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

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[1]*Enteric Neuromuscular Disorders and Visceral Pain Center, Division of Gastroenterology, Department of Internal Medicine, and* [2]*Department of Neuroscience and Cell Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas*

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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 α_{1C}-subunit of *Ca*$_v$*1.2b* 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 $\alpha_{1C}1b$ promoter that increased the acetylation of histone H3 lysine 9 (H3K9) in the core promoter region. Vasoactive intestinal peptide (VIP) treatment of naïve muscularis externae swiftly recruited CREB-binding protein (CBP) to the $\alpha_{1C}1b$ promoter and dissociated HDAC3 from this region to initiate transcription. The CBP interaction with the $\alpha_{1C}1b$ promoter was transient, but the dissociation of HDAC3 persisted to sustain H3K9 hyperacetylation and increase in transcription. Intraperitoneal treatment of adult naïve rats with butyrate mimicked the effects of neonatal colon inflammation. We concluded that neonatal inflammation upregulates VIP in the colon muscularis externae, which modulates epigenetic events at the $\alpha_{1C}1b$ promoter to activate $\alpha_{1C}1b$ gene transcription. Inflammatory insult in early life may be one of the etiologies of smooth muscle dysfunction in adult life, which contributes to the altered motility function in patients with diarrhea-predominant IBS.

cell signaling; motility; smooth muscle

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 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 α_{1C}-subunit of Ca$_v$1.2b (L-type) channels ($\alpha_{1C}1b$) in smooth muscle cells that enhances smooth muscle contractility (10). However, the underlying cellular and molecular mechanisms of modulation of $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ 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 α_{1C}-subunit of Ca$_v$1.2b channels (10). It remains unknown whether the neonatal inflammatory insult upregulates $\alpha_{1C}1b$ and *Vip* genes independently or it upregulates *Vip*, which, in turn, epigenetically upregulates $\alpha_{1C}1b$ in adult life. We tested the hypothesis that neonatal inflammatory insult upregulates the expression of *Vip*, which, in turn, epigenetically upregulates the expression of $\alpha_{1C}1b$. We found that only about half of the neonates subjected to a severe inflammatory insult showed a significant increase in expression of the α_{1C}-subunit of Ca$_v$1.2b 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 $\alpha_{1C}1b$ 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, (Ala[11,22,28])-VIP (VPAC1 receptor agonist), and (p-Chloro-D-Phe[6],Leu[17])-VIP (VPAC1 and VPAC2 receptor antagonist) 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 (Indiana, 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 CO_2 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% O_2-95% CO_2 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 and were incubated in a 37°C CO_2 incubator. All treated tissues were snap-frozen in liquid nitrogen and stored at −80°C in a freezer.

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 H3 (Lys18), and 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 α_{1C}*1b* promoter were used for real-time PCR. Fold differences in precipitated DNA were normalized against input. Primers specific to the α_{1C}*1b* promoter are as follows: ChIP-α_{1C}-CREB1F: 5′-GGC ATG GAG TAG GTG ACG AC-3′, ChIP-a1c-CREB1R: 5′-TTG GGG AGC TTA TTG GAC TG-3′ (−2731/−2596); ChIP-α_{1C}-CREB2F: 5′-AAG GTT TTG GGT CTC AAG CA-3′, ChIP-α_{1C}-CREB2R, 5′-GTA TAG AGC AGG GGC TGG TG-3′ (−2388/−2231); ChIP-α_{1C}-CREB3F: 5′-TCT GCG TTC TGT CTG AGG TG-3′, ChIP-α_{1C}-CREB3R: 5′-TGG CTT TAA GCA AGG GTG TC-3′ (−1711/−1455); α_{1C}P-ChIP-2F: 5′- GTGCAATAATGGGGAT-CAGG-3′, α_{1C}P-ChIP-2R: 5′-TGGTGGTTTTCCTCTGGAAC-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 α_{1C}*1b* 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

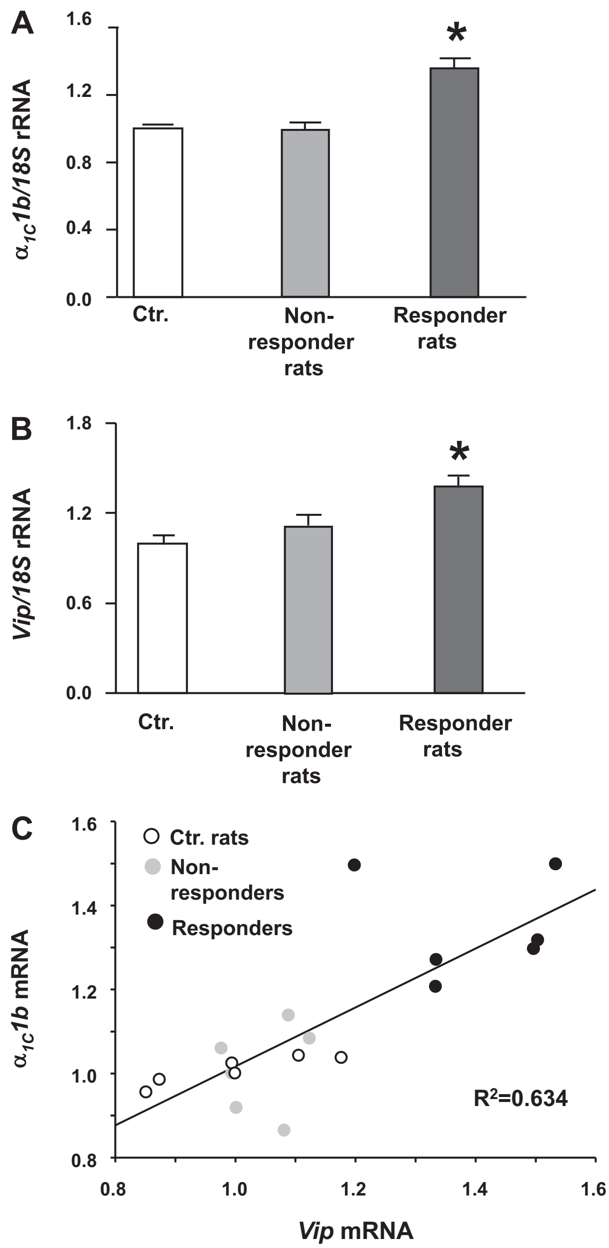

Fig. 1. Colon inflammatory insult in neonate rats upregulates α_{1C}*1b* and *Vip* mRNA levels. In responder rats (*A*) α_{1C}*1b* mRNA was upregulated in colon muscularis externae of 6 out of 12 receiving neonatal inflammatory insult. mRNA was quantitated by real-time RT-PCR; 18S rRNA served as internal control. *B*: *Vip* mRNA expression was elevated in colonic muscularis externae of responder rats but not in nonresponder rats. *C*: positive correlation between α_{1C}*1b* and *Vip* mRNA levels in colon muscularis externae. $F = 27.7$; $R^2 = 0.634$; $N = 6$. *$P < 0.05$ 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 $\alpha_{1C}1b$ mRNA in colon muscularis externae. We reported previously that neonatal inflammation upregulates expression of the pore-forming α_{1C}1b subunit of L-type ($Ca_v1.2b$) 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 $\alpha_{1C}1b$ genes in rats subjected to neonatal inflammation. Six out of twelve rats receiving neonatal inflammatory insult showed significant elevation of $\alpha_{1C}1b$ mRNA, compared with rats receiving vehicle treatment (1.4 ± 0.07 vs. 1.0 ± 0.02, $P <$ 0.05) (Fig. 1*A*). 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 $\alpha_{1C}1b$ mRNA did not differ from vehicle-treated rats, were called "nonresponder" rats (Fig. 1*A*). 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. 1*B*), suggesting that the elevation of VIP may underlie the increase of $\alpha_{1C}1b$ transcription. The expression of *Vip* mRNA significantly correlated with the expression of $\alpha_{1C}1b$ mRNA (Fig. 1*C*, $F = 27.7$, $R^2 = 0.634$, $p < 0.001$). 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 α_{1C}1b 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 $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ (Fig. 2*C*) or *Vip* (Fig. 2*D*) on PND 17.

Identification of the transcription start sites for the rat Cacna1c gene. Cacna1c has several tissue-specific transcription start sites that lead to three major α_{1C} isoforms: α_{1C}1a, α_{1C}1b, and α_{1C}1c (7). A previous report found that α_{1C}1b is the major isoform in human colon smooth muscle (56). To identify the transcription start sites of *Cacna1c* in colonic muscularis externae of rats and hence locate the $\alpha_{1C}1b$ promoter, we performed 5′ rapid amplification of cDNA end by using 5′/3′ RACE Kit, 2nd Generation (Roche) and *Cacna1c*-specific primers (see MATERIALS AND METHODS). Sequence analysis identified two transcription start sites (CCTTTC-CAA**G**CAGTTTTT**G**GCCAATGGTCAAT, TSS in bold) for exon 1b (E1b) (Fig. 3*A*). 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 $\alpha_{1C}1b$ promoter of IBS-like rats. We reported previously that VIP induces pore-forming α_{1C}-subunit expression of $Ca_v1.2b$ channels by transient phosphorylation of CREB in human colonic circular smooth muscle cells (62).

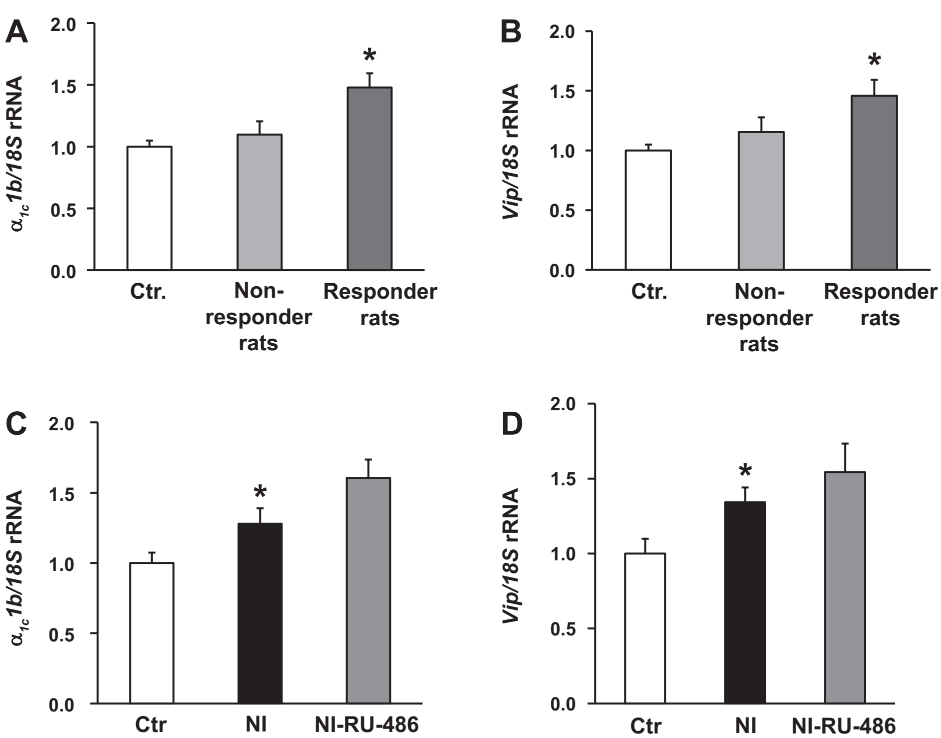

Fig. 2. Neonatal inflammation elevates $\alpha_{1C}1b$ and *Vip* mRNA levels in postnatal day (PND) 17 rats. *A*: $\alpha_{1C}1b$ mRNA level increased in colon muscularis externae of responder rats. *B*: *Vip* mRNA expression was augmented in responder rats. *C* and *D*: RU-486 treatment did not block the increase of *Vip* or $\alpha_{1C}1b$ mRNA on PND 17. $N = 6$. *$P < 0.05$ vs. control.

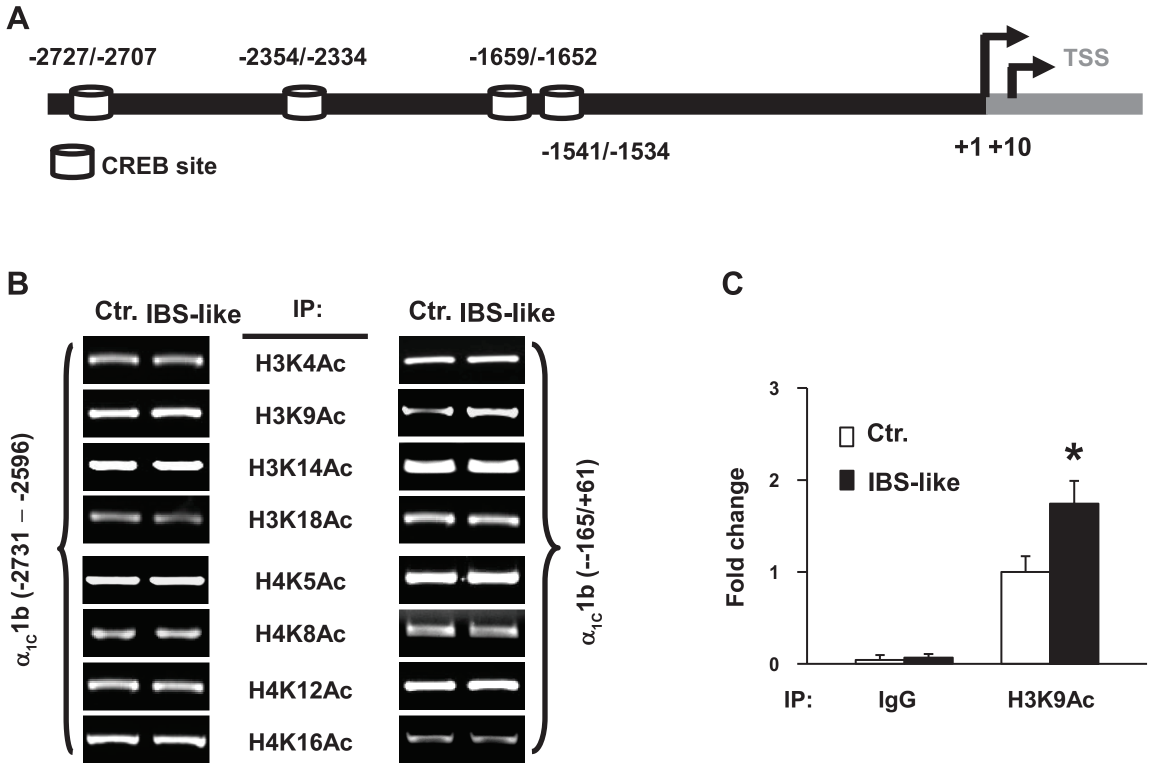

Fig. 3. Histones H3 and H4 acetylation around the $\alpha_{1C}1b$ promoter in colon muscularis externae of responder rats. *A*: schematic of the rat $\alpha_{1C}1b$ promoter with CREB-binding sites. Arrows indicate transcription start sites (TSS). *B*: acetylation of histones H3 and H4 at the $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ 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.

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 α_{1C}-subunit of $Ca_v1.2b$ channels ($\alpha_{1C}1b$) by epigenetic modifications to induce colonic motor dysfunction in adult life.

Based on our newly identified transcription start sites of $\alpha_{1C}1b$ in rats, MatInspector software (Genomatrix, Germany) found four putative CREB-binding motifs in the rat $\alpha_{1C}1b$ promoter (−2,727/−2,707, −2,354/−2,334, −1,659/−1,652, and −1,541/−1,534) (Fig. 3*A*). 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 (−1,711/−1,455), and one for the core promoter region (−165/+61) (Fig. 3*A*). Preliminary screening by ChIP followed by conventional PCR showed that CBP predominately 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 $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ promoter in colon muscularis externae of control and IBS-like rats (Fig. 3*B*). ChIP-qPCR showed that neonatal inflammation significantly upregulated H3K9 acetylation at the $\alpha_{1C}1b$ core promoter region of IBS-like vs. control rats (1.7 ± 0.3 vs. 1.0 ± 0.2 control, $P < 0.05$) (Fig. 3*C*). 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. 3*B*). 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 $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ core promoter, we performed ChIP-qPCR assay by using antibodies against CBP, HDAC3, and RNAP II. CBP interaction with the $\alpha_{1C}1b$ promoter was not modulated by neonatal inflammatory insult (Fig. 4*A*), but HDAC3 association with the $\alpha_{1C}1b$ promoter was markedly suppressed (1.0 ± 0.2 vs. 0.15 ± 0.21, $P < 0.05$) (Fig. 4*B*), suggesting that HDAC3 dissociation from the $\alpha_{1C}1b$ promoter may play an important role in upregulating histone H3K9 acetylation. We also looked at HDAC1 and HDAC4 interaction with the $\alpha_{1C}1b$

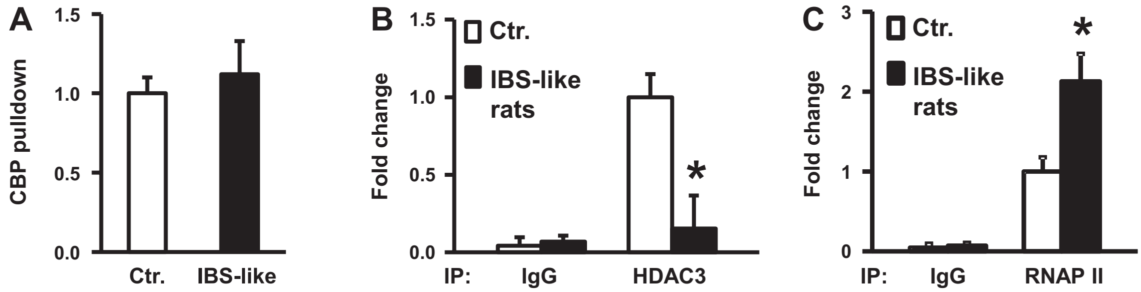

Fig. 4. CREB-binding protein (CBP), histone deacetylase 3 (HDAC3), and RNA polymerase II (RNAP II) interaction with the CREB-binding site (−2,731/−2,596) or core promoter (−165/+61) of $\alpha_{1C}1b$ gene in colon muscularis externae of control and IBS-like rats. *A*: CBP interaction with the $\alpha_{1C}1b$ promoter was not altered in IBS-like rats. *B*: HDAC3 association with the $\alpha_{1C}1b$ core promoter was dramatically suppressed in IBS-like rats. *C*: RNAP II binding to the $\alpha_{1C}1b$ 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.

core promoter, and no changes were observed (data not shown). RNA polymerase II binding to the $\alpha_{1C}1b$ core promoter was significantly elevated (Fig. 4*C*), indicating $\alpha_{1C}1b$ gene activation due to relaxed chromatin resulting from H3K9 hyperacetylation.

Time course of VIP modulation of CBP and HDAC3 association with the rat $\alpha_{1C}1b$ promoter in colon muscularis externae. Because *Vip* and $\alpha_{1C}1b$ genes were concurrently upregulated in IBS-like rats, the key question is whether VIP is responsible for the epigenetic modifications of the $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ promoter. VIP treatment for 15 min rapidly recruited a large amount of CBP to the CREB-binding site of $\alpha_{1C}1b$ (Fig. 5*A*). At the same time, HDAC3 was markedly dissociated (Fig. 5*B*), but histone H3K9 acetylation (Fig. 5*C*) and RNAP II association (Fig. 5*D*) 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. 6*A*), but HDAC3 dissociation from the $\alpha_{1C}1b$ promoter persisted (Fig. 6*B*). H3K9 acetylation was significantly increased (Fig. 6*C*), and RNAP II association was markedly upregulated after 24-h treatment with VIP (Fig. 6*D*). Apparently, rapid and brief CBP interaction with the $\alpha_{1C}1b$ promoter set the stage for histone modifications by dissociation of HDAC3 from the core promoter region.

We also found that (Ala[11,22,28])-VIP, a selective VPAC1 receptor agonist, significantly suppressed HDAC3 binding and upregulated H3K9Ac and RNAP II association with the $\alpha_{1C}1b$ promoter in rat colon muscularis externae (Fig. 7*A*). In addition, the increase of H3K9Ac and RNAP II and decrease of HDAC3 interaction with the $\alpha_{1C}1b$ promoter were almost completely blocked by (p-Chloro-D-Phe^6,Leu^{17})-VIP, a specific VIP receptor antagonist (Fig. 7*B*), underscoring the role of VIP in epigenetic modifications at the $\alpha_{1C}1b$ promoter.

In vivo butyrate treatment significantly augments $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ promoter and $\alpha_{1C}1b$ mRNA level in colonic muscularis externae.

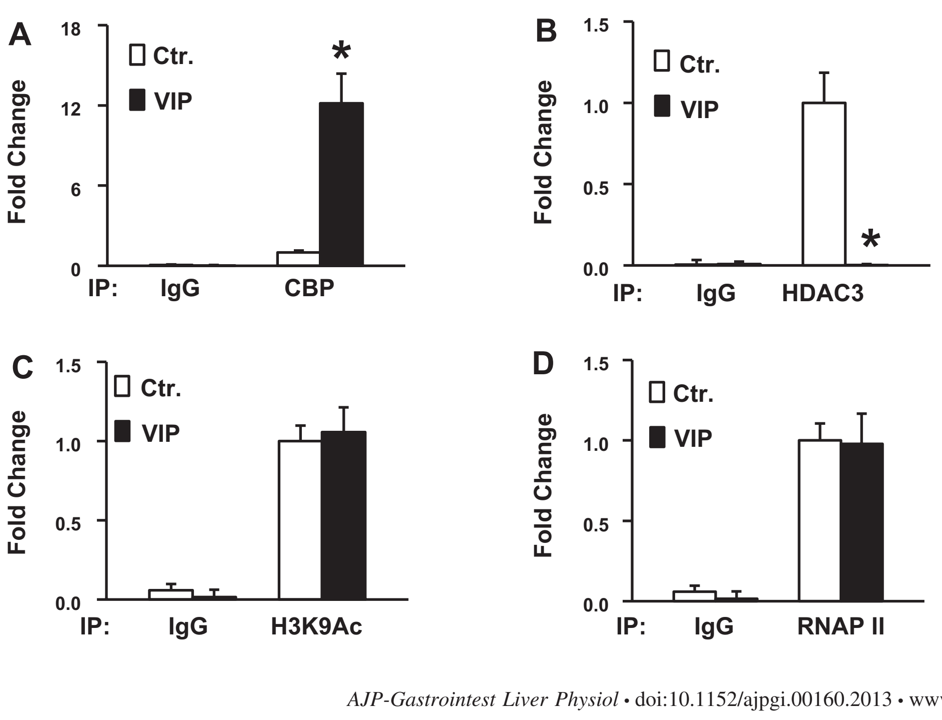

Fig. 5. 15-min vasoactive intestinal peptide (VIP) treatment rapidly recruits CBP to the $\alpha_{1C}1b$ promoter. Colon muscle strips from naïve rats were incubated with 100 nM VIP for 15 min. ChIP-qPCR assays were performed to assess CBP, H3K9Ac, HDAC3, and RNAP II binding at the $\alpha_{1C}1b$ promoter. *A*: CBP association with the CREB site (−2,731/−2,596) of $\alpha_{1C}1b$ promoter was markedly upregulated by 15-min VIP treatment. *B*: H3K9Ac at the $\alpha_{1C}1b$ 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 $\alpha_{1C}1b$ promoter remained unchanged after VIP treatment for 15 min. $N = 3$. $^*P < 0.05$ vs. control.

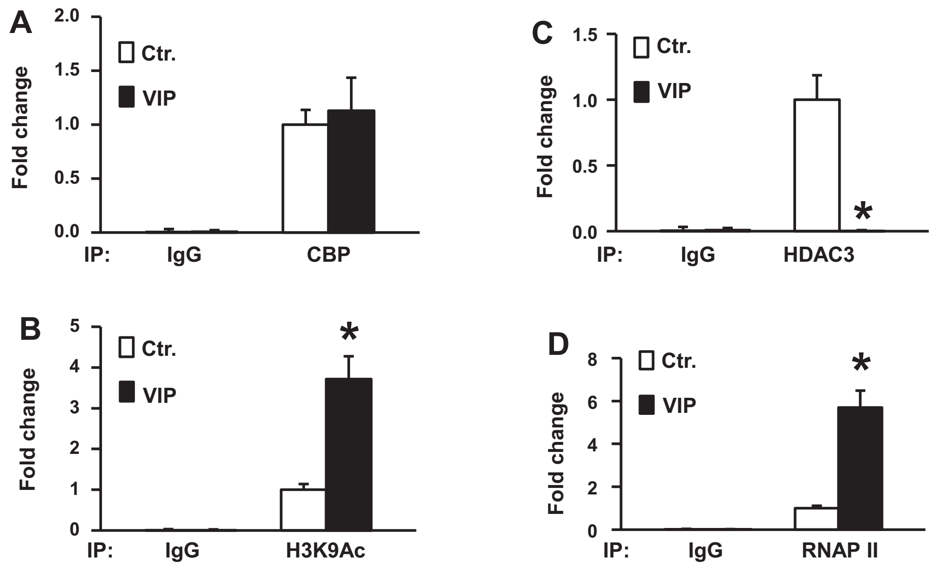

Fig. 6. Incubation of colon muscularis externae with VIP for 24 h increases H3K9 acetylation and dissociates HDAC3 at the $\alpha_{1C}1b$ core promoter (−165/+51). Colon muscularis externae tissues from naïve rats were incubated with 100 nM VIP for 24 h. ChIP-qPCR assays were performed to quantify CBP, H3K9Ac, HDAC3, and RNAP II association with the $\alpha_{1C}1b$ promoter. *A*: CBP association with the CREB-binding motif went back to constitutive level after 24-h VIP incubation. *B*: histone H3K9 acetylation was markedly elevated by 24-h VIP treatment. *C*: HDAC3 interaction with the $\alpha_{1C}1b$ core promoter was dramatically suppressed by VIP treatment for 24 h. *D*: RNAP II binding with the $\alpha_{1C}1b$ core promoter was significantly augmented after incubation with VIP for 24 h. Rabbit IgG was used for mock precipitation. $N = 3$. *$P < 0.05$ vs. control.

Butyrate treatment significantly upregulated $\alpha_{1C}1b$ mRNA level (Fig. 8*A*). ChIP-qPCR results showed that H3K9Ac and RNAP II were significantly elevated and HDAC3 binding was significantly downregulated at the $\alpha_{1C}1b$ core promoter (Fig. 8*B*).

DISCUSSION

Our present findings, along with those of others (1, 4, 10, 11, 14, 59, 72), show that a severe nonspecific inflammatory insult

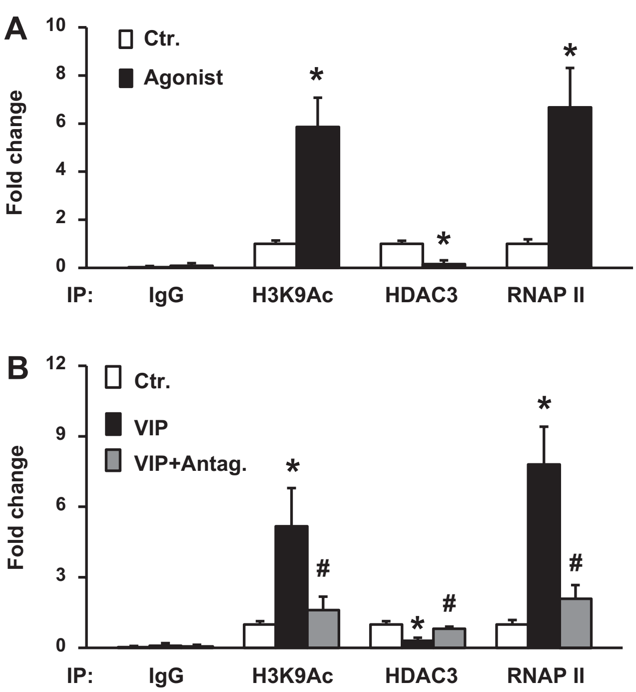

Fig. 7. Effects of VIP receptors VPAC1 agonist and $VPAC_{1/2}$ antagonist on epigenetic modifications at the $\alpha_{1C}1b$ promoter. Colon muscle strips from naïve rats were treated for 24 h before snap-freezing in liquid nitrogen. DNA bound to proteins was immunoprecipitated by ChIP assays and quantified by real-time PCR. *A*: VPAC1 agonist upregulated H3K9Ac and RNAP II but suppressed HDAC3 association at the $\alpha_{1C}1b$ core promoter. *B*: effects of VIP on H3K9Ac, HDAC3, and RNAP II binding to the $\alpha_{1C}1b$ core promoter were reversed by $VPAC_{1/2}$ antagonist. $N = 3$. *$P < 0.05$ vs. control. #$P < 0.05$ vs. VIP.

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, however, that the neonatal programming targeted specifically the *Vip* gene in the colon myenteric plexus; the expression of enzymes, ChAT, and nNOS regulating the expression of the neurotransmitters ACh and NO in the colon wall were not affected. The epigenetic programming is known to be tissue, cell type, and gene specific (25), but the underlying mechanisms of specificity remain unknown.

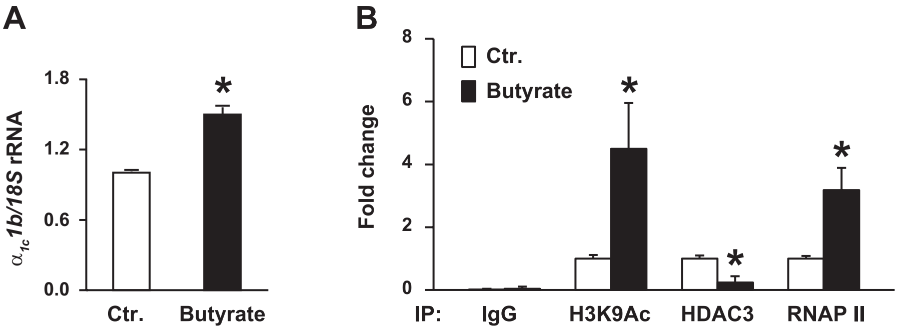

Fig. 8. Sodium butyrate (SB) enhances $\alpha_{1C}1b$ gene transcription by modulating epigenetic modifications at the $\alpha_{1C}1b$ promoter. 6-wk-old naïve rats were given daily i.p. injection of 20 mM SB in saline for 5 days. Control rats received saline. Colon muscularis externae were collected 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 $\alpha_{1C}1b$ mRNA expression. *B*: butyrate upregulated H3K9Ac and RNAP II but decreased HDAC3 binding to the $\alpha_{1C}1b$ promoter. $N = 4$. $^*P < 0.05$ vs. control.

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 the gastric-responsive primary afferents in adult life (73). However, an increase of corticosterone was not responsible for the upregulation of *Vip*; the blockade of glucocorticoid receptors by RU-486 did not block the upregulation of *Vip* or $\alpha_{1C}1b$ 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 type of infective agent may not be an independent risk factor for the development of FBDs. Our findings show that nonspecific inflammation by TNBS caused organ dysfunction in adult life. Our findings that the upregulation of *Vip* and $\alpha_{1C}1b$ occurred within a week after the inflammatory insult support the appearance of childhood FBDs. By contract, 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 $\alpha_{1C}1b$ 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 α_{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

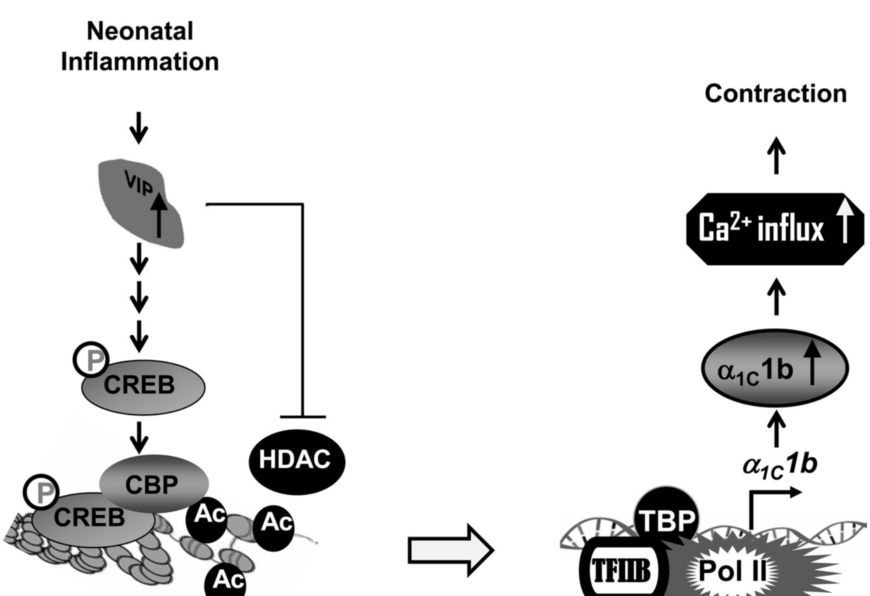

Fig. 9. Cartoon summarizing epigenetic modulation of $\alpha_{1C}1b$ promoter by increase of VIP in rats subjected to an inflammatory insult as neonates and its effect on smooth muscle contractility. VIP transiently phosphorylated CREB to recruit histone acetyltransferase (CBP) to $\alpha_{1C}1b$ promoter to induce histone H3K9 acetylation and enhance transcription of *$Ca_v1.2b$*. VIP concurrently dissociated HDAC3 from the core promoter region of $\alpha_{1C}1b$ promoter to maintain H3K9 hyperacetylation that sustained the increase in α_{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; TFIIB, transcription factor IIB; Pol II, RNA polymerase II.

A by H-89 prevents the transcription of $\alpha_{1C}1b$ (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 $\alpha_{1C}1b$ 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 α_{1C}-subunit of $Ca_v1.2b$ channels in smooth muscle cells. VIP treatment phosphorylated CREB, which recruited HAT (CBP) to the $\alpha_{1C}1b$ promoter to initiate transcription process. At the same time, VIP dissociated HDAC3 from the core promoter region of $\alpha_{1C}1b$ promoter. The phosphorylation of CREB was transient, but the dissociation of HDAC3 from $\alpha_{1C}1b$ 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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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. conception and design of research; S.K.S. edited and revised manuscript; S.K.S. approved final version of manuscript.

REFERENCES

1. **Al-Chaer ED, Kawasaki M, Pasricha PJ.** A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 119: 1276–1285, 2000.
2. **Apley J, Hale B.** Children with recurrent abdominal pain: how do they grow up? *Br Med J* 3: 7–9, 1973.
3. **Bale TL, Baram TZ, Brown AS, Goldstein JM, Insel TR, McCarthy MM, Nemeroff CB, Reyes TM, Simerly RB, Susser ES, Nestler EJ.** Early life programming and neurodevelopmental disorders. *Biol Psychiatry* 68: 314–319, 2010.
4. **Barreau F, Cartier C, Ferrier L, Fioramonti J, Bueno L.** Nerve growth factor mediates alterations of colonic sensitivity and mucosal barrier induced by neonatal stress in rats. *Gastroenterology* 127: 524–534, 2004.
5. **Bradford K, Shih W, Videlock EJ, Presson AP, Naliboff BD, Mayer EA, Chang L.** Association between early adverse life events and irritable bowel syndrome. Clinical gastroenterology and hepatology: the official clinical practice. *J Am Gastroenterol Assoc* 10: 385–390; e381–e383, 2012.
6. **Bush A.** COPD: a pediatric disease. *COPD* 5: 53–67, 2008.
7. **Cheng X, Pachuau J, Blaskova E, Asuncion-Chin M, Liu J, Dopico AM, Jaggar JH.** Alternative splicing of Cav1.2 channel exons in smooth muscle cells of resistance-size arteries generates currents with unique electrophysiological properties. *Am J Physiol Heart Circ Physiol* 297: H680–H688, 2009.
8. **Chey WY, Jin HO, Lee MH, Sun SW, Lee KY.** Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abdominal pain and diarrhea. *Am J Gastroenterol* 96: 1499–1506, 2001.
9. **Chitkara DK, van Tilburg MA, Blois-Martin N, Whitehead WE.** Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review. *Am J Gastroenterol* 103: 76–774; quiz 775, 2008.
10. **Choudhury BK, Shi XZ, Sarna SK.** Gene plasticity in colonic circular smooth muscle cells underlies motility dysfunction in a model of postinfective IBS. *Am J Physiol Gastrointest Liver Physiol* 296: G632–G642, 2009.
11. **Christianson JA, Bielefeldt K, Malin SA, Davis BM.** Neonatal colon insult alters growth factor expression and TRPA1 responses in adult mice. *Pain* 151: 540–549, 2010.
12. **Clarke JM, Young GP, Topping DL, Bird AR, Cobiac L, Scherer BL, Winkler JG, Lockett TJ.** Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats. *Carcinogenesis* 33: 197–202, 2012.
13. **Cortes JE, Curns AT, Tate JE, Parashar UD.** Trends in healthcare utilization for diarrhea and rotavirus disease in privately insured US children $<$5 years of age, 2001–2006. *Pediatr Infect Dis J* 28: 874–878, 2009.
14. **Coutinho SV, Plotsky PM, Sablad M, Miller JC, Zhou H, Bayati AI, McRoberts JA, Mayer EA.** Neonatal maternal separation alters stress-induced responses to viscerosomatic nociceptive stimuli in rat. *Am J Physiol Gastrointest Liver Physiol* 282: G307–G316, 2002.
15. **Darnaudery M, Maccari S.** Epigenetic programming of the stress response in male and female rats by prenatal restraint stress. *Brain Res Rev* 57: 571–585, 2008.
16. **de Wit MA, Koopmans MP, Kortbeek LM, Wannet WJ, Vinje J, van Leusden F, Bartelds AI, van Duynhoven YT.** Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology. *Am J Epidemiol* 154: 666–674, 2001.
17. **Delvaux M, Denis P, Allemand H.** Sexual abuse is more frequently reported by IBS patients than by patients with organic digestive diseases

or controls. Results of a multicentre inquiry French Club of Digestive Motility. *Eur J Gastroenterol Hepatol* 9: 345–352, 1997.
18. **Drong AW, Lindgren CM, McCarthy MI.** The genetic and epigenetic basis of type 2 diabetes and obesity. *Clin Pharmacol Ther* 92: 707–715, 2012.
19. **Drossman DA.** Abuse, trauma, and GI illness: is there a link? *Am J Gastroenterol* 106: 14–25, 2011.
20. **Faulk C, Dolinoy DC.** Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. *Epigenetics* 6: 791–797, 2011.
21. **Flores AR, Szilagyi PG, Auinger P, Fisher SG.** Estimated burden of rotavirus-associated diarrhea in ambulatory settings in the United States. *Pediatrics* 125: e191–e198, 2010.
22. **Fukumoto S, Tatewaki M, Yamada T, Fujimiya M, Mantyh C, Voss M, Eubanks S, Harris M, Pappas TN, Takahashi T.** Short-chain fatty acids stimulate colonic transit via intraluminal 5-HT release in rats. *Am J Physiol Regul Integr Comp Physiol* 284: R1269–R1276, 2003.
23. **Furness JB.** Types of neurons in the enteric nervous system. *J Auton Nerv Syst* 81: 87–96, 2000.
24. **Geeraerts B, Van Oudenhove L, Fischler B, Vandenberghe J, Caenepeel P, Janssens J, Tack J.** Influence of abuse history on gastric sensorimotor function in functional dyspepsia. *Neurogastroenterol Motil* 21: 33–41, 2009.
25. **Gluckman PD, Hanson MA, Cooper C, Thornburg KL.** Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med* 359: 61–73, 2008.
26. **Gwee KA, Leong YL, Graham C, McKendrick MW, Collins SM, Walters SJ, Underwood JE, Read NW.** The role of psychological and biological factors in postinfective gut dysfunction. *Gut* 44: 400–406, 1999.
27. **Holladay SD, Smialowicz RJ.** Development of the murine and human immune system: differential effects of immunotoxicants depend on time of exposure. *Environ Health Perspect* 108, *Suppl* 3: 463–473, 2000.
28. **Howell S, Poulton R, Talley NJ.** The natural history of childhood abdominal pain and its association with adult irritable bowel syndrome: birth-cohort study. *Am J Gastroenterol* 100: 2071–2078, 2005.
29. **Kamath PS, Hoepfner MT, Phillips SF.** Short-chain fatty acids stimulate motility of the canine ileum. *Am J Physiol Gastrointest Liver Physiol* 253: G427–G433, 1987.
30. **Kendler KS, Gardner CO, Prescott CA.** Toward a comprehensive developmental model for major depression in women. *Am J Psychiatry* 159: 1133–1145, 2002.
31. **Klooker TK, Braak B, Painter RC, de Rooij SR, van Elburg RM, van den Wijngaard RM, Roseboom TJ, Boeckxstaens GE.** Exposure to severe wartime conditions in early life is associated with an increased risk of irritable bowel syndrome: a population-based cohort study. *Am J Gastroenterol* 104: 2250–2256, 2009.
32. **Kovarik JJ, Tillinger W, Hofer J, Holzl MA, Heinzl H, Saemann MD, Zlabinger GJ.** Impaired anti-inflammatory efficacy of n-butyrate in patients with IBD. *Eur J Clin Invest* 41: 291–298, 2011.
33. **Kruis W, Azpiroz F, Phillips SF.** Contractile patterns and transit of fluid in canine terminal ileum. *Am J Physiol Gastrointest Liver Physiol* 249: G264–G270, 1985.
34. **Lembo T, Munakata J, Mertz H, Niazi N, Kodner A, Nikas V, Mayer EA.** Evidence for the hypersensitivity of lumbar splanchnic afferents in irritable bowel syndrome. *Gastroenterology* 107: 1686–1696, 1994.
35. **Leroi AM, Bernier C, Watier A, Hemond M, Goupil G, Black R, Denis P, Devroede G.** Prevalence of sexual abuse among patients with functional disorders of the lower gastrointestinal tract. *Int J Colorectal Dis* 10: 200–206, 1995.
36. **Li Q, Sarna SK.** Nuclear myosin II regulates the assembly of preinitiation complex for ICAM-1 gene transcription. *Gastroenterology* 137: 1051–1060; e1051–1053, 2009.
37. **Longstreth GF, Wolde-Tsadik G.** Irritable bowel-type symptoms in HMO examinees Prevalence, demographics, and clinical correlates. *Dig Dis Sci* 38: 1581–1589, 1993.
38. **Lupien SJ, McEwen BS, Gunnar MR, Heim C.** Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nat Rev Neurosci* 10: 434–445, 2009.
39. **Martinez FD.** The origins of asthma and chronic obstructive pulmonary disease in early life. *Proc Am Thorac Soc* 6: 272–277, 2009.
40. **McDonald C, Murray RM.** Early and late environmental risk factors for schizophrenia. *Brain Res Rev* 31: 130–137, 2000.
41. **McKendrick MW, Read NW.** Irritable bowel syndrome—post salmonella infection. *J Infect* 29: 1–3, 1994.
42. **Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K, Seckl JR.** Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. *J Neurosci* 20: 3926–3935, 2000.
43. **Mearin F, Perez-Oliveras M, Perello A, Vinyet J, Ibanez A, Coderch J, Perona M.** Dyspepsia and irritable bowel syndrome after a Salmonella gastroenteritis outbreak: one-year follow-up cohort study. *Gastroenterology* 129: 98–104, 2005.
44. **Mertz H, Naliboff B, Munakata J, Niazi N, Mayer EA.** Altered rectal perception is a biological marker of patients with irritable bowel syndrome. *Gastroenterology* 109: 40–52, 1995.
45. **Naliboff BD, Munakata J, Fullerton S, Gracely RH, Kodner A, Harraf F, Mayer EA.** Evidence for two distinct perceptual alterations in irritable bowel syndrome. *Gut* 41: 505–512, 1997.
46. **Neal KR, Hebden J, Spiller R.** Prevalence of gastrointestinal symptoms six months after bacterial gastroenteritis and risk factors for development of the irritable bowel syndrome: postal survey of patients. *BMJ* 314: 779–782, 1997.
47. **Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y.** The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* 87: 953–959, 1996.
48. **Palsson OS, Morteau O, Bozymski EM, Woosley JT, Sartor RB, Davies MJ, Johnson DA, Turner MJ, Whitehead WE.** Elevated vasoactive intestinal peptide concentrations in patients with irritable bowel syndrome. *Dig Dis Sci* 49: 1236–1243, 2004.
49. **Pont SJ, Carpenter LR, Griffin MR, Jones TF, Schaffner W, Dudley JA, Arbogast PG, Cooper WO.** Trends in healthcare usage attributable to diarrhea, 1995–2004. *J Pediatr* 153: 777–782, 2008.
50. **Pont SJ, Grijalva CG, Griffin MR, Scott TA, Cooper WO.** National rates of diarrhea-associated ambulatory visits in children. *J Pediatr* 155: 56–61, 2009.
51. **Pryce CR, Aubert Y, Maier C, Pearce PC, Fuchs E.** The developmental impact of prenatal stress, prenatal dexamethasone and postnatal social stress on physiology, behaviour and neuroanatomy of primate offspring: studies in hesus macaque and common marmoset. *Psychopharmacology (Berl)* 214: 33–53, 2011.
52. **Qu R, Tao J, Wang Y, Zhou Y, Wu G, Xiao Y, Hu CY, Jiang X, Xu GY.** Neonatal colonic inflammation sensitizes voltage-gated sodium channels via upregulation of cystathionine beta-synthetase expression in rat primary sensory neurons. *Am J Physiol Gastrointest Liver Physiol* 304: G763–G772, 2013.
53. **Reik W, Dean W, Walter J.** Epigenetic reprogramming in mammalian development. *Science* 293: 1089–1093, 2001.
54. **Rice F, Harold GT, Boivin J, van den Bree M, Hay DF, Thapar A.** The links between prenatal stress and offspring development and psychopathology: disentangling environmental and inherited influences. *Psychol Med* 40: 335–345, 2010.
55. **Ritchie J.** Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. *Gut* 14: 125–132, 1973.
56. **Saada N, Dai B, Echetebu C, Sarna SK, Palade P.** Smooth muscle uses another promoter to express primarily a form of human Ca(V)1.2 L-type calcium channel different from the principal heart form. *Biochem Biophys Res Commun* 302: 23–28, 2003.
57. **Saps M, Lu P, Bonilla S.** Cow's-milk allergy is a risk factor for the development of FGIDs in children. *J Pediatr Gastroenterol Nutr* 52: 166–169, 2011.
58. **Saps M, Pensabene L, Di Martino L, Staiano A, Wechsler J, Zheng X, Di Lorenzo C.** Post-infectious functional gastrointestinal disorders in children. *J Pediatr* 152: 812–816; e811, 2008.
59. **Schwetz I, McRoberts JA, Coutinho SV, Bradesi S, Gale G, Fanselow M, Million M, Ohning G, Tache Y, Plotsky PM, Mayer EA.** Corticotropin-releasing factor receptor 1 mediates acute and delayed stress-induced visceral hyperalgesia in maternally separated Long-Evans rats. *Am J Physiol Gastrointest Liver Physiol* 289: G704–G712, 2005.
60. **Seckl JR.** Prenatal glucocorticoids and long-term programming. *Eur J Endocrinol* 151, *Suppl* 3: U49–U62, 2004.
61. **Sethi AK, Sarna SK.** Contractile mechanisms of canine colonic propulsion. *Am J Physiol Gastrointest Liver Physiol* 268: G530–G538, 1995.
62. **Shi XZ, Choudhury BK, Pasricha PJ, Sarna SK.** A novel role of VIP in colonic motility function: induction of excitation-transcription coupling in smooth muscle cells. *Gastroenterology* 132: 1388–1400, 2007.

63. **Shi XZ, Sarna SK.** Gene therapy of Cav1.2 channel with VIP and VIP receptor agonists and antagonists: a novel approach to designing promotility and antimotility agents. *Am J Physiol Gastrointest Liver Physiol* 295: G187–G196, 2008.
64. **Soret R, Chevalier J, De Coppet P, Poupeau G, Derkinderen P, Segain JP, Neunlist M.** Short-chain fatty acids regulate the enteric neurons and control gastrointestinal motility in rats. *Gastroenterology* 138: 1772–1782, 2010.
65. **Spiller RC.** Role of infection in irritable bowel syndrome. *J Gastroenterol* 42, *Suppl* 17: 41–47, 2007.
66. **Talge NM, Neal C, Glover V; Early Stress, Translational Research and Prevention Science Network: Fetal and Neonatal Experience on Child and Adolescent Mental Health.** Antenatal maternal stress and long-term effects on child neurodevelopment: how and why? *J Child Psychol Psychiatry* 48: 245–261, 2007.
67. **Talley NJ, Fett SL, Zinsmeister AR, Melton LJ, 3rd.** Gastrointestinal tract symptoms and self-reported abuse: a population-based study. *Gastroenterology* 107: 1040–1049, 1994.
68. **Vinolo MA, Rodrigues HG, Hatanaka E, Sato FT, Sampaio SC, Curi R.** Suppressive effect of short-chain fatty acids on production of proinflammatory mediators by neutrophils. *J Nutr Biochem* 22: 849–855, 2011.
69. **Waffarn F, Davis EP.** Effects of antenatal corticosteroids on the hypothalamic-pituitary-adrenocortical axis of the fetus and newborn: experimental findings and clinical considerations. *Am J Obstet Gynecol* 207: 446–454, 2012.
70. **Wang Z, Zang C, Rosenfeld JA, Schones DE, Barski A, Cuddapah S, Cui K, Roh TY, Peng W, Zhang MQ, Zhao K.** Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat Genet* 40: 897–903, 2008.
71. **Weaver IC.** Shaping adult phenotypes through early life environments. *Birth Defects Res C Embryo Today* 87: 314–326, 2009.
72. **Winston J, Shenoy M, Medley D, Naniwadekar A, Pasricha PJ.** The vanilloid receptor initiates and maintains colonic hypersensitivity induced by neonatal colon irritation in rats. *Gastroenterology* 132: 615–627, 2007.
73. **Winston JH, Sarna SK.** Developmental origins of functional dyspepsia-like gastric hypersensitivity in rats. *Gastroenterology* 144: 570–579; e573, 2013.