T-type calcium channels contribute to colonic hypersensitivity in a rat model of irritable bowel syndrome

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The symptoms of irritable bowel syndrome (IBS) include significant abdominal pain and bloating. Current treatments are empirical and often poorly efficacious, and there is a need for the development of new and efficient analgesics aimed at IBS patients. T-type calcium channels have previously been validated as a potential target to treat certain neuropathic pain pathologies. Here we report that T-type calcium channels encoded by the $Ca_V3.2$ isoform are expressed in colonic nociceptive primary afferent neurons and that they contribute to the exaggerated pain perception in a butyrate-mediated rodent model of IBS. Both the selective genetic inhibition of $Ca_V3.2$ channels and pharmacological blockade with calcium channel antagonists attenuates IBS-like painful symptoms. Mechanistically, butyrate acts to promote the increased insertion of $Ca_V3.2$ channels into primary sensory neuron membranes, likely via a post-translational effect. The butyrate-mediated regulation can be recapitulated with recombinant $Ca_V3.2$ channels expressed in HEK cells and may provide a convenient in vitro screening system for the identification of T-type channel blockers relevant to visceral pain. These results implicate T-type calcium channels in the pathophysiology of chronic visceral pain and suggest $Ca_V3.2$ as a promising target for the development of efficient analgesics for the visceral discomfort and pain associated with IBS.

analgesia | visceral nociceptor | sensitization | trafficking

Irritable bowel syndrome (IBS) is one of the most prevalent lower gastrointestinal (GI) tract disorders, affecting ~20% of the population in developed countries. Despite high prevalence and considerable impairment of quality of life, current treatments for IBS are empirical and often poorly effective, and the disorder remains a challenge to clinicians (1). IBS is characterized by abdominal pain and discomfort associated with abnormal bowel functions. Although different etiologies have been proposed, it is generally accepted that IBS is multifactorial and that there are likely multiple molecular targets relevant to innovative drug development strategies (2). Among these, there is considerable interest in dysregulation of the brain–gut pain neuraxis and specific subtypes of ion channels in primary afferent neurons that mediate the detection of nociceptive stimuli and transmission to the CNS (3). Moreover, in a number of animal models of chronic pain, the pathological remodeling of ion channel expression patterns has been linked to the hyperexcitability of primary afferent nociceptors (4, 5).

A number of ionic conductances contribute to neuronal firing, including voltage-gated calcium channels, which uniquely both shape action potentials and influence neuronal excitability. In mammals, 10 pore-forming calcium channel α_1 subunit genes have been identified, three of which, $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$, form low-voltage-activated (LVA) T-type calcium channels that are activated by weak depolarizations and generally act to control excitability (6, 7). Although much is known concerning T-type calcium channel tissue distribution, a complete description of their physiological roles has been limited by a lack of subtype-selective pharmacological agents. With the molecular identification of the Ca_V3 genes, genetic elimination of T-type isoforms has been possible by both targeted antisense (AS; refs. 8 and 9) and gene knockout (KO) approaches (10–12). Together with the discovery of T-type channel modulators (13–16), there is an emerging consensus for a major pronociceptive function played by the $Ca_V3.2$ subtype toward somatic pain (17). To date, a role for T-type calcium channels in visceral pain perception and especially in models of GI tract pathologies has yet to be established.

In the present study, we used an AS knockdown strategy to explore the contribution of T-type calcium channels to visceral pain in the context of a model of noninflammatory colonic hypersensitivity (18). The model reproduces the elevated colonic butyrate concentration often found in IBS patients resulting from a peculiar butyrogenic enteric flora. The results show that the genetic or pharmacological blockade of $Ca_V3.2$ channels prevents the development of colonic hypersensitivity. Moreover, we found that an increase in T-type channel density in the colonic visceral nociceptors coincides with the development of colonic hypersensitivity. This effect can be recapitulated in vitro by treating cultures of sensory neurons or HEK cells expressing recombinant $Ca_V3.2$ channels with butyrate. Mechanistically, we find that a posttranslational modulation of $Ca_V3.2$ channels contributes to increased current amplitude. Overall, our results suggest that the $Ca_V3.2$ T-type calcium channel represents a potential target for the development of analgesics aimed at visceral discomfort and pain associated with IBS.

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Results

$Ca_V3.2$ Expression and Functionality in Colonic Nociceptors. After 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) injection into the colon wall, a subset of sensory neurons within the DRGs were found to be retrogradely labeled in the thoracolumbar and in the lumbosacral levels (Fig. S1; refs. 19 and 20). Immunohistochemistry confirmed that the majority of labeled neurons lacked IB4 reactivity and were mainly peptidergic unmyelinated fibers [calcitonin gene-related peptide (CGRP) costaining and minimal Neurofilament 200 costaining; Fig. S1]. Further staining with a subtype-specific polyclonal antibody against the rat $Ca_V3.2$ channel (8) revealed that a subset of the colonic nociceptors expressed $Ca_V3.2$ channels (Fig. 1*A*). The specificity of $Ca_V3.2$ immunoreactivity was confirmed by a lack of staining in DRGs from $Ca_V3.2$-KO mice (ref. 12; Fig. 1*B*). To provide functional evidence for $Ca_V3.2$ expression, whole cell calcium currents were recorded from retrogradely labeled colonic dissociated neurons from naive rats. DiI-labeled cells (e.g., Fig. 1*C*) expressed both LVA T-type currents and high-voltage-activated (HVA) currents (Fig. 1 *D* and *E*). The T-type calcium currents activated near −70 mV and peaked near −35 mV. In contrast, HVA currents were evoked at potentials above −35 mV and peaked at approximately −10 mV. Overall, the T-type current density was 3.0 ± 0.6 pA/pF (n = 33), and the HVA current density was 68 ± 11 pA/pF (n = 13). T-type currents in somatic DRGs exhibit $Ca_V3.2$-like properties and are blocked by low concentrations of nickel ions (10, 16). Similarly, T-type currents from colonic nociceptors were found to be highly sensitive to blockade by nickel (IC_{50} = 3.8 μM, n = 8; Fig. 1 *F* and *G*).

Silencing of $Ca_V3.2$ Attenuates Colonic Hypersensitivity. Chronic visceral hypersensitivity is an important pathological symptom in IBS. Here, we used a model representative of this pathology wherein colonic hypersensitivity is induced by intracolonic injections of sodium butyrate (18). This treatment, mimicking the elevated colonic butyrate concentration found in a proportion of IBS patients, induces robust visceral hypersensitivity and referred lumbar cutaneous allodynia within 3 d after treatment without inflammation-induced mucosal damage in the colon. The consequences of $Ca_V3.2$ channel repression in this model was evaluated in vivo. We first validated that the surgery and the DiI injections did not modify the butyrate-induced colonic hypersensitivity (Fig. S2) and then examined the effect of a $Ca_V3.X$ AS knockdown procedure. Fig. 2*A* shows that mismatch, AS-$Ca_V3.1$-, and AS-$Ca_V3.3$-treated animals exhibited a clear butyrate-induced hypersensitivity characterized by the decrease of threshold to colorectal distention (CRD) compared with the control value (18). In contrast, AS-$Ca_V3.2$ treatment prevented butyrate-induced hypersensitivity without modifying colonic sensitivity of rats that received saline instead of butyrate (Fig. 2*B*). The lack of effect in

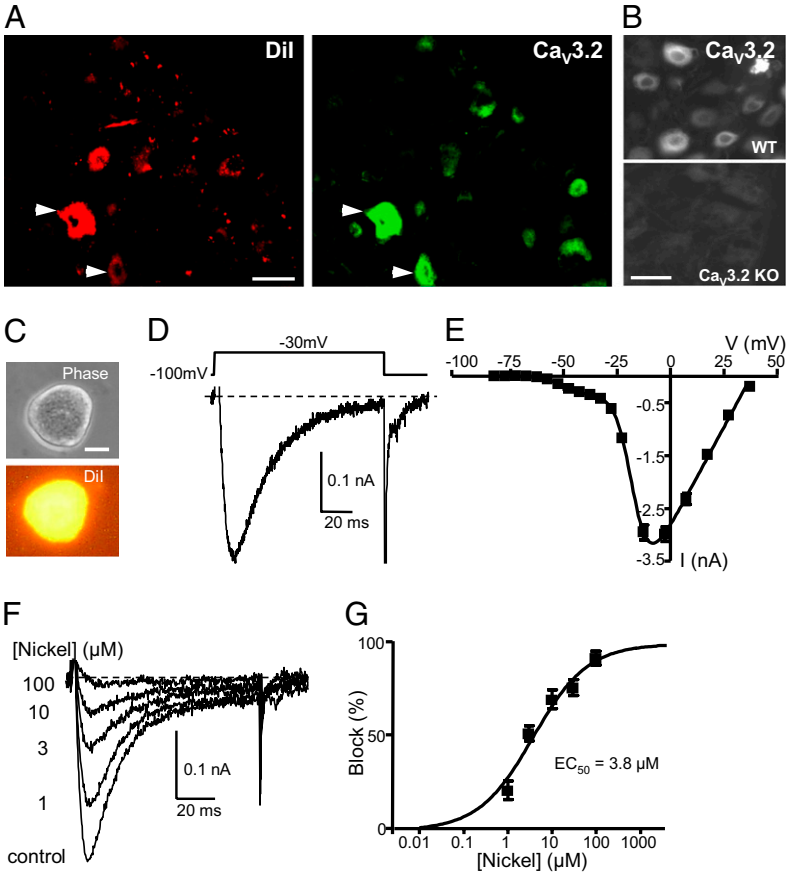

Fig. 1. T-type calcium channels from colonic sensory neurons. (*A*) Histological identification of rat DRG colonic sensory neurons (DiI positive, *Left*) labeled with $Ca_V3.2$ polyclonal antisera (*Right*). Arrowheads indicate DiI-positive neurons strongly expressing $Ca_V3.2$ channels. (*B*) Staining of lumbar DRG sections from wild-type (WT) and $Ca_V3.2$ KO mice, showing no detectable immunoreactivity in the $Ca_V3.2$ KO mice. (*A* and *B*: Scale bars, 40 μm.) (*C*) Images of a dissociated colonic nociceptor cell soma (*Upper*, phase contrast; *Lower*, epifluorescence; scale bar: 10 μm). (*D*) Typical T-type current recording from a DiI-positive DRG neuron evoked by a test pulse from −100 to −30 mV. (*E*) Mean calcium current/voltage relationship in colonic nociceptors showing the presence of a small T-type current component and robust HVA currents. (*F*) Traces of colonic T-type currents before and after application of increasing concentrations of nickel ions. (*G*) Dose–response curve of nickel.

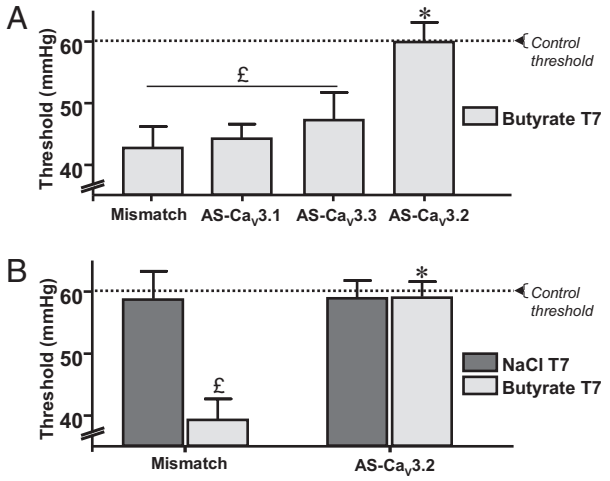

Fig. 2. Effect of T-type channel knockdown on butyrate-induced colonic hypersensitivity. Thresholds to CRD measured 7 d (T7) after the beginning of saline (dark bars) or butyrate (gray bars) enemas. Control threshold (18) is represented by the dotted line. (*A*) Butyrate-treated animals received IT mismatch ODN, AS-Ca$_V$3.1, AS-Ca$_V$3.2, or AS-Ca$_V$3.3 ODN AS. There was no significant difference in the mean thresholds of mismatch, AS-Ca$_V$3.1-, or AS-Ca$_V$3.3-treated animals. In all these conditions the threshold to CRD were statistically different from control values. (£, $P < 0.05$). The AS-Ca$_V$3.2 oligonucleotide reversed the butyrate-induced colonic hypersensitivity and restored the threshold to CRD test to those of naive animals. *$P < 0.05$ compared with mismatch, AS-Ca$_V$3.1, or AS-Ca$_V$3.3 groups). (*B*) Influence of AS-Ca$_V$3.2 on the colonic sensitivity of saline- and butyrate-treated animals. AS-Ca$_V$3.2 treatment did not modify the threshold to CRD in saline-treated animals but did reverse the hypersensitivity in butyrate-treated animals. *$P <$ 0.05 compared with butyrate-mismatch group; £, $P < 0.05$ compared with NaCl-mismatch group.

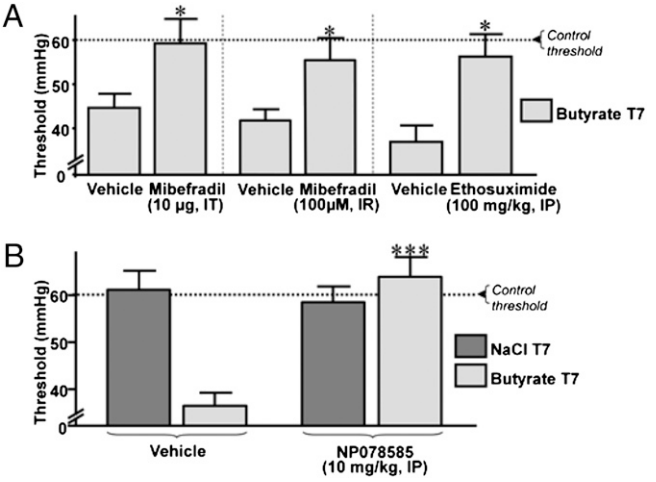

Fig. 3. Effect of administration of calcium channel antagonists on colonic hypersensitivity. (*A*) Thresholds to CRD were determined 7 d (T7) after beginning of butyrate treatment. Effect of IT (10 µg per rat, 20 min before CRD), IR (1 mL of 100 µM solution 40 min before CRD) injection of vehicle or mibefradil. Similarly, effect of intraperitoneal (IP) injection of vehicle or ethosuximide (100 mg/kg) 30 min before CRD. (*B*) Effect of IP injection of vehicle or NP078585 (10 mg/kg) 30 min before CRD in butyrate- or saline-treated rats. *$P < 0.05$, ***$P < 0.001$ compared with saline-treated group.

control animals indicates that Ca$_V$3.2 channels do not significantly participate to colonic sensitivity under healthy conditions.

The colonic hypersensitivity was further studied by using blockers (mibefradil and ethosuximide) known to block T-type channels among other channels and by using NP078585, a unique antagonist exhibiting high-affinity block for T and N types versus P/Q and L types (21). Intrathecal (IT) administration of mibefradil reversed the butyrate-mediated colonic hypersensitivity (Fig. 3*A*). Topical application of mibefradil (at a dose efficacious after intraplantar injections in a neuropathic pain model; ref. 16) to the colonic mucosa to access the sensory neurons endings similarly reversed the butyrate-mediated hypersensitivity. By using systemic administration to be closer to clinical practice, i.p. injection of ethosuximide or NP078585 produced a robust antihyperalgesic effect (Fig. 3 *A* and *B*), abolishing butyrate-induced hypersensitivity without affecting the normal sensitivity in control animals (Fig. 3*B*).

Butyrate Up-Regulates T-Type Channels in Nociceptors. To evaluate whether an alteration of T-type current properties parallels the development of colonic hypersensitivity, we analyzed ex vivo calcium currents from DRGs isolated from saline- and butyrate-treated rats. The treatment did not alter the DRGs' mean cell size (Fig. 4*A*); however, T-type current amplitude was clearly increased in colonic nociceptors from the butyrate-treated animals (Fig. 4*B*). Comparison of current densities showed a significant increase of LVA currents (Fig. 4*C*), but not of HVA currents (Fig. 4*D*). Consistent with a selective effect on T-type channels from colonic neurons, analysis of DiI-negative cells (15- 30 µm in diameter; putative nociceptors) did not reveal any butyrate-mediated effect (Fig. 4 *E* and *F*). The increased T-type current density in DiI-positive neurons was not linked to detectable changes in T-type current biophysical properties (Fig. S3).

Butyrate Treatment Increases Neuronal T-Type Current Density in Vitro. At high concentrations, butyrate is known to increase colonic mucosal permeability (22) facilitating the access of luminal factors (including butyrate itself) to sensory nerve endings. To assess whether butyrate can directly act on sensory neurons, we treated DRG cells in vitro. To select a concentration of butyrate that might replicate the dose seen by sensory nerves in the colonic mucosa, we estimated that the 200-mM butyrate enema leads to a stable concentration at least 50-fold lower. Moreover, studies examining butyrate effects in vitro often use concentrations up to 10 mM (23–25); thus, we tested a 5-mM butyrate treatment for 2 d. The analysis was performed on DiI-positive as well as on DiI-negative cells similar to the ex vivo approach. Butyrate significantly increased T-type calcium current density in both DiI-positive and negative neurons (Fig. 5 *A* and *B*). Similar to that for the ex vivo data, HVA currents were not affected (Fig. S4). The T-type current density increase (approximately threefold) was similar to that observed for DiI-positive neurons ex vivo. These results suggest that butyrate by itself can up-regulate T-type calcium currents in nociceptors, which in turn may contribute to the butyrate-mediated hypersensitivity. In support, current-clamp recording on colonic DRGs treated with butyrate in vitro showed that action potential thresholds were significantly lowered. In addition, low threshold spikes after hyperpolarization, a signature of T-type channel activity (7), were more frequent after butyrate treatment (Fig. S5).

Mechanism of T-Type Channels Up-Regulation. Transcriptional machinery is known to be affected by butyrate (26–28), and differences in the promoter regions of calcium channel subtypes might explain the selective modification of T-type channels over other subtypes. We tested in vitro butyrate treatment on recombinant channels expressed from plasmids with a similar cytomegalovirus (CMV) promoter. Recombinant T-type currents were expressed by using a Ca$_V$3.2 cDNA, and HVA currents were examined with a Ca$_V$2.2 subunit cDNA encoding the major N-type current found in nociceptors. Butyrate was applied 24 h after transfection

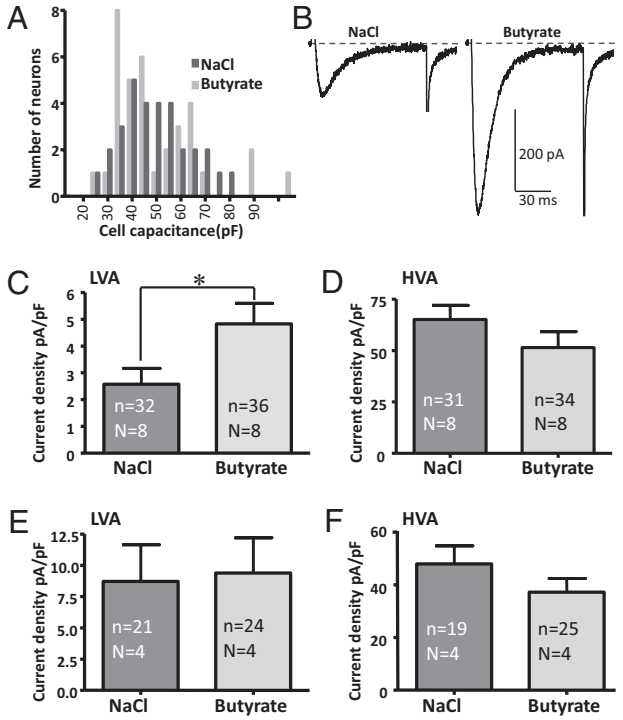

Fig. 4. Increased functional expression of LVA T-type calcium currents in colonic sensory neurons in butyrate-treated rats. (*A*) Distribution of colonic sensory neuron size in the cells sampled for the electrophysiological analysis presented here (mean capacitance: NaCl, 49.7 ± 16 pF; butyrate, 50.8 ± 19 pF). (*B*) Typical traces of T-type currents from DiI-positive neurons from saline- and butyrate-treated animals. Currents were evoked by 100-ms pulses from −100 to −35 mV. (*C* and *D*) T-type (*C*) and HVA (*D*) calcium current density in DiI-positive neurons from saline-treated (dark bar) or butyrate-treated (gray bar) animals. (*E* and *F*) Same analysis as in *C* and *D* but from DiI-negative neurons presumably not innervating the colon. $*P < 0.05$ compared with NaCl.

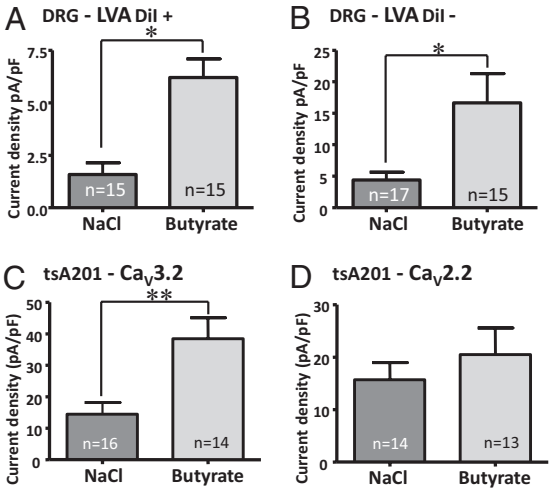

Fig. 5. Effect of a 48-h treatment with 5 mM butyrate in vitro of sensory neuron cultures or HEK cells expressing recombinant calcium channel. (*A* and *B*) Effect of butyrate on LVA current density from DiI-positive (*A*) and DiI-negative (*B*) neurons. (*C* and *D*) Effect of butyrate on transfected $Ca_V3.2$ *(C)* or $Ca_V2.2$ *(D)* channels. Note that as for native calcium channels, butyrate selectively increased recombinant $Ca_V3.2$-mediated T-type currents. $*P < 0.05$, $**P < 0.01$ compared with NaCl.

into tsA201 cells, and recordings were performed 3 d after transfection. Butyrate treatment was found to selectively increase $Ca_V3.2$ current density without affecting $Ca_V2.2$ N-type currents (Fig. 5 *C* and *D*). Confocal imaging of a recombinant epitope-tagged $Ca_V3.2$ (29) confirmed that cell surface channel expression was increased by butyrate treatment (Fig. S6). Together, these observations appear to minimize the involvement of a transcriptional mechanism. We next challenged whether the blockade of protein synthesis or downstream posttranslational processing could prevent butyrate effects in colonic nociceptors. Because most of the pharmacological tools used to block these processes are toxic to cell survival and are generally used over short time frames, we first measured the time course of the butyrate effect. This analysis revealed that the increase in T-type current density began within 3 h after butyrate application and was nearly maximal by ~12 h (Fig. 6*A*). We subsequently tested whether protein synthesis blockade by 10 μM anisomycin (previously shown to inhibit protein synthesis in DRGs; ref. 30) prevented the effects of butyrate at 12 h. Fig. 6*B* shows that anisomycin treatment did not modify the butyrate-induced up-regulation of T-type current-density; thus, a major direct effect on transcription is unlikely. However, we cannot completely rule out transcriptional effects, as quantitative RT-PCR analysis showed that a 12- to 18-h butyrate treatment resulted in a twofold increase of $Ca_V3.2$ mRNA in DRG cultures containing colonic and noncolonic afferents (Fig. S7).

Protein trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus and then to the plasma membrane involves highly regulated processes, and butyrate has been shown to function as a molecular chaperone for certain ion channels (23). To investigate mechanisms downstream of protein synthesis, we used brefeldin A, an inhibitor of anterograde protein transport from the ER to the Golgi previously shown to prevent membrane trafficking of ion channels (31). Fig. 6*C* shows that the T-type current increase was significantly attenuated by 1 μM brefeldin A, suggesting that butyrate acts to increase T-type channel trafficking to the cell membrane. It should be noted that when brefeldin A was used (±butyrate), the T-type current density was smaller than in control conditions (without brefeldin A), suggesting that both basal- and butyrate-stimulated channel insertion were impaired at some level by this agent.

Discussion

Combining gene silencing in spinal sensory ganglia and pharmacological approaches in vivo, we describe a selective pronociceptive role for $Ca_V3.2$ T-type channels toward visceral pain in the context of a colonic hypersensitivity model that mimics the pathophysiology of IBS. The effect is consistent with an increase in functional $Ca_V3.2$ channels in colonic afferent DRG neurons induced by butyrate treatment. This notion was confirmed by analyzing T-type current density in isolated cell bodies of identified colonic afferent neurons. Indeed, colonic nociceptors that are located at the thoracolumbar and sacral levels (19, 20, 32, 33) were both found to express the $Ca_V3.2$ channels and to display small whole-cell T-type currents under control conditions. In contrast, in animals with induced colonic hypersensitivity, T-type current density was significantly increased, supporting their role as major pain signal amplifiers in primary afferent neurons.

Our findings agree with the notion that peripheral T-type channels contribute to somatic pain conditions of distinct etiologies (8, 9, 13, 16, 34–36). In the present study, we further show that specific $Ca_V3.2$ channel expression in colonic nociceptors is crucial to visceral pain hypersensitivity. Localized pharmacological blockade corroborates the AS data and suggests that $Ca_V3.2$ channels exert their pronociceptive role at nociceptor-free endings in the colon mucosa as well as in the spinal ganglia. Some previous studies have documented heterogeneous expression of T-type currents in visceral nociceptors, such as in the urinary tract (37). To date, T-type currents have not been described in colonic

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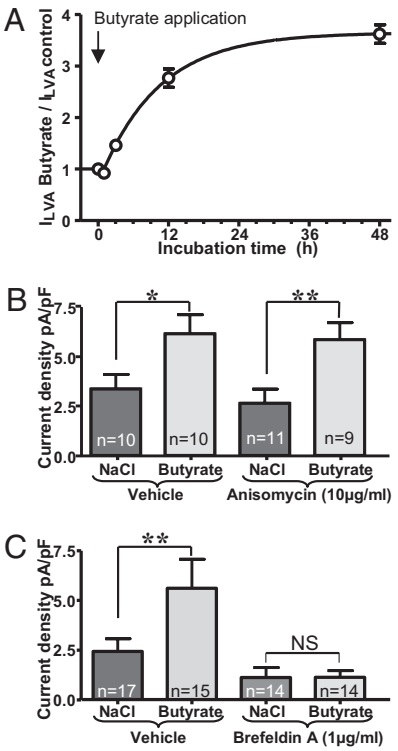

Fig. 6. Effect of protein synthesis blockade or disruption of the Golgi apparatus on the butyrate-induced T-type calcium channel functional expression on DRG culture in vitro. (*A*) Kinetic of the butyrate effect (5 mM) on T-type channel expression in DiI-positive cells. (*B*) Inhibition of protein synthesis with 10 μg/mL anisomycin for 12 h did not alter the butyrate stimulatory effect on T-type calcium channel expression in DiI-positive cells. (*C*) Inhibition of posttranslational cell machinery with 1 μg/mL brefeldin A for 12-h blocked T-type calcium channel up-regulation by butyrate. $*P < 0.05$, $**P < 0.01$ compared with NaCl.

retrogradely identified nociceptors, although a contribution of T-type channels to colonic pain has been suggested (38).

A large fraction of nociceptors appear silent under healthy conditions but become sensitized under painful situations (39). The physiological implications of sensitization are predicted to be a lower threshold for action potential firing and sustained spontaneous activity. A hyper-excitability related to increased T-type currents has been shown in somatic nociceptors (9, 34, 35), and the increase that we describe in colonic nociceptors likely contributes to the hypersensitivity as suggested by the lowering of action potential threshold in butyrate-treated DRGs in vitro. Ion channel plasticity has been documented in visceral nociceptors, with disequilibrium between excitatory and inhibitory currents. For example, sodium currents are up-regulated in experimental colitis (19), and transducers of mechanical and chemical stimuli are activated in models of chronic visceral pain (3, 40). Moreover, certain potassium channel subtypes are down-regulated (41). Although we found that $Ca_V3.2$ up-regulation appears crucial toward the butyrate-induced colonic hypersensitivity, it is important to note that the relative contributions of other ion channels remain to be similarly addressed.

To better understand the cellular mechanisms of butyrate on T-type channels, we evaluated whether there was a direct action in vitro. A direct action would be consistent with the increased mucosal permeability induced by butyrate (22) and support the notion of increased access of butyrate to nociceptors endings. Our results demonstrate that in vitro butyrate treatment resulted in a significant up-regulation of T-type current density. Several mechanisms have been described for butyrate action that might be relevant to our observations. For example, short-chain fatty acids, including butyrate, are agonists of the GPR41 and GPR43 G-protein-coupled receptors (GPCRs) present in the colon (25). However, from a kinetic perspective, the butyrate effects in our experiments are not compatible with an action on GPCRs. In agreement, neither acute butyrate application on DRGs nor incubation of <2–3 h was found to alter T-type currents. The kinetics of T-type current up-regulation is more compatible with augmented channel biosynthesis and/or insertion into the plasma membrane. Interestingly, butyrate is known to affect both of these processes including that of transcriptional activation (27, 28, 42). Examining these possibilities, we initially tested whether recombinant T-type $Ca_V3.2$ and N-type $Ca_V2.2$ channels expressed under identical promoters were affected by butyrate as expected from its known action on the CMV promoter (27). The results showing a specific action on $Ca_V3.2$ channels decreases the likelihood of a direct transcriptional effect. This notion was also tested on native currents by blocking protein biosynthesis. The negative result further suggests that butyrate likely acts downstream of T-type channel transcription to promote increased T-type current density. Nevertheless, quantitative RT-PCR showed that butyrate increased $Ca_V3.2$ transcription, leaving the possible implication of a transcriptional effect, although probably to a smaller extent. Given that butyrate can act as a molecular chaperone for some membrane proteins (23, 24) and that only a fraction of $Ca_V3.2$ subunits synthesized are directed to the plasma membrane (43), we hypothesized that butyrate might promote $Ca_V3.2$ cell surface insertion. With the use of brefeldin A, our results are consistent with a trafficking mechanism and are reminiscent of the action of certain neurotrophic factors on $Ca_V3.2$ channels in chick nodose neurons (44).

In summary, our results suggest the $Ca_V3.2$ T-type channels as a promising target for the development of unique analgesics aimed at the visceral discomfort and pain associated with IBS. The up-regulation of $Ca_V3.2$ channels appears to be directly involved in the development of the pathology and may be a common feature of many somatic and visceral pain syndromes. Selective molecules for calcium channels are beginning to emerge (17, 45, 46), and the currently limited therapeutic arsenal for treating visceral pain may benefit from these new pharmacological agents.

Methods

Behavior. Experiments were performed according to the recommendations of the International Association for the Study of Pain and approved by the local ethical committee. Induction of colonic hypersensitivity with butyrate enemas was performed in rats as published (18). Colonic visceral sensitivity was evaluated by monitoring the threshold for abdominal cramping events after distention of the colon with a balloon inserted at 7 cm to the anal margin and gradually inflated with a barostat (*SI Methods*).

Retrograde Labeling. Laparotomy was performed under isoflurane anesthesia to gain access to the pelvic organs. The fluorescent tracer DiI (50 mg/mL in DMSO) was injected in the distal colon wall (7 cm from anal margin) as described (19, 32). For the control condition, a saline solution was injected. For the next 3 d, the animals received either the butyrate or saline treatments, and CRD was performed 7 d after the beginning of enemas. Similarly, for ex vivo experiments, sensory neurons were prepared from rats at day 7 (T7).

IT ODN Administration. AS ODNs targeting each of the rat Ca_V3 sequences (AS-Ca_V3-1, AS-Ca_V3-2, AS-Ca_V3-3,) and a control mismatch were injected IT (12.5 μg per rat) twice daily for 4 d (days 1–4), by using a protocol previously shown to effectively reduce T-type channel expression in dorsal root ganglia at the lumbar area and the adjacent zones (sacral and thoracolumbar junction; ref. 10).

In Vivo Pharmacology. Pharmacological treatments were performed at day 7 after beginning the butyrate enemas. For mibefradil, a first group of animals received an IT injection of vehicle or mibefradil (10 μg per rat; ref. 47) 20 min before the CRD test. To determine whether mibefradil could have a peripheral effect on the sensory neuron endings in colonic mucosa, a group of

animals received 1 mL enemas of saline or mibefradil (100 μM) intrarectally (IR) 30 min before the CRD test. Ethosuximide (10 mg/kg; ref. 47), its vehicle, NP078585, or its vehicle were administered IP 30 min before CRD test on either saline- or butyrate-treated animals.

Electrophysiological Recordings. At T7 DRG cell bodies were isolated from DiI injected rats. For ex vivo experiments, all recordings were completed within 6 h of plating. The experimenter was blinded to the saline or butyrate treatments until the completion of data analysis. For in vitro effects of butyrate, DRG neurons were prepared similarly from animals that had not received any colonic treatment in vivo. Butyrate (5 mM) or vehicle were added directly to the DRG culture 2 h after plating. Similar treatment was performed on tsA201 cells expressing recombinant $Ca_V3.2$ or $Ca_V2.2$ channels. In all cases, patch-clamp recordings were made at room temperature from DRGs and transfected tsA201 cells (*SI Methods*).

Immunohistochemistry. Immunohistochemistry was performed 7 d after DiI injection. DRG were snap frozen, embedded in OCT, and sectioned by using a cryostat. Section were incubated overnight with a polyclonal anti-$Ca_V3.2$ (ref. 10; 1/5,000 dilution), then washed and incubated with an Alexa 488-coupled secondary antibody (Invitrogen; 1/500 dilution). Images were obtained with a CCD camera connected to an inverted microscope (*SI Methods*).

Statistics. Results are presented as the mean ± SEM. Data were compared with Student *t* test (electrophysiology) or by one-way ANOVA analysis followed by a Tukey post hoc test (behavior).

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