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. First published July 25, 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 α1C-subunit of Cav1.2b 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 promotes gene transcription. Inflammatory insult 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 that neonatal inflammation increases the reactivity of colon smooth muscle cells to acetylcholine (ACh), accelerates colonic 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 α1C-subunit of Cav1.2b (L-type) channels (α1C/lb) in smooth muscle cells that enhances smooth muscle contractility (10). However, the underlying cellular and molecular mechanisms of modulation of α1C/lb 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 α1C/lb in colonic 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 α1C-subunit of Cav1.2b channels (10). It remains unknown whether the neonatal inflammatory insult upregulates α1C/lb and Vip genes independently or if it upregulates Vip, which, in turn, epigenetically upregulates α1C/lb in adult life. We tested the hypothesis that neonatal inflammatory insult upregulates the expression of Vip, which, in turn, epigenetically upregulates the expression of α1C/lb. We found that only about half of the neonates subjected to a severe inflammatory insult showed a significant increase in expression of the α1C-subunit of Cav1.2b 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 α1C/lb promoter to increase the acetylation of histone H3 lysine 9 (H3K9) in the core promoter region to increase transcription.

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 colon 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 that neonatal inflammation increases the reactivity of colon smooth muscle cells to ACh, accelerates colonic transit, and increases defecation rate, resulting in diarrhea-like conditions in adult life (10).
MATERIALS AND METHODS

Reagents. VIP, (Ala11,22,28)-VIP (VPAC1 receptor agonist), and (p-Chloro-D-Phe4,Leu7)-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 carbogenated Krebs solution with 5% O2-95% CO2 mix (36). After removal of the mucosal/submucosal layers, the remaining muscularis externae were cut into small pieces and placed in high glucose DMEM (HyClone, South Logan, UT) containing 10% FBS, antibiotics, and treatment reagent 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′-GCG ATG GAG TAG GTG AGC AC-3′, ChIP-α1C-CREB1R: 5′-TTG GGG AGC TTA TTG GAC TC-3′ (−2731 to −2596); ChIP-α1C-CREB2F: 5′-AAG GTT TTG GCT CTC AAG CA-3′, ChIP-α1C-CREB2R: 5′-GTA TAG AGC AGG GGC TGG TC-3′ (−2388 to −2221); ChIP-α1C-CREB3F: 5′-TCT GCC TTC TGT AGG TTG-3′, ChIP-α1C-CREB3R: 5′-TGT CTT TAA GCA AGG GTG TC-3′ (−1711 to −1455); α1C-P-ChIP-2F: 5′- GTGCAATATCGGAGATCAGG-3′, α1C-P-ChIP-2R: 5′-TGTTGGTTTTCCTCTGGAA-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

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Fig. 1. Colon inflammatory insult in neonate rats upregulates α1C1b and Vip mRNA levels. In responder rats (A) α1C1b mRNA was upregulated in colon muscularis externae of 6 out of 12 receiving neonatal inflammatory insult. mRNA was quantitated by real-time RT-PCR. 18S rRNA served as internal control. B: Vip mRNA expression was elevated in colon muscularis externae of responder rats but not in nonresponder rats. C: positive correlation between α1C1b and Vip mRNA levels in colon muscularis externae. F = 27.7; R² = 0.634; N = 6. *P < 0.05 vs. control (Ctr.).
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 expression of the pore-forming α₁c1b subunit of L-type (Ca,1.2b) 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, R² = 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 α₁c1b protein in the gastric fundus muscularis externa (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 Caacna1c gene. Caacna1c 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 Caacna1c 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 Caacna1c-specific primers (see MATERIALS AND METHODS). Sequence analysis identified two transcription start sites (CCTTTCCAAAGCAATTTTGCCAAATGTTCAAT, 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 Ca,1.2b 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 a1c1b mRNA on PND 17. N = 6. *P < 0.05 vs. control.
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. 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 α1C1b subunit of Ca_{1.2}b 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: −1,651 to −1,455, one for the core promoter region; −2,388 to −2,231, one for binding sites 3 and 4 together; and −2,731 to −2,596, two for binding sites 1 and 2. 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. 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 RNAPII. CBP interaction with the α1C1b promoter was marked by HDAC3 dissociation from the α1C1b promoter and reversed by HDAC3 association with the α1C1b promoter (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.
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.

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**Fig. 4.** CREB-binding protein (CBP), histone deacetylase 3 (HDAC3), and RNA polymerase II (RNAP II) interaction with the CREB-binding site (−2.731/−2.596) or core promoter (−165/+61) of α1C1b gene in colon muscularis externae of control and IBS-like rats. A: CBP interaction with the α1C1b promoter was not altered in IBS-like rats. B: HDAC3 association with the α1C1b core promoter was dramatically suppressed in IBS-like rats. C: RNAP II binding to the α1C1b core promoter was upregulated in colon muscularis externae of IBS-like rats. ChIP-qPCR was used to quantify precipitated DNA. Fold change was normalized against input. N = 3. *P < 0.05 vs. control.

**Fig. 5.** 15-min vasoactive intestinal peptide (VIP) treatment rapidly recruits CBP to the α1C1b promoter. Colon muscle strips from naïve rats were incubated with 100 nM VIP for 15 min. ChIP-qPCR assays were performed to assess CBP, H3K9Ac, HDAC3, and RNAP II binding at the α1C1b promoter. A: CBP association with the CREB site (−2.731/−2.596) of α1C1b promoter was markedly upregulated by 15-min VIP treatment. B: H3K9Ac at the α1C1b core promoter (−165/+61) was not altered in colon muscularis externae after incubation with VIP for 15 min. C: HDAC3 binding was downregulated by VIP treatment for 15 min. D: RNAP II interaction with the α1C1b promoter remained unchanged after VIP treatment for 15 min. N = 3. *P < 0.05 vs. control.
Butyrate treatment significantly upregulated \( \alpha_{1C}lb \) mRNA level (Fig. 8A). ChIP-qPCR results showed that H3K9Ac and RNAP II were significantly elevated and HDAC3 binding was significantly downregulated at the \( \alpha_{1C}lb \) 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 (various stages of fetal and neonatal development periods), and the intensity of stressor (20, 27, 53).
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 during pre- and 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 upregulation of αC1β mRNA expression on PND 17.

Childhood infections resulting in diarrhea are prevalent (16). The annual episodes of diarrhea in US children under 5 yr of age range from 20 to 35 million; 220,000 of these infections are severe enough to result in hospitalization (49, 50). The development of functional bowel disorders in infants does not appear to be specific to any single type of bacterial or viral infection; rotavirus-, campylobacter-, salmonella-, and shigella-infected children were included among those who developed IBS or FBD (13, 21, 58). A recent study identified cow’s milk allergy as a risk factor for the development of functional bowel disorders in children (57). Together, these findings 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 inflammatory insult upregulates αC1β 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 αC1C-subunit by concurrently phosphorylating CREB associated with the αC1β 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. 8. Sodium butyrate (SB) enhances αC1b gene transcription by modulating epigenetic modifications at the αC1b promoter. 6-wk-old naïve rats were given daily i.p. injection of 20 mM SB in saline for 5 days. Control rats received saline. Colon muscularis externae were collected 3 h after the last injection, snap-frozen in liquid nitrogen, smashed, and processed for real-time RT-PCR analysis of mRNA expression and ChIP-qPCR analysis of protein-DNA interaction. A: butyrate increased αC1b mRNA expression. B: butyrate upregulated H3K9Ac and RNAP II but decreased HDAC3 binding to the αC1b promoter. N = 4. *P < 0.05 vs. control.

Fig. 9. Cartoon summarizing epigenetic modulation of αC1b 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 αC1b promoter to induce histone H3K9 acetylation and enhance transcription of Ca.1.2b. VIP concurrently dissociated 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
A by H-89 prevents the transcription of $\alpha_{1C}lb$ (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 RNAP II 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 $\alpha_{1C}lb$ 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 increase of 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 dysfunction 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 $\alpha_{1C}$-subunit of Ca$_{\text{L,2b}}$ channels in smooth muscle cells. VIP treatment phosphorylated CREB, which recruited HAT (CBP) to the $\alpha_{1C}lb$ promoter to initiate transcription process. At the same time, VIP dissociated HDAC3 from the core promoter region of $\alpha_{1C}lb$ promoter. The phosphorylation of CREB was transient, but the dissociation of HDAC3 from $\alpha_{1C}lb$ persisted to sustain the increase in transcription. The dissociation of HDAC3 hyperacetylated 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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