Developmental origins of colon smooth muscle dysfunction in IBS-like rats

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Li Q, Winston JH, Sarna SK. Developmental origins of colon smooth muscle dysfunction in IBS-like rats. Am J Physiol Gastrointest Liver Physiol 305: G503–G512, 2013. doi:10.1152/ajpgi.00160.2013.—Epidemiological studies show that subsets of adult and pediatric patients with irritable bowel syndrome (IBS) have prior exposures to psychological or inflammatory stress. We investigated the cellular mechanisms of colonic smooth muscle dysfunction in adult rats subjected to neonatal inflammation. Ten-day-old male rat pups received 2,4,6-trinitrobenzene sulfonic acid to induce colonic inflammation. Colonic circular smooth muscle strips were obtained 6 to 8 wk later. We found that about half of the neonate pups subjected to inflammatory insult showed a significant increase in expression of the pore-forming αC1b-subunit of Ca1.2b channels in adult life. These were the same rats in whom Vip mRNA increased in the colon muscularis externae. Additional experiments showed reduced interaction of histone deacetylase (HDAC) 3 with the HDAC3 from this region to initiate transcription. The CBP interaction with HDAC3 persisted to sustain H3K9 hyperacetylation and increase in expression of the pore-forming αC1b-subunit of Ca1.2b channels in adult life. This was the same rats in whom Vip mRNA increased in the colon muscularis externae. Additional experiments showed reduced interaction of histone deacetylase (HDAC) 3 with the HDAC3 from this region to initiate transcription. The CBP interaction with HDAC3 persisted to sustain H3K9 hyperacetylation and increase in expression of the pore-forming αC1b-subunit of Ca1.2b channels in adult life.

INFLAMMATION AND PSYCHOLOGICAL STRESS in early life are recognized risk factors for the development of complex diseases, such as hypertension, metabolic syndrome, asthma, chronic obstructive pulmonary disease, and neurological disorders, in adult life (3, 6, 18, 25, 39). Epidemiological studies in adults and children show that functional bowel disorders (FBD) also belong to the class of complex diseases; subsets of adult and pediatric patients with FBD have a history of severe enteritis, abuse, or trauma (2, 5, 9, 17, 19, 24, 28, 31, 35, 37, 67). Clinical studies have demonstrated that the pain threshold to colonic or gastric distension is lower in patients with FBD than in healthy control subjects, suggesting that the sensitization of primary afferent neurons may contribute to the symptom of pain in these patients (34, 44, 45, 55). However, the cellular and molecular mechanisms of organ dysfunction that lead to abdominal pain and motility dysfunction in patients with FBD remain largely unknown. The major obstacles are the lack of availability of living tissue from visceral organs and limitations on the application of experimental stressors to humans. In the absence of direct clinical data, preclinical studies in rodents have provided scientific evidence that colon inflammation/irritation in neonates sensitizes the primary afferent neurons by modulating the expression of select genes encoding nociceptive proteins (1, 4, 10, 11, 14, 59, 72). Animal models show also that neonatal inflammation increases the reactivity of colon smooth muscle cells to acetylcholine (ACH), accelerates colon transit, and increases defecation rate, resulting in diarrhea-like conditions in adult life (10).

Our understanding of the cellular and molecular mechanisms by which a severe adverse psychological or inflammatory insult in early life causes organ dysfunction in adult life is still evolving. During fetal and neonatal development periods, the epigenome inherited from the parents programs the expression of genes in each cell type to impart phenotype. However, this programming may change in the face of alterations in the fetal and neonatal microenvironments to protect the growing organisms. Such reprogramming may persist in adult life to cause organ dysfunction. An inflammatory insult in neonates activates the immature neuroendocrine system that triggers the epigenome to alter the expression of genes vulnerable at the time of the insult. An earlier study found that a severe, but not mild/moderate, neonatal inflammatory insult upregulates expression of the pore-forming αC1-subunit of Ca1.2b (L-type) channels (αC1b) in smooth muscle cells that enhances smooth muscle contractility (10). However, the underlying cellular and molecular mechanisms of modulation of αC1b to cause motility dysfunction remain unknown.

Vasoactive intestinal peptide (VIP) is a neurotransmitter of the myenteric inhibitory motor neurons. In addition, VIP regulates the expression of αC1b in colon smooth muscle cells (62, 63). Earlier findings show that neonatal inflammation also upregulates the expression of VIP in the colon muscularis externae at the same time that it upregulates the αC1-subunit of Ca1.2b channels (10). It remains unknown whether the neonatal inflammatory insult upregulates αC1b and VIP genes independently or it upregulates VIP, which, in turn, epigenetically upregulates αC1b in adult life. We tested the hypothesis that neonatal inflammatory insult upregulates the expression of VIP, which, in turn, epigenetically upregulates the expression of αC1b. We found that only about half of the neonates subjected to a severe inflammatory insult showed a significant increase in expression of the αC1-subunit of Ca1.2b channels in adult life. These were the same rats in whom Vip mRNA increased in the colon muscularis externae. Additional experiments showed that VIP reduced the interaction of histone deacetylase 3 (HDAC3) with αC1b promoter to increase the acetylation of histone H3 lysine 9 (H3K9) in the core promoter region to increase transcription.

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MATERIALS AND METHODS

Reagents. VIP, (Ala112228)-VIP (VPAC1 receptor agonist), and (p-Chloro-o-Phe-Leu17)-VIP (VPAC1 and VPAC2 receptor antagonists) were purchased from Bachem Americas (Torrance, CA), and sodium butyrate and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were from Sigma (St. Louis, MO).

Animals. Male Sprague Dawley rats were used in all experiments. The institutional animal care and use committee at the University of Texas Medical Branch at Galveston approved all procedures performed on animals.

Five-day-old and six-week-old male Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). For neonatal inflammatory insult, TNBS (130 mg/kg, dissolved in 200 μl saline containing 10% ethanol) was injected intraluminally 2 cm into the colon of male pups on postnatal day 10 (PND 10). The animals were kept in a head-down position while the anus was held closed for 1 min to prevent leakage. Rats in the control group received 200 μl saline. Six to eight weeks later, animals were euthanized by CO2 inhalation to obtain tissues.

Six- to eight-week-old adult male rats were used for in vivo treatment with sodium butyrate. One milliliter of sodium butyrate solution (20 mM sodium butyrate plus 134 mM sodium chloride) was given daily to each rat by intraperitoneal (i.p.) route for 5 days. Control rats received daily injection of 1 ml saline (154 mM NaCl). Animals were euthanized 3 h after the last treatment. Colonic muscle strips were collected, snap-frozen in liquid nitrogen, and stored at −80°C.

Preparation and treatment of rat colon muscularis externae. Full-thickness colon tissues were immersed in carbogenerated Krebs solution containing 10% FBS, antibiotics, and treatment reagent (20 mM sodium butyrate and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were kept in a head-down position while the anus was held closed for 1 min to prevent leakage. Rats in the control group received 200 μl saline. Six to eight weeks later, animals were euthanized by CO2 inhalation to obtain tissues.

Identification of transcription start sites of the rat Cacna1c gene. To identify the transcription start sites of the rat Cacna1c gene, 5′ rapid amplification of cDNA ends (RACE) was performed by using the 5′/3′ RACE Kit, 2nd Generation (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. The following primers were used: Cacna1c-SP1: 5′-TCT TGG GTT TCC CAT ACT GC-3′, Cacna1c-SP2: 5′-GCT GTG TGG AAC TGA CGG TA-3′, Cacna1c-SP3: 5′-CCA GCA CTG CCC ATT AAC TT-3′. The RACE products were cloned into pGEM-T vector (Promega, Madison, WI) for sequence analysis. Numbering is relative to the upstream transcription start sites (G) in exon 1b.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (36). Antibodies used for immunoprecipitation are as follows: Histone H3 acetyl Lys 4, Lys 9, Lys 14, Histone H4 acetyl Lys 5, Lys12, Lys16, HDAC3, and RNA pol II polyclonal antibodies (Active Motif, Carlsbad, CA); anti-mouse CREB-binding protein (CBP)-NT rabbit polyclonal, anti-acetyl-Histone H4 (Lys 8) rabbit antiserum (Millipore, Temecula, CA). Precipitated DNA, SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and primers specific to the α1C1b promoter were used for real-time PCR. Fold differences in precipitated DNA were normalized against input. Primers specific to the α1C1b promoter are as follows: ChIP-α1C-CREB1F: 5′-GTC GAG TAG GTG ACG AC-3′, ChIP-α1C-CREB1R: 5′-TTG GGG AGC TTA TTG GAC TG-3′ (−2731/−2596); ChIP-α1C-CREB2F: 5′-AAG GGT TTG GGC CCT ACG CA-3′, ChIP-α1C-CREB2R: 5′-GTA TAG AGC AGG GGG TGG TG-3′ (−2388/−2221); ChIP-α1C-CREB3F: 5′-TCT GCG TTC TGT AGG TG-3′, ChIP-α1C-CREB3R: 5′-TGT CTT TTA GCA AGG GTG TC-3′ (−1711/−1455); α1C-P-ChIP-2F: 5′- GTGCAAATATGGGGATCAGG-3′, α1C-P-ChIP-2R: 5′-TGGTGTTTTTCTCTGGAA-3′ (−165/+61).

Real-time RT-PCR. Total RNA was extracted by using RNeasy Mini Kit (QIAGEN, Valencia, CA). One microgram of total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Quantification of Vip and α1C1b mRNA levels by real-time PCR was performed with a StepOnePlus Thermal Cycler and TaqMan probe and primers (Applied Biosystems). 18S rRNA was quantified as internal control for the amount and quality of cDNA. All samples were assayed in triplicate in an Optical 96-well reaction plate with Optical Adhesive Covers in
a 20-μl volume containing 5 μl (2 μl for 18S rRNA) diluted cDNA (1:5 dilution in water).

Statistics. All data are expressed as means ± SE and analyzed by two-tailed Student’s t-test or by one-way ANOVA followed by Fisher post hoc analysis, considering $P < 0.05$ as significant.

RESULTS

Neonatal inflammatory insult upregulates Vip and α1C1b mRNA in colon muscularis externae. We reported previously that neonatal inflammation upregulates the pore-forming α1C1b subunit of L-type (Ca1.2b) calcium channels and VIP in the muscularis externae of rats subjected to neonatal inflammation (10). In this study, we used real-time RT-PCR to investigate correlation between the upregulation of VIP and α1C1b genes in rats subjected to neonatal inflammation. Six out of twelve rats receiving neonatal inflammatory insult showed significant elevation of α1C1b mRNA, compared with rats receiving vehicle treatment (1.4 ± 0.07 vs. 1.0 ± 0.02, $P < 0.05$) (Fig. 1A). We called these “responder” or irritable bowel syndrome-like, or “IBS-like”, rats. The remaining six rats that received neonatal inflammatory insult, but in whom the α1C1b mRNA did not differ from vehicle-treated rats, were called “nonresponder” rats (Fig. 1A). Importantly, all responder rats showed significant upregulation of Vip mRNA in colonic muscularis externae (1.4 ± 0.04 vs. 1.0 ± 0.02, $P < 0.05$). The nonresponder rats maintained normal level of Vip mRNA (Fig. 1B), suggesting that the elevation of VIP may underlie the increase of α1C1b transcription. The expression of Vip mRNA significantly correlated with the expression of α1C1b mRNA (Fig. 1C, $F = 27.7, R^2 = 0.634, p < 0.001$). Neonatal inflammation did not affect the mRNA expression of enzymes ChAT (1.2 ± 0.2 vs. 1.0 ± 0.1) and neuronal nitric oxide synthase (nNOS) (1.2 ± 0.19 vs. 1.0 ± 0.08) that respectively regulate the synthesis of ACh and NO. In addition, neonatal inflammation did not alter the expression of α1C1c1b protein in the gastric fundus muscularis externae (1.0 ± 0.08 vs. 0.92 ± 0.14 IBS-like, $n = 12$).

We investigated whether the phenomenon of responder and nonresponder rats began right after the neonatal insult or developed in adult life. We found that out of 12 rat pups receiving TNBS on PND 10, six had elevated α1C1b and Vip mRNA on PND 17, whereas the mRNA expression in the remaining six did not differ from the untreated pups (Fig. 2, A and B). Glucocorticoid overexposure in early life is a major risk factor for organ dysfunction in adult life (38, 51, 54, 73). However, we found that the blockade of glucocorticoid receptors by RU-486 from PND 10 to PND 17 did not block the upregulation of α1C1b (Fig. 2C) or Vip (Fig. 2D) on PND 17.

Identification of the transcription start sites for the rat Ca1Na1c gene. Ca1Na1c has several tissue-specific transcription start sites that lead to three major α1C isoforms: α1C1a, α1C1b, and α1C1c (7). A previous report found that α1C1b is the major isoform in human colon smooth muscle (56). To identify the transcription start sites of Ca1Na1c in colonic muscularis externae of rats and hence locate the α1C1b promoter, we performed 5’ rapid amplification of cDNA end by using 5’/3’ RACE Kit, 2nd Generation (Roche) and Ca1Na1c-specific primers (see MATERIALS AND METHODS). Sequence analysis identified two transcription start sites (CCTTTCCAGACGATTTTGGGCAAATGCAAT, TSS in bold) for exon 1b (E1b) (Fig. 3A). There is a GC-rich region (~750/−350, numbering is relative to the upstream TSS in exon 1b) but no TATA box or CCAAT box found in 5’ flanking region.

Neonatal inflammation upregulates histone H3 lysine 9 acetylation in α1C1b promoter of IBS-like rats. We reported previously that VIP induces pore-forming α1C1b-subunit expression of Ca1.2b channels by transient phosphorylation of CREB in human colonic circular smooth muscle cells (62).

![Fig. 2. Neonatal inflammation elevates α1C1b and Vip mRNA levels in postnatal day (PND) 17 rats. A: α1C1b mRNA level increased in colon muscularis externae of responder rats. B: Vip mRNA expression was augmented in responder rats. C and D: RU-486 treatment did not block the increase of Vip or α1C1b mRNA on PND 17. N = 6. *P < 0.05 vs. control.](https://doi.org/10.1152/ajpgi.00160.2013)
Phosphorylation of CREB at Ser133 is essential for recruiting CBP to drive the transcription of target genes. CBP is a histone acetyltransferase (HAT) that acetylates all four core histones in nucleosomes (47). Therefore, we hypothesized that neonatal colon inflammation upregulates the expression of Vip in the muscularis externae of the distal colon. VIP, in turn, enhances transcription of the gene encoding the α1C-subunit of Ca2+ channels (α1C1b) by epigenetic modifications to induce colonic motor dysfunction in adult life.

Based on our newly identified transcription start sites of α1C1b in rats, MatInspector software (Genomatrix, Germany) found four putative CREB-binding motifs in the rat α1C1b promoter (−2,727/−2,707, −2,354/−2,334, −1,659/−1,652, and −1,541/−1,534) (Fig. 3A). We designed four sets of primers, one each for binding sites 1 (−2,731/−2,596) and 2 (−2,388/−2,231), one for binding sites 3 and 4 together (−1,659/−1,534), and one for the core promoter region (−165/+61) (Fig. 3A). Preliminary screening by ChIP followed by conventional PCR showed that CBP predominantly binds to CREB site 1 (−2,731/−2,596), whereas RNA polymerase II (RNAP II) binds to the core promoter (−165/+61). Therefore, we examined CBP association in −2,731/−2,596 region and histone acetylation, HDAC binding, and RNAP II interaction at −165/+61 region of α1C1b promoter to decipher epigenetic modifications. We used ChIP followed by conventional (for screening) and real-time PCR (qPCR) assays to examine acetylation status at histone H3 lysine residues 4, 9, 14, and 18 and histone H4 lysine residues 5, 8, 12, and 16 in the α1C1b promoter in colon muscularis externae of control and IBS-like rats (Fig. 3B). ChIP-qPCR showed that neonatal inflammation significantly upregulated H3K9 acetylation at the α1C1b core promoter region of IBS-like vs. control rats (1.7 ± 0.3 vs. 1.0 ± 0.2 control, P < 0.05) (Fig. 3C). No difference was observed between nonresponder and control rats (data not shown). No significant effect was observed at any of the other lysine residues of H3 and H4 histones examined at the CBP binding site or the core promoter region (Fig. 3B). Acetylation of histone H3 lysine 9 generally associates with unfolding of chromatin and transcription initiation, thereby positively influencing gene expression (70). The remaining experiments focused on H3K9 acetylation in the core promoter region.

Neonatal inflammation suppresses HDAC3 interaction with the α1C1b promoter. Histone acetylation is catalyzed by HAT and reversed by HDAC (70). To identify specific HAT and HDACs that might be responsible for increased H3K9 acetylation at the α1C1b core promoter, we performed ChIP-qPCR assay by using antibodies against CBP, HDAC3, and RNAP II. CBP interaction with the α1C1b promoter was not modulated by neonatal inflammatory insult (Fig. 4A), but HDAC3 association with the α1C1b promoter was markedly suppressed (1.0 ± 0.2 vs. 0.15 ± 0.21, P < 0.05) (Fig. 4B), suggesting that HDAC3 dissociation from the α1C1b promoter may play an important role in upregulating histone H3K9 acetylation. We also looked at HDAC1 and HDAC4 interaction with the α1C1b promoter with no significant change (Fig. 4C).

Fig. 3. Histones H3 and H4 acetylation around the α1C1b promoter in colon muscularis externae of responder rats. A: schematic of the rat α1C1b promoter with CREB-binding sites. Arrows indicate transcription start sites (TSS). B: acetylation of histones H3 and H4 at the α1C1b promoter in colon muscularis externae of control and irritable bowel-syndrome (IBS)-like rats. Immunoprecipitated DNA by indicated antibodies was amplified by PCR, separated in 2% agarose gel, and visualized by ethidium bromide. Among 8 lysine residues of histones H3 and H4 examined, only H3K9 acetylation was enhanced. C: chromatin immunoprecipitation (ChIP)-qPCR quantification shows that H3K9Ac at the α1C1b core promoter region (−165/+61) was significantly upregulated in colon muscularis externae of IBS rats compared with that of control rats. IgG was used for mock immunoprecipitation (IP). Fold change was normalized against input. N = 3. *P < 0.05 vs. control.
core promoter, and no changes were observed (data not shown). RNA polymerase II binding to the α1C1b core promoter was significantly elevated (Fig. 4C), indicating α1C1b gene activation due to relaxed chromatin resulting from H3K9 hyperacetylation.

**Time course of VIP modulation of CBP and HDAC3 association with the rat α1C1b promoter in colon muscularis externae.** Because VIP and α1C1b genes were concurrently upregulated in IBS-like rats, the key question is whether VIP is responsible for the epigenetic modifications of the α1C1b promoter in these rats. To address this question, we incubated rat colon muscularis externae with 100 nM VIP for 15 min or 24 h and assessed epigenetic modifications of the α1C1b promoter. VIP treatment for 15 min rapidly recruited a large amount of CBP to the CREB-binding site of α1C1b (Fig. 5A). At the same time, HDAC3 was markedly dissociated (Fig. 5B), but histone H3K9 acetylation (Fig. 5C) and RNAP II association (Fig. 5D) with the core promoter region were not significantly altered at this time. CBP interaction with the CREB-binding site was back to the constitutive level after 1 h of VIP treatment (data not shown). After 24-h incubation, CBP association with the CREB-binding site stayed at the constitutive level (Fig. 6A), but HDAC3 dissociation from the α1C1b promoter persisted (Fig. 6B). H3K9 acetylation was significantly increased (Fig. 6C), and RNAP II association was markedly upregulated after 24-h treatment with VIP (Fig. 6D). Apparently, rapid and brief CBP interaction with the α1C1b promoter set the stage for histone modifications by dissociation of HDAC3 from the core promoter region.

We also found that (Ala11,22,28)-VIP, a selective VPAC1 receptor agonist, significantly suppressed HDAC3 binding and upregulated H3K9Ac and RNAP II association with the α1C1b promoter in rat colon muscularis externae (Fig. 7A). In addition, the increase of H3K9Ac and RNAP II and decrease of HDAC3 interaction with the α1C1b promoter were almost completely blocked by (p-Chloro-d-Phe6,Leu17)-VIP, a specific VIP receptor antagonist (Fig. 7B), underscoring the role of VIP in epigenetic modifications at the α1C1b promoter.

**In vivo butyrate treatment significantly augments α1C1b gene expression in rat colonic muscularis externae.** Finally, we treated naïve rats with 20 mM sodium butyrate (daily i.p. injection for 5 days), a well-known natural HDAC inhibitor, to suppress HDAC activity and examined histone modifications at the α1C1b promoter and α1C1b mRNA level in colonic muscularis externae.
Butyrate treatment significantly upregulated α1C/1b mRNA level (Fig. 8A). ChIP-qPCR results showed that H3K9Ac and RNAPII were significantly elevated and HDAC3 binding was significantly downregulated at the α1C/1b core promoter (Fig. 8B).

**DISCUSSION**

Our present findings, along with those of others (1, 4, 10, 11, 14, 59, 72), show that a severe nonspecific inflammatory insult to the distal colon during neonatal development may be a common etiology for the concurrent sensitization of primary afferent neurons and increase of smooth muscle reactivity to ACh in adult life. It is noteworthy that the adult-life maladaptive effects of adverse events during pre- and postnatal development are complex; they depend on the type of stressor (e.g., psychological, inflammatory, or chemical), the time of application of the stressor (various stages of fetal and neonatal development periods), and the intensity of stressor (20, 27, 53). For example, mild/moderate TNBS insult to the distal colon applied on PND 10 in rat pups did not cause smooth muscle dysfunction in adult life, but severe inflammatory insult did (10). Clinical studies also noted a correlation between the severity of IBS symptoms and the severity of abuse/trauma in early life (19, 37).

About 25% of adult subjects with severe enteric infections go on to develop lasting symptoms of IBS. The severity and duration of infection and concurrent psychological disorder or recent trauma are risk factors for the development of postinfectious IBS (26, 41, 43, 46, 65). These cofactors were not required for organ dysfunction in adult life when the inflammatory insult occurred in neonatal rats. The persistent changes in organ function in response to early-life stress occur largely by epigenetic programming during cellular differentiation in the neuroendocrine and immune systems in early life (25, 27). However, only half of the adult rats subjected to identical inflammatory neonatal insult developed smooth muscle dysfunction. The reasons for differential outcome to neonatal insult remain unknown although genetic factors or reversal of epigenetic marks by environmental factors, such as nutrition and postnatal maternal care, are likely to contribute to this phenomenon (25, 42, 71). It is noteworthy that the adult-life maladaptive effects of adverse events during pre- and postnatal development are complex; they depend on the type of stressor (e.g., psychological, inflammatory, or chemical), the time of application of the stressor (various stages of fetal and neonatal development periods), and the intensity of stressor (20, 27, 53). For example, mild/moderate TNBS insult to the distal colon applied on PND 10 in rat pups did not cause smooth muscle dysfunction in adult life, but severe inflammatory insult did (10). Clinical studies also noted a correlation between the severity of IBS symptoms and the severity of abuse/trauma in early life (19, 37).

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An inopportune increase of glucocorticoids during pre- and postnatal development has been identified as a key factor for the development of neuroendocrine dysfunction in adult life (38, 51, 54, 60, 69, 73). A recent study found that the inopportune increase of corticosterone on PND 15 in rats sensitizes postnatal development has been identified as a key factor for the development of neuroendocrine dysfunction in adult life (73). However, an increase of corticosterone was not responsible for the gastric-responsive primary afferents in adult life (73).

Our findings show that neonatal inflammatory insult upregulates $\alpha_{1C}1b$ mRNA expression. By contrast, several other complex diseases following early-life stress appear after maturity or may express following another severely stressful challenge (25).

Our findings show that neonatal inflammatory insult upregulates $\alpha_{1C}1b$ mRNA expression indirectly by upregulating VIP expressed in enteric neurons (23). The precise reasons as to why the epigenetic mechanisms in this case targeted a gene expressed in the enteric neurons rather than the one expressed in colon smooth muscle cells remain unknown. Human and animal studies show that the central neurons are highly susceptible to early-life adverse events resulting in neuropsychiatric disorders, including schizophrenia (40), attention deficit hyperactivity disorder (66), and major depression (15, 30, 51). Neonatal inflammation also modulates the expression of genes encoding nociceptive proteins and ion channels in primary afferent and spinal cord neurons (4, 11, 52, 72, 73). Our findings show that neonatal inflammation also targets the enteric neurons to upregulate VIP expression. It is noteworthy that VIP levels are elevated also in the plasma and mucosal biopsy tissue of patients with IBS (48).

ChIP assay showed that VIP upregulates expression of the $\alpha_{1C}$-subunit by concurrently phosphorylating CREB associated with the $\alpha_{1C}1b$ promoter and dissociating HDAC3 from the core promoter region (Fig. 9). CREB phosphorylation was transient, lasting for less than an hour. Nevertheless, it is essential to trigger transcription; the blockade of protein kinase

Fig. 9. Cartoon summarizing epigenetic modulation of $\alpha_{1C}1b$ promoter by increase of VIP in rats subjected to an inflammatory insult as neonates and its effect on smooth muscle contractility. VIP transiently phosphorylated CREB to recruit histone acetyltransferase (CBP) to $\alpha_{1C}1b$ promoter to induce histone H3K9 acetylation and enhance transcription of $Ca_{1.2}b$. VIP concurrently dissociated HDAC3 from the core promoter region of $\alpha_{1C}1b$ promoter to maintain H3K9 hyperacetylation that sustained the increase in $\alpha_{1C}1b$ expression, which resulted in increase of Ca$^{2+}$ influx to enhance smooth muscle contraction (62). Sequence of arrows represent multiple intermediate steps in the phosphorylation of CREB by VIP. TBP, TATA-binding protein; TFII, transcription factor IIIB; Pol II, RNA polymerase II.
A by H-89 prevents the transcription of α1C1b (62). By contrast, the dissociation of HDAC3 persists for at least 24 h after exposure to VIP. HDAC3 dissociation from the core promoter allowed increase in acetylation of H3K9 to relax the chromatin and allow greater access of transcription factors and RNAPII to the core promoter region that enhanced transcription.

Sodium butyrate is a short-chain fatty acid produced by the fermentation of undigested carbohydrates in the colon. Butyrate has multiple functions in the colon, including homeostasis, trophic, and anticancer effects (12, 32, 68). The action of butyrate as an HDAC inhibitor mediates several of the above roles. We found that intraperitoneal administration of butyrate to naïve adult rats mimicked the epigenetic modulation of α1C1b promoter by neonatal inflammation or VIP treatment. Another study found that in vivo or in vitro treatment of myenteric neurons with butyrate significantly increased the proportion of ChAT-immunoreactive neurons, without affecting the nNOS-reactive neurons (64). The increase of ChAT-immunoreactive neurons increased the ACh content of the colon. Taken together, the increased ACh release and increase of smooth muscle reactivity to ACh would enhance colonic motor function to accelerate colonic transit. Indeed, studies in humans and animals found that intraluminal administration of short-chain fatty acids stimulates the ultrapropulsive giant migrating contractions to induce the sensation of urgency in human subjects and faster colonic transit in rats (22, 29). Giant migrating contractions are ultrapropulsive contractions that cause mass movements (33, 61). It is noteworthy that the amplitude and frequency of giant migrating contractions are increased in patients with diarrhea-predominant IBS (8). Taken together, the above findings suggest butyrate treatment as a potential therapeutic option to accelerate colon transit in patients with constipation.

In conclusion, our findings show that a severe inflammatory insult in early life causes smooth muscle dysfunction that starts within 1 wk of insult and persists in adult life. Such dysfunc-
tion occurs in half the rats subjected to the same insult. In responder rats, the inflammatory insult upregulated the expression of VIP in the muscularis externae, which, in turn, upregulated expression of the pore-forming α1C-subunit of Cav1.2b channels in smooth muscle cells. VIP treatment phosphorylated CREB, which recruited HAT (CBP) to the α1C1b promoter to initiate transcription process. At the same time, VIP dissociated HDAC3 from the core promoter region of α1C1b promoter. The phosphorylation of CREB was transient, but the dissociation of HDAC3 from α1C1b persisted to sustain the increase in transcription. The dissociation of HDAC3 hyper-acetylated H3K9 in the core promoter region. Intraperitoneal treatment of adult naïve rats with butyrate mimicked the effects of neonatal colon inflammation. It appears that a severe inflammatory insult in early life might be a common etiology of hypersensitization of primary afferent neurons and smooth muscle dysfunction, which respectively contribute to the symptoms of abdominal pain and altered bowel dysfunction in patients with diarrhea-predominant IBS. IBS is a complex disease. Our findings relate to diarrhea-predominant IBS. It remains unknown whether an inflammatory or psychological stressor at a different stage of antenatal development would mimic colonic motor dysfunction in patients with constipation-predominant IBS.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: Q.L. and J.H.W. performed experiments; Q.L. and J.H.W. analyzed data; Q.L. and S.K.S. interpreted results of experiments; Q.L. prepared figures; Q.L. drafted manuscript; S.K.S. concept and design of research; S.K.S. edited and revised manuscript; S.K.S. approved final version of manuscript.

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