Peripheral contribution of NGF and ASIC1a to colonic hypersensitivity in a rat model of irritable bowel syndrome

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Key Messages
• IBS is associated with idiopathic colonic hypersensitivity.
• NGF is involved in visceral pain development, presumably via regulation of ion channels such as ASICs.
• We tested the hypothesis that NGF contributes to colonic hypersensitivity via ASICs modulation.
• The effect of NGF on ASICs expression and on colonic pain was assessed in rats with butyrate-induced colonic hypersensitivity.
• Our results show that the development of butyrate-induced colonic hypersensitivity results from a NGF-dependent ASIC1a over expression in nociceptive colonic neurons.
• NGF-ASIC1a interplay may have a pivotal role in the sensitization of colonic sensory neurons and should be considered as a potential new therapeutic target for IBS treatment.

Abstract
Background Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder associated with idiopathic colonic hypersensitivity (CHS). However, recent studies suggest that low-grade inflammation could underlie CHS in IBS. The pro-inflammatory mediator nerve growth factor (NGF) plays a key role in the sensitization of peripheral pain pathways and several studies have reported its contribution to visceral pain development. NGF modulates the expression of Acid-Sensing Ion Channels (ASICs), which are proton sensors involved in sensory neurons sensitization. This study examined the peripheral contribution of NGF and ASICs to IBS-like CHS induced by butyrate enemas in the rat colon.

Methods Colorectal distension and immunohistochemical staining of sensory neurons were used to evaluate NGF and ASICs contribution to the development of butyrate-induced CHS. Key Results Systemic injection of anti-NGF antibodies or the ASICs inhibitor amiloride prevented the development of butyrate-induced CHS. A significant increase in NGF and ASIC1a protein expression levels was observed in sensory neurons of rats displaying butyrate-induced CHS. This increase was specific of small- and medium-diameter L1 + S1 sensory neurons, where ASIC1a was co-expressed with NGF or trkA in CGRP-immunoreactive somas. ASIC1a was also overexpressed in retrogradely labeled colon sensory neurons. Interestingly, anti-NGF antibody administration prevented ASIC1a overexpression in sensory neurons of butyrate-treated rats. Conclusions
Inferences Our data suggest that peripheral NGF and ASIC1a concomitantly contribute to the development of butyrate-induced CHS NGF-ASIC1a interplay may have a pivotal role in the sensitization of colonic sensory neurons and as such, could be considered as a potential new therapeutic target for IBS treatment.

Keywords acid-sensing ion channel, animal model, colonic hypersensitivity, nerve growth factor, sensory neurons.

Abbreviations: ASIC, acid sensing ion channel; CGRP, calcitonin-gene related peptide; CHS, colonic hypersensitivity; CRD, colorectal distension; DEG/ENaC, degenerin/epithelial sodium channel; DRG, dorsal root ganglia; IBS, irritable bowel syndrome; KO, knock-out; NGF, nerve growth factor; TrkA, receptor tyrosine kinase A.

INTRODUCTION

Chronic colonic hypersensitivity (CHS) is a key feature of irritable bowel syndrome (IBS), a common functional gastrointestinal disorder, which strongly impairs patients’ quality of life.1 Despite studies pointing to peripheral and central mechanisms of visceral afferent sensitization,2-8 CHS etiopathogenesis in IBS remains poorly understood.

Several mediators have been shown to sensitize visceral nociceptive afferents.4 Among them, the nerve growth factor (NGF) plays a crucial role in the peripheral mechanisms leading to the development of inflammatory visceral hypersensitivity, as demonstrated in clinical and preclinical studies.5-10 Exogenous administration of NGF11,12 or systemic blockade of NGF expression modulates colonic sensitivity in rodent models of inflammatory visceral pain.13,14 Nerve growth factor contribute to the sensitization of nociceptive neurons through the induction of neuroplastic changes that are notably characterized by an increased expression of proteins involved in nociception, such as neuropeptides, G protein-coupled receptors and voltage-gated ion channels.15-17 Acid-Sensing Ion Channels (ASICs) are up-regulated by NGF.18,19 Acid-Sensing Ion Channels are channels of the degenerin/epithelial sodium channel (DEG/ENaC) family and are composed of ASIC1 (ASIC1a and ASIC1b), ASIC2 (ASIC2a and ASIC2b), ASIC3 and ASIC4 subunits, assembling into homo- or heterodimers.20-24 They are expressed both in the nervous system and in the gastrointestinal tract,18,25-27 where they contribute to nociceptive processes by sensing changes in proton concentration.28-30 Protons are highly suspected of triggering pain and/or hypersensitivity through the activation of cationic currents in nociceptors.31-34

While IBS has been considered for years as a sine materia pathology, most recent insights suggest that low-grade inflammation could underlie its symptomatology (for reviews, see Refs. 35-37) and NGF might play a critical role in this process.5,48 Therefore, we hypothesized that NGF could play a pro-nociceptive role in IBS pathophysiology by modulating ASICs. To test this hypothesis, we used a relevant rat model of IBS-like CHS induced by repeated butyrate colonic enemas, which combines a decrease in colorectal distension (CRD) pain thresholds and the absence of macroscopic or histological modifications of rat colonic mucosa.39 Using this model, we examined (i) the peripheral contribution of NGF and ASICs to IBS-like CHS, (ii) changes in NGF and ASICs expression in dorsal root ganglia (DRG) sensory neurons of butyrate-treated rats and (iii) the effect of NGF blockade on ASICs expression in DRG sensory neurons. Our results suggest that butyrate-induced CHS could involve a NGF-mediated peripheral up-regulation of ASIC1a expression in colonic DRG sensory neurons, further sensitizing colonic nociceptors.

METHODS

All experiments were performed in accordance with the ethical guidelines of the International Association for the Study of Pain,30 EU guidelines, the regulations of the French Agriculture and Forestry Ministry, and in agreement with local ethical committee (no’s. B63-113.15, CE02-13, and CE03-13).

Animals

Male Sprague-Dawley rats [Charles River, France] weighting 225-250 g were acclimatized to laboratory conditions for 1 week prior any experiment. Animals were housed 4 per cage on a 12-hours light/dark cycle with ad libitum access to food and water.

Butyrate-induced CHS

Colonic hypersensitivity was induced in rats by butyrate enemas in the distal colon as previously described.39,41-43 Briefly, colonic enemas were performed using a 2 mm Fogarty catheter introduced rectally at 7 cm from the anal margin. Each animal received six enemas of 1 mL of a 200 mM sodium n-butyrate diluted in saline (Sigma-Aldrich, Lyon, France, pH7), or saline (NaCl 0.9%, pH7). The six enemas were performed over 4 days, as follows: first enema in the evening (6 pm), then twice daily (8 am and 6 pm) for 2 days, and last enema in the morning (8 am).

Colorectal distention test

Experimental design All experiments were performed on the last day of butyrate or saline enemas, allowing time for the develop-
ment of a strong and persistent butyrate-induced CHS. Animals were randomly assigned to the different experimental groups using the Latin square block method, i.e. we assigned one animal per treatment group (butyrate or saline) to a single block, and were blind regarding group treatments. Each experimental series used a different set of animals.

**Evaluation of colonic sensitivity**

Colonic hypersensitivity was evaluated using the CRD test, which allows assessment of pharmacological drugs effects in vivo without requiring surgery (i.e. for electromyographic monitoring of abdominal muscle contraction), thus preserving visceral physiological status in animals. Mechanical distension during CRD test does not alter the colonic mucosa of distended-rats (Matricon et al., see Fig. S1). Colonic hypersensitivity was assessed in 24 h-fasted rats by measuring the intra-colonic pressure required to induce characteristic abdominal contractions. Distension probes were prepared using a 2 mm Fogarty catheter cut at 9 cm. A 2 cm length flexible latex balloon was ligated to the tip of the catheter. Following volatile anesthesia (2% isofluorane; Baxter, Maurepas, France) and feces removal, the probe was inserted intra-rectally at 7 cm from the anal margin, and the catheter taped to the base of the tail. After 5 min of recovery, rats were placed in the middle of a 40 x 40 cm polymethyl acrylate box and the catheter was connected to an electronic barostat apparatus (Synectics Visceral Stimulator, Medtronic, Boulougne-Billancourt, France). Balloon pressure was gradually increased from 0 to 80 mmHg during 10 min and pain behavior analyzed up to the cut-off pressure (80 mmHg). This procedure allows a consistent evaluation of CHS as previously shown. Typically, following an exploration phase, animals adopted a prostration behavior up to the appearance of characteristic abdominal contractions associated to an elevation of the posterior part of the animal’s body. Pain threshold values were assessed by the same trained experimenters in order to limit inter-experimenter variability. Pain threshold was defined as the pressure necessary to induce the first long-lasting abdominal cramp observed, with rats staying prostrated and not resuming an exploratory behavior up to the cut-off signal. Animals displaying feces during the experiment were excluded from analysis, as feces could interfere with balloon volume.

**In vivo treatments**

**Subchronic administration of anti-NGF antibodies**

Three independent experiments were performed in order to investigate the effect of anti-NGF treatment on butyrate-induced CHS and on ASIC expression in the DRG of butyrate-treated rats.

**Experiment 1:** Assessment of the effect of anti-NGF antibodies treatment on CRD pain thresholds. Following each enema, butyrate- (n = 16) and saline-treated rats (n = 16) received one intraperitoneal injection of either an anti-NGF antibody (1 : 2000, 2 mL kg^{-1}, Sigma-Aldrich, n = 8 per group) or a control isotype antibody (1 : 2000, 2 mL kg^{-1}, Sigma-Aldrich, n = 5 per group) as previously described. With respect to the time frame used for the CRD experiment, rats were sacrificed on the day of the last injection (the procedure starting at 1 pm) and lumbar L1 and sacral S1 DRG [L1 + S1], which contain the somas of colonic sensory neurons, were dissected and processed for immunoblotting.

**Experiment 3:** Assessment of the effect of anti-NGF antibodies treatment on ASIC1a expression by immunohistochemistry in L1 + S1 DRG. Following each enema, butyrate- (n = 4) and saline-treated rats (n = 4) received one intraperitoneal injection of anti-NGF antibody (1 : 2000, 2 mL kg^{-1}, Sigma-Aldrich) as previously described. Rats were sacrificed and DRG were dissected on the day of the last injection (the procedure starting at 1 pm) and processed for immunohistochemistry.

**Subchronic administration of NGF**

To investigate the effect of NGF on ASIC1a expression in sensory neurons, saline-treated rats (n = 12) were subchronically injected with NGF (10 ng in 0.1% BSA, 1 mL kg^{-1}, Sigma-Aldrich, n = 6) or its vehicle (n = 6) as previously described on a time frame similar to the one used for subchronic treatment with anti-NGF antibodies. Rats were sacrificed and L1 + S1 DRG were dissected on the day of the last injection (the procedure starting at 1 pm) and processed for immunoblotting.

**Administration of the ASICs inhibitor amiloride**

To investigate ASICs contribution to butyrate-induced CHS, butyrate-treated rats (n = 8) were injected intravenously with amiloride (1, 3 or 6 mM, Tocris, Bristol, UK) or saline (100 µL per 100 g) 10 min before CRD test. Behavioral assessment of amiloride effects was performed following administration at in vivo doses (e.g. 1 mM corresponding to a commonly used dose of 0.25 µg kg^{-1} in order to avoid smooth muscle relaxation, which could interfere with CRD test).

**Analysis of ASICs mRNA expression**

**mRNA extraction**

Molecular analysis of ASICs mRNA expression used a new set of rats. Lumbarosacral L1 + S1 DRG were rapidly dissected on ice from butyrate- or saline-treated rats (n = 5), frozen in liquid nitrogen and preserved at -80 °C. Total mRNA was extracted using a phenol/chloroform method and quantified using a spectrophotometer at 260 nm. mRNA purity and integrity were confirmed by electrophoresis in a 2% agarose gel containing ethidium bromide.

**RT-PCR experiments**

Total mRNA (2 µg) was reverse-transcribed using the First-Strand cDNA Synthesis Kit (GE Healthcare, Amersham, UK). Semi-quantitative PCR analysis was performed in the linear cDNA amplification phase using the housekeeping ribosomal gene L32 as a reference. All primers are listed in Table 1. After migration in a 2% agarose gel containing ethidium bromide, the intensity of the PCR product was measured using Kodak Digital Science 1D Image Analysis Software (Kodak, Maisons-Alfort, France). All experiments were performed in duplicate.

**Analysis of ASIC1a protein expression**

Immunoblotting was performed using a mini-gel apparatus and binding was revealed by electrochemiluminescence using the Pierce ECL kits (Pierce, Brevieres, France). Lumbarosacral L1 + S1
Table 1 Sequence of primers for rat mRNA quantification

<table>
<thead>
<tr>
<th>Names</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASIC1a</td>
<td>5′-ACA GAT GTC TGA AAA GCA G-3′</td>
<td>5′-CAT GGT AAC AGC ATT GCA GGT GC-3′</td>
<td>a</td>
</tr>
<tr>
<td>ASIC1b</td>
<td>5′-ATG CCG TGC GGT TGT CCC-3′</td>
<td>5′-CAT GGT AAC AGC ATT GCA GGT GC-3′</td>
<td>b</td>
</tr>
<tr>
<td>ASIC2a</td>
<td>5′-CCA AAT TCC ACA TCC GGA CCC G-3′</td>
<td>5′-CCA GTC CCA TCT CTT AGG ACC GG-3′</td>
<td>c</td>
</tr>
<tr>
<td>ASIC2b</td>
<td>5′-CTC TCA TGC AGC CCA AGT TC-3′</td>
<td>5′-CTG TCC CAG AAA TAC CCC AGG AC-3′</td>
<td>d</td>
</tr>
<tr>
<td>ASIC3</td>
<td>5′-ACC AAG CCC AGA CCC AGC CCT CC-3′</td>
<td>5′-TTG GTG ACT CTG ATG CCC AG-3′</td>
<td>f</td>
</tr>
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DRG from butyrate- or saline-treated rats injected with anti-NGF or a control isotype antibody (see Effect of subchronic administration of anti-NGF antibodies: Experiment 2) or from saline-treated rats injected with NGF or its vehicle (see Effect of subchronic administration of NGF) were rapidly removed and dissected on ice and homogenized in 300 µL of ice-cold lysis buffer containing 20 µM leupeptin and 100 IU/mL aprotinin (Sigma-Aldrich). The protein concentration of tissue lysates was determined with the BCA protein assay kit from Interchim (Montluçon, France). Proteins were separated using 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioTrace, VWR, Fontenay-sous-Bois, France). Membranes were blocked for 1 h at room temperature (RT) in Tris Phosphate Buffer containing 0.1% Tween (TBS-T) and 5% of non-fat dry milk, and incubated overnight at 4 °C with the appropriate antibodies. A rabbit polyclonal anti-ASIC1a antibody (Alpha Diagnostics International, San Antonio, TX, USA) and a mouse monoclonal anti-β-actin antibody (Lab Vision, Brie brières, France) were used at 1:1000 dilutions. Blots were washed three times in TBS-T and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies (Pierce). The signal was developed in ECL solution (SuperSignal West Pico or Femto chemiluminescent substrate) for 10 s and visualized with the BCA protein assay kit from Interchim (Montluçon, France). Membranes were blocked for 1 h at room temperature (RT) in Tris Phosphate Buffer containing 0.1% Tween (TBS-T) and 5% of non-fat dry milk, and incubated overnight at 4 °C with the appropriate antibodies. A rabbit polyclonal anti-ASIC1a antibody (Alpha Diagnostics International, San Antonio, TX, USA) and a mouse monoclonal anti-β-actin antibody (Lab Vision, Brie brières, France) were used at 1:1000 dilutions. Blots were washed three times in TBS-T and incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies (Pierce). The signal was developed in ECL solution (SuperSignal West Pico or Femto chemiluminescent substrate) for 10 s and visualized with the BCA protein assay kit from Interchim (Montluçon, France).

### Immunohistochemical detection of NGF and ASIC1 proteins

At all stages of immunohistochemical experiments, samples from both butyrate- and saline-treated animals were processed concomitantly to ensure similar conditions of dissection, preparation and analysis. All slices processed for immunohistochemistry carried the two conditions in a randomized fashion (butyrate on top and saline on bottom, or vice-versa), to ensure that the samples were processed similarly. NGF and ASIC1 specific detection by the anti-NGF and the anti-ASIC1 antibody respectively was supported by the observation of a sole and specific Western Blot band and the absence of immunostaining in negative controls (omitting primary or secondary antibodies).

### ASIC1a immunohistochemical detection in retrogradely labeled DRG colonic neurons

Retrograde labeling of DRG colonic neurons ASIC1a immunohistochemical detection in retrogradely-labeled DRG colonic neurons used a new set of rats. Butyrate- or saline-treated rats (n = 5) were anesthetized with a ketamine (100 mg kg⁻¹; Imalgène®1000, Merial, Lyon, France) solution and xylazine (10 mg kg⁻¹; Rompun 2%, Bayer Pharma, Loos, France) mixture in saline. A 1 cm abdominal incision was made to access the distal colon, and 4 injections of 5 µL of a 3% Fluorogold® (Sigma-Aldrich) solution were performed using a 25 µL Hamilton syringe. The abdominal incision was sutured, antibiotic ( Aureomycin Monot 3% pomade, Merck, Semoy, France) was applied, and the animals allowed recovering.
ASIC1a immunohistochemical detection Seven days following colonic Fluorogold™ injections, immunohistochemistry was performed on lumbosacral L1 + S1 DRG. DRG were fixed 2 h in a 4% PFA in PBS solution (pH 7.4) at 4 °C and embedded in paraffin. Five micrometer-thick sections were deparaffinized and incubated in a 5% donkey serum blocking solution in TBS-T for 1 h at RT. Sections were then incubated overnight at 4 °C with a rabbit polyclonal anti-ASIC1a antibody (1 : 500, Alpha Diagnostic International) that was revealed by a Cy3-conjugated donkey anti-rabbit secondary antibody (1 : 500, Jackson Laboratories, Suffolk, UK) for 2 h at RT. Images were acquired using a Zeiss LSM 510 confocal microscope (40× objective, numerical aperture 1.3). Background staining provided by secondary antibodies was determined by omitting primary antibodies and was subtracted from complete primary + secondary immunohistochemical detection.

Statistical analysis

Results are expressed as mean ± SEM. Colorectal distension pain thresholds values were analyzed using a two-way ANOVA followed by a Bonferroni post-hoc test to compare treatment groups. Molecular biological and immunohistochemical data were analyzed using a one-way ANOVA followed by a Student-Neuman-Keuls post-hoc test. Differences were considered significant for $P < 0.05$.

RESULTS

NGF contribution to butyrate-induced colonic hypersensitivity

To assess the peripheral contribution of NGF to IBS-like CHS, we administered anti-NGF antibodies, which do not cross the brain-blood barrier, or non-specific isotypes (IgG) to butyrate- (CHS model) or saline-treated rats (control). In animals receiving IgG, CRD pain thresholds of butyrate-treated rats were significantly lower than those treated with saline (42 ± 4 mmHg vs 57 ± 4 mmHg, respectively; $P < 0.05$; Fig. 1A), reflecting the expected butyrate-induced CHS.$^{39,41-43}$ In con-

Figure 1 Nerve growth factor (NGF) involvement in butyrate-induced colonic hypersensitivity. (A) Butyrate enemas decreased colorectal distension pain thresholds compared to saline enemas, reflecting butyrate-induced colonic hypersensitivity (CHS). Butyrate-induced CHS was reversed by concomitant administration of anti-NGF antibodies, but not of non-specific IgG isotypes [lgG] to butyrate- (CHS model) or saline-treated rats (control). In animals receiving IgG, CRD pain thresholds of butyrate-treated rats were significantly lower than those treated with saline (42 ± 4 mmHg vs 57 ± 4 mmHg, respectively; $P < 0.05$; Fig. 1A), reflecting the expected butyrate-induced CHS.$^{39,41-43}$ In con-

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contrast, in animals receiving anti-NGF antibodies, butyrate had no effect on CRD pain threshold [55 ± 6 mmHg vs 56 ± 5 mmHg for butyrate- vs saline-treated animals, respectively; P > 0.05, Fig. 1A], suggesting that peripheral NGF contributes to the development of butyrate-induced CHS.

We then investigated whether this contribution could arise from an increased expression of NGF at peripheral level. Accordingly, we semi-quantified NGF expression in L1 + S1 DRG neurons, known to innervate the distal colon and to be activated by CRD, and to be activated by butyrate and saline-treated rats. We observed a significant increase in NGF expression levels in lumbosacral DRG neurons of butyrate-treated rats when compared with saline-treated controls [27 ± 7 vs 54 ± 4, respectively; P < 0.05, Fig. 1B]. Therefore, these data suggest that butyrate-mediated CHS could be partially mediated by a peripheral NGF-dependent mechanism occurring in lumbosacral L1 + S1 DRG neurons.

**ASIC1a contribution to butyrate-induced colonic hypersensitivity**

Acid-Sensing Ion Channels contribute to nociceptive processes by sensing protons and have been shown to participate at peripheral level to CHS of inflammatory origin. To assess whether peripheral ASICs could participate to CHS in our model, we first investigated the effect of amiloride, a non-selective ASICs antagonist, on CRD pain thresholds in butyrate-treated rats. The mean CRD pain threshold of butyrate-treated animals receiving an intravenous injection of amiloride at the concentration of 6 mM was significantly increased and higher than that of butyrate-treated animals receiving the vehicle solution [65 ± 6 mmHg vs 42 ± 2 mmHg, respectively; P < 0.05; Fig. 2A]. The transient receptor vanilloid 1 (TRPV1) is another channel involved in acid sensing by sensory neurons and TRPV1 has been shown to be modulated by NGF and to participate in visceral sensitization. To rule out the possibility that TRPV1 might contribute to CHS development in our model, CRD was performed in butyrate-treated animals injected with the TRPV1 antagonist capsazepine. No effect was observed on CRD pain thresholds [Fig. S1]. These data suggest that ASICs expressed at the periphery could play a role in the development of butyrate-induced CHS.

To investigate which ASIC subtypes could be involved in this mechanism, we used semi-quantitative RT-PCR in L1 + S1 DRG to detect potential changes in ASIC subunits mRNA levels induced by butyrate treatment. Our results showed increased ASIC1a [0.99 ± 0.14 vs 1.73 ± 0.35, respectively; 74.7% increase, P < 0.001] and ASIC1b [1.22 ± 0.16

**Figure 2** ASICs involvement in butyrate-induced colonic hypersensitivity. (A) Dose-effect of amiloride (1, 3 and 6 mM, i.v.) on colonic hypersensitivity (CHS) in butyrate-treated rats. Butyrate-treated rats injected with vehicle had decreased colorectal distension pain thresholds compared to saline-treated rats (dashed line). Butyrate-induced CHS was reversed with the dose of 6 mM amiloride n = 8 per group; *P < 0.05. (B) Increased ASIC1a and ASIC1b mRNA levels in lumbosacral DRG neurons of butyrate-treated rats compared to saline-treated rats. No change was found when assessing ASIC2a, ASIC2b and ASIC3 mRNA levels. The intensity of ASIC PCR products was normalized to that of the ribosomal mRNA L32. All experiments were performed in duplicate. n = 5 per group **P < 0.01, ***P < 0.001.
vs 1.81 ± 0.19, respectively; 48.4% increase, \( P < 0.01 \) mRNA levels in L1 + S1 DRG of butyrate-treated rats when compared to saline-treated rats [Fig. 2B]. In contrast, no change was detected for ASIC2a (0.99 ± 0.05 vs 1 ± 0.06), ASIC2b (0.97 ± 0.11 vs 0.81 ± 0.07) or ASIC3 (1 ± 0.08 vs 0.91 ± 0.03) subunits (Fig. 2B). We thus focused our attention on ASIC1a and ASIC1b subunits in the following experiments.

To confirm ASIC1a and ASIC1b increased levels in butyrate-treated rats, we quantified their protein expression using immunohistochemistry in L1 + S1 lumbosacral DRG neurons. Compared to rats receiving saline, the proportion of ASIC1a-IR neurons was significantly higher in L1 + S1 DRG neurons of butyrate-treated rats (0.42 ± 0.03 vs 0.53 ± 0.03, respectively; 26% increase, \( P < 0.05 \), Fig. 3A), but, importantly, not in thoracic DRG neurons (Fig. S2), which do not innervate the lower part of the gastrointestinal tract and were used as a negative control. In contrast, no statistical difference in the percentage of ASIC1b-IR neurons could be evidenced between butyrate- and saline-treated rats. At least 250 cells were measured for each group. Scale = 100 \( \mu m \); \( n = 4 \) per group; *\( P < 0.05 \).

Figure 3 Increased density of ASIC1a expressing neurons in L1 + S1 DRG of butyrate-treated rats. Quantification of ASIC1a- [A] and ASIC1b- [B] immunoreactive neurons in L1 + S1 DRG. The percentage of ASIC1a-IR neurons was significantly increased in butyrate-treated rats when compared to saline-treated rats. This increase was specific of small-diameter neurons (cross-sectional area <500 \( \mu m^2 \)). In contrast, no statistical difference in the percentage of ASIC1b-IR neurons could be evidenced between butyrate- and saline-treated rats. At least 250 cells were measured for each group. Scale = 100 \( \mu m \); \( n = 4 \) per group; *\( P < 0.05 \).
3.72 ± 0.15 ASIC1a-IR/m², respectively; 34% increase, $P < 0.01$, Fig. 4D). Moreover, this increase was specific of small- (3.95 ± 0.20 vs 5.22 ± 0.22 ASIC1a-IR/m², saline- vs butyrate-treated; 32% increase, $P < 0.01$) and medium- (1.99 ± 0.08 vs 2.32 ± 0.06 ASIC1a-IR/m², saline- vs butyrate-treated; 17% increase, $P < 0.01$) diameter neurons (Fig. 4D).

Altogether, these results show that ASIC1a expression was specifically increased in colonic L1 + S1 DRG neurons following butyrate treatment, suggesting their contribution to butyrate-mediated CHS.

**ASIC1a overexpression in lumbosacral DRG neurons is NGF-dependent**

Our results show that butyrate treatment is associated with an increase in NGF and ASIC1a expression levels in L1 + S1 DRG neurons. To investigate whether NGF mediates ASIC1a overexpression in sensory DRG neurons, we first assessed ASIC1a co-expression with NGF or its specific receptor trkA in small-diameter L1 + S1 lumbosacral DRG neurons expressing Calcitonin Gene Related Peptide (CGRP), a marker of nociceptive neu-
rons. Using ASIC1a-NGF-CGRP and ASIC1a-trkA-CGRP triple immunolabeling and confocal microscopy, we observed a strong overlap of ASIC1a staining with NGF or trkA staining in CGRP-positive DRG neurons of both saline- and butyrate-treated rats (Fig. 5).

Knowing that NGF can modulate the expression of several ion channels involved in pain mechanisms, we then analyzed ASIC1a protein expression in L1 + S1 DRG neurons of saline-treated rats following subchronic treatment with NGF. We found that NGF administration resulted in an increase of ASIC1a protein ($1.06 \pm 0.31 \text{ vs } 4.85 \pm 0.40$, vehicle- vs NGF-injected, 358% increase, $P < 0.001$, Fig. 6).

We also analyzed ASIC1a protein expression in L1 + S1 DRG neurons of butyrate-treated rats following subchronic administration of anti-NGF antibodies or IgG. While an expected increase ($1.95 \pm 0.14 \text{ vs } 2.63 \pm 0.20$, saline- vs butyrate-treated, 32% increase, $P < 0.05$, Fig. 7A) in ASIC1a protein levels was observed in butyrate-treated animals receiving IgG, anti-NGF treatment prevented this up-regulation ($2.63 \pm 0.20 \text{ vs } 1.43 \pm 0.17$, butyrate-treated rats, anti-NGF vs IgG, $P < 0.05$, Fig. 7A). We further showed no difference in the proportion of ASIC1a-IR neurons (all size: $44.87 \pm 1.31 \text{ vs } 45.08 \pm 1.59$, saline- vs butyrate-treated, Fig. 7B) in L1 + S1 DRG of butyrate-

Figure 5 Colocalization of ASIC1a and nerve growth factor (NGF) or its receptor trkA in CGRP-positive L1 + S1 DRG neurons of butyrate-treated rats. Examples of L1 + S1 DRG neurons stained with a triple labeling for ASIC1a + NGF + CGRP or ASIC1a + trkA + CGRP in butyrate- [A and C] and saline-treated rats [B and D]. ASIC1a [red] was frequently co-expressed with NGF or trkA [green] in sensory DRG neurons expressing CGRP [white]. Insets in the top row indicate neurons imaged at higher magnification below. Arrows indicate triple-labeled neurons. Scale bar = 50 µm [top row] and 25 µm (subsequent rows).
and saline-treated rats receiving anti-NGF antibodies, regardless of DRG cell bodies diameter.

In conclusion, our data support the hypothesis that an NGF-dependent ASIC1a overexpression in small L1 + S1 sensory colonic DRG neurons underlies the development of butyrate-induced CHS.

DISCUSSION

Irritable bowel syndrome is a visceral pain syndrome of multifactorial origin, which involves both central and peripheral mechanisms. Its pathophysiology is notably characterized by abdominal pain or discomfort in the absence of tissue injury. While colonic hypersensitivity is often proposed as a biological marker for IBS, with patients displaying lower sensory thresholds to colorectal balloon distension, studies have failed so far to find clear neuroanatomical or neuromolecular basis of these sensory alterations. At the periphery, CHS could arise from primary afferent fibers sensitization. It has been suggested that peripheral sensitization of colonic afferents might involve NGF following neuro-immune interactions between enteric neurons and mast cells. Indeed, during colonic inflammation, NGF stimulates neuropeptides expression and immune cells activity to mediate neurogenic inflammation. While no evident macroscopic signs of inflammation could be associated with IBS, recent findings suggest that low-grade inflammation could contribute to its pathophysiology, in which NGF could play a significant role.

To test the hypothesis of NGF involvement in IBS pathophysiology, we used a clinically relevant rat model of IBS, which combines CHS induced by colonic butyrate enemas and absence of macroscopic or histological modifications of colonic mucosa. We showed in this model that peripheral neutralization of NGF prevented the development of CHS through modulation of ASIC1a expression in nociceptive peptidergic neurons innervating the colon. Specifically, our data showed that NGF expression is increased in lumbosacral L1 + S1 DRG of rats with butyrate-induced non-inflammatory CHS. Peripheral neutralization of NGF with anti-NGF antibodies administered systemically, which fail to cross the brain-blood barrier, blocked this CHS, suggesting that CHS induced in our
model depends on NGF-dependent peripheral mechanisms. Nerve growth factor was previously involved in peripheral sensitization under inflammatory conditions, including the development of inflammatory CHS induced by tri-nitrobenzene sulfonic acid (TNBS). However, our study is the first to report NGF involvement in a non-inflammatory model.

Mechanisms of primary afferents sensitization mediated by NGF are notably associated to an increased membrane expression of several ion channels expressed on nociceptors, such as ASIC3 and TRPV1, which are known to contribute to inflammatory colonic pain. Accordingly, we hypothesized that these channels could be involved downstream of NGF and mediate CHS in our model. We found that blocking ASICs with amiloride improved butyrate-mediated CHS. However, administration of capsazepine, a specific TRPV1 antagonist, had no effect on CRD thresholds of butyrate-treated rats. This result suggests that TRPV1 involvement may not be relevant in non-inflammatory CHS. Due to amiloride poor specificity, molecular investigation of butyrate-mediated changes in ASICs expression was then performed and showed an overexpression of ASIC1a mRNA in L1 + S1 DRG. On the other hand, ASIC1b, ASIC2a/b and ASIC3 mRNA expression levels were not altered in our model. In contrast to the contribution of these ASIC subunits to inflammatory visceral pain, ASIC1a overexpression was mostly specific of small-diameter sensory neurons. In our model, neonatal capsaicin or CGRP antagonist injections have been shown to prevent butyrate-induced CHS development in adult rats and we observed that NGF was consistently present in DRG neurons expressing CGRP. These observations suggest that increased NGF levels in nociceptive peptidergic C-fibers leads to butyrate-induced CHS, presumably by a process involving upregulation of ASIC1a expression. Accordingly, we demonstrated that subchronic NGF treatment can induce ASIC1a protein overexpression in L1 + S1 DRG neurons and that ASIC1a protein overexpression in L1 + S1 DRG neurons of butyrate-treated animals was prevented by anti-NGF antibodies administration. These results support the view of an NGF-dependent ASIC1a modulation in butyrate-induced CHS, which may involve the activation of the NGF high-affinity receptor trkA and associated downstream signaling pathways, similarly to what has been shown in vitro for TRPV1 and the NGF-trkA-PI3K-MAPK system. Ma et al. have found that NGF can activate the NGF-p75-JNK-p38-MAPK pathway to enhance ASIC3 gene transcription in inflammatory conditions. Nerve growth factor upregulates ASIC3 expression and increases its activity, leading to an enhanced excitability of small-diameter sensory neurons. As confocal microscopy indicates a strong co-localization of NGF, trkA and ASIC1a in peptidergic CGRP-positive C-fibers, we hypothesize that a similar mechanism involving ASIC1a underlies butyrate-induced CHS development. Additional experiments are required to decipher the downstream signaling pathways in our model and to determine whether NGF acts via trkA to enhance the sensitivity of colonic afferent fibers by up-regulating the expression/activity of ASIC1a.

In summary, our data provide new insights into the pathophysiological mechanisms of IBS-like CHS in our model, indicating that NGF can sensitize colonic...
nociceptive afferents through specific ASIC1a activation in non-or low-grade inflammatory conditions. These findings identify the interplay between NGF and ASIC1a as a potential new drug targeted mechanism for IBS treatment.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Effect of capsazepine in the CRD test.

**Figure S2.** ASIC1a expression in non-colonic sensory neurons.
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