 μmol/kg FC (Fig. 9, G and H) compared with animals treated with vehicle alone (Fig. 9, A and B). The same was true in the myenteric plexus of the mouse colon when we compared animals treated with 10 μmol/kg FC (Fig. 10, D and E) with animals treated with the vehicle (Figs. 10, A and B). Neuronal morphology, as assessed with PGP 9.5, was also unchanged in animals treated with vehicle (Figs. 1C and 10C), 10 μmol/kg (Figs. 9F and 10F), or 20 μmol/kg (Fig. 9F) FC in the myenteric plexus of the ileum and colon, respectively.

Because contractility was altered by FC treatment in vitro (Fig. 6), we investigated whether intestinal transit was altered in FC-treated animals. Gastrointestinal transit was assessed by the passage of Evans blue dye front after 15 min was decreased by FC in a dose-dependent manner and reached significance at 20 μmol/kg FC compared with vehicle-treated animals (1-way ANOVA, P = 0.026; Fig. 11A). We also noted that the resting length of the small intestine that was traveled by the Evans blue dye front was significantly reduced by FC (Fig. 11), whereas the latency of colonic bead expulsion (Fig. 11B) or the length of the mouse colon was altered by either dose of FC treatment (Fig. 11D).

As a recent study of glial ablation observed similar changes in intestinal motility in concurrence with a decrease in the proportion of myenteric NOS-expressing neurons (2), we assessed the number of myenteric NOS-immunoreactive neurons in both the ileum and colon of animals treated in vivo with FC (20 μmol/kg; n = 4) or an equivalent volume of vehicle (n = 3) (Fig. 12, A and B). No significant differences in the number of NOS-immunoreactive neurons per myenteric ganglion were noted between the two groups in either the mouse ileum or colon (Fig. 12C; P = 0.53 for the ileum and P = 0.33 for the colon, Student’s t-test).
DISCUSSION

The aim of this study was to examine whether FC can be used as a tool to elucidate the role of enteric glia in physiological functions of the intestine. We have demonstrated that FC specifically alters energy-dependent processes in enteric glia, including glucose metabolism in cultured rat enteric glia and dipeptide uptake in both the guinea pig and mouse. We also demonstrated that FC selectively increased the expression of pERK1/2 in guinea pig ileal enteric glia. In addition, we have shown that FC treatment leads to effects on intestinal motility in mice both in vitro and in vivo, supporting a role for enteric glia in intestinal physiology. These effects include a decrease in baseline tone and stimulated contractility in the ileum as well as a reduction in gastrointestinal transit but not colonic transit. We did not observe any changes in colonic ion transport, nor did we observe any evidence of macroscopic or microscopic inflammation, or any changes in glial and neuronal morphology, or in the number of NOS-immunoreactive cells in FC-treated tissues (ileum or colon). To our knowledge, this is the first study describing the use of a selective gliotoxin to cause enteric glial dysfunction. Under the conditions of our study, we did not observe any inflammation in the gastrointestinal tract, enabling us to suggest that enteric glia play a physiological role in the regulation of intestinal motility.

A recent model of glial ablation has used an adoptive transfer of hemagglutinin-specific CD8\(^+\) T cells into adult transgenic mice expressing hemagglutinin in enteric glia (2). In this model, the authors observed a mild disruption of the enteric glial network by CD8\(^+\) T cells, resulting in a decrease in GFAP expression, an increase in glial apoptosis, and an increase in T cell infiltration into myenteric ganglia; however, these destructive changes were not severe enough to cause an overt macroscopic inflammation (2). These authors also observed changes in intestinal motility and chemical coding of the jejunal myenteric plexus in transgenic animals, such that EFS-induced relaxation, intestinal transit, and gastric emptying were all decreased in parallel to a decrease in the proportion of NOS-expressing neurons (2). In the present study, we also observed a decrease in gastrointestinal transit, in agreement with the results of Aube et al. (2). We did not measure EFS-induced relaxation but instead observed a decrease in basal tone and EFS-induced contractility in the ileum. We examined the number of myenteric neurons expressing nitric oxide in the ileum and colon and found no changes in the pattern of

Fig. 4. In vitro incubation of the guinea pig ileum with FC caused an increase in immunoreactivity of the phosphorylated form of ERK1/2 (pERK1/2). Isolated segments of the guinea pig ileum (n = 5) were incubated in vitro with FC (30, 100, or 300 \(\mu\)M) or vehicle. Myenteric plexus preparations were stained with antibodies directed toward pERK1/2. Tissues treated with vehicle (Ai) exhibited little kinase immunofluorescence, whereas 30 \(\mu\)M FC (Ai) caused a slight increase in immunoreactivity, and 100 \(\mu\)M FC (Aii) caused a marked increase in kinase immunoreactivity. Maximal stimulation of pERK1/2 occurred at the 100 \(\mu\)M dose of FC, as kinase immunoreactivity was reduced in ileal segments incubated with 300 \(\mu\)M FC (Aiv) compared with those incubated with 100 \(\mu\)M FC. pERK1/2 pixel intensity for vehicle and FC-treated animals was assessed using image analysis (B). A significant increase in pERK1/2 pixel intensity was observed in 100 \(\mu\)M FC-treated ileal segments (Kruskal-Wallis test, *P = 0.04, with results expressed as a percentage of the vehicle pixel intensity). pERK1/2-immunoreactive cells were all enteric glia, as they double labeled with the glial specific marker S-100\(\beta\) (C; 100 \(\mu\)M dose of FC). Scale bars = 50 \(\mu\)m.
expression in our study, suggesting that the effects we observed are attributable to an action primarily on enteric glia. Aube et al. also observed that transgenic animals displayed an increase in the in vivo paracellular permeability to FITC-dextran (2). In the present study we did not observe any changes in epithelial resistance in FC-treated animals. The differences noted between these studies are likely a result of the method of glial ablation and regional differences between the ileum and jejunum; however, consistent changes in vivo intestinal motility were observed in both studies, suggesting that enteric glial dysfunction leads to a decrease in intestinal motility and that enteric glia play a significant role in enteric neurotransmission.

Glucose Uptake and Metabolism in Enteric Glia

We observed a pronounced decrease in glucose metabolism in cultured rat enteric glial cells treated with FC compared with vehicle alone. Although little information is available regarding glucose metabolism in enteric glia, astrocytic glucose metabolism has been extensively studied, the first step in which is the phosphorylation of glucose by the rate-limiting enzyme hexokinase (6, 57, 59). FC dose dependently decreases glucose metabolism in cultured astrocytes (22).

There are two possible reasons for the decrease in phosphorylated 2-NBDG that we have observed in FC-treated enteric glia. Either the uptake of glucose by enteric glia is reduced, or hexokinase activity is decreased. It is unlikely that glucose uptake in enteric glia is decreased. Glucose is mainly derived from extracellular sources, and in astrocytes it is taken up through facilitated diffusion (35, 55). Because this process does not rely on ATP, but on a passive diffusion gradient, it is likely that decreasing enteric glial metabolism with FC will not inhibit glucose uptake, especially not at the early time point of 1 h chosen in this study. Therefore, it is most likely that
hexokinase activity is decreased in glial cells treated with FC, as hexokinase activity is dependent on ATP levels (57, 59). These data suggest that FC has a robust inhibitory effect on energy-dependent glucose metabolism in enteric glia.

Dipeptide Uptake in Organotypic Cultures

In the present study, we observed a significant decrease in enteric glial uptake of the fluorescent dipeptide Ala-Lys-AMCA in FC-treated organotypic cultures of the guinea pig and mouse colonic myenteric plexus. We also observed a significant decrease in AMCA fluorescence in enteric glia in FC-treated preparations from the mouse ileum, whereas the guinea pig ileum was not studied. Enteric glia in the guinea pig, rat, and mouse have been shown to express PEPT2, a high-affinity dipeptide transporter that mediates the uptake of Ala-Lys-AMCA in these cells (45). PEPT2 is part of a family of di- and tripeptide transporters that couple peptide transport to the movement of protons down an electrochemical gradient (19) and is expressed by astrocytes (11). Ala-Lys-AMCA has been previously used to study dipeptide uptake in both astrocytes and enteric glia (11, 45). In astrocytes, Dieck et al. (11) found that Ala-Lys-AMCA uptake by PEPT2 was ATP dependent, as it was almost completely abolished by rotenone, an inhibitor of the mitochondrial respiratory chain and CCCP, an uncoupler of oxidative phosphorylation. Similarly, in our study, inhibition of the TCA cycle with FC pretreatment significantly reduced Ala-Lys-AMCA uptake in vivo could lead to an accumulation of these peptide fragments and subsequent alterations in neurotransmission. Because we saw no changes in the macrophage uptake of dipeptide or the neuronal uptake of dipeptide in intrinsic primary afferent neurons in the guinea pig, we conclude that...
the effect of FC is specific to glia. Taken together, the FC-mediated decreases in glucose metabolism and dipeptide uptake indicate that FC can specifically inhibit energy-dependent processes in enteric glia.

Activation of pERK1/2

In the present study, we observed that treatment of isolated preparations of the guinea pig ileum with FC caused a concentration-dependent increase in enteric glial immunoreactivity of pERK1/2, a member of the mitogen-activated protein kinase family. Cellular stressors such as proinflammatory cytokines (36, 51), oxygen-glucose deprivation (25, 56), and reactive oxygen species (53) induce ERK phosphorylation and activation in both astrocytes and neurons in culture and in situ. In our study, pERK1/2 immunoreactivity was solely confined to enteric glia, indicating that FC specifically targets these cells. Depending on the cell type and the context, the increase in pERK1/2 may represent either a proapoptotic signal (25, 51, 56) or a prosurvival and differentiation signal (25, 51). Indeed, pERK1/2 activation in astrocytes has been linked to reactive gliosis or an increase in GFAP expression and astrocyte proliferation in CNS injury models (23). However, because we did not observe concomitant inflammation or changes in glial and neuronal morphology in response to FC administration, it is unlikely that activation of this kinase represents a signal for apoptosis, although we did not test for glial apoptosis directly. Taken together, the activation of pERK1/2 uniquely in enteric glia indicates the specificity of FC for these cells.

Role of Enteric Glia and Glial Heterogeneity

Enteric glia may participate in neurotransmission as well as in the regulation of the extracellular environment in the ENS (9, 46). Enteric glia receive “synaptic-like contacts” from enteric neurons (15), and previous studies have demonstrated that these cells express receptors for neurotransmitters, such as the purinergic P2Y2 and P2X7 receptors and the adrenergic α2A receptor (29, 39, 54, 61). There is evidence that enteric glia, like astrocytes, can also regulate the concentration of extracellular K⁺ (5, 20, 45) and the uptake and inactivation of neurotransmitters such as GABA (12) in the ENS. It is possible that these mechanisms may contribute to the changes in smooth muscle contractility, basal ileal tone, and gastrointestinal transit that we have observed in FC-treated mice, because FC alters the energy-dependent uptake of K⁺ and neurotransmitters such as GABA and glutamate in the brain (13, 21, 31, 32, 42, 60), although we have no direct evidence to support this in the ENS.

It is puzzling, however, that FC-mediated dysfunction of glia would not equally affect the neuronal circuitry underlying
motility in the colon vs. the ileum. Similarly, we were surprised that ion transport was not affected by FC treatment. The Ussing chamber studies were conducted in the colons of the same mice where we observed changes in smooth muscle contractility in the ileum, suggesting that it was not due to drug dosing. It is possible that enteric glia in the myenteric and the submucosal plexuses are differentially susceptible to FC or that glia in these two regions have heterogeneous functions that lead to altered susceptibility to FC (44), even though dipeptide uptake studies do not provide evidence for this contention. In the glial ablation studies of Bush et al. (8), a similar difference was noted between the ileum, which was sensitive to glial ablation, and the colon, which was not, despite transgene expression in both regions. In further support of glial heterogeneity, in a previous study (39), we observed widespread expression of the α2A adrenergic receptor on enteric glia of the mouse colon, whereas only a few glial cells in the mouse ileum expressed this receptor. Taken together, these data indicate that there is regional heterogeneity between enteric glia of the ileum and colon, although the functional significance of this remains unclear.

Cellular Morphology and Intestinal Inflammation

In the present study, we did not see any changes in neuronal or glial morphology in either the ileum or colon of mice treated with FC compared with vehicle-treated animals. In accordance with this observation, intracerebral administration of up to 5 nmol FC into the lateral ventricle or local injection of up to 1 nmol FC directly into the cortex do not cause any evident changes in GFAP immunoreactivity (32–34, 60). At these doses, no increase in the thickness of GFAP-positive processes is observed, nor is there any evident loss of astrocytes (32). However, these authors still observed that recovery of extracellular K⁺ was impaired after neuronal stimulation (32) and that cortical ATP levels were significantly decreased (33), demonstrating that although astrocyte morphology is not altered by low-dose FC, defects in astrocyte metabolism still inhibit their ability to regulate the extracellular environment (34).

In the present study, we did not observe any macroscopic damage, any change in MPO activity (an indicator of granulocyte infiltration), or any microscopic indications of inflam-
information in any of the FC-treated animals. Therefore, the dose of FC that we have chosen to use in vivo in the present study was not high enough to cause degenerative and inflammatory changes in the ENS. We can be confident, therefore, that the changes we have observed in enteric glial glucose metabolism, dipeptide uptake, pERK1/2 activation, and intestinal motility are not due to intestinal inflammation.

Specificity of FC Actions on Enteric Glia and Astrocytes

FC is preferentially taken up by astrocytes and more efficiently inhibits the TCA cycle in these cells (13, 58); however, at higher doses, neurons can also be adversely affected (31, 42). Furthermore, FC can also inhibit aconitase in isolated endothelial (43) and epithelial cell lines (16). Our 2-NBDG

Fig. 10. Long-term treatment with FC did not cause any changes in the morphology of neurons or enteric glia in the myenteric plexus of the mouse colon. Mice were given twice daily injections of FC (10 μmol/kg) or vehicle, and the morphology of neurons and glial cells in the myenteric plexus of the mouse colon was visualized using the neuronal marker PGP 9.5 and the enteric glial cell markers GFAP and S-100β. A–C: vehicle-treated animals (n = 3–4). D–F: animals treated with 10 μmol/kg FC (n = 3–4). No changes in the distribution or the intensity of staining of any of these markers were noted. Scale bars = 50 μm.

Fig. 11. FC caused a decrease in small intestinal length and transit but no changes in colonic length and transit time. Mice were given twice daily injections of FC (10 μmol/kg FC, n = 10–12; 20 μmol/kg FC, n = 5–6) or vehicle (n = 15–16). Gastrointestinal transit was assessed by using the passage of Evans blue dye (A). The distance traveled by the Evans blue front was dose dependently decreased in mice treated with FC and was significantly decreased in mice treated with 20 μmol/kg FC compared with vehicle-treated animals (1-way ANOVA, *P = 0.026). Small intestinal length (B) was also significantly decreased in animals treated with 20 μmol/kg FC (1-way ANOVA, P = 0.0030, **significantly decreased compared with both vehicle and 10 μmol/kg FC-treated animals). Colonic transit was assessed using a colonic bead expulsion assay (C). Neither the latency of colonic bead expulsion nor the length of the colon (D) was altered by either dose of FC compared with vehicle-treated controls.
phosphorylation data and our dipeptide uptake data in both guinea pigs and mice, as well as our guinea pig pERK1/2 data, suggest that this dose of 100 μM FC maximally inhibits energy-dependent processes in enteric glia. Because we performed our studies on glucose uptake in isolated, cultured enteric glial cells, it is possible that nonspecific effects of FC on glucose metabolism exist or that nonspecific inhibitory effects on ileal smooth muscle account for the ileal shortening that we observed. This explanation is implausible, however, because dipeptide uptake in neurons or in macrophages was not altered, as the expression of pERK1/2 immunoreactivity was only enhanced in enteric glia and not smooth muscle or neurons after FC treatment and as we did not observe any changes in colonic transit or colonic length of FC-treated animals. Therefore, it is unlikely that we are observing solely a nonspecific effect on ileal smooth muscle. Furthermore, because secretion in response to EFS was unchanged in FC-treated tissues and because FC had no apparent dose-dependent effect on ion transport, this would suggest that FC does not have nonspecific effects on the neuronal circuitry underlying secretion. In addition, because forskolin and carbachol are known to act directly on the epithelium (7), FC does not appear to have any nonspecific effects on epithelial cells. Because Gardner et al. (16) performed their experiments on a cell line derived from a human epithelial lung carcinoma, it is possible that these differences are a result of the aberrant phenotype of the carcinoma, species differences, or regional differences between the lung and the gut. Rist et al. (43) observed that FC had effects on immortalized rat endothelial cells. We did not perform any tests for FC effects on the microvasculature; however, because the submucosa contains a large number of blood vessels and because FC did not alter ion transport, it is unlikely that FC is solely having nonspecific effects on the microvasculature. Therefore, it is most likely that the changes we have observed in FC-treated tissues are due to the inhibition of enteric glial function and thus indicate that these cells modulate intestinal motility.

Because mice received intraperitoneal injections of FC before both in vivo and in vitro studies on intestinal motility and contractility, it is possible that FC could have altered intestinal motility through effects on spinal cord astrocytes, thus modulating extrinsic neuronal input to the ENS (2). However, as we did not observe any symptoms suggesting central FC toxicity, such as seizures (24, 41), and as high local concentrations of FC in the CNS are required to affect astrocytes (42) as discussed above, it is likely that the site of FC action in this study is in the ENS.

In conclusion, we have observed that the gliotoxin FC alters energy-dependent processes in enteric glia, such as glucose metabolism and dipeptide uptake in vitro. In addition, we have demonstrated that FC causes an increase in pERK1/2 activation specifically in enteric glia. We have also shown that this gliotoxin causes alterations in stimulated contractility in vitro as well as a decrease in gastrointestinal transit in vivo, consistent with a recent report that utilizes an immunological method of glial ablation (2). Because we did not observe any signs of inflammation in our study, we conclude that these changes are independent of inflammation and that enteric glia play a modulatory role in the ENS.

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Fig. 12. Long-term treatment with FC did not cause any changes in the number of nitric oxide synthase (NOS)-immunoreactive myenteric neurons in the mouse ileum or colon. Mice were given twice daily injections of FC (20 μmol/kg; n = 4) or vehicle (n = 3). Myenteric neurons were visualized with an antibody directed toward NOS. A: ganglion from the mouse ileum of a vehicle-treated animal. B: ganglion from the mouse ileum of a FC-treated animal. Scale bars = 50 μm. The numbers of NOS-immunoreactive neurons per myenteric ganglion in both the ileum and colon were not significantly different between FC- and vehicle-treated mice (Student’s t-test, P = 0.53 for the ileum and P = 0.33 for the colon; C).
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