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Electrical and Mechanosensitive Activity of the Mouse Fetal Enteric Nervous System

Richard J. Amedzrovi Agbesi,^{*} Nick Spencer,[†] Tim Hibberd,[‡] and Nicolas R. Chevalier[§]
*Laboratoire Matière et Systèmes Complexes UMR 7057,
Université Paris Cité / CNRS and College of Medicine and Public Health,
Flinders University(Adelaide, South Australia)*

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Gut motility undergoes a switch from myogenic to neurogenic control in late embryonic development. Here, we report on the first electrical events underlying neurogenic motility in the developing mouse, using a transgenic line expressing the GCamp6f reporter in neural crest cell derivatives. We find that both nitrergic inhibitory and cholinergic excitatory neurons exhibit spontaneous calcium activity and that this activity requires L-type calcium channels and intracellular store-operated calcium channels (IP3R). This spontaneous electrical activity translates in terms of motility in periodic, high-frequency contraction complexes, which are inhibited by tetrodotoxin. We demonstrate that the ENS is mechanosensitive from the earliest stages on (E14.5) and that this behavior is tetrodotoxin and 2-APB resistant, but requires synaptic transmission. We extend our result on spontaneous L-type channel-dependent spontaneous activity and TTX-resistant mechanosensitivity to the adult colon. Our results shed light on the critical transition from myogenic to neurogenic motility in the developing gut.

I. INTRODUCTION

The gastrointestinal tract is a tube with the complex task of propelling content along its length to allow sequential flow and facilitate the mixing and transport of nutrients to the gut wall for absorption[1]. These functions are made possible by the coordinated action of smooth muscle, interstitial cells of Cajal, and the enteric nervous system(ENS)[2–5]. The ENS is an extensive network of neurons and glial cells connected to extrinsic autonomic and primary afferent innervation[6]. The ENS arises from the neural crest cells, mostly from the vagal region of the neural tube[7]. During embryonic development in mice, neural crest-derived cells migrate into the developing foregut around E9.5 and populate it in a wave-like pattern[8, 9]. The ENS performs its function by responding to mechanical and chemical cues[10–12]. Myenteric neurons, whether isolated or in situ, can transduce the mechanical forces that act on the gut wall into action potential firing, including changes in length (strain), tension (stress), and compression[13, 14]. In the colon of mature mice, KCa_{1.1} and transient receptor potential(TRP) have been demonstrated to be mechanosensitive channels[15]. The embryonic mouse has been found to respond to electrical field stimulation and this response was mediated by L, N, P/Q, and R calcium channels[16]. Pharmacological studies have revealed that the main intestinal motility patterns in mature animals - segmentation, peristalsis, and migrating motor complex all depend on neuronal activity[17, 18]. In the mouse embryo, the first motor activity at E13.5 is purely myogenic and

takes the form of spontaneous circular muscle contractile waves that propagate along the intestine. The ENS is present in the whole gut from E14.5 but remains in a state of functional quiescence until E18.5 when the first neurogenic motility patterns can be detected[19, 20]. The developmental transition from myogenic to neurogenic patterns yields great insight into the basic mechanisms by which the ENS affects motility. In the chicken embryo, our group has found that the ENS gives rise to a descending, inhibitory, mechanosensitive coupling of the circular and longitudinal muscle layers. This antagonism gives rise to a new motor pattern, the migrating motor complex(MMC). The MMC has the same frequency as longitudinal smooth muscle contractions, which act as a trigger for this motor pattern[21]. The polar, descending character of neural inhibition gives rise to the stereotyped “ascending contraction-descending inhibition” response to mechanical stimulation, known as “the law of the intestine”[22]. We wondered to what extent the results we obtained in chicken applied to a mammalian model, the mouse. We take advantage here of a mouse model expressing the GCamp fluorescent intracellular calcium reporter in neural crest-derived cells. We study the development of ENS calcium activity from E18.5-P3, which to the best of our knowledge has not been examined so far. We were in particular keen on preserving an intact muscular activity (imaging without muscle inhibitors like nicardipine) to observe the coupling of contractions with neuronal activity. The embryonic gut is small, enabling the study of relatively expansive ENS areas and network-wide effects that may not be evident from visualizing only a few ganglia, as is typical of adult gut studies. Because ENS electrical activity in the embryo was sensitive to mechanical stimulation, we further investigated which ion channels could relay this mechanosensitive response.

^{*} richard-junior.amedzrovi-agbesi@etu.u-paris.fr

[†] nicolas.spencer@flinders.edu.au

[‡] tim.hibberd@flinders.edu.au

[§] nicolas.chevalier@paris7.jussieu.fr

II. MATERIALS AND METHOD

A. Ethics

All experiments were performed in accordance with the ethics and guidelines of INSERM and CNRS.

B. Animal Model

Mice were hosted at the Institut Jacques Monod animal husbandry. The Cre reporter mice Gt(ROSA)26 Sortm1(EYFP)Cos₄₃ is referred to as GCampfl/fl. A transgenic mouse line in which the transgene is under the control of the 3-kb fragment of the human tissue plasminogen activator (Ht-PA) promoter Tg(PLATcre)116Sdu16, was referred to as Ht-PA::Cre. GCampfl/fl males were crossed with Ht-PA::Cre females to generate embryos carrying the calcium fluorescent reporter in neural crest cells and their derivatives. The day a plug was observed following mating overnight was defined as E0.5. The day of birth was considered as post-natal day P0=E19.5. The mice were dissected at E18.5(embryos were taken at 18.5 days of gestation), P1, and P3. The gastrointestinal tracts were dissected from the stomach to the hindgut in phosphate-buffered saline (PBS) with Ca²⁺ and Mg²⁺, and the mesentery was removed.

C. Calcium Imaging and Analysis

After dissection, a 0.2 mm diameter nylon thread was inserted in the lumen of a ~ 2 cm long gut segment. The end of the thread was inserted non-invasively in a block of soft PDMS (Sylgard XXX) in a way that the nylon gently pressed the gut segment against the bottom of a 30mm diameter petri dish(Greiner). This method has been detailed in [23]. It allows muscle contractions to take place but prohibits bulk movements of the sample in and out of the imaging field. The gut was covered with 5 mL of Dulbecco's Modified Eagle Medium(DMEM) supplemented with 1% penicillin and constantly bubbled with 95% O₂ - 5% CO₂ at $36.5 \pm 1^\circ\text{C}$. Imaging of the gut was captured using an inverted spinning disk confocal microscope (Olympus) with x10 objective, the illumination wavelength was set to 488 nm. Fluorescence was collected within the range of 492-630 nm, and the acquisition rate was set to 2 Hz. The video duration was 2 to 3 minutes. Drugs were administered from a stock solution by directly adding them to the petri dish under the microscope. To ensure proper mixing, the solutions were gently agitated by an up-and-down movement using a 1 mL pipette. To analyze calcium transients, the region of interest(ROI) was selected over the ENS area. The pixel intensity changes (calcium transients) over time were obtained using the 'findpeaks' function in MATLAB. We set the minimal peak prominence, and minimal and maximal peak width to take into consideration. The number

of calcium flashes is the total number of peaks measured over the region of interest. We next assessed the area of ENS (mm²) in the imaging field using the internodes thresholding method in Fiji. Normalized calcium activity was finally reported as the total number of flashes / per unit area ENS / per unit time.

D. Immunohistochemistry

Samples were fixed for 1h in 4% PFA in PBS, and washed 3 times. They were then dehydrated for 24 h in 30% sucrose solution in water. 1-2 cm pieces of the samples were embedded in OCT and frozen with a -80°C freezer pack. Frozen longitudinal sections were performed on a Leica cryostat machine set to -20°C at 14 μm thickness. Thin slice sections were blocked and permeated in 1% BSA and 0.1% triton in PBS overnight. The antibodies used were AntiCav1.2(ACC003AN7125 - alomone labs), AF 647(α rabbit), Cy3(α rabbit), Choactase(E-7, AF 546 conjugated), NOS1(A-11, AF 647 conjugated).

E. Statistics and Reproducibility

All sample numbers indicated in this article correspond to different embryos (guts, biological replicates). For mice, data were collected from a minimum of 3 different litters per age group to ensure robustness. Each group presented in this study comprises a minimum of n=5 samples, ensuring an adequate sample size for statistical analysis. Statistical analysis was performed with 2-way ANOVA and t-tests and was considered significant at $p < 0.05$.

III. RESULTS

A. Embryonic Enteric Nervous System Calcium Activity

Veratridine(a depolarizer that causes persistent opening of the voltage-gated Na⁺ channels) and tetrodotoxin(a Na⁺ channel blocker) were used to excite and inhibit the ENS, respectively. Veratridine(10 μM) increased the spontaneous calcium activity of E18.5(Fig. 1a, S1)neurons by more than 10-fold(Fig. 1b). Tetrodotoxin(1 μM) abolished the stimulating effect of veratridine and almost no neuronal calcium activity was recorded after application(Fig. 1b, S1). These results indicate that the observed calcium signals stem from enteric neural electric activity.

Calcium activity in enteric neural crest cells has been reported at earlier stages (E11.5-E14.5)[16, 24] and is absent in the period E14.5-E18.5. We first examined how calcium activity evolved in the late fetal to early postnatal period from E18.5 - P3 small intestine. The average

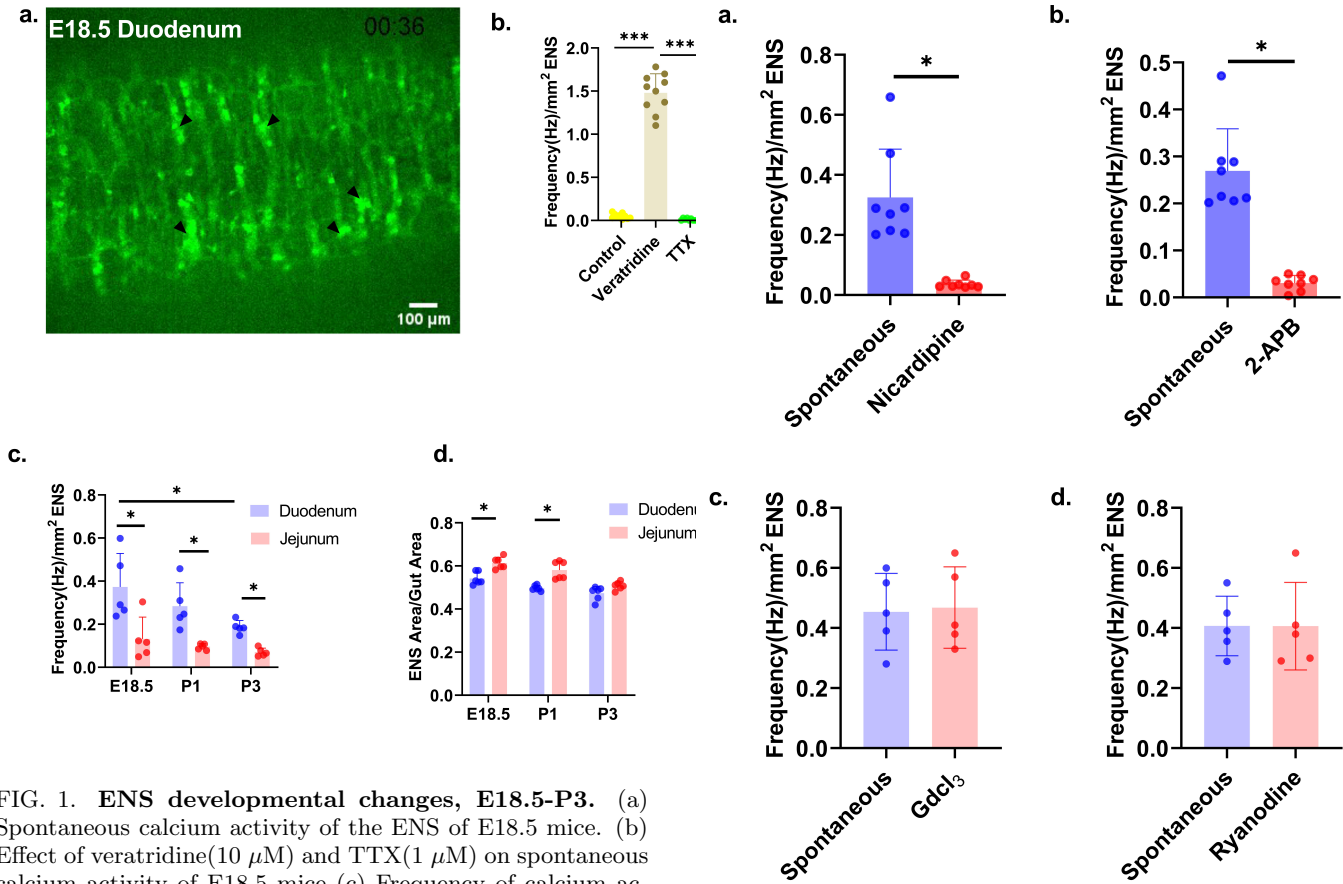


FIG. 1. **ENS developmental changes, E18.5-P3.** (a) Spontaneous calcium activity of the ENS of E18.5 mice. (b) Effect of veratridine(10 μM) and TTX(1 μM) on spontaneous calcium activity of E18.5 mice (c) Frequency of calcium activity per unit area of the ENS of E18.5 to P3 mice. (d) Fractional ENS area covered to the area of mice gut from E18.5 to P3.

frequency of calcium activity per unit area of ENS decreased from E18.5 to P3 in both the duodenum and jejunum(Fig. 1c); this decrease was statistically significant for the duodenum between E18.5 and P3. The frequency of calcium activity per unit area of the duodenum was always a factor ~ 2 higher than in the jejunum at each stage considered (Fig. 1c). We note that the fraction area occupied by the ENS was lower in the duodenum than in the jejunum (Fig. 1d), consistent with previous reports[25]. Nicardipine(10 μM , n=8) - an L-type calcium channel blocker almost completely abolished spontaneous calcium activity(Fig. 2a, S2). 2-APB(20 μM , n=8), a known inhibitor of IP3R and store-operated Ca^{2+} (SOC) channels significantly reduced the frequency of spontaneous calcium activity of the ENS(Fig. 2b, S3). In contrast, GdCl_3 (100 μM , n=5) - a transient receptor potential(TRP) channel blocker, and ryanodine (10 μM , n=5) - RyRs receptors blocker found on the endoplasmic reticulum had no effect on calcium activity(Fig. 2c,d). Nicardipine is a known smooth muscle blocker. It is not expected that this drug acts directly on enteric neurons, yet was effective in blocking spontaneous patterns of neuronal activity. To investigate the possibility of a direct neuronal action of nicardipine, we investigated the pres-

FIG. 2. **Voltage-Gated Calcium Channels Responsible for Spontaneous Calcium Activity of the ENS.** (a-d) Effects of nicardipine(10 μM), 2-APB(20 μM), GdCl_3 (100 μM), and ryanodine(10 μM) on spontaneous calcium activity respectively.

ence of L-type calcium channels on neurons in the myenteric plexus. Immunohistochemistry studies revealed the presence of the L-type voltage-gated calcium channel, $\text{CaV}_{1.2}$ on populations of myenteric neurons that also contained ChAT or NOS immunoreactivity. (Fig. 3).

B. Mechanosensitivity of the Embryonic Mouse

Because the ENS plays a key role in mediating the mechanosensitive response of the gut[5, 15], we were interested in finding out if mechanosensitive properties are established at the fetal and early postnatal ages(E18.5-P3). We applied mechanical force using a 0.3 mm diameter blunt glass probe to gently pinch the gut wall. Intracellular calcium concentration in the ENS promptly rose to high levels upon pinching(Fig. 4b). We observed contraction of the circular smooth muscle(n=7/10) that was synchronous with the intracellular calcium rise in the ENS(S4). We investigated what ion channels and

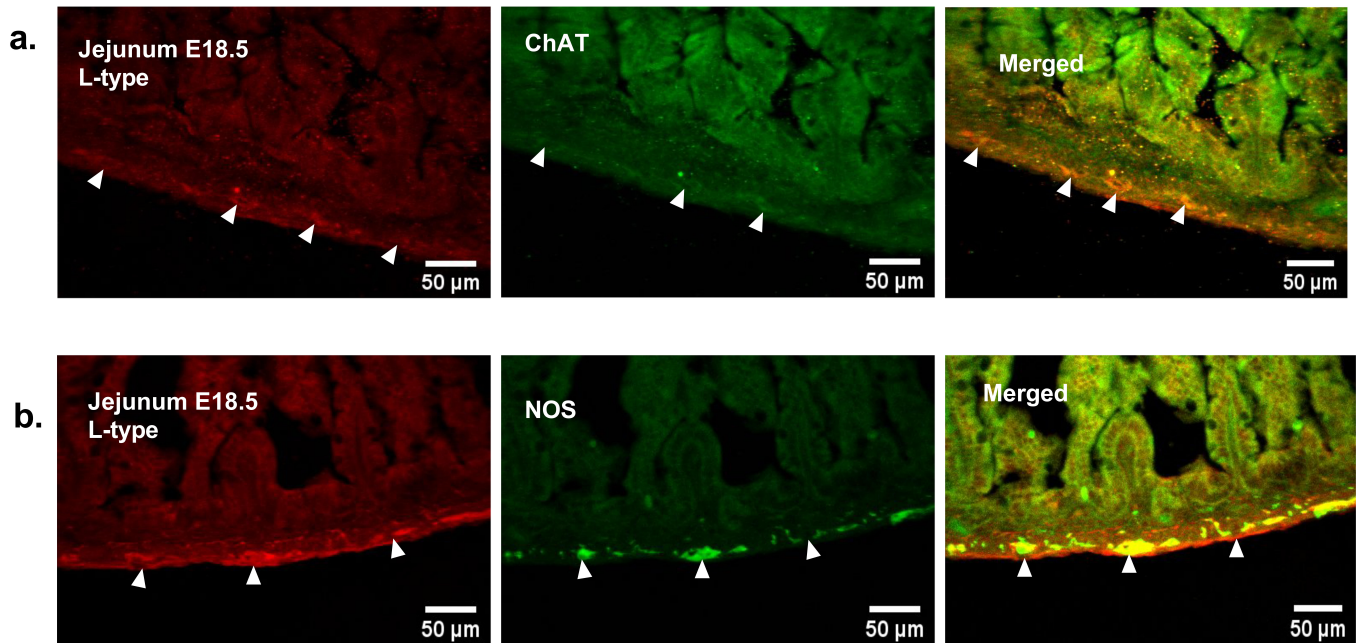


FIG. 3. **Presence of L-type, NOS, and ChAT labeled enteric neurons in E18.5.** the white arrows point to labeled myenteric nerve cell bodies (a,b) L-type colocalization with nitrenergic(NOS) and cholinergic(ChAT) neurons in the jejunum of E18.5 mouse.

molecular pathways could mediate the mechanosensitive behavior of the fetal ENS. The three compounds that abrogated spontaneous calcium activity, 2-APB(100 μ M, n=10), nifedipine(10 μ M, n=10), and TTX(1 μ M, n=10) had no effect on the mechanosensitive calcium flash when the gut was pinched(Fig. 4a, S5). We further tested NOLA(100 μ M, n=5) - an inhibitor of nitric oxide synthase and GdCl₃(100 μ M, n=5) - a TRP channel blocker; neither of these compounds inhibited the calcium uprise in the ENS upon pinching(Fig. 4a, S5). We finally found that the same mechanosensitive response was also present at E14.5, a stage at which the ENS does not present any spontaneous calcium activity(Fig. 4c). Blocking synaptic transmission(Mg^{2+} (10 μ M), Ca^{2+} (0.25 μ M)(n=3) of the ENS abolished the pinch-induced calcium flash. Spontaneous ENS calcium activity was also significantly reduced(n=3).

C. Relation between ENS spontaneous calcium activity and motility

Motility is purely myogenic at stages E13.5 to E17.5. We were interested in understanding how the ENS affects motility when the first spontaneous neurogenic activity settles in at E18.5. We further wondered if the contractile response to pinching would differ for an active or inactive (TTX-inhibited) ENS. E18.5 motility is characterized by a propagating circular contraction and longitudinal contraction. The duodenum(n=4/10), and jejunum(n=5/10) showed an alternating high(with a dom-

inant frequency > 18 cpm) and low(with a dominant frequency < 10 cpm) frequency of contraction(Fig. 5a, S6). The periods of high-frequency contractility occurred at a rate of 0.55 ± 0.1 cpm and lasted for about 60 ± 5 s. After TTX(1 μ M) was added, the alternating high and low frequency wave pattern was abolished(Fig. 5b). This pattern, therefore, represents one of the first neurally mediated motility patterns of E18.5 mice. The gut in TTX showed a rhythmic contraction with a frequency of 11 ± 2 cpm, i.e. comprised between the high and low dominant frequencies when TTX was introduced (Fig. 5b). There was a circular contraction in response to the pinch after TTX(1 μ M) was added.

D. Mechanosensitivity and Spontaneous Calcium Activity of the Adult Mouse

The embryonic gut responds to pinching by an increased calcium activity even in the presence of TTX. We were curious to find out if this mechanism is conserved in the adult mouse. We applied mechanical force using a 0.3 mm diameter blunt glass probe to gently pinch the gut wall of the mouse colon. We observed an instantaneous increase in calcium activity(Fig.6a, S7) in the ENS(n=3). Instantaneous calcium increase of the colon ENS(n=3) persisted in the presence of TTX(1 μ M)(Fig.6b, S8). The region of the gut pinched is a few millimeters away from the point imaged. We blocked synaptic transmission(Mg^{2+} (10 μ M), Ca^{2+} (0.25 μ M) [26]) as a proof of concept that pinch-induced calcium activity requires chemi-

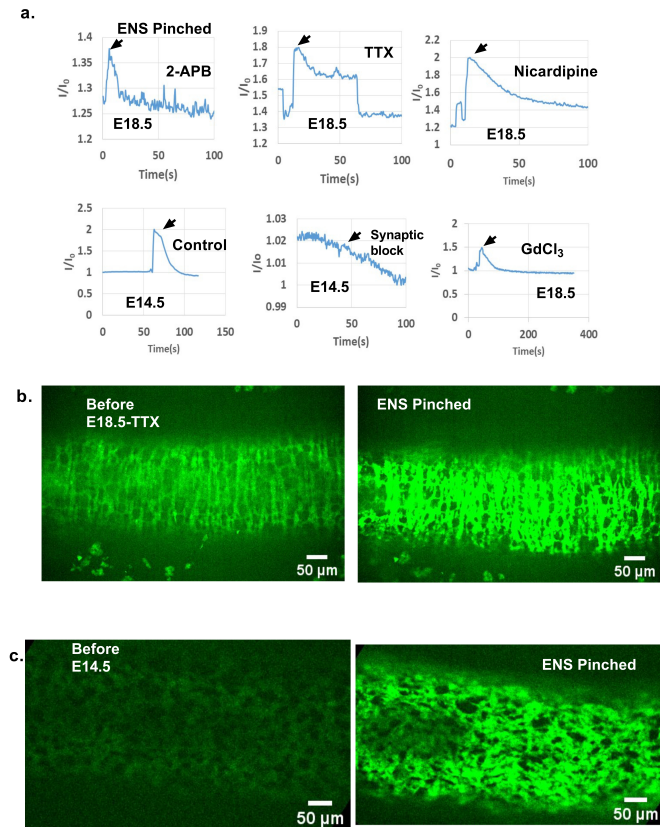


FIG. 4. **Pinching effect of ENS.** ENS intensity change when pinched (a) after 2-APB(100 μ M), TTX(1 μ M), nicardipine(10 μ M), Krebs solution([26]), GdCl₃(10 μ M), were added. (b) Calcium flash of the ENS when pinched after TTX(1 μ M) was added (c) E14.5 jejunum response to pinching.

cal synapses. No flash or increased calcium activity of the ENS was evident(S9) in these conditions. Spontaneous ENS calcium activity was significantly reduced($n=3$). Contraction of the smooth muscles persisted when synaptic transmission was blocked and a pinched-induced contraction of the smooth muscles was recorded($n=1/3$). We also found that nicardipine(10 μ M), as in the embryonic mouse, almost completely abolished spontaneous calcium activity in the adult mouse($n=7$) colon(Fig.6c).

IV. DISCUSSION

In this study, we showed that spontaneous neuronal calcium activity is present in the E18.5 mouse small intestine and that the frequency of this activity decreased as the mouse aged from E18.5-P3. This spontaneous calcium activity requires L-type calcium channels (Fig. 2, S2), intracellular store-operated calcium channels, and IP3R (2, S3). Consistent with this, L-type calcium channels immunoreactivity was identified in both excitatory and inhibitory neurons (Fig. 3). L-type channel

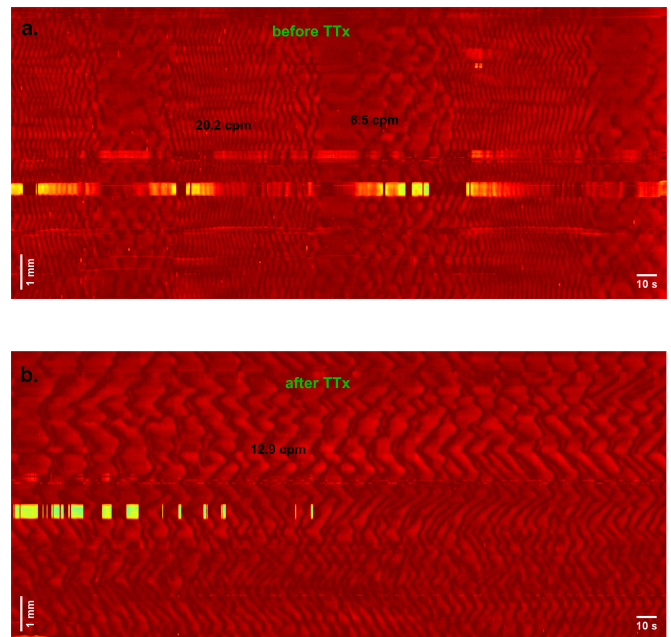


FIG. 5. **Motility patterns of E18.5.** (a) Alternating high and low frequency wave pattern. (b) Rhythmic contraction after TTX(1 μ M) was added.

dependence of spontaneous activity was further observed in the adult colon (Fig.6). We identified that the fetal ENS can transduce mechanical force into calcium activity. Mechanical probing promptly increased intracellular calcium in ENS cells (Fig. 4, S7). This behavior was from E14.5 (Fig. 4), was TTX resistant both in the fetal and adult gut (Fig. 6), but was inhibited when synaptic transmission was blocked (Fig. 4). We detected a motility pattern of alternating high- and low-frequency circular smooth muscle contractions that disappeared when tetrodotoxin was added at E18.5 (Fig. 5). Spontaneous calcium activity has been recorded in many parts of the developing central nervous system and is known to control essential aspects of development [27–32]. We found that E18.5 mouse enteric neurons in the duodenum and jejunum present spontaneous calcium activity. This activity is TTX-dependent and mediates a motility pattern characterized by alternating high and low contractile frequencies in the duodenum and jejunum. These results corroborate and complement previous electrophysiological and motility observations at E18.5 [19]. As this neurally mediated motility pattern develops just prior to birth, it presumably facilitates absorption of milk-derived nutrients during weaning; it might prefigure neurogenic motility patterns like segmentation or migrating motor complexes that are crucial in mature animals [1, 2]. We note that the spontaneous neuronal activity we observe at E18.5 is distinct from the early calcium events observed in neuronal and migrating enteric neural crest cells [16]. This latter activity has been shown to peak at E12.5

and disappear by E16.5, and to be driven by purinergic signaling [16].

We found that calcium activity was higher in the duodenum than in the jejunum at each of the stages considered (E18.5-P3). Because enteric neurons colonize the duodenum first and mature earlier in more rostral regions of the gut [8, 33], this higher activity in the duodenum probably results from increased ion channel expression or developmental changes in ion channel properties. Neurogenic motility in the hindgut for example only develops from stage P10 [19]; the amplitude of calcium response to electrical stimulation from E15.5 and E18.5 duodenum has been reported to be higher than those from the colon at the same ages [16]. Unexpectedly, we found that spontaneous calcium activity decreased from E18.5-P3 in the duodenum and jejunum. This observation parallels studies in the central nervous system showing that spontaneous depolarizing potentials are observed during the first eight postnatal days in the hypothalamus, but decrease from P9 to P12 and disappear by P12.2 [27].

L-type channels are prominently expressed in smooth muscle [34–37] and are often used in electrophysiological and calcium imaging studies of the ENS to limit movements of intestinal preparations. Our protocol constrained movements by gently pressing the gut against the Petri dish bottom and we were thus able to monitor the effects of L-type channels on spontaneous neural activity. Strikingly, we found that nifedipine application almost completely abolished spontaneous activity, both in the fetal E18.5 mouse foregut and the adult mouse colon. Because L-type calcium channels are present on both NOS and ChAT neurons, these results support a direct neuronal effect of nifedipine and further indicate that the spontaneous ENS calcium activity we observe stems from both NOS and ChAT neurons. Given the widespread use of L-type channel blockers to treat hypertension[38, 39], it is noteworthy that they have extensive repercussions on the ENS as well.

We further found that 2-APB abolished spontaneous neural activity at E18.5, indicating the prominent role played by intracellular calcium stores. We note that 2-APB affects spontaneous ENS activity and smooth muscle(both spontaneous and pinch-induced contraction), and not pinch-induced ENS activity. Synaptic blocking affects both spontaneous and pinch-induced ENS activity and not pinch-induced smooth muscle contraction. This helped us to dissociate ENS and smooth muscle mechanosensitivity response.

Distension results in mechanical deformation of the cell body of neurons and changes in calcium activity. Mechanosensitive neurons detect mechanical distension and relay the bioelectric signal to excitatory or inhibitory effector neurons [40]. Recent studies suggest that a broad population of enteric neurons respond to mechanical stress by a change in their firing behavior and or

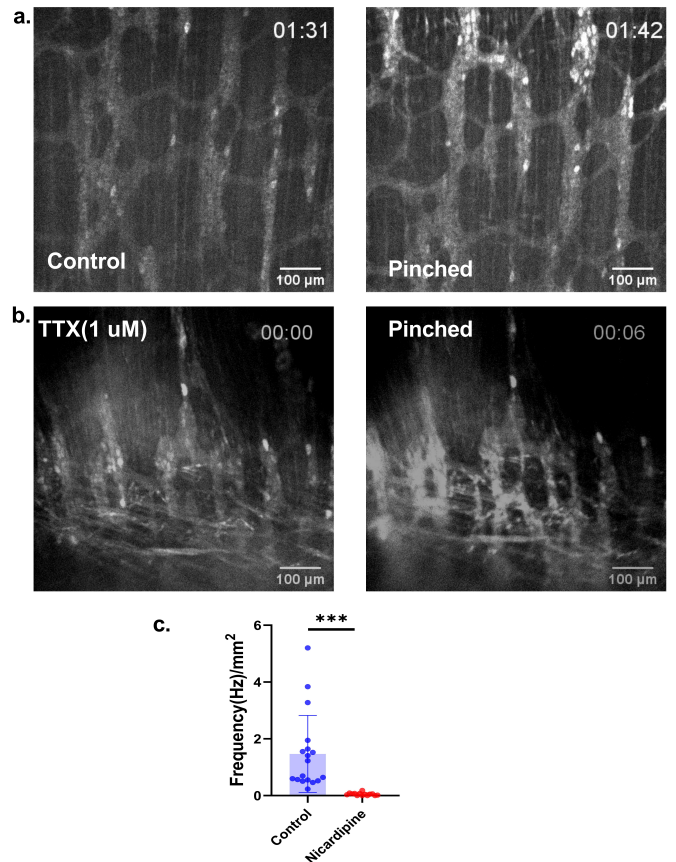


FIG. 6. Spontaneous and Pinch-Induced Calcium Activity of the Adult Mouse Colon. (a) Calcium activity of adult mouse colon before and when pinched(S7). (b) Calcium activity in the presence of TTX($1 \mu\text{M}$) before and when pinched(S8). (c) Effects of nifedipine($10 \mu\text{M}$) on spontaneous calcium activity($n=7$, we looked at different regions of the colon of seven different mice).

frequency [13, 41]. Intestinal distension in the small and large intestines of adult mouse colon affects most of the neurons in the ENS by locally modulating their levels of intracellular calcium ions[15]. Similar observations were made in primary cultures of neurons from the esophagus [42]. What triggers the increase of intracellular calcium ions in response to mechanical stress in the late embryonic and early postnatal ages remain to be fully understood. Candidates of ion channels considered were Piezo1[43], which is widely expressed in enteric neurons and considered mechanosensitive, TRP[15], which has been shown to be involved in mechanosensitivity, L-type channel, and intracellular SOC, which we have shown to be involved in spontaneous calcium activity. In the colon of adult mice, distension-induced regulation of intracellular calcium was shown to be dependent on KCa1.1 channels activity [15]. We found that when the embryonic gut was pinched, the ENS responded with a conspicuous increase in intracellular calcium. Because

whole-organ mechanical stimulation elicits both an instantaneous myogenic contractile response and a compression of the ENS, we could not dissociate whether the ENS calcium increase was due to muscle contraction, direct ENS mechanical stimulation, or both. Strikingly, this response was resistant to GdCl_3 , 2-APB, nicardipine, and tetrodotoxin, and was present even at very early embryonic stages (E14.5). We further demonstrated that this TTX-resistant response is present in the adult colon. Our results allude to the fact that the increase of intracellular calcium in the ENS might be a generic reaction resulting from compression-induced disruption of the endoplasmic reticulum within cells, that triggers a calcium release even in the presence of 2-APB. Similarly, electric field stimulation-induced calcium transients still persisted after the addition of a cocktail of antagonists to block all five types of voltage-gated calcium channels in E12-E18 mice [16]. Our results suggest that mechanical (and perhaps electrical) stimulation can trigger calcium release by cytoplasmic phenomena that are independent of voltage-gated channels at the membrane. An interesting result that needed exploring was the calcium flash in the presence of TTX at the early embryonic and late postnatal stages. It has been well established

that nerve conduction along axons in the ENS cannot occur without Na^+ channels[44–46]. We were curious to find out if this result is observed in the adult mouse as it poses a potential alternative mechanism for cell-cell propagation. Increased calcium activity persisted in the ENS of adult mouse colon when pinched in the presence of TTX. This indicates synaptic axonal propagation even when Na^+ channels were blocked. One possibility is a different mechanism that could mediate cell-cell propagation without Na^+ channels. It has been found in our work and other works of ENS[15] and CNS[44, 45], the presence of TTX-resistant neurons. Although three to five times slower than the kinetics of fast TTX sensitive neurons[44], could be a possible means of synaptic propagation in the presence of TTX.

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