Modulation of visceral hypersensitivity by glial cell line-derived neurotrophic factor family receptor α-3 in colorectal afferents

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Modulation of visceral hypersensitivity by glial cell line-derived neurotrophic factor family receptor α-3 in colorectal afferents. Am J Physiol Gastrointest Liver Physiol 300: G418–G424, 2011. First published December 30, 2010; doi:10.1152/ajpgi.00456.2010.—Irritable bowel syndrome is characterized by colorectal hypersensitivity to colorectal distension before and after intracolonic treatment with 2,4,6-trinitrobenzene sulfonic acid (TNBS). Baseline responses to colorectal distension did not differ between C57BL/6 and TNBS-induced visceral hypersensitivity in GFRα3 knockout (KO) mice. The proportion of GFRα3 immunopositive thoracolumbar and lumbosacral colorectal dorsal root ganglion neurons was significantly elevated 2 days after TNBS treatment. In single fiber recordings, responses to colorectal afferent endings in C57BL/6 mice were significantly increased (sensitized) after exposure to an inflammatory soup, whereas responses to stretch did not sensitize in GFRα3 KO mice. These findings suggest that enhanced GFRα3 signaling in visceral afferents may contribute to development of colorectal hypersensitivity.

Irritable bowel syndrome; growth factors; visceral pain

MATERIALS AND METHODS

Animals. Experiments were conducted with C57BL/6 mice (Taconic, Germantown, NY) and GFRα3 knockout (KO) mice weighing 20–30 g (backcrossed onto the C57BL/6 genetic background for >10 generations). GFRα3 KO mice were graciously provided by Dr. Jeffrey Milbrandt, Washington University School of Medicine. The genetic status of mice was confirmed by PCR genotyping as described in Honma et al. (15). Mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility with free access to water and food. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Behavioral assessment of colorectal hypersensitivity was carried out by the investigators in the Rodent Behavioral Assay Core in the animal facility.

Colorectal distension and sensitivity. All surgical procedures were performed under 2–3% isoflurane anesthesia (Hospira, Lake Forest, IL). Following hair removal and disinfection of the surgical area, the
lower right abdominal musculature was exposed. Stripped ends of two sets of sterilized Teflon-coated stainless steel wire (Cooner Wire Sales, Chatsworth, CA) were inserted into the peritoneal musculature and secured in place with sutures. The other ends were tunneled subcutaneously to the back of the head (see Ref. 10 for details). Wounds were sutured and treated with a local anesthetic ointment. Buprenorphine (0.9 μg/mouse) was administered subcutaneously for pain management. After electrode implantation, mice were housed separately.

Following a 4- to 5-day recovery period from surgery, baseline responses to colorectal distension (CRD) were obtained. As previously described (10, 20, 31), mice were exposed to 2% isoflurane for brief sedation to allow balloon insertion. A lubricated polyethylene balloon (length, 1.5 cm; diameter, 0.9 cm) was inserted transanally to 0.5 cm beyond the anal sphincter and taped securely to the tail. Movement of mice was restricted by placement in a plastic tube (20 cm) and allowed to recover from isoflurane for 30 min. CRD consisted of constant-pressure phasic balloon inflation (15, 30, 45, and 60 mmHg, 10 s) every 3 min, three times at each distension pressure. Electromyographic (EMG) activity was recorded from the peritoneal musculature, amplified, and rectified. The rectified EMG was quantified as area under the curve using the modulus program in the Spike 2 software [Cambridge Electronic Design (CED), Cambridge, UK] (see Ref. 10 for details). Responses to CRD were taken as the difference in EMG during distension minus the resting EMG (recorded in the 10-s period immediately before each distension) normalized as percentage of the baseline response to 60 mmHg CRD.

After baseline responses were determined, mice were anesthetized (2–3% isoflurane), and either 0.2 ml of saline or TNBS (10 mg/ml; Sigma-Aldrich, St. Louis, MO) in 50% ethanol was given transanally using an oral feeding tube inserted 20–30 mm in the colon from the anus. Responses to CRD were subsequently determined 2, 7, 10, and 14 days after intracolonic treatment.

Colorectal compliance was measured in GFR3 KO and C57BL/6 mice (n = 4 each) by measuring the volume of fluid required to produce distending pressures of 15, 30, 45, and 60 mmHg. Each mouse was subjected to three trials, first while sedated with isoflurane and the third after recovery from sedation.

Immunohistochemistry. To label DRG neurons innervating the colorectum, mice were anesthetized (2–3% isoflurane), a laparotomy was performed, and 50 mg/ml (in 100% DMSO) of 1,1’-diodecyl-3,3',3'-tetramethylindocarbocyanine methane sulfonate (DiI; Molecular Probes, Eugene, OR) was injected in one to three sites within the distal colorectum (~6 μl/site). Visible leakage of DiI was removed with a cotton swab. All wounds were sutured, and mice were treated as above and housed separately for 14 days before intracolonic instillation of vehicle or TNBS (as above). At different times after intracolonic treatment, mice were deeply anesthetized with ketamine/xylazine (87.5/12.5 mg/kg ip) and transcardially perfused with saline followed by a cold fixative containing 4% paraformaldehyde in 0.2% picric acid and 0.1 M phosphate buffer (pH 7.4). Thoracolumbar (T11-L1) and lumbosacral (L6-S2) DRG were removed and postfixed in the same fixative for 4–5 h at 4°C. After cryoprotection for 10 min at 14,000 rpm at 4°C, and protein concentration of the supernatant was determined using a Pierce 660 nm protein assay kit (Thermo Scientific, Rockford, IL). Protein samples were heat-denatured in Laemmli sample buffer solution (Bio-Rad, Hercules, CA) and stored at ~80°C until use.

Samples (35 μg) were subject to electrophoresis for protein separation on 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (GE Healthcare, Piscataway, NJ). Membranes were incubated overnight at 4°C with artemin antibody (Santa Cruz, Santa Cruz, CA) diluted in TBS solution containing 5% skim milk. Artemin binding was visualized using a horseradish peroxidase-conjugated donkey anti-goat antibody (Santa Cruz) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Band intensity was quantified using a Fuji Las-1000 imaging system (Fujifilm, Valhalla, NY) and normalized to GAPDH immunoreactivity on blots probed with anti-GAPDH antibody (Santa Cruz) after removing artemin binding using a stripping reagent (Thermo Scientific).

In vitro colon-pelvic nerve preparation. As previously described (14, 18), mice were killed by inhalation of CO2 followed by exsanguination after perforating the right atrium. The colorectum with major pelvic ganglion and pelvic nerve attached was removed, and the colorectum was opened longitudinally along the antimesenteric border and placed in modified Krebs solution on ice [in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO3, 1.3 NaH2PO4, 1.2 MgSO4(H2O)2, 2.5 CaCl2, 11.1 d-glucose, 2 butyrate, and 20 acetate] bubbled with carbogen (95% O2–5% CO2) to which was added the L-type calcium channel antagonist nifedipine (4 μM; to block spontaneous muscle contractions) and indomethacin (3 μM; to block endogenous prostaglandin synthesis). The tissue preparation was pinned flat mucosal-side-up in a custom-made organ bath. The pelvic nerves were extended from the tissue chamber and laid on a mirror in a separate recording chamber isolated by a moveable wall with a small hole allowing passage of the nerves. The recording and the tissue chambers were filled with paraffin oil and perfused with the Krebs solution at 33–34°C, respectively. The pelvic nerve was teased into fine bundles ~10 μm thick by fine forceps after peeling the sheath surrounding the nerve to expose the nerve trunk.

An individual nerve bundle was placed on a platinum-iridium recording electrode, and electrical signals generated by fibers were amplified, filtered, and sampled via a 1401 interface (sample rate: 20 kHz; CED), which allowed signal monitoring on Spike2 software. A reference electrode was placed in the tissue chamber. To identify mechanosensitive receptive fields, the mucosal surface was stroked with a brush, after which responses to circumferential stretch and stroking of the receptive field with calibrated nylon filaments (10 mg force) were determined. Circumferential stretch was achieved using custom-built claws attached at 1-mm intervals along the length of the antimesenteric edge of the colorectum and fixed to a rigid plastic block whose displacement was regulated by a servo-controlled force actuator (series 300B dual mode servo system; Aurora Scientific, Toronto, Canada). As described previously (14), this produces a homogeneous circumferential stretch (slow ramped force from 0 to 170 mM at 5 mN/s) and allows mathematical conversion of the circumferential force to intraluminal pressure [pressure = 2-force/ (LD), where L is colon length and D is the circumference]. The forces applied convert to pressures between 0 and 45 mmHg distension.
Pelvic nerve afferent sensitization after application of an inflammatory soup (IS) was investigated in GFRα3 KO and C57BL/6 mice. The IS consisted of bradykinin, PGE2, serotonin, and histamine (all at 10 μM) with pH adjusted to 6.0. Only stretch-sensitive receptive endings were studied. IS was applied for 3 min, and responses to stretch were compared before and 2 min after IS washout. At least 5 min separated successive ramped stretch of the colon. All recorded data were saved to a personal computer and analyzed by Spike2 software.

Myeloperoxidase activity assay. Myeloperoxidase (MPO) activity was evaluated as a biochemical indication of inflammation. Mice were killed under deep isoflurane anesthesia, and the distal colorectum (~2 cm) was removed as above at varying times after intracolonic treatment, rinsed in saline, and weighed. Finely minced colon was homogenized in potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and the tissue suspensions were frozen and thawed three times. The supernatant was collected after centrifugation for 5 min at 13,000 rpm and mixed with phosphate buffer containing o-dianisidine dihydrochloride (Sigma-Aldrich) and 0.0005% hydrogen peroxide. The samples underwent three freeze-thaw cycles and were centrifuged two times and loaded, along with MPO standards (Calbiochem, San Diego, CA), on a 96-well plate. The samples and standards were reacted with o-dianisidine dihydrochloride (Sigma) and read on a plate reader at 460 nm every 20 s for 15 min. The slope for each standard was calculated, plotted, and used to calculate units of MPO activity/tissue weight for each sample.

Fig. 1. Responses to colorectal distension. A: visceromotor responses to distension did not differ between C57BL/6 and glial cell line-derived neurotrophic factor family receptor α-3 (GFRα3) knockout (KO) mice [F(1,112) = 1.07, P = 0.304]. Relative to intracolonic treatment with saline (B), 2,4,6-trinitrobenzene sulfonic acid (TNBS, C) significantly increased visceromotor responses to distension on all days of testing [TNBS, n = 6, F(4,20) = 6.32, P = 0.002]. Responses to distension in saline-treated mice were slightly, but significantly, increased on day 10 of testing relative to baseline [saline, n = 7, overall F(4,24) = 5.37; Holm-Sidak post hoc test, t = 4.58, P < 0.05]. Data are expressed as means ± SE of responses normalized to the pretreatment baseline response to 60 mmHg. D: areas under the stimulus-response curves (AUC) derived from the data presented in B and C. Visceral hypersensitivity produced by TNBS significantly differed in magnitude across days after treatment [F(1,44) = 8.09, P = 0.0160].

Fig. 2. TNBS-produced visceral hypersensitivity is significantly attenuated in GFRα3 KO mice. Intracolonic treatment with TNBS (B) but not saline (A) significantly increased visceromotor responses to distension [saline, n = 7, overall F(4,24) = 3.65; Holm-Sidak post hoc test, t = 1.78, P > 0.05; TNBS, n = 7, F(4,24) = 9.10, P < 0.001]. Data are expressed as means ± SE of responses normalized to the pretreatment baseline response to 60 mmHg. C: AUC derived from the data presented in A and B. Visceral hypersensitivity produced by TNBS in GFRα3 KO mice significantly differed in magnitude across days after treatment [F(1,48) = 10.43, P = 0.007]. D: comparison of TNBS-induced stimulus-response curves (AUC) between C57BL/6 (from Fig. 1D) and GFRα3 KO mice. TNBS induced significantly greater visceral hypersensitivity in C57BL/6 mice than in GFRα3 KO mice [F(1,44) = 4.96, P = 0.048].
RESULTS

TNBS-induced visceral hypersensitivity. We first examined baseline responses to CRD in C57BL/6 and GFRα3 KO mice. Figure 1A reveals equivalent visceromotor responses in these two genotypes [\(F(1,112) = 1.07, P = 0.304\)]. Subsequently, the effect of intracolonic TNBS treatment on responses to distension was investigated. Figures 1 and 2 summarize data collected before (baseline) and 2, 7, 10, and 14 days after intracolonic administration of either saline (as control) or TNBS in C57BL/6 (Fig. 1, B–D) and GFRα3 (Fig. 2, A–C) KO mice. Relative to intracolonic instillation of saline, TNBS produced a robust colorectal hypersensitivity in C57BL/6 mice on all days of testing after treatment [\(F(4,20) = 6.32, P = 0.002\); Fig. 1C]. Repeated CRD in saline-treated mice produced no colorectal hypersensitivity except on day 10 [overall \(F(4,24) = 5.37\); Holm-Sidak post hoc test, \(t = 4.58, P < 0.05\); Fig. 1B]. The TNBS-treated group differed significantly from the saline-treated group [\(F(1,44) = 8.09, P = 0.016\); Fig. 1D].

Similarly, intracolonic instillation of TNBS produced colorectal hypersensitivity in GFRα3 KO mice on days 7, 10, and 14 after treatment [\(F(4,24) = 9.10, P < 0.001\); Fig. 2B], but not on day 2 after treatment. Repeated CRD in saline-treated GFRα3 KO mice produced no colorectal hypersensitivity [overall \(F(4,24) = 3.65\); Holm-Sidak post hoc test, \(t = 1.78, P > 0.05\); Fig. 2A]. An area under the stimulus-response curves analysis revealed a significant difference between saline- and TNBS-treated groups of GFRα3 KO mice [\(F(1,48) = 10.43, P = 0.007\); Fig. 2C]. Although intracolonic instillation of TNBS produced colorectal hypersensitivity in both genotypes, the magnitude of hypersensitivity in GFRα3 KO mice was significantly attenuated relative to that produced in C57BL/6 mice [\(F(1,44) = 4.96, P = 0.048\); Fig. 2D].

These differences in colorectal hypersensitivity produced by TNBS could reflect differences in colorectal compliance or the magnitude of inflammation produced in the two genotypes. Colorectal compliance measurements revealed no difference between GFRα3 KO and C57BL/6 mice [\(F(1,6) = 0.348, P = 0.577\)]. Figure 3 shows that neither baseline nor intracolonic GFRα3 KO mice produced any differences in colorectal compliance or the magnitude of inflammation produced in the two genotypes. Intracolonic saline treatment did not [\(F(1,12) = 0.48, P = 0.501\)], whereas TNBS did [\(F(2,15) = 33.79, P < 0.001\)], significantly increase MPO activity in either saline-treated or TNBS-treated mice [\(F(2,15) = 4.96, P = 0.038\)].}

Data analysis. Results were analyzed by one-way, two-way, or repeated-measures two-way ANOVA followed by Bonferroni or Holm-Sidak’s post hoc multiple comparisons as appropriate using SigmaPlot version 9.0 (Systat Software, San Jose, CA). \(P < 0.05\) was considered statistically significant.

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The number of DiI-labeled colorectal neurons and artemin immunopositive neurons comprised 54.3 ± 2.0 and 58.8 ± 3.9% of thorocolumbar and lumbrosacral DiI-labeled neurons, respectively. These percentages significantly increased to 71.0 ± 1.5% [thorocolumbar: F(4,15) = 5.43, P = 0.007] and 71.2 ± 3.5% [lumbrosacral: F(4,15) = 3.35, P = 0.038] on day 2 after TNBS treatment (Fig. 4C) and returned to control thereafter. Associated with upregulation in the receptor, a modest, non-significant increase in colon artemin protein was observed following intracolonic TNBS treatment relative to saline treatment (Fig. 5).

**Fig. 5.** Effect of intracolonic TNBS treatment on artemin content in the colorectum. Data are derived from Western blots quantified by densitometry and presented as percentage relative to artemin tissue content in naïve mice.

**GFRα3 and artemin expression following TNBS treatment.** Expression of GFRα3 in colorectal DRG neurons and artemin in colorectal tissue were examined by immunohistochemistry and Western blotting, respectively. Figure 4, A and B, shows the numbers of DiI-labeled colorectal DRG neurons in thorocolumbar and lumbrosacral ganglia, respectively, before and after TNBS treatment and the numbers that were also GFRα3 immunopositive. The number of DiI-labeled colorectal neurons in thorocolumbar and lumbrosacral DRG did not significantly differ either before or after TNBS treatment. GFRα3-immunopositive neurons comprised 54.3 ± 2.0 and 58.8 ± 3.9% of thorocolumbar and lumbrosacral DiI-labeled neurons, respectively. The maximum pre-IS response.

**DISCUSSION**

In the present study, we evaluated the involvement of GFRα3 signaling in the mouse colorectum. Neither baseline responses to CRD nor responses of pelvic nerve afferent fibers to circumferential stretch differed in GFRα3 KO mice relative to C57BL/6 control mice, suggesting that colorectal mechanotransduction was unaffected by the deletion of GFRα3. Accordingly, GFRα3 appears not to play an important role in normal colorectal mechanotransduction. However, the magnitude of colorectal hypersensitivity in GFRα3 KO mice was...
significantly attenuated relative to C57BL/6 mice after intracolonic instillation of the inflammmogen TNBS. In support, stretch-responsive muscular and muscular-mucosal pelvic nerve afferent endings recorded from colorectums of GFRα3 KO mice failed to sensitize when exposed to an IS. Together, these findings reveal a role for GFRα3 in modulating the excitability of colorectal afferents, suggesting that artemin and GFRα3 normally contribute to processes of sensitization of the colorectal innervation.

Previous reports have documented the expression of GFRα3 in rodent and human DRG sensory neurons, implicating GFRα3 in nociceptive processing (19, 28). We found that ~55–60% of thoracolumbar and lumbosacral colorectal DRG neurons expressed GFRα3, a proportion that increased significantly in both pathways of colorectal innervation 2 days after TNBS treatment, a time when colorectal inflammation is maximal in this model. We did not find a corresponding significant increase in colorectal artemin, although artemin tended to increase modestly after TNBS treatment. In a related study (25), artemin mRNA in mouse colorectum was significantly increased twofold after TNBS treatment, consistent with the trend seen here. In addition, artemin mRNA significantly increased in inflamed skin produced by injection of Freund’s complete adjuvant. This increase corresponded with the period of complete Freund’s adjuvant-induced behavioral hyperalgesia and with an increase in GFRα3 mRNA in DRG neurons (23). In clinical studies, artemin and GFRα3 are significantly overexpressed in chronic pancreatitis, correlating with pain severity (9). The augmentation of GFRα3 expression in the present study was early and transient after intracolonic TNBS treatment, consistent with the development but not maintenance of colorectal inflammation and hypersensitivity. TNBS-produced colorectal hypersensitivity was absent in GFRα3 KO mice on day 2 after treatment and significantly attenuated thereafter compared with C57BL/6 mice. This time course of events mirrors the changes in GFRα3 in C57BL/6 mice after TNBS treatment, suggesting that the inability of GFRα3 KO mice to develop colorectal hypersensitivity is related to the absence of the receptor and not associated with colorectal inflammation, which did not differ between the two genotypes. We thus suggest that GFRα3 upregulation, associated perhaps with a tissue increase in its endogenous ligand artemin, is important for the development, but not maintenance, of TNBS-induced colorectal hypersensitivity.

The present findings add to growing evidence that GFRα3 signaling in sensory neurons is an important modulator of nociceptive transduction. Artemin has been shown to potentiate capsaicin-evoked Ca2+ influx and calcitonin gene-related peptide release in isolated DRG neurons (30) and to sensitize thermal responses of nociceptors in an ex vivo preparation (23). In addition, artemin overexpression in skin produces by injection of Freund’s complete adjuvant induced behavioral hyperalgesia in mice (9). The augmentation of GFRα3 expression in the present study was early and transient after intracolonic TNBS treatment, consistent with the development but not maintenance of colorectal inflammation and hypersensitivity. TNBS-produced colorectal hypersensitivity was absent in GFRα3 KO mice on day 2 after treatment and significantly attenuated thereafter compared with C57BL/6 mice. This time course of events mirrors the changes in GFRα3 in C57BL/6 mice after TNBS treatment, suggesting that the inability of GFRα3 KO mice to develop colorectal hypersensitivity is related to the absence of the receptor and not associated with colorectal inflammation, which did not differ between the two genotypes. We thus suggest that GFRα3 upregulation, associated perhaps with a tissue increase in its endogenous ligand artemin, is important for the development, but not maintenance, of TNBS-induced colorectal hypersensitivity.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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