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Neonatal Colon Insult Alters Growth Factor Expression and TRPA1 Responses in Adult Mice

Julie A. Christianson^{*,†}, Klaus Bielefeldt, Sacha A. Malin, and Brian M. Davis

Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, Pittsburgh, PA 15261

Abstract

Inflammation or pain during neonatal development can result in long-term structural and functional alterations of nociceptive pathways, ultimately altering pain perception in adulthood. We have developed a mouse model of neonatal colon irritation (NCI) to investigate the plasticity of pain processing within the viscerosensory system. Mouse pups received an intracolonic administration of 2% mustard oil (MO) on postnatal days 8 and 10. Distal colons were processed at subsequent timepoints for myeloperoxidase (MPO) activity and growth factor expression. Adult mice were assessed for visceral hypersensitivity by measuring the visceromotor response during colorectal distension. Dorsal root ganglion (DRG) neurons from adult mice were retrogradely labeled from the distal colon and calcium imaging was used to measure transient receptor potential vanilloid 1 (TRPV1) and ankyrin 1 (TRPA1) responses to acute application of capsaicin and MO, respectively. Despite the absence of inflammation (as indicated by MPO activity), neonatal exposure to intracolonic MO transiently maintained a higher expression level of growth factor messenger RNA (mRNA). Adult NCI mice displayed significant visceral hypersensitivity, as well as increased sensitivity to mechanical stimulation of the hindpaw, compared to control mice. The percentage of TRPA1-expressing colon afferents was significantly increased in NCI mice, however they displayed no increase in the percentage of TRPV1-immunopositive or capsaicin-sensitive colon DRG neurons. These results suggest that early neonatal colon injury results in a long-lasting visceral hypersensitivity, possibly driven by an early increase in growth factor expression and maintained by permanent changes in TRPA1 function.

Introduction

Early experience has been shown to have a profound impact on the prevalence of functional bowel disorders. Restricted fetal growth (birth weight < 2500g) significantly increases the incidence of irritable bowel syndrome (IBS) and a birth weight less than 1500g accelerates the age of symptom onset [9], most likely due to an immature gastrointestinal tract at birth [10]. Gastric suctioning at birth, most commonly performed in response to respiratory distress, has also been associated with functional bowel disorders later in life [4]. Outside of the gastrointestinal tract, noxious stimulation in the neonatal somatosensory system, whether due

Correspondence to: Julie Christianson, Department of Anatomy and Cell Biology, University of Kansas Medical Center, MS 3051, 2106 Olathe Boulevard, Kansas City, KS 66160, Phone: (913) 588-9899, Fax: (913) 588-5677, jchristianson@kumc.edu.

[†]Current address: Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS 66160

Neonatal exposure to intracolonic mustard oil increases growth factor expression, produces visceral hypersensitivity and alters functional TRPA1 expression within colon DRG neurons.

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to circumcision [56] or routine heel lancing [36], can produce both short-term and long-lasting changes in pain processing [25].

Animal models have been employed to study the impact of early experience in both somatic and visceral sensory systems. Neonatal stress in rats, generated by either maternal separation [7] or unpredictable shock [58], produces many of the symptoms observed in IBS patients, including visceral hyperalgesia [20], enhanced colonic motility [52] and increased epithelial permeability [6]. Nerve growth factor (NGF) [5] and corticotropin releasing factor [52] have been implicated in the initiation and maintenance of altered colonic sensitivity and permeability. Directly irritating the colon of neonatal rats by using either noxious stimuli, e.g. mustard oil [2], 0.5% acetic acid [60], or balloon distension [2;35] produces adult visceral hyperalgesia, as well as changes in the sensory innervation of the colon. These changes include an increase in the number of hypogastric nerves that respond to colonic distension in adults [35] and an increase in protein expression for transient receptor vanilloid 1 (TRPV1) in dorsal root ganglion (DRG) neurons innervating the distal colon [60].

TRPV1 is widely expressed among DRG neurons innervating the colon [48;18;57], bladder [27], pancreas [24], stomach and duodenum [62]. Disruption of TRPV1, either by genetic deletion or pharmacological blockade, diminishes baseline viscerosensory input [11;30] and reduces inflammatory-induced visceral hyperalgesia [30;29;42]. NGF and artemin have been shown to dramatically increase following acute inflammation [19;3;41] and are capable of increasing the expression of and/or potentiate the activation of TRPV1 on sensory neurons, both *in vivo* [28;3] and *in vitro* [47;41;51]. A related TRP channel, transient receptor potential ankyrin 1 (TRPA1), has recently been shown to be required for proper viscerosensory transduction and is widely expressed among DRG neurons that specifically innervate the colon [12]. Endogenous growth factor application also potentiates TRPA1 activation *in vitro* [40].

The goal of the current study is to determine whether TRP channels and growth factors play a role in neonatal viscerosensory plasticity. To address this hypothesis, we have developed a mouse model of neonatal colon irritation (NCI) that displays lifelong visceral hyperalgesia. Following a transiently maintained increase in growth factor expression in the neonatal colon, we observed an increased percentage of TRPA1-expressing DRG neurons innervating the distal colon, as well as an altered interaction between TRPA1 and TRPV1. Some of these data have been presented in abstract form [16].

Methods

Animals

Experiments were performed on male C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) born and housed in the Department of Laboratory Animal Resources at the University of Pittsburgh Medical Center. Mice received water and food *ad libitum*. All research performed conformed to NIH guidelines in accordance with the guidelines specified by the University of Pittsburgh Medical Center Animal Care and Use Protocols and the Committee for Research and Ethical Issues of IASP.

Neonatal colon insult

To induce neonatal colon insult, mice received an intracolonic instillation of 10 μ L of 2% mustard oil (MO; allyl isothiocyanate, Sigma, St. Louis, MO) suspended in saline, via a 26-gauge gavage feeding needle attached to a Hamilton syringe on postnatal days 8 and 10. The mustard oil solution was continually agitated to maintain a constant suspension. A separate cohort only received MO on postnatal day 8. A water-based lubricant was liberally applied to the perianal region prior to instillation of MO to avoid sensitization of surrounding somatic

tissues. Mice were held briefly (less than 5 minutes) in a secondary container and observed for any adverse effects (e.g. rectal bleeding) before being returned to their home cages. With the exception of the mice used for mRNA and MPO analysis, all mice remained undisturbed until weaning on postnatal day 21.

Three separate control paradigms were tested to determine the effects of handling procedures used to induce NCI. The first cohort, termed “naïve” in this report, consisted of mice born and bred in the same animal facility as the NCI mice and exposed only to handling procedures normally experienced by naïve animals, i.e. cage changes, weaning. The second cohort, termed “saline”, received the exact same treatment as NCI mice, but received intracolonic saline rather than MO on postnatal days 8 and 10. The third cohort, termed “lubricant”, received only the application of water-based lubricant and short-term separation from the dam, but no administration of intracolonic solution. These three cohorts were tested for responses to colorectal distension and somatic behavioral tests.

Electromyographic electrode implantation and colorectal distension

The visceromotor response (VMR) to colorectal distension (CRD) was evaluated in 8-week old naïve (n=37), NCI (P8 and 10, n=18; P8 only, n=5), saline (n=9) and lubricant-only (n=4) mice. Electrode implantation and CRD testing were performed as previously described [14]. The bare ends of two Teflon-coated stainless steel wires (3mm; Grass Technologies, West Warwick, RI) were inserted into the right lateral abdominal musculature, secured via 7–0 prolene sutures, tunneled subcutaneously to a small incision made in the nape of the neck and externalized for access during testing. Mice were housed singly and allowed to recover for a minimum of 4 days before undergoing testing.

Prior to testing, mice were briefly sedated with inhaled isoflurane and a custom- made polyethylene plastic cylinder (length, 1.5cm; diameter, 0.8cm) was inserted through the anus until the proximal end of the balloon was 0.5cm from the anal verge (total balloon insertion, 2cm) and secured to the tail with tape. The mouse was then placed into a Broome-style rodent restraint (Kent Scientific, Torrington, CT), the free ends of the electrode wires were attached to the differential amplifier, and the mice were allowed to recover from anesthesia for 30 minutes. CRD was produced by inflating the balloon with air from a compressed nitrogen tank equipped with a dual-stage low delivery pressure regulator and a separate pressure monitor used to regulate the pressure inside of the balloon. Each pressure (15, 30, 45, 60 and 75mmHg) was applied three times for 20 seconds with a 4-minute rest period in between. A custom-made distension control device (The University of Iowa Medical Instruments, Iowa City, IA) was used to control the gas flow through the system. EMG electrode activity was amplified, filtered and recorded on a personal computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK) for off-line analysis. CRD responses were quantified by measuring the area under the curve for the entire distension period divided by the length of the distension and expressed as a percent of baseline activity (prior to CRD). No statistical difference was measured between groups of similarly-treated mice tested at different times, therefore all of the data from each treatment group was combined for the final analysis.

Behavioral analysis

Naïve (n=13), NCI (n=13), saline-treated (n=8) and lubricant only-treated (n=7) mice were tested for thermal sensitivity using the paw withdrawal latency to radiant heat [26] and for mechanical sensitivity using von Frey monofilaments. For all assays, the operator was blinded to the treatment group. Mice were placed on a 30°C heated glass surface in individual chambers (10×10×13cm) of a 16-chamber Plexiglass container (IITC, Woodland Hills, CA). Animals were acclimated to the apparatus for 30 minutes before testing. Alternating hindpaws were tested a total of three times per day and the latency to hind paw withdrawal from the stimulus

was recorded within 0.01 second. Following thermal testing, the mice were transferred to a wire mesh-top table and allowed to acclimate for 30 minutes. A 1g von Frey monofilament was applied a total of three times to the middle plantar surface of each hind paw to determine the percent withdrawal response. Values from the left or right hindpaw did not significantly differ from one another and were therefore averaged together for each mouse. Measurements were taken for three consecutive days (following two days of acclimatization testing) and averaged together for each mouse. No statistical difference was measured between groups of similarly-treated mice tested at different times using parametric or non-parametric statistical analysis, therefore all of the data from each treatment group was combined for the final analysis.

Myeloperoxidase assay and histological examination

Myeloperoxidase (MPO) activity was measured in distal colon from P8 naive mice; P9 (n=6), P11 (n=8) and P18 (n=6) NCI mice; and P11 saline-treated mice (n=4). Briefly, mice were overdosed with inhaled isoflurane and the distal 1.5 cm of colon was dissected and the fecal matter, excess blood vessels and mesentery were removed. The tissue was weighed, added to a beaker containing 1mL 0.5% Hexadecyltrimethylammonium bromide (HTAB; Sigma) and finely minced using spring scissors. The solution was transferred to a 15mL centrifuge tube, along with another 2mL HTAB, and sonicated for 10 seconds before being homogenized for 30 seconds. Another 2mL HTAB was added and the tube was placed on dry ice until all samples were similarly collected. The samples underwent three freeze thaw cycles, were centrifuged twice and loaded, along with MPO standards (Calbiochem, San Diego, CA), onto a 96-well plate. The samples and standards were reacted with O-dianisidine dihydrochloride (Sigma) and read on a plate reader at 460nm every 20 seconds for 15 minutes. The slope for each standard reading was calculated and plotted and the slope of those values was used to calculate the units of MPO activity/tissue weight for each sample.

Histological assessments were made on paraffin-embedded cross-sections of distal colon stained with hematoxylin and eosin from P8 naive mice (n=2), P9 (n=3) and P11 (n=3) NCI mice, and P11 saline-treated mice (n=3) as described by Neurath et al., [44]. The degree of inflammation was graded semiquantitatively from 0 to 4 (0, no signs of inflammation; 1, very low level; and 2, low level of leukocytic infiltration; 3, high level of leukocytic infiltration, high vascular density, thickening of the colon wall; 4, transmural infiltrations, loss of goblet cells, high vascular density, thickening of the colon wall). Grading was done in a blinded fashion by the same pathologist.

mRNA extraction and RT-PCR

Growth factor mRNA levels in the distal colon (neonates = 1.5cm; adults = 2.5cm) were evaluated on postnatal days 8, 14, 18 and 42 in both naive and NCI mice (n = 4–5 for each age and treatment). RNA extraction and real-time PCR were performed as previously described [43;41]. Mice were overdosed with inhaled isoflurane and transcardially perfused with ice cold 0.9% saline. The distal 1.5cm (neonates) or 2.5cm (adults) of the colon was dissected and the fecal matter removed. The tissue samples were homogenized in 1–2mL Trizol (Invitrogen, Carlsbad, CA) and RNA was extracted using chloroform and phase lock gel tubes (Eppendorf, Hamburg, Germany) and precipitated in isopropanol overnight at –80°C. The RNA was washed with 75% EtOH and resuspended in Rnase-free water. RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and quantity was calculated using the 260nm absorbance reading from a spectrophotometer. Extracted RNA was treated with DNase (Invitrogen) to remove genomic DNA and 1µg was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen). Negative control reactions were run without RNA to test for contamination.

SYBR Green PCR amplification was performed using an Applied Biosystems (Foster City, CA) 5700 real-time thermal cycler. Threshold cycle (Ct) values were recorded as a measure of initial template concentration and relative fold changes in RNA levels were calculated by the $\Delta\Delta C_t$ method using GAPDH as a reference standard [37]. Primer sequences are listed in Table 1.

Retrograde labeling of colon afferents

All surgical procedures were performed under sterile conditions in a designated animal surgery area as previously described [18]. Mice were anesthetized by inhaled isoflurane and a laparotomy was made to expose the pelvic viscera. Using a Hamilton microsyringe (33 gauge needle), a single 5 μ L injection of Alexa Fluor 488-conjugated cholera toxin- β (CTB, 2%; Invitrogen) was made beneath the serosal layer in the distal region of the colon, at the level of the dome of the urinary bladder. The colon was swabbed to remove any excess of tracer and the wound was sutured shut. We have previously released 5 μ L of CTB onto the serosal surface of the colon (and adjacent tissues) at the same level as the injections and only 1-2 labeled cells per ganglion were observed indicating minimal issues with dye leakage using this procedure [18].

Immunohistochemistry

Immunohistochemistry with TRPV1 antiserum (1:4000; Neuromics, Minneapolis, MN; cat# RA14113) in retrogradely-labeled DRG neurons was performed and analyzed as previously described [17;18]. At four days post-operatively, animals were overdosed with inhaled isoflurane and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4. Paired T13 or L6 DRG were dissected and embedded together into 10% gelatin. The embedded tissue was post-fixed for 1 hour at 4°C, cryoprotected in 25% sucrose at 4°C overnight, and sectioned at 35 μ m on a sliding microtome. Every sixth section was processed for immunohistochemistry. Briefly, sections were washed three times with 0.1M PB and blocked for 1 hour in 0.1M PB containing 5% normal horse serum and 0.25% Triton X-100. Sections were then incubated overnight at room temperature with primary antiserum to TRPV1 (1:4000; Neuromics, Minneapolis, MN; cat# RA14113), a purified rabbit polyclonal antibody developed against a synthetic peptide (EDAEVFKDSMVPGEK) corresponding to amino acids 824-838 of rat capsaicin receptor and conjugated to KLH. Sections were washed three times with 0.1M PB and incubated for two hours with secondary donkey anti-rabbit CY5 antisera (1:300, Jackson ImmunoResearch, West Grove, PA). Sections were washed three times in 0.1M PB and mounted in 0.1% gelatin onto Superfrost slides. Specificity of staining was established by testing TRPV1 antiserum on DRG sections from respective knockout mice (not shown).

Sections were viewed using a 40X objective on a Leica confocal microscope (Leica Microsystems; Wetzlar, Germany). For each tissue section, a 16 μ m thick optical segment was captured and divided into four optical planes and the number of CTB-positive cells and the number of immunopositive CTB-positive cells were counted in the first and last plane and averaged together. Any CTB-positive neurons present in both sections were not counted to ensure that large cells were not disproportionately represented as recommended by the stereological technique contained in Pakkenberg and Gundersen [45].

Cell culture and calcium imaging

Colon neurons from eight-week old naive and NCI mice were back-labeled as described above. After a recovery period of 7–10 days, mice were overdosed with inhaled isoflurane and perfused with ice-cold Ca²⁺/Mg²⁺-free Hanks' Balanced Salt Solution (HBSS; Invitrogen) and thoracolumbar (TL; T12-L1) and lumbosacral (LS; L5-S1) DRG were dissected and separately prepared for culture as previously described [39]. Dissociated cells were resuspended in F12 media (Invitrogen) containing 10% FBS and penicillin/streptomycin (50U/ml) and plated onto

laminin (0.1mg/ml) and poly-D-lysine (5mg/ml) coated glass coverslips. Cells were incubated overnight at 37°C and imaged the following day as previously described [41]. Prior to imaging, the cells were incubated in HBSS containing 10mg/ml BSA (Sigma) and 2μM fura-2 (Molecular Probes) for 30 minutes at 37°C. Coverslips were mounted on an inverted Olympus (Thornwood, NY) microscope stage with HBSS buffer flowing at 5ml/min, controlled by a gravity flow system (Warner Instruments, Hamden, CT). Perfusate temperature was maintained at 30°C using a heated stage and an in-line heating system (Warner Instruments). Chemicals were delivered with a rapid-switching local perfusion system. Firmly attached, CTB-positive neurons were identified using a 480nm filter and chosen as regions of interest using Simple PCI software (Compix Imaging Systems, Sewickley, PA). Unlabeled, adjacent cells were also identified and imaged. All fields were first tested with a brief application (4s) of 50mM K⁺ (high K⁺) to ensure that cells were healthy and responsive. Following a five-minute recovery period, 1μM capsaicin (Sigma) or 100μM mustard oil (MO; Sigma) was applied for five or ten seconds, respectively. 10mM capsaicin in 1-methyl-2-pyrrolidinone was used as a stock solution; 1μM capsaicin was made fresh daily in HBSS. 100mM mustard oil in 1-methyl-2-pyrrolidinone was made fresh daily and diluted to 100μM using HBSS. Absorbance data at 340nm and 380nm were collected at one frame per second. Responses were measured as the ratio of 340/380nm excitation and 510nm emission ($\Delta F_{340/380}$; DG4, Sutter Instruments, Novato, CA). Peak responses $>0.1 \Delta F_{340/380}$ were included in the analysis and were easily distinguishable from optical noise ($<0.02 \Delta F_{340/380}$). The prevalence of capsaicin or MO responsive colon afferents was determined as a percentage of total healthy (high K⁺-responsive) CTB-positive cells. Any cell with significantly diminished Fura-2 signal over the duration of the experiment or that did not recover to baseline prior to the second agonist application was not included in the analysis. Ca²⁺ response peak was calculated using Microsoft Excel as a measure of peak Ca²⁺ signal.

Statistics

Calculations were made using Microsoft Excel and statistical analysis was performed using 1-way or 2-way repeated measures analysis of variance (ANOVA) followed by Bonferroni's posttest (GraphPad Prism, GraphPad Software, La Jolla, CA). All data are expressed as mean $\pm$ SEM. A *P* value of less than 0.05 was considered significant.

Results

Neonatal colon insult produces visceral and somatic hypersensitivity in adult mice

To determine whether early exposure to intracolonic mustard oil can induce long-lasting hypersensitivity similar to that shown in rats [2], eight-week old naive and mice treated neonatally with MO only on P8 or on both P8 and 10 (hereafter referred to as NCI mice) were tested for VMR during CRD. Indeed, EMG activity of the abdominal muscles of NCI mice was significantly higher than in naive mice, with greater than half of the NCI mice exceeding the 95% confidence interval of the naive group (Figure 1A-B). NCI mice similarly exhibited a greater VMR over the entire distension series compared to mice treated with MO only on P8 (Figure 1B). Mice that were treated intracolonic with saline or only received application of water-based lubricant were also tested for their VMR to CRD. Neither treatment (perianal application of lubricant with or without intracolonic administration of saline on P8 and 10) significantly affected VMR, compared to naive mice (Figure 1C).

Previous studies have shown that perturbations of the viscerosensory system can result in changes in somatic sensitivity [53;63], therefore we assessed the effect of NCI on thermal and mechanical responses in the hindpaw of adult mice. When tested for their responsiveness to noxious thermal stimuli, NCI mice showed a trend toward shortened withdrawal latencies than naive mice using the Hargreave's method, however the difference was not statistically

significant (Figure 2A). Saline- and lubricant-treated mice did not show any change in thermal sensitivity (Figure 2A). Mechanical sensitivity was tested by measuring paw withdrawal frequency in response to a 1.0g von Frey microfilament. NCI mice exhibited a significantly higher withdrawal frequency than naive mice (Figure 2B). Saline- and lubricant-treated mice displayed a trend towards higher withdrawal frequency, but the difference was not significant (Figure 2B).

Neonatal mustard oil application does not induce colonic inflammation

To determine whether intracolonic instillation of MO produced an acute inflammatory response, MPO assays and histological analysis was performed on distal colon from naive (P8), NCI-treated mice on P9 (24 hours after the first MO instillation) and P11 (24 hours after the second MO instillation), and saline-treated mice on P11 (24 hours after the second saline instillation). MPO is abundantly released upon neutrophil activation [33] and is commonly reported as a measure of the severity of visceral inflammation in rodents. MPO activity was not significantly elevated 24 hours following the first (P9) or second neonatal MO (P11) application (Figure 3a). Intracolonic saline administration had no effect on MPO activity when assessed 24 hours after the second saline instillation (data not shown). MPO activity was also not significantly elevated one week later (P18), indicating a lack of delayed inflammatory response to neonatal MO instillation (Figure 3a). Blinded histological examination of hematoxylin and eosin-stained paraffin sections of distal colon from corresponding treatment groups similarly revealed a lack of diffuse inflammation. Representative sections revealed an intact epithelium without mucin depletion, lamina propria expansion or neurophilic infiltration of the lamina propria for all specimens (Figure 3b-d).

Intracolonic mustard oil transiently maintains elevated neonatal-level of growth factor expression

Growth factors have long been known to play a major role in the development and postnatal maintenance of sensory neurons. Recently they have been shown to directly enhance the responses of dissociated sensory neurons to capsaicin [41;51], as well as reduce withdrawal latency to noxious thermal stimuli in the absence of injury or inflammation [41]. To determine whether growth factor expression may be affected by NCI or contribute to the long-lasting visceral hypersensitivity, real-time PCR was performed on neonatal and adult distal colons of naive and NCI mice to measure relative mRNA levels of NGF, neurotrophin-3 (NT-3), glial cell line-derived neurotrophic factor (GDNF), artemin (ART) and neurturin (NRTN). The mRNA levels of all growth factors tested were significantly higher in naive neonatal colon (postnatal day 8, prior to MO instillation) than in naive adult colon (Figure 4A). On postnatal day 14 expression levels decreased, but were still significantly higher than adult levels. By postnatal day 18, the expression levels for each growth factor in naive colons were not significantly different from naive adult levels (Figure 4A). Comparisons between naive and NCI colons at these timepoints revealed a significantly higher mRNA expression level of NGF, NT-3 and GDNF in NCI colons than in naive colons on postnatal day 18 (Figure 4B). This was a transient maintenance of elevated growth factor expression, as all levels were not significantly different between naive and NCI colons on postnatal days 20 and 42 (Figure 4B).

Neonatal colon irritation does not affect the percentage of TRPV1-positive colon afferents

Several studies have provided evidence that capsaicin-responsive afferents in the mouse are responsive to acute distension of the colon [54;38]. Mice with a null mutation in TRPV1, the receptor for capsaicin [13;21], have significantly reduced VMR under naïve conditions, as well as following zymosan-induced colonic inflammation [30]. To determine if changes in TRPV1 might underlie the observed visceral hypersensitivity in our model of NCI, as was reported for a rat model of neonatal colon irritation [60], we evaluated the percentage of colon DRG neurons

that responded to capsaicin or expressed TRPV1-immunoreactivity. The distal colons of adult naive and NCI mice were subserosally injected with Alexa Fluor-conjugated CTB to back-label colon-specific neurons in the DRG (hereby referred to as “colon neurons”, not to be confused with the intrinsic innervation of the colon). The relative abundance of CTB-positive DRG neurons was similar to that reported by previous studies using CTB to identify colon neurons (LS > TL) [18;15;57]. The DRG were then processed for either immunohistochemistry or dissociated and used for calcium imaging studies with capsaicin or MO to functionally assess the percent of TRPV1- and TRPA1-expressing colon neurons, respectively. Nearly all TL (T12-L1) colon neurons in naive (100%; n=56 cells) and NCI mice ($94.7\% \pm 3.6$; n=41 cells) responded to $1\mu\text{M}$ capsaicin (Figure 5B). Likewise, nearly all TL colon neurons in either naive (100%) or NCI mice ($95.8\% \pm 4.2$) expressed TRPV1-immunoreactivity (Figure 6). The percentage of LS (L5-S1) colon neurons responding to capsaicin was similarly high in both naive ($84.4\% \pm 5.2$; n=90 cells) and NCI mice ($91.2\% \pm 3.8$; n=99 cells) (Figure 5B), however the peak response of capsaicin-induced calcium transients in LS neurons was significantly smaller than that observed in TL neurons in both naive and NCI mice (Figure 5D). The percentage of LS colon DRG neurons expressing TRPV1-immunoreactivity was not significantly different between naive ($61.5\% \pm 6.0$) and NCI mice ($53.0\% \pm 2.9$; Figure 6), however the percentages were significantly lower than when measuring capsaicin-responsiveness with calcium imaging, indicating that the percentage of LS colon neurons expressing functional TRPV1 is higher than the percentage that is observed via immunohistochemistry. Regardless of the assay, the anatomical and functional expression of TRPV1 did not appear to be affected by NCI.

Neonatal colon irritation increases the percentage of mustard oil-responsive colon afferents

TRPA1 is expressed exclusively within the TRPV1-population of afferents [55;23] and has recently been shown to modulate mechanotransduction in cutaneous sensory neurons [34], as well as contribute to visceral hypersensitivity following colitis [61]. To investigate whether functional expression of TRPA1 is modulated following NCI, we evaluated MO-induced calcium transients using calcium imaging [31;8]. Nearly all TL CTB-positive neurons responded to acute application of $100\mu\text{M}$ MO in both naive ($95.5\% \pm 5.0$; n=20 cells) and NCI mice (100%; n=32 cells; Figure 5A). Significantly fewer LS colon neurons were MO-responsive, compared to TL; however, NCI mice displayed a significantly larger percentage of LS CTB-positive neurons ($55.2\% \pm 6.3$; n=82 cells) than did naive ($36.7\% \pm 4.2$; n=56 cells; Figure 5A). The peak response of MO-induced calcium transients were significantly smaller in the LS colon neurons compared to TL, for both naive and NCI mice (Figure 5C). Colon neurons from mice treated only with lubricant as neonates had a slight, but not significant, increase in the percentage of MO-responsive LS neurons compared to naive (data not shown). Taken together, these data suggest that the percentage of colon neurons expressing functional TRPA1 is significantly increased in the LS ganglia of NCI mice, compared to naive.

Neonatal colon irritation prevents capsaicin-dependent desensitization of TRPA1

Recent studies from the Hargreaves' laboratory have shown that coexpression of TRPV1 is vital for proper activation and inactivation of TRPA1 [1;49;50]. To determine if NCI altered interactions between TRPV1 and TRPA1, colon-specific neurons were exposed to capsaicin followed ten minutes later by MO, and vice versa. As previously mentioned, nearly all TL naive and NCI colon neurons responded to an application of MO (Figure 5A). However, when capsaicin was applied ten minutes prior to MO application, only $55.3\% \pm 11.6$ of naive and $67.4\% \pm 8.4$ of NCI TL neurons responded to MO, confirming previous reports that activation of TRPV1 can desensitize TRPA1 receptors [1;49] (Figure 5A). Prior application of capsaicin also significantly reduced the percentage of MO-responsive LS naive afferents from $36.7\% \pm 4.2$ to $19.2\% \pm 5.6$ (Figure 5A). However, the percentage of MO-responsive NCI LS afferents after prior capsaicin exposure ($52.7\% \pm 9.1$) was essentially identical to that observed when

MO was applied first ($55.2\% \pm 6.3$), suggesting that NCI had disrupted TRPV1-mediated desensitization of TRPA1 (Figure 5A). Interestingly, colon neurons that responded only to MO (and not also capsaicin) were observed only among LS neurons and when MO was applied first and were modestly increased following NCI ($6.9\% \pm 5.1$ vs. $1.1\% \pm 1.1$; data not shown), suggesting that NCI may have enhanced the capacity of TRPA1 activation to mediate desensitization of TRPV1. Despite the retention of detectable MO-induced calcium transients in LS NCI colon neurons, prior exposure to capsaicin significantly reduced the peak calcium response to MO for all treatment groups, suggesting that TRPV1 activation was still capable of at least partially desensitizing TRPA1 (Figure 5C).

When the agonist presentation was reversed (MO then capsaicin), prior application of MO did not affect the percent of TL capsaicin-responsive neurons in either naive or NCI mice (Figure 5B), nor did it significantly alter the peak response to capsaicin (Figure 5D). In contrast, prior application of MO significantly reduced the percentage of capsaicin-responsive LS afferents in both naive ($84.4\% \pm 5.2$ to $64.1\% \pm 5.0$) and NCI mice ($91.2\% \pm 3.8$ to $67.2\% \pm 4.7$; Figure 5B). The peak capsaicin-induced response was not significantly altered by a prior MO application in either naive or NCI LS colon neurons, however the peak response in NCI LS colon neurons was significantly higher than naive (Figure 5D). These results suggest that NCI appears to differentially disrupt the ability of TRPV1 and TRPA1 activation to desensitize the other receptor in LS colon neurons.

Discussion

Previous studies of early perturbations to the viscerosensory system have revealed long-lasting hypersensitivity in visceral organs subjected to neonatal injury or insult [2;20;35;56;52;60]. Changes in primary afferent sensitivity [35], as well as upregulation of pain-related molecules [60], have been documented in these animal models; however, the current study is the first to show an increase in the percentage of colon neurons expressing functional TRPA1, as well as a diminished cross-sensitization between TRPV1 and TRPA1. We hypothesize that this altered expression results from high growth factor expression observed following NCI, which thereby permanently sensitizes those primary afferents innervating the colon.

Balloon distension of the colon has long been used as a standard method of evaluating colorectal hypersensitivity [14]. Similar to other studies of NCI in rat [2;35;60], mice that received MO on postnatal days 8 and 10 exhibited significantly increased visceromotor responses than naive mice or those treated with MO only on postnatal day 8. More than half of the NCI mice exceeded the 95% confidence interval of the mean VMR of the naive group and no NCI mice exhibited visceral hyposensitivity, which was observed in a subset of NCI rats in a previous study [59]. This observation validates our model as one of neonatally-induced life long hypersensitivity, as well as emphasizes the importance of multiple insults to produce such a long-lasting change in nociceptive processing. Importantly, we also showed that breaking down the treatment into separate components (by infusing intracolonic saline or only applying lubricant), was not sufficient to permanently alter visceromotor responses to CRD. The observation of increased hindpaw mechanical sensitivity in the NCI mice may be due to sensitization of central circuits, suggesting a role for higher-order convergence in the manifestation of both visceral and somatic hypersensitivity with this model. Alternatively, the neonatal insult may have altered the intrinsic stress response system of the mice, thereby rendering it more sensitive to future perturbations in general. This has previously been observed in models of maternal separation [20;52;58] and is supported by the trend towards increased mechanical sensitivity exhibited by mice treated with saline or lubricant only, which were also briefly removed from the dam during neonatal treatments.

Our laboratory and others have shown that peripheral inflammation or injury can increase local growth factor production in either neonates [19;5] or adults [3;41]. Although we did not measure an overt inflammatory response to intracolonic MO, we cannot rule out that a mild, short-lasting neurogenic inflammation was present following MO instillation. We observed a transient maintenance of growth factor expression following neonatal MO instillation. Increases in growth factor expression, both *in vivo* [28;3] and *in vitro* [47;41], have been shown to increase the expression of and potentiate the activation of TRPV1 on sensory neurons. Similar potentiation of TRPA1 has been observed following *in vitro* application of growth factors [40]. The transient maintenance of increased growth factor expression in NCI colons may have influenced the increase in functional TRPA1 expression among colon afferents in adult NCI mice. Not only did we observe a significant increase in the percentage of MO-responsive neurons in NCI mice, those neurons maintained their responsiveness to MO even after an initial application of capsaicin, unlike naive mice. The inability of TRPV1 activation to completely desensitize TRPA1 could contribute to increased afferent sensitivity, particularly in light of work from Hargreaves' laboratory showing the intimate relationship between the two receptors in mediating sensory neuron responsiveness [1;49;50]. The observation of altered TRPA1 function only among LS colon neurons, and not TL, could be due to differences in growth factor receptor expression between these two populations. The co-receptor for artemin (GFR α 3) is expressed by all TL colon neurons, yet is expressed by only half of all LS colon neurons (unpublished observation). Therefore, it is possible that disproportionate expression of other growth factor receptors between TL and LS colon neurons could underlie the LS-specific changes in TRPA1 function. Further investigation is required to determine a direct role for increased growth factor production in NCI-induced visceral hypersensitivity and altered TRPA1 function.

How the increase in TRPA1 expression/function translates into increased mechanical sensitivity is not clear. Recent studies suggest that TRPA1 is necessary for the development of visceral hyperalgesia following colonic inflammation [61] and that it can also modulate mechanotransduction in cutaneous sensory neurons [46;22;32;34]. Although TRPV1 has never been shown to directly transduce mechanical stimuli, mice lacking TRPV1 display reduced visceral mechanosensation [30]. Considering the wide range of endogenous molecules that open or modulate these channels (e.g. products of lipid metabolism, free radicals, protons), a role for TRPV1 and TRPA1 in visceral mechanosensation most likely occurs through both channels, either acting together or individually, enhancing the nociceptive tone of primary afferents. It will be important to determine whether the observed increase in functional TRPA1 is due to increased expression of the receptor among afferents that normally do not express TRPA1 at functional levels, NCI-induced changes in downstream signaling pathways that mediate TRPV1/TRPA1 desensitization, and/or posttranslational modification increasing the phosphorylation state and accessibility of TRPA1 at the cell membrane.

In conclusion, we have presented data from a mouse model of neonatal colon irritation, showing that early exposure to intracolonic mustard oil induces a transient maintenance of developmentally-high growth factor expression within the neonatal colon, followed by increased visceral sensitivity and altered functional TRPA1 expression within colon-specific DRG neurons. That these changes occurred in the absence of overt inflammation indicate that relatively mild perturbations of the colon, when they occur early in life, can have long lasting consequences for visceral sensitivity. This suggests that aggressive measures to limit activation of visceral afferents should be considered during procedures on neonatal patients.

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Figure 1.

NCI on postnatal days 8 and 10 produces visceral hypersensitivity in adulthood. A) Examples of EMG recordings of the VMR to graded balloon distension of the colon in naïve (left) and NCI mice (right). B) Graph depicting the VMR of adult naïve (black), NCI on P8 only (gray) and NCI on P8 and 10 (open) mice at increasing balloon pressures. NCI on P8 and 10 resulted in significantly higher VMR than NCI on P8 or naïve ($P < 0.05$, two-way ANOVA), particularly at the highest pressures measured (* $P < 0.05$, ** $P < 0.01$ Bonferroni's posttest). C) Treating neonatal mice with a perianal application of water-based lubricant (with or without intracolonic saline) did not influence the VMR during adulthood.

Figure 2.

NCI increases mechanical sensitivity in the hindpaw of adult mice. A) No differences in thermal sensitivity were observed between naive mice and those treated with MO, saline or only lubricant as neonates. B) NCI mice had a significantly higher paw withdrawal frequency to a 1g von Frey monofilament than naive mice ($P < 0.05$, one way ANOVA and Bonferroni's posttest).

Figure 3.

Neonatal MO application did not induce colonic inflammation. A) MPO activity was measured in distal colon from naive (P8) and NCI mice on P9 and P11, corresponding to 24 hours following each intracolonic MO application (indicated by gray arrows) and on P18. MPO activity was not significantly elevated during NCI (P9 and P11) or one week following MO instillation (P18). Histological examination was performed on hematoxylin and eosin-stained paraffin sections of distal colon from naive (P8, B) and NCI-treated mice on P9 (C) and P11 (D). No evidence of inflammation was observed in NCI treated mice either by MPO measurements or histological scoring. Scale bar measures 100 μ m.

Figure 4.

NCI increases growth factor mRNA expression in the colon. Growth factor mRNA was measured prior to and following NCI. A) On P8, the colon expresses significantly higher levels of all growth factors examined, compared to adult mice ($*** P < 0.0001$, two-way ANOVA and Bonferroni's posttest). All levels remained significantly elevated on P14, but were not significantly different from adult by P18. B) Comparisons between naïve and NCI mice at each timepoint revealed a significant elevation in NGF, NT-3 and GDNF mRNA expression on P18 in NCI mice ($** P < 0.01$, $*** P < 0.0001$ vs. naïve, two-way ANOVA and Bonferroni's posttest).

Figure 5.

NCI significantly increases the percentage of colon neurons expressing functional TRPA1. Calcium imaging was performed on colon neurons retrogradely labeled by an injection of Alexa Fluor-conjugated CTB into the distal colon to measure responses to capsaicin and mustard oil (MO). A) Nearly all TL colon neurons responded to an application of 100 μ M MO in both naïve (solid circle) and NCI (open circle) mice. Significantly fewer LS neurons responded to MO in both naïve and NCI mice, however significantly more MO-responsive LS neurons were observed in NCI mice than naïve. Following an initial application of 1 μ M capsaicin, a significant reduction in the percentage of MO-responsive neurons was observed for all cohorts with the exception of LS neurons in NCI mice. B) Nearly all TL and LS colon afferents in both naïve and NCI mice were responsive to 1 μ M capsaicin. A prior exposure to 100 μ M MO resulted in a significant decrease in the percentage of capsaicin-responsive LS afferents in both naïve and NCI mice. C) The mean peak response to MO was significantly larger in TL than in LS neurons in both naïve and NCI mice. Prior exposure to capsaicin significantly reduced the peak response in all populations tested. D) The mean peak response to capsaicin was also significantly larger in TL than in LS neurons in both naïve and NCI mice. Prior exposure to MO did not significantly affect the peak capsaicin response within any cohort, however LS NCI colon neurons had a significantly higher peak response to capsaicin following MO than naïve. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$, two-way ANOVA and Bonferroni posttest.

Figure 6.

NCI did not affect the percentage of TRPV1-immunopositive colon neurons. TRPV1 immunohistochemistry was performed in colon neurons retrogradely labeled by an injection of Alexa Fluor-conjugated CTB into the distal colon. Photomicrographs depicting CTB-positive (A), TRPV1-positive (B), and an overlay of both populations (C) in L6 DRG show a majority of colon-specific neurons express TRPV1 (arrows). D) No significant difference in the population of CTB/TRPV1-positive neurons was observed between naïve and NCI mice.

Table 1

Primer sequences used for real-time PCR assays

Gene	Forward primer (5' → 3')	Reverse primer (5' → 3')
NGF	ACACTCTGATCACTGCGTTTTG	CCTTCTGGGACATTGCTATCTGT
NT-3	CCTGTGGGTGACCGACAAG	GATCTCCCCAGCACTGTGA
GDNF	AGCTGCCAGCCCAGAGAATT	GCACCCCGATTTTGC
Artemin	GGCCAACCCTAGCTGTTCT	TGGGTCCAGGGAAGCTT
Neurturin	TGAGGACGAGGTGTCCTCCT	AGCTCTTGACGCGTGTGGTA
GAPDH	ATGTGTCCGTCGTGGATCTGA	ATGCCTGCTTCACCACCTTCTT