Shifting into high gear: how interstitial cells of Cajal change the motility pattern of the developing intestine

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Running title: From smooth-muscle to pacemaker driven contractile waves

Keywords
Interstitial cells of Cajal, embryo, intestine, contraction wave, smooth muscle, motility, calcium imaging, pacemaker

New & Noteworthy
- We reveal a sharp transition from smooth muscle to Interstitial Cell of Cajal driven motility in the chicken embryo, leading to higher frequency, more rhythmic contractile waves.
- We predict the transition to happen between 12 and 14 embryonic weeks in humans.
- We image for the first time the onset of ICC activity in an embryonic gut by calcium imaging.
- We show the first KIT and ANO1 in-situ hybridization micrographs in the embryonic chicken gut

Abstract

The first contractile waves in the developing embryonic gut are purely myogenic, they only involve smooth muscle. Here, we provide evidence for a transition from smooth-muscle to Interstitial Cell of Cajal (ICC) driven contractile waves in the developing chicken gut. In-situ hybridization staining for ANO1, a known ICC marker, shows that ICCs are already present throughout the gut as from embryonic day 7 (E7). We devised a protocol to reveal ICC oscillatory and propagative calcium activity in embryonic
gut whole-mount and find that the first steady calcium oscillations in ICCs occur at E14. We show that the activation of ICCs leads to an increase in contractile wave frequency, regularity, directionality and velocity between E12 and E14. We finally demonstrate that application of the c-KIT antagonist imatinib mesylate in organ culture specifically depletes the ICC network and inhibits the transition to a regular, rhythmic wave pattern. We compare our findings to existing results in the mouse and predict that a similar transition should take place in the human fetus between 12 and 14 weeks of development. Together, our results point to an abrupt physiological transition from smooth-muscle mesenchyme self-initiating waves to ICC-driven motility in the fetus, and clarify the contribution of ICCs to the contractile wave pattern.

Introduction

The interstitial cells of Cajal (ICC) are the pacemakers of the intestine (21). They constitute a network of anastomosing cells that present spontaneous, rhythmic oscillations of their membrane potential, called “slow waves” (25, 35, 39). Electrical coupling of these cells to the circular smooth muscle paces the generation of circular contractile waves essential for digestion. They are also electrically connected to the enteric nervous system (ENS), acting as an intermediate between the ENS and its effector, the smooth muscle (19). ICCs have attracted considerable attention both for their fundamental “clock-cell” property (21), as well as for their potential involvement in conditions like Hirschsprung disease, pyloric stenosis, or chronic intestinal pseudo-obstruction (10).

ICCs and smooth muscle both originate from the mesoderm (20). ICCs have been detected as early as E13.5 in the mouse embryo (31), i.e., at the same time as circular smooth muscle differentiation in the midgut. They reach a mature morphology only around E18.5 in the mouse (31), stage at which the first slow waves could be recorded by microelectrode impalement (3, 31). Using W/W’ ICC-depleted mutant mice, Roberts et al. concluded (31) that the first circular smooth muscle contractile waves starting at E13.5 are not generated by ICCs. The calcium waves underlying these smooth muscle contractile waves have been recently imaged in the chicken embryo (6, 17). Motility of the adult W/W’ mouse has further been investigated both in-vivo (9) and ex-vivo (29). All of these studies indicate that smooth muscle alone has the capability to spontaneously generate contractile waves. This myogenic phase precedes any influence of the enteric nervous system on motility because the latter only becomes active later in development (E18.5 in mice (31), E16 in the chicken (5)). The ICCs are not the only cells that can produce rhythmic motility patterns in the gut: it has recently been shown (32, 33) that the enteric nervous system can also generate rhythmic patterns of coordinated neuronal firing leading to rhythmic patterns of electrical activity (EJPs and IJPs) and contractility in the smooth muscle. Adult W/W’ ICC-depleted mutant mice still display exhibit prominent enteric neurogenic activity and cyclical motor complexes still occur (34).

In a recent publication (5), we had measured an abrupt change in the speed of circular smooth muscle contractile waves between E12 and E14 in the chicken intestine, leading us to speculate that a developmental transition to ICC-driven waves could occur around this embryonic stage. The emergence
of slow waves in the mouse embryo has been examined by microelectrode single-cell
electrophysiological measurements (3, 31, 40). In this report, we strived to measure the effect of ICCs on
motility in the embryo by time and space resolved techniques: calcium imaging and spatiotemporal
contractility maps. Given the lack of information on the development of ICCs in the chicken embryo, we
also performed in-situ hybridization for the cell surface receptor ANO1, revealing the ultrastructure of
this cell network at early embryonic stages in this important animal model. We compare our results to
electrophysiological (3, 31) and motility (9, 29) studies in the mouse, discuss their implications for
humans, and conclude that interstitial cells of Cajal cells lead to an abrupt transition in the motility
pattern of the developing gut.

Results

Interstitial cells of Cajal are present throughout the chicken GI tract as from E7

We detected the presence of interstitial cells of Cajal by in-situ hybridization for the surface receptor
ANO1 (also called TMEM16A) and KIT. The ANO-1 chloride channel has been shown in the mouse (14) to
be a specific maker of ICCs. ANO-1 positive cells were present in the entire lower GI tract (duodenum,
jejunum, ileum, hindgut and caeca) as from E7 and at all later stages (Fig.1a). The number of guts
stained were: E7 n=5, E8 n=3, E9 n=3, E10 n=3, E12 n=4, E14 n=2. The stomach was only stained in the
muscular region; the mesentery, the cloaca and any remaining umbilical stalk tissue were not stained.
KIT staining could only be detected as from E10 (Fig.1b). ANO1 and KIT staining patterns were identical
as from E10.
Figure 1. ANO1 (TMEM16A) and KIT in-situ hybridization. (a) Whole-mount images for ANO-1. St= stomach, duod = duodenum, jej = jejunum, il = ileum, hg = hindgut. (b) Whole-mount images for KIT. (c) Magnified regions of TMEM16A staining imaged by differential-interferential contrast, in the duodenum, jejunum and ileum at E14, the scale bar is the same for all images, the black line indicates the rostro-caudal axis.

Higher magnification differential-interferential contrast (DIC) imaging of the ANO1 in-situ hybridized guts showed a net of dark, interconnected cells, that were located just above the circular muscle layer and elongated along the rostro-caudal axis of the gut (Fig.1c). At E14 the longitudinal layer was differentiated and we could confirm that the ANO1-positive cells were located between the longitudinal and circular muscle layers, i.e. at the level of the myenteric plexus (MP). These ANO1-positive cells are therefore ICC-MP. The ICCs presented similar morphologies at E10 (Fig.S1).

A specific calcium-sensitive dye whole mount loading protocol reveals the electrical activity of ICCs

We next devised a protocol to monitor the calcium activity of ICCs with the calcium-sensitive probe Fluo4-AM. Short-time (10 min) application of this dye to transverse sections of early (E9) embryonic gut had allowed us to visualize calcium waves propagating across the contractile smooth muscle layer (6). Here, we loaded ~1 cm long whole-mount segments of demensenterized E12-E14 guts at the same
concentration, but for significantly longer times (4 hours) and in the presence of higher (0.1%) quantities of the cell permeant Kolliphor EL (for details see Materials & Methods). The samples were immobilized as shown in Fig. 2a inset (see Materials & Methods for details). A representative video of the calcium activity observed following this preparation is shown in Video S1.

Active (=presenting a time‐dependent fluorescence) cells had a stellate morphology, and were slightly elongated along the rostro-caudal axis of the gut (Fig.2a). They formed a discontinuous net, probably because of non-uniform penetration of the calcium dye. We found that the calcium activity consisted of regular spikes of fluorescence that propagated along the rostro-caudal axis (Fig. 2b). Although the explants presented contractions (see Video S1), the active cells were not contractile. The frequency of calcium oscillations at age E14 (2.2 ± 0.5 cpm, n=16 samples from n=9 embryos, uncertainty is ± SD) and E16 (3.1 ± 0.5 cpm, n=8 samples from n=5 embryos) corresponded to the frequency of circular smooth muscle contractions as recorded in a physiological bath at these two stages (Fig.2c). In particular, the calcium spike frequency was significantly higher at E16 than at E14, just like the circular contraction frequency (Fig.2c). We further found that the oscillations were insensitive to nicardipine (Fig.2d-e), a Ca\(^{2+}\) L-type channel blocker. This is unlike smooth muscle calcium transients, which critically depend on L-type channels (18). Independence on L-type calcium channel is a characteristic of oscillations in ICCs (21).

Fluo4-AM penetrated in the ganglia and inter-ganglionic fibers of the myenteric plexus (MP), so that the morphology of this part of the enteric nervous system could be clearly visualized following calcium dye loading (Video S2). However, none of the ganglia or inter-ganglionic fibers presented any activity, i.e. the fluorescence labeling was constant in time. Importantly, the active cells were in the same plane as the myenteric plexus, but never co-localized with ENS structures. On the basis of the morphological and physiological characteristics of the transients and of the cells that produced them (summarized in Table 1), we can conclude that the active cells are neither smooth muscle, nor enteric neurons, nor glia, and can only be stellate-shaped pacemaker ICC-MP present at the level of the myenteric plexus.
Figure 2. Calcium activity in interstitial cells of Cajal. (a) Still shot from Video S1, E14 duodenum. The ICCs appear as brighter cells with a stellate morphology. Inset: Setup to immobilize the gut for calcium imaging. The nylon thread inserted in the lumen gently presses the gut against the dish bottom. (b) Fluorescence (calcium) dynamics in three cells circled in (a), showing both the rhythmic and propagative nature of the transients. (c) Comparison of the frequency of calcium spikes recorded by calcium imaging, and of the contraction frequency obtained from video recording of guts in a physiological bath. *: statistically significant difference, p<0.05, one way ANOVA test. (d) Representative effect of nicardipine on calcium oscillations. (e) Frequency change induced by nicardipine for n=7 samples of E14 duodenum and proximal jejunum, from n=4 different embryos. The frequency change is not significantly different from 0. (f) Percent active samples for E12, E14 and E16 embryos. The number of embryos at each stage is n=5 E12, n=13 E14, n=7 E16. An active sample is defined as having at least one region with one cell showing sustained fluorescence oscillations.
Table 1: Properties of the cells observed (Fig. 1) compared to other cells in the intestines. Green indicates a correspondence with properties of the cells we observed.

<table>
<thead>
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<th>Morphology</th>
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<td>0.2-0.4 cpm</td>
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<td>Honeycomb</td>
<td></td>
<td></td>
<td>At MP</td>
<td>Yes</td>
</tr>
<tr>
<td>ICC-MP</td>
<td>Stellate</td>
<td>Longitudinal</td>
<td>1.5-3 cpm</td>
<td>No</td>
<td>At MP</td>
<td>No</td>
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Propagating calcium activity in ICCs can first be detected at E14

We examined the calcium activity of ICCs at age E12, E14 and E16. We defined a sample as active if it presented at least one cell with sustained oscillations after examining 3 different regions of the sample. The same protocol for calcium indicator loading was applied to all samples so that their activity could be compared; E12 and E14 guts were loaded on the same days and in the same 12-well plate. Activity could be detected in 1/15 samples at E12, in 25/44 samples at E14 and in 9/17 samples at E16 (Fig. 2f).

Activation of ICCs is concomitant with a change of contractile wave speed, frequency and regularity

We next examined the contractile wave pattern at E12 and E14, at the time where calcium activity in ICCs was first observed. Spatiotemporal diagrams at E12 and E14 show conspicuous differences (Fig.3a). Contractile waves at E12 are multi-directional, irregular, and relatively slow. In contrast, at E14, they become regular, keep the same direction for prolonged periods of time, and travel at a relatively higher speed. By compounding data from experiments at embryonic (7) and fetal (5) stages we found that the frequency of contractile waves increased linearly between E8 and E16, at a rate of 0.22 cpm/day (Fig.3b). The speed of contractile waves exhibited a pronounced discontinuity between E12 and E14 (Fig.3c), increasing from 25 µm/sec to 125 µm/sec, a five-fold difference.
Specific ICC inhibition by imatinib mesylate abolishes the transition to ICC-driven motility at E12-E14

We finally asked whether the transition to regular contractile waves happening between E12 and E14 could be inhibited by specifically targeting ICCs. Imatinib mesylate is a known inhibitor of the c-KIT surface receptor and has been successfully used to inhibit ICC development in the mouse by Beckett et al. (3). We first examined the effects of imatinib on ICCs, smooth muscle cells and the ENS by performing in-situ hybridization and RTqPCR on E12 intestines cultured for 3 days in the presence or in absence of Imatinib (Fig.4). We placed 1-2 cm long segments of E12 alternately in control medium (with vehicle only) or in 10 µM imatinib medium; this procedure guaranteed that control and drug-treated samples were from the same rostro-caudal region along the GI tract (see Materials & Methods). Imatinib treatment induced a decrease in the level of TMEM16A (Fig.4a) and KIT transcripts (Fig.4d), suggesting a decrease in the number of ICCs. No significant changes were observed on the transcript levels of SOX10 (Fig.4b,e) and SMC-specific genes, such as αSMA (Fig.4f) and SM22 (Fig.4c,g), indicating that the ENS network and the smooth muscle cells were not affected by imatinib.
Figure 4: Whole-mount in situ hybridization of E12 intestine treated or not with imatinib (10 μM) for 3 days. Control (left panel) and imatinib-treated (right panel) intestines were processed for TMEM16A (a), SOX10 (b) and SM22 (c) riboprobes. TMEM16A staining is lower in imatinib-treated intestine compared to Control segment. ENS network (SOX10 staining) and smooth muscle (SM22 staining) are unaffected upon Imatinib treatment. (d-g) Quantification of transcript levels by RT-qPCRs in control (Control) and imatinib-treated intestines (10 μM and 20 μM). Imatinib treatments induce a decrease in the level of KIT (d). No significant changes were observed in the level of SOX10 (ENS marker)[e] and aSMA/SM22 (smooth muscle marker)[f, g]. Data were normalized to GAPDH and RPLPO expression. Normalized expression levels were converted to fold changes. *P<0.05; ns, not significant. Values are presented as the mean ± SD of n = 4 control-, n = 4 10 μM - and 20 μM - imatinib treated intestines. *P < 0.05; **P < 0.01 by two-tailed Mann–Whitney tests, comparisons are performed with the control condition.

We then examined the effects of imatinib on motility. We first tested the acute effects of this drug on contractile activity at E14, when ICCs are already active. We found that imatinib at concentrations of 1 μM and 10 μM did not have any acute effects on the contractile wave pattern (Fig.5a, n=5, E14 duodenum) for up to 30 minutes. This indicates that imatinib at these concentrations does not have an immediate action on ion channels that mediate contractility (e.g. calcium channels). Imatinib did however have a marked effect when applied for 3 days on E12 guts in culture, in the critical period when ICCs become active. In control samples (n=18), the frequency (Fig.5e) and contractile wave speed (Fig.5f) significantly increased after 3 days of culture, reflecting the physiological changes happening in-ovo (Fig.3b-c). In particular, the contractile wave pattern of control samples after culture exhibited a high degree of regularity and directionality (Fig. 5b, top), like E14 intestines (Fig.3a). This echoes findings by other investigators that the ICC network develops normally in cultured, isolated mouse intestines (40).
Figure 5. Effect of imatinib mesylate on the motility during the Cajal transition. (a) Representative spatiotemporal maps of acute effects probed on E14 duodenum (n=5 embryos probed). (b) Spatiotemporal maps of control (n=18/18) and imatinib-treated segments featuring very irregular contractions (n=13/18). (c) Representative Fourier-transforms of a horizontal line along the spatio-temporal diagram of control and imatinib-treated samples. (d) Compared intensity of the first peak of the Fourier transform (n=18 control, n=18 imatinib). (e,f) Evolution of the frequency and speed of contractile waves at E12 (n=10) and after 3 day culture of the midgut segments in DMEM with vehicle alone (control, DMSO, n=18) or in imatinib 10µM (n=18) *: statistically significant difference, p<0.05, two-tailed Mann-Whitney test.
The contractile pattern of imatinib-treated samples was characterized by a conspicuous lack of rhythmicity (Fig.5b bottom). We characterized this by performing time Fourier transforms of the spatiotemporal diagrams (Fig.5c). Whereas control samples presented a distinct peak (black arrowhead) with harmonics, the peak was either weak and broad in 5/18 or non-existent in 13/18 imatinib-treated samples. The intensity of the Fourier peak was significantly reduced in imatinib treated samples (Fig.5d). Motility in imatinib samples were characterized by long periods of quiescence, interspersed by single or trains of contractions. The average frequency between trains of contractions was 0.9 ± 0.7 cpm (n=18, Fig.5e) and was significantly lower than in controls (2.9 ± 1.3 cpm). The contraction velocity was higher in imatinib-treated samples (418 ± 316 µm/sec, Fig.5f) than in their control counterpart (266 ± 270 µm/sec), although this was not statistically significant. Video S3 illustrates the contractile activity of a pair of control and imatinib-treated guts after 3 days in culture. We also tested whether imatinib could have side-effects on E7 guts cultured for 3 days, i.e., at a time interval where ICCs are presumably not yet active. We found that the spatiotemporal maps, frequency and speed of contractile waves in control and imatinib treated samples did not differ (Fig.S2), indicating that imatinib 10 µM did not affect purely myogenic (smooth-muscle driven) contractility.

Discussion

We showed by in-situ hybridization for the cell-surface receptor ANO1 that ICCs are present at least as from E7 in the whole lower gastrointestinal tract of the chicken, and presented the first images of this cell network in the avian embryonic gut (Fig.1). We next devised a protocol for whole-mount calcium indicator loading of gut explants that allowed us to record what we identified as ICC oscillatory and propagative calcium activity (Fig.2). We found that calcium activity in ICCs starts at E14. Comparison of the contractile wave activity at E12 and E14 shows that the start of ICC activity coincides with the onset of rhythmic and coordinated contractions that propagated as a coherent wavefront along the intestine, with a stabilization of contractile wave rhythm, propagation direction, and also with an increased propagation speed (Fig.3). Subjecting E12 guts to the c-KIT inhibitor imatinib mesylate in culture depleted ICCs (Fig.4) and abolished the physiological transition to regular, rhythmic contractile waves (Fig.5).

Lecoin et al. (20) were the first to perform c-kit radioactive probe in-situ hybridization in the chicken embryonic gut. They found a weak signal at E7 and provided cross-section images at E9 indicating the presence of c-KIT positive cells at this stage. Using alkaline phosphatase detection to reveal KIT and ANO1 in-situ hybridization, we found that KIT was present in the entire GI tract at E10 but not E7, and that ANO1 positive cells were present at least as from E7, i.e. at the same time or soon after smooth muscle differentiation at E5-E6 (4, 7, 26). This difference between KIT and ANO-1 may be due to the fact that transcriptional levels of ANO-1 in ICCs are known to be higher than for KIT (24). Our results are consistent with studies in the mouse (3, 31), where ICCs were found as from E14.5, i.e. only a day after smooth muscle differentiation in the midgut at E13.5. We found that ICCs organize as a continuous net, located at the level of the myenteric plexus. The morphology of the ICC network in chicken resembles
that revealed by antibody labeling in the mouse embryo (3, 31, 40); this is unlike the enteric nervous system, which has a very different morphology in these two species.

Ca$^{2+}$ imaging has been used to visualize the activity of ICCs in numerous investigations (1, 2, 27, 41), but the results presented here are to the best of our knowledge the first in-situ calcium recordings of ICCs in a developing organism, and in particular of their very first glimpses of activity in this organism’s life. We have found that sustained calcium electrical oscillations start in these cells at stage E14. Electrophysiological recordings indicate that the first slow waves in the mouse intestine arise at stage E18-E19 (3, 31), and are fully developed by two days after birth (23). These developmental times are consistent with the 4-7 day advance of developmental events in the chicken embryo compared to the mouse (see table in (5) for examples). We summarize important milestones in the development of gut motility in the chicken in Fig. 6a. Given the week-day equivalence of human and chicken intestinal development (5), we predict that a transition to ICC-driven motility should take place between 12 and 14 weeks of development in the human embryo.

Figure 6. (a) Important milestones in the development of gut motility in the chicken embryo. Given the observed week-day equivalence (5) of many of these events in human and chicken intestinal development, a similar timeline is expected to hold for the human embryonic gut, in weeks of development. (b) Focus on the underlying cellular differentiation events that underpin the ICC motility transition. *: some contractility in ICCs has been observed by Thuneberg (15) but remains to be confirmed.

We found that the appearance of calcium activity in ICCs at E14 led to drastic changes in the circular smooth muscle contractile wave pattern: waves became more rhythmic (more regular), kept the same direction for prolonged periods of time, and had an increased propagation speed (from 25 µm/sec to 125 µm/sec from E12 to E14). Several methods have been developed to deplete or inactivate the ICC
network: irradiation after methylene blue staining (22), antibodies directed towards c-kit (3, 37, 40), imatinib mesylate (3), and, last but obviously not least, the W/WV mutant which lacks the c-KIT receptor (25, 39). All of these alterations have been shown to suppress slow waves in the murine gut. The W/WV mutant is viable and the effects of ICC depletion on GI motility in the adult mouse have been examined by radiography of barium sulfate gavaged mice and subsequent electrophysiology & manometry (9). These investigators found that whereas contractions in control mice were rhythmic and pushed the contrast fluid aborally, movements in W/WV mice were erratic, with irregular smooth muscle action potentials travelling both orally and aborally leading to weakened overall bolus propulsion. This picture resonates with the erratic contractile wave pattern we obtained after treatment of E12 gut with the c-KIT inhibitor for 3 days (Fig.4d right). Parsons et al. recently reported (29) spatiotemporal D-maps of W/WV and of the Wsh/Wsh mouse; they find very high velocity (“lines of simultaneity”), irregular and multidirectional contractions. The overall frequency of the contractions was ~3 times lower in these mutants (~10-15 cpm) than in control mice (~45 cpm). Our results are in line with these findings, as we also find that the E12 guts treated with imatinib for three days (and which hence are a model of a gut with dysfunctional ICs) present very high velocity, irregular contractions. Physiologically, ICC activation at E14 coincides with a sharp increase in the velocity of contractile waves compared to E12 (Fig.3c). We speculated that this increase may be due to the transition from a trigger-wave regime (28) at early embryonic stages (E7-E12) to an ICC-driven phase wave (28) regime at fetal stages (E14+). Since waves in chains of coupled oscillators depend on electrical phase coupling between adjacent cells, we intuited that they may propagate at higher speeds than trigger waves. We therefore expected ICC-inhibition by imatinib to result in a lower propagation speed compared to controls, but it is in fact the opposite we observed. The increased speed of contraction between E12 and E14 may therefore not be a direct consequence of ICC activation, but rather involve other phenomena like increased gap-junction conductance. As a matter of fact, very early E5-E6 embryonic gut (7), W/WV mice (29) or vascular smooth muscle (30) also feature very high velocity contractions, but none of them contain active ICs. ICCs and smooth muscle are closely related as they both originate from the mesoderm (20) and simply blocking c-kit signaling has been shown to revert ICCs to a smooth-muscle phenotype (36). This plasticity reinforces the view that the early smooth-muscle mesenchyme (E6-E12) should be viewed as one unit that has both contractile and contraction-initiating (pace-making) properties (Fig.6b left). It separates in a Kit- & ANO1-positive (ICCs) and a Kit and ANO1-negative population (smooth muscle). The first population (ICCs) loses the ability to contract and specializes in generating the regular electrical impulses that initiate contractions. The second (smooth muscle) in turn loses the ability to generate its own regular electrical impulses, and retains contractility. While ICCs can be observed as from E7, they only become active in the interval E12-E14. This phase separation and subsequent activation (Fig.6b right) drives a major change in the rhythmicity and directionality of contractions. In contrast, spatiotemporal maps from E6 to E12 were qualitatively quite similar. We therefore suggest the term “ICC transition” to refer to this abrupt motility regime change happening in the developing fetal gut. Important questions are: how common is this separation in contractile vs pacemaking population in other smooth-muscle lined organs (16) ? In cancer tumors (8) ? What factors trigger this separation? Later in gut development the enteric nervous system becomes active (at E16 in the chicken and E18.5 in the mouse), triggering the emergence of the migrating motor complex (5, 31) and of the asymmetric ascending-contraction
descending-inhibition barometric reflex (5). Because different cell types become active at distinct times during ontogenesis, the developing embryo represents a unique laboratory to understand the sequence of events and the mechanisms leading to mature physiological reflexes.

**Materials & Methods**

**Chicken gut samples**

Fertilized chicken eggs were purchased from EARL Morizeau (Chartres, France, breeder Hubbard, JA57 hen, I66 rooster, yielding type 657 chicks). The eggs were incubated at 37.5 °C in a humidified chamber for 7 to 16 days. The gastrointestinal tracts were dissected from hindgut to stomach, the mesentery and Remak’s nerve were removed.

**Whole-mount in situ hybridization**

Whole-mount in situ hybridization analyses were carried out as described using newly isolated antisense ANO1 riboprobes (4, 12), SOX10 (4), SM22 (11). Chick ANO1 cDNA also known as TMEM16A or DOG1 was isolated from E6 stomach RNA extract using specific primer pair of predicted chick ANO1 (Gene Bank ID: XM_004941529.3; forward: 5’-AAG AGA AAG CAG ATG CGG- 3’, reverse: 5’-GCA ACA TAG AAG ATG GGT GTG- 3’). The resulting 517 base pair (bp) fragment was cloned into the pGEM-T Easy plasmid (Invitrogen) and sequenced to verify identity. Dissected GI tissues were fixed in 4% paraformaldehyde for 1 h at room temperature, washed in PBS, gradually dehydrated in methanol in order to store the samples at −20 °C for at least one night. For whole-mount in situ hybridization experiments, tissues were gradually rehydrated in PBS, washed in PBT (PBS, 0.1% Tween) and incubated for 1 h in 6% hydrogen peroxide (Sigma, France). Samples were next permeabilized by treatment with proteinase K (10 µg/ml) for 10 min, washed with glycine in PBT and fixed in 4% paraformaldehyde/0.2% gluteraldehyde in PBT for 20 min. Tissues were then hybridized with antisense ANO1 digoxigenin-labeled (Roche) riboprobes overnight at 70 °C. After post-hybridization washes at 70 °C, tissues were incubated in 10% sheep serum for 2.5 h at room temperature and finally mixed with preabsorbed anti-digoxigenin coupled with alkaline phosphatase antibody (Roche) overnight at 4°C. The complexes were detected with BM Purple, a chromogenic substrate for alkaline phosphatase (Roche). Whole-gut images (Fig.1a) were acquired with a Canon D500 digital camera fixed to a tripod; magnified images (Fig.1c, S1) were obtained by pressing excised ~1 cm long gut segments between two microscope glass slides spaced out by 200 µm. The compressed guts were imaged with an inverted Leica microscope in DIC (differential interferential contrast) mode.

**Quantitative real-time RT-PCR**

Total RNA was extracted from cultured intestines with or without Imatinib with the HighPure RNA Isolation Kit (Roche). Reverse transcription (RT) were performed using the Verso cDNA Synthesis Kit (Thermo Scientific) and quantitative real-time PCR was performed using the LightCycler technology (Roche Diagnostics). PCR primers (Fig.S3) were designed using the LightCycler Probe Design software-
2.0. Four individual intestines were individually analyzed for each condition. Levels of KIT, αSMA, SM22 and SOX10 transcripts were determined with the LightCycler analysis software (version 3.5) relative to standard curves. Data were presented as the expression levels of KIT, αSMA, SM22 and SOX10 relative to the expression of the reference genes GAPDH and RPLPO. The relative mRNA expression was calculated using the 2-ΔΔCT method (13, 26).

**Calcium imaging**

1-cm long segments from the proximal half of the midgut (duodenum and jejunum) were collected. For calcium indicator loading, 50 μg of Fluo4-AM (Thermofisher, F14201) were dissolved in 10 μL DMSO; this solution was added to 2 ml of DMEM GlutaMAX (Thermofisher, 4.5 g/L D-glucose, sodium pyruvate, Ca²⁺ 1.8 mM, Mg²⁺ 0.8 mM) containing 1% penicillin-streptomycin (PS), 25 mM HEPES and 0.1% Kolliphor EL (Sigma). Each segment was placed in a separate well containing 200 μL of this loading solution, in a 12-well plate; the atmosphere above the wells was maintained at 95% O₂, 5% CO₂ by constant perfusion of the plate with carbogen, in a 37°C incubator. Samples were incubated for 4 hours – this unusually long incubation time as well as the high Kolliphor concentration (0.1%) were key to reliably obtain signal in ICCs in this whole-mount preparation protocol. When comparing the calcium activity at different ages (Fig.2c), gut segments of different aged embryos (E12-E16) were incubated in the same loading solution and plate and their activity compared after 4 hour incubation.

Immobilization of the samples for imaging is illustrated in Fig.2a inset. A thin plastic rod (nylon fishing thread, 150 μm diameter for E12, 200 μm for E14 and E16) was inserted in the lumen of each segment, and one end of the rod was pinned to a ~ 1 cm³ slab of PDMS secured to the bottom of a 35 mm Petri Dish. The rod delicately pressed the preparations against the petri dish. Friction of the gut with the dish prevented excessive motion of the cells due to gut contractions. Samples were imaged with an inverted spinning-disk confocal microscope (Olympus or Zeiss) at magnification x20, laser excitation 488 nm, emission filter 512 nm, 2 Hz imaging frequency, at 37°C. Images were collected from one confocal plane (no z-stack). The samples were immersed in 3 mL of DMEM that had been saturated with carbogen just prior to sample imaging. For experiment with L-type calcium channel blockers, time-lapse was set for ~10 min; nicardipine (10 μM) was added after ~5 min recording. Resulting movies and calcium fluctuations were analyzed with ImageJ.

**Recording of contractile activity**

Physiological bath video recordings of gut activity were performed following previously established techniques (5). Briefly, gut segments were pinned vertically to the wall of a Sylgard-lined chamber filled with 40 mL DMEM at 37°C constantly bubbled with carbogen. Guts were recorded with a video camera (1 Hz time-lapse) after a 30 min equilibration period, up to 6 segments were recorded simultaneously. The contractile activity – speed and frequency - was analyzed using spatiotemporal maps and the “Reslice “function on rectangular ROIs encompassing the gut segments (5, 7). The speed was sometimes directly measured from the movie when a good quality map could not be obtained. Acute effects of imatinib mesylate (Sigma) were tested by adding increasing concentrations from a 14.4 mg/mL concentrate solution in DMSO.
Organ Culture

Immediately after dissection, E12 proximal midguts (duodenum ad jejunum) were cut in ~1 cm long segments, the segments were alternatively placed in the control medium (DMEM + 0.04 % dmso) or in the imatinib medium (DMEM + 10 µM imatinib). This procedure guaranteed that control and imatinib-treated sample pairs originated from the same gut region and therefore initially had similar contractile properties. Each segment was placed in 1 mL of medium in a 35 mm Petri dish; all samples were incubated at 37°C in a 95% O₂ + 5% CO₂ atmosphere by constant carbogen perfusion. We previously (18) found this atmosphere to be suitable for chicken fetal gut development and growth. Medium and drug were replaced after 1.5 days culture. After 3 day culture, the contractile activities of the control and imatinib-treated segments were monitored in the physiological bath setup described above.

Supplementary Videos

Supplementary Information and Videos can be accessed at the repository (private link):
https://figshare.com/s/148c4857ccbce94a783e

Supplementary Information: Fig. S1 Magnified regions of TMEM16A staining at E10; Fig.S2 Effect on motility of imatinib mesylate 10 µM on E7 guts for 3 days in culture; Fig.S3 Gallus Gallus primers for RT-PCR.

Video S1: Calcium activity of ICCs in E14 duodenum

Video S2: Enteric nerve static labeling by Fluo4-AM and absence of colocalization with active (oscillating) cells in E14 duodenum

Video S3: Activity of a pair of control (C) and imatinib (I) treated guts.

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Author contributions

NRC led the project, obtained funding, performed experiments, analyzed data, synthesized data and wrote the paper; YA discovered calcium activity in embryonic ICCs, performed experiments and analyzed data; AG, CT performed experiments; PSB and SF performed experiments, critically appraised the paper and co-wrote the revision.

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**Circular Smooth Muscle Mesenchyme**
- Contractile
- Some pace-making
- Mechanosensitive

**Phase separation**
- Differentiation & Activation

**ICC Activation**
- Regularity & Rhythm

**Longitudinal Smooth Muscle**
- Cross-direction contractions

**Enteric Nervous System**
- Longitudinal-circular coupling & polarized peristaltic reflex & migrating motor complex

**Smooth Muscle**
- Kit- and & ANO1 negative
- Elongated
- No pace-making
- Contractile
- Mechanosensitive

**Gut Tube Closure**
First spontaneous contractile waves

**Myogenic**
- Day 6

**Neurogenic**
- Day 12
- Day 14
- Day 16
- ?

**Future**