Development of electrical rhythmicity in the murine gastrointestinal tract is specifically encoded in the tunica muscularis

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1. Interstitial cells of Cajal (ICCs) have been identified as pacemaker cells in the gastrointestinal (GI) tracts of vertebrates. We have studied the development of ICCs in pacemaker regions and the onset of electrical rhythmicity in the gastric antrum, small bowel and proximal colon of the mouse.
2. ICCs, as detected by c-Kit immunofluorescence, were found during embryogenesis in regions of the GI tract that eventually become pacemaker areas. Prior to birth, these cells were organized into well-structured networks, and by the end of the embryonic period the morphology of ICC networks in pacemaker regions appeared very similar to that observed in adult animals.
3. Electrical rhythmicity was recorded prior to birth (by E18) in the proximal GI tract (stomach and jejunum), and this activity developed to adult-like behaviour within a week after birth. In the ileum and proximal colon rhythmicity developed after birth, and adult-like characteristics were apparent within the first week.
4. Post-junctional responses of smooth muscles to neural inputs could be recorded at birth, and stimulation of intrinsic nerves often led to oscillatory activity resembling slow waves for up to several minutes following brief stimuli. Nerve stimulation augmented spontaneous activity in the proximal portions of the GI tract and elicited rhythmic activity temporarily in quiescent tissues of the distal GI tract.
5. ICCs and rhythmicity developed in an apparently normal manner in tissues isolated at birth and placed in organ culture. These data suggest that the tunica muscularis provides a suitable microenvironment for the development of ICCs and rhythmicity without the need for extrinsic stimuli.
6. Treatment of small intestinal tissues taken from embryos at E15 with neutralizing c-Kit antibodies abolished ICC development and the organization of ICCs into networks that typically occurs during the late embryonic period. Treatment of muscles taken from newborn animals with c-Kit antibodies blocked postnatal development of ICCs, disrupted already established and functional ICC networks, and rendered muscles electrically quiescent.
7. In summary, ICC networks develop in the pacemaker regions of the murine GI tract before birth. Development and organization of ICCs of the myenteric plexus region into networks precedes the development of electrical rhythmicity. Post-natal development of electrical rhythmicity is mainly characterized by enhancement of the amplitude and frequency of slow waves. The development of ICCs and electrical rhythmicity persists *in vitro*. ICCs appear to be necessary for the initiation of electrical rhythmicity. These findings provide further evidence for the pacemaker role of ICCs.

Several studies have suggested that interstitial cells of Cajal (ICCs) generate electrical rhythmicity and serve as intermediates in neurotransmission in the gastrointestinal (GI) tract (see reviews by Thuneberg, 1982; Christenssen, 1992; Daniel & Berezin, 1992; Sanders, 1996). This hypothesis has been tested by experiments in which (i) ICCs were removed by dissection (e.g. Suzuki, Prosser & Dahms, 1986; Hara, Kubota & Szurszewski, 1986; Smith, Reed & Sanders, 1987*a*,*b*); (ii) ICCs were lesioned by cytotoxic chemicals thought to have specificity for these cells (Thuneberg, Johanson, Rumenssen & Anderson, 1983; Ward, Burke & Sanders, 1990; Liu, Thuneberg & Huizinga, 1994); (iii) the excitability properties and responses to drugs were directly studied in isolated ICCs (Langton, Ward, Carl, Norell & Sanders, 1989; Lee & Sanders, 1993; Publicover, Hammond & Sanders, 1993); (iv) responses to neurotransmitters were studied with immunohistochemical techniques (Young, McConalogue, Furness & de Vente, 1993; Shuttleworth, Xue, Ward, de Vente & Sanders, 1993); and (v) ICC development was blocked by manipulating the c-Kit signalling pathway (Maeda *et al.* 1992; Ward, Burns, Torihashi & Sanders, 1994; Huizinga, Thuneberg, Kluppel, Malysz, Mikkelsen & Bernstein, 1995; Torihashi, Ward, Nishikawa, Nishi, Kobayashi & Sanders, 1995; Ward, Burns, Torihashi, Harney & Sanders, 1995; Burns, Lomax, Torihashi, Sanders & Ward, 1996). Although all of these studies have supported the proposed functional roles for ICCs, much has yet to be learned about basic mechanisms of rhythmicity and transduction of neural inputs, the specific contributions of ICCs and smooth muscle cells to these behaviours, and the factors that regulate the development of ICCs and the formation of ICC–smooth muscle networks and enteric neuron–ICC–smooth muscle motor units (see Burns *et al.* 1996).

A powerful means of investigating the physiological role of ICCs is to manipulate the development of these cells and the formation of ICC networks (see Sanders, 1996 for review). In order to design such studies properly, it is necessary to understand the time course of, and the factors that regulate, development. Developmental studies, however, have been hampered by the lack of specific cellular labels for ICCs. Rigorous identification of these cells has required ultrastructural studies (see Christensen, 1992). Faussone-Pellegrini (1984, 1985) studied the development of ICCs using transmission electron microscopy, but the conclusions that ICCs developed well after birth are inconsistent with the observation that electrical rhythmicity can be measured at or before birth (Torihashi, Ward & Sanders, 1997). Use of ultrastructural criteria may not be the best means of following the development of ICCs because the features commonly associated with the mature phenotype may not develop as early as cellular function. The recent finding that ICCs express c-Kit protein makes it possible to follow the development of these cells back into the embryonic period and to investigate the relationship between the development of ICCs and the initiation of electrical rhythmicity. We have found that c-Kit expression in the small bowel begins midway through gestation. Cells within the tunica muscularis that express c-Kit develop before birth within the myenteric plexus region and form characteristic ICC networks (IC-MY). After birth, ICCs develop within the region of the deep muscular plexus, forming networks of IC-DMP (Torihashi *et al.* 1997).

In the present study we have compared the time courses of development of ICC networks and electrical rhythmicity and responses to motoneuron stimulation in pacemaker regions throughout the GI tract. We have also tested whether ICCs develop *in vitro* and whether this is accompanied by the initiation and maintenance of electrical rhythmicity. We have further tested whether disruption of the ICC network by blocking the c-Kit signalling pathway in organ culture causes loss of electrical rhythmicity.

METHODS

Animals

Pregnant BALB/c mice at precisely known stages of gestation (Harlan Sprague–Dawley, Indianapolis, IN, USA) were purchased at least 1 week prior to use. Mice were killed at days 15–19 of gestation, and fetuses (E15–19) were removed for morphological and electrophysiological investigations. Mice ranging from neonates to 30 days old were also used. Animals were anaesthetized by chloroform inhalation and exsanguinated by decapitation following cervical dislocation. Fetuses removed from pregnant animals were also anaesthetized by chloroform and exsanguinated by cervical dislocation followed by decapitation. The use and treatment of animals was approved by the Institutional Animal Use and Care Committee at the University of Nevada.

Immunohistochemistry

Tissues from the stomach (greater curvature of the fundus, corpus and antrum), jejunum, ileum and colon were pinned onto the base of a Sylgard (Dow Corning) dish, mucosal side up. The tissues were opened and contents washed with Krebs–Ringer–bicarbonate (KRB) solution. The mucosa was removed by sharp dissection and the remaining muscularis tunica fixed in acetone (4 °C, 10 min). Following fixation preparations were washed for 30 min in phosphate-buffered saline (PBS; 0·1 M, pH 7·4). Non-specific antibody binding was reduced by incubation in 10% rabbit serum for 1 h at room temperature (21–22 °C). Tissues were incubated overnight at 4 °C with a monoclonal antibody raised against c-Kit protein (ACK2; 5 μg ml^{-1} in PBS; Gibco BRL). Immunoreactivity was detected using FITC-conjugated secondary antibody (FITC-anti rat; Vector Laboratories, Burlingame, CA, USA; 1:100 in PBS, 1 h, room temperature). Control tissues were prepared in a similar manner, either omitting ACK2 or the secondary antibody from the incubation solution. For cryostat sections, tissues were dehydrated in graded sucrose solutions (5–20% sucrose in 0·1 M PBS), embedded in a 2:1 solution of 20% sucrose in PBS and Tissue Tek (Miles, Naperville, IL, USA) and frozen in isopentane pre-cooled in liquid nitrogen. Cryostat sections were cut at 10 μm thickness and fixed in acetone. Cryostat sections were then treated in a similar manner to whole mounts. Tissues were examined with a conventional Leitz fluorescence microscope or with a Bio-Rad MRC 600 (Hercules, CA, USA) confocal microscope with an excitation

wavelength appropriate for FITC (488 nm). Confocal micrographs are digital composites of Z-series scans of ten to fifteen optical sections through a depth of 10–35 μm. Final images were constructed with Comos software (Bio-Rad).

Electrophysiological studies

Segments of gastric antrum, jejunum, ileum and proximal colon were isolated and opened along the mesenteric border. Lumenal contents were removed with KRB solution. After removing the mucosa, muscle strips (6 mm × 2 mm for fetal tissues and 10 mm × 4 mm for neonates) were cut and pinned to the Sylgard floor of a recording chamber with the mucosal side of the circular muscle facing upward. Alternatively tissues were taken at different time periods in organ culture and placed in the recording chamber. Parallel platinum electrodes were placed on either side of the muscle strips to evoke neural responses. Electrical recordings of tissues from the small intestine were made in the presence of nifedipine (1 μM) to reduce muscle contraction. Nifedipine was not added during experiments on gastric or colonic tissues.

Circular muscle cells were impaled with glass microelectrodes filled with 3 M KCl and having resistances of 30–50 MΩ. Transmembrane potential was measured using a high input impedence amplifier (WPI S-7071, Sarasota, FL, USA) and outputs were displayed on an oscilloscope. Electrical signals were recorded on magnetic tape (Racal 40S, Southhampton, UK). Neural responses were elicited by square wave pulses of electrical field stimulation (EFS; Grass S48), 1–20 Hz for 1 s, 0·5 ms in duration at supramaximal voltage. Data are expressed as means ± standard error of the mean. Differences in the data were evaluated by Student's t test; P values less than 0·05 were taken as a statistically significant difference. The number of cells from which recordings were made is denoted by n. The number of animals from which the n was obtained is also provided.

Organ culture

Embryonic tissues at E15 were removed from the small intestine. These tissues were too small to perform further dissection, so they were cultured as intact segments. Segments of gastric antrum, jejunum, ileum and colon were isolated from newborn animals (less than 1 day old, approximately 50% had not suckled) and opened along the mesenteric border. Jejunums were also isolated from day 10 (D10), day 15 (D15) and day 20 (D20) animals. Lumenal contents were removed with KRB solution and the mucosa was removed by sharp dissection. Muscle strips (5 mm × 2 mm) were cut and pinned to the base of a sterile tissue culture chamber slide lined with Sylgard, with the mucosal side of the circular muscle facing upward. Tissues were washed four times with KRB solution and placed in M199 medium (Sigma) containing penicillin (200 u ml^{-1}), streptomycin (200 mg ml^{-1}) and amphotericin B (0·5 mg ml^{-1}), washed another four times and incubated at 37 °C (90% humidity and 95% O_2–5% CO_2) for up to 10 days with culture medium being changed every second day. Some tissues were incubated in M199 medium containing ACK2 (c-Kit neutralizing antibody; 5 μg ml^{-1}). ACK2 was not added to chamber slides containing control tissues. Other control experiments were performed in which non-immune serum (Hyclone, Logan, UT, USA) was included in the incubation medium to control for possible non-specific effects of antibody.

Solutions and drugs

Muscles were maintained in KRB solution (37·5 ± 0·5 °C; pH 7·3–7·4) containing (mM): Na^+, 137·4; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·2; Cl^-, 134; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; and dextrose, 11·5; bubbled with 97% O_2–3% CO_2. Solutions of nifedipine (Sigma) prepared in ethanol at 10 mM were diluted to 1 μM where stated.

RESULTS

Immunohistochemistry

Immunohistochemical experiments were performed on segments of muscle from various parts of the GI tract at different stages of development to characterize the distribution of c-Kit-positive cells. Whole mount preparations revealed that c-Kit-like immunoreactivity (c-Kit-LI) was located within specific populations of cells in the stomach, jejunum, ileum and proximal colon. In the stomach we characterized cells with c-Kit-LI during the period of E15 to birth. Cells with c-Kit-LI were observed at E15 in the fundus, corpus and antrum. In the fundus only spindle-shaped cells running with the long axis of the muscle fibres of the circular and longitudinal muscle layers were observed (intramuscular ICCs; IC-IM). By E17–18 these cells were well defined and densely distributed in both muscle layers (Fig. 1*A*). Cells with c-Kit-LI were found in the myenteric regions (myenteric plexus ICCs; IC-MY) of the corpus and antrum. These cells had rounded bodies and multiple processes (Fig. 1*B–D*). Spindle shaped cells were also observed within the circular and longitudinal muscle layers, but the density of these cells was less than in the fundus as previously noted in adult animals (Burns *et al.* 1996). At birth cells with c-Kit-LI were located at the level of the myenteric plexus, forming networks in the corpus and antrum (Fig. 2*A*). Long spindle-like cells with c-Kit-LI were also distributed within the circular and longitudinal muscle layers. The morphology of these cells was similar to intramuscular ICCs (IC-IM) of adult animals described previously (Burns *et al.* 1996). Since we could detect no differences in the structure of IC-MY networks and the distribution of IC-IM at birth with that of adult animals (see Burns *et al.* 1996), we did not characterize gastric ICCs during the neonatal period.

ICC distribution and morphology in the small intestine during the embryonic period (i.e. from E15 to birth) were similar to that previously described (Torihashi *et al.* 1997). At birth we observed a well-developed network of cells with c-Kit-LI in the myenteric plexus region (IC-MY) of the jejunum (Fig. 2*B*) and ileum (Fig. 2*C*). These cells are thought to provide pacemaker input in the small intestine (Ward *et al.* 1994; Huizinga *et al.* 1995). IC-MY had ovoid nuclei, prominent perinuclear cytoplasm, and multiple fine processes extending in various directions. Primary and secondary processes overlapped processes of neighbouring cells to form a loose network. At the level of light microscopy, the morphology of these cells and the structure of the networks they formed were indistinguishable from the adult morphology (see Ward *et al.* 1994). There were also no obvious differences in the networks of ICCs observed in jejunum and ileum. Cells with c-Kit-LI were not observed at the level of the deep muscular plexus in embryos or at birth. Previous reports have described the development of these cells several days after birth (Faussone-Pellegrini, 1984; Torihashi *et al.* 1995).

Cells with c-Kit-LI were also observed in the myenteric region of the proximal colon at E15. At E16 these cells were tightly packed with short processes (Fig. 1*E*). By E19, cells with c-Kit-LI had spread apart and the intercellular extensions were lengthened and became more prominent. Thus, as the colon developed the network of ICCs appeared to become less dense with longer processes between cells. This trend continued through to birth (Fig. 2*D*), and the network in the myenteric region in the colon appeared to have a similar morphology to IC-MY of the small intestine. We also confirmed the presence of ICCs along the submucosal surface of the circular muscle layer (IC-SM). As previously noted IC-SM were not present at birth (Torihashi *et al.* 1995). By day 20 IC-SM were sparsely distributed with long processes extending along the long axis of the circular muscle. The long processes appeared to connect cells in a serial manner, and shorter processes appeared to interconnect parallel cells.

Development of electrical activity in different regions of the gastrointestinal tract

Electrical activity was recorded from representative rhythmic GI muscles (i.e. antrum of the stomach; jejunum and ileum

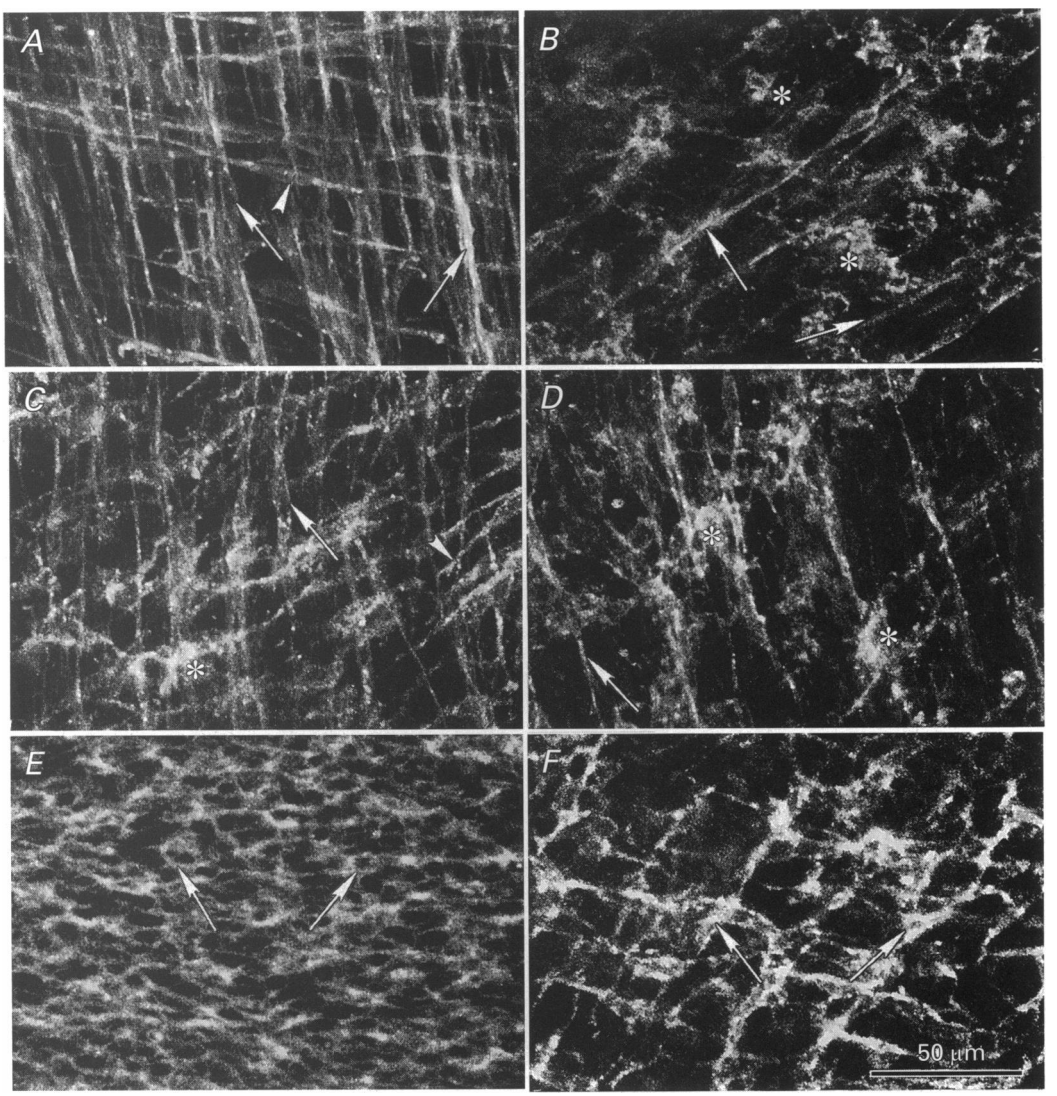

Figure 1. c-Kit-like immunoreactivity in whole mounts of stomach and colon of embryos

A, a confocal composite image of 11 optical sections through the tunica muscularis of an E17 fetus. Long spindle-shaped c-Kit-immunopositive cells (IC-IM) were observed within the circular (arrows) and longitudinal (arrowhead) muscle layers of the fundus. *B*, in the antrum fewer IC-IM (arrows) were observed than in the fundus, and c-Kit-immunopositive cells (IC-MY) were also observed at the level of the myenteric plexus (*). *C* and *D*, spindle-shaped IC-IM (arrows) and IC-MY (*) in the corpus and antrum, respectively, from animals at E18. *E* and *F*, c-Kit-immunopositive cells in the proximal colon at E16 and E19, respectively. At E16, c-Kit-positive cells were densely packed (arrows) and short projections between cells were observed. By E19 processes between c-Kit-positive cells (arrows) had extended to form a loose network. Scale bar, 50 μm; applies to all panels.

of the small intestine; and proximal colon) during the final 5 days of gestation up to 10 days postpartum. Electrical rhythmicity was initiated, developed in frequency and amplitude, and reached adult-like activity in all regions studied within this period.

Gastric antrum. Circular muscle cells from the antrums of E15–19 fetuses and from day 1 (D1) and day 10 (D10) postpartum animals were impaled with microelectrodes. At E15 and E16 circular cells had resting potentials averaging -42 ± 2 mV ($n = 30$ from 6 animals). Spontaneous depolarizations were observed in one of six tissues tested (amplitude, 4 mV; duration, 11 s; frequency, 1 cycle min^{-1}). The resting membrane potential (V_{rest}) increased with age such that at E19, V_{rest} of circular muscle cells averaged -57 ± 2 mV ($P < 0{\cdot}001$), and spontaneous slow wave activity was observed in each muscle. Slow waves averaged $6{\cdot}8 \pm 2{\cdot}8$ mV in amplitude, $7{\cdot}2 \pm 2{\cdot}6$ s in duration, and occurred at a frequency of $2{\cdot}6 \pm 1{\cdot}2$ cycles min^{-1} ($n = 15$ from 3 animals). The V_{rest} of circular smooth muscle cells from the antral region was unchanged at D0 (i.e. -57 ± 3 mV; $P > 0{\cdot}5$), but the amplitude of slow waves increased significantly to an average of $17{\cdot}4 \pm 2{\cdot}6$ mV (i.e. $P < 0{\cdot}05$ compared with E19 fetuses). The duration and frequency of antral slow waves was not significantly different from that of E19 fetuses ($8{\cdot}9 \pm 1{\cdot}0$ s and $1{\cdot}1 \pm 0{\cdot}2$ cycles min^{-1}, respectively; $n = 45$ from 9 animals; Fig. 3). At D1 postpartum, the resting membrane potential recorded from circular muscle cells was $-60{\cdot}2 \pm 2{\cdot}9$ mV and slow waves were $20{\cdot}4 \pm 5{\cdot}4$ mV in amplitude and

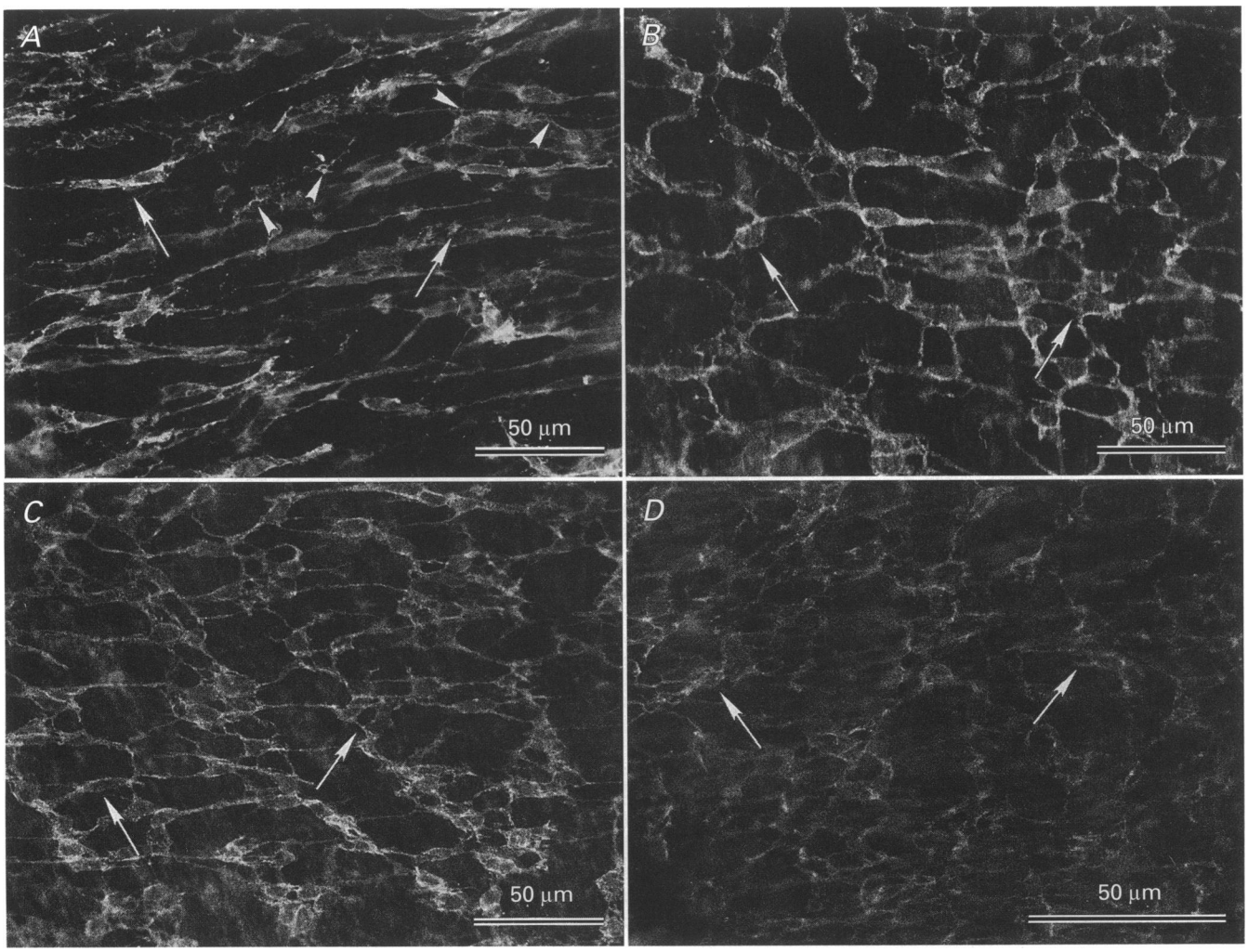

Figure 2. Conventional immunofluroescence revealing c-Kit-immunopositive cells in the antrum, jejunum, ileum and proximal colon of D0 neonates

In the antrum spindle-like cells (*A*; arrows) were observed within the circular muscle layer (IC-IM). Cells with multiple processes (arrowheads) were located at the level of the myenteric plexus (IC-MY). In the jejunum and ileum c-Kit-immunopositive cells with several processes extending out from the cell body in different directions were observed at the level of the myenteric plexus (IC-MY; *B* and *C*; arrows). Primary and secondary processes often contacted one another, forming a loose network. *D*, c-Kit-immunopositive cells in the proximal colon (arrows). These cells were located at the level of the myenteric plexus.

9·2 ± 2·8 s in duration. Slow waves occurred at a frequency of 1·4 ± 0·4 cycles min^{-1} ($n = 24$ from 4 animals). By D10 V_{rest} and slow waves were similar to these parameters in D30 adult animals (see also Burns *et al.* 1996).

Jejunum. Slow wave activity was recorded from circular muscle cells of the jejunum at E19, as previously reported (Torihashi *et al.* 1997). V_{rest} in these muscles averaged −58 ± 2 mV and slow waves averaging 11·0 ± 0·4 mV in amplitude and 2·5 ± 0·1 s in duration were recorded ($n = 18$ from 3 animals). The amplitude of jejunual slow waves increased with age, and at birth (D0) the V_{rest} of circular muscle cells averaged −57 ± 1 mV and slow waves 11·3 ± 1·1 mV in amplitude and 2·8 ± 0·2 s in duration occurred at a frequency of 15·5 ± 0·9 cycles min^{-1} ($n = 74$ from 14 animals). By D1 the V_{rest} of the circular muscle cells averaged −59 ± 3 mV and slow waves 12·5 ± 0·7 mV in amplitude occurred at a frequency of 29·0 ± 3·0 cycles min^{-1} ($n = 36$ from 6 animals). At D6 V_{rest} had become more polarized, averaging −65 ± 2 mV and slow wave amplitude increased to 19·4 ± 1·5 mV ($n = 60$ from 12 animals). At D9 slow waves increased in amplitude to an average of 24·7 ± 3 mV ($n = 22$ from 4 animals), and these events were not different from slow waves recorded from D30 and older adult animals (cf. Ward *et al.* 1995 and Fig. 4).

Ileum. Electrical rhythmicity in the small intestine developed sequentially, with more distal regions developing after the proximal small bowel. Slow wave activity was absent in circular muscle layer of the ileum at birth in tissues of all but two of twelve animals (Fig. 5). The small oscillations recorded in the two spontaneously active muscles were 4 and 14 mV in amplitude. The lack of slow wave activity in the ileum was not a consequence of differences in V_{rest} because ileal cells at D0 had an average V_{rest} of −58 ± 2 mV ($n = 68$ from 12 animals; $P > 0·5$ when compared with jejunal cells at D0). Slow waves developed after birth in the ileum, and by D3 the V_{rest} averaged −64 ± 2 mV and slow waves averaging 12·2 ± 1·8 mV in amplitude and at a frequency of 17·5 ± 2·1 cycles min^{-1} were recorded in each muscle tested ($n = 45$ from 7 animals; Fig. 5). At D6 V_{rest} averaged −66 ± 2 mV and slow waves 28·5 ± 1·5 mV in amplitude were recorded at a frequency of 16·8 ± 0·5 cycles min^{-1} ($n = 36$ from 6 animals). By D9 the V_{rest} averaged −73 ± 1 mV and slow waves with an amplitude of 31·2 ± 4·1 mV occurred at a frequency of 28 ± 1·5 cycles min^{-1} ($n = 28$ from 5 animals). These slow wave parameters recorded were not different from slow waves previously recorded from D30 adult animals (Ward *et al.* 1994, 1995).

Proximal colon. Due to the difficulties in removing the mucosa from the proximal colon prior to birth, the electrical activity in this region of the GI tract was studied only after birth. At birth the circular muscle layer of the proximal colon had an average V_{rest} of −58 ± 3 mV ($n = 40$ from 5 animals) and typical tissues were electrically quiescent. In two muscles small, irregular oscillations in membrane potential (2–3 mV in amplitude) were recorded. At D1

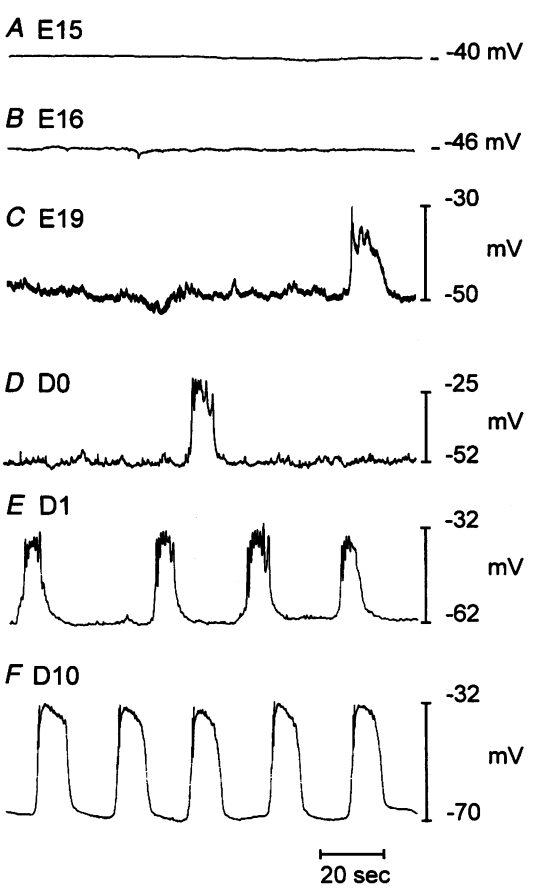

Figure 3. Development of electrical activity in the circular muscle layer of the murine gastric antrum

Electrical activities of muscles from E15, E16 and E19 fetuses and from D0, D1 and D10 neonates are shown. Muscles from E15 and E16 were not spontaneously active (*A* and *B*); however, by E19 slow waves were apparent (*C*). Resting membrane potential became more polarized and the frequency of slow waves increased from D0 to D10 (*D–F*). Activity of D10 animals was similar to that of adults.

resting membrane potential averaged -53 ± 2 mV ($n = 12$ from 3 animals). Regular oscillatory activity was not observed, but some cells displayed small oscillations in potential similar to those observed on D0 (see Fig. 6). At day 6 resting potential averaged -52 ± 2 mV ($n = 24$ from 4 animals). Spontaneous electrical activity consisting of action potential complexes interspersed with several minutes of quiescence were recorded in one of four muscles. At D8, V_{rest} averaged -53 ± 2 mV ($n = 30$ from 6 animals) and the frequency of spike complexes had increased to 1 complex every $2{\cdot}8 \pm 0{\cdot}6$ min. EFS evoked an inhibitory junction potential followed by a spike complex in muscles of D6 and D8 animals (see below). D10 muscles of the proximal colon had average resting potentials of -52 ± 3 mV and displayed regular spike complexes (36 ± 2 mV in amplitude and occurring at a frequency of $2{\cdot}7 \pm 0{\cdot}3$ cycles min^{-1}) that were indistinguishable from the typical pattern of activity in D20 animals and older adult animals (average resting potential -50 ± 1 mV, with spike complexes 48 ± 3 mV in amplitude and occurring at a frequency of $3{\cdot}1 \pm 0{\cdot}1$ cycles min^{-1}; $n = 8$ from 8 animals; see Bywater, Small & Taylor, 1989; Burns, Torihashi, Harney, Sanders & Ward, 1995). We did not record slow wave activity from the murine colon, as can be recorded from larger mammals (see Smith *et al.* 1987*a*).

Responses to neural stimulation

We found that just prior to the development of rhythmicity in GI muscles, oscillatory activity could be induced via excitatory neural inputs. For example, in the antrum slow wave activity was irregular in fetal muscles (see Fig. 3), but EFS, using short duration (0·5 ms) pulses to activate intrinsic nerves, evoked postjunctional responses that were similar in waveform to slow waves. In muscles displaying rhythmicity EFS induced slow wave-like events (Fig. 7). Although circular muscle cells in the jejunum were electrically quiescent at E16 and E17 as previously reported (Torihashi *et al.* 1997), excitatory junction potentials (EJPs) could be elicited by EFS (Fig. 7). From an average resting potential of -54 ± 3 mV, EFS evoked EJPs averaging $12{\cdot}6 \pm 0{\cdot}3$ mV in amplitude ($n = 16$ from 3 animals). At E18 resting membrane potential averaged -56 ± 2 mV ($n = 16$ from 3 animals) and small irregular oscillations in membrane potential were noted. EFS evoked EJPs averaging $19{\cdot}0 \pm 1{\cdot}7$ mV in amplitude ($n = 8$ from 3 animals). Brief

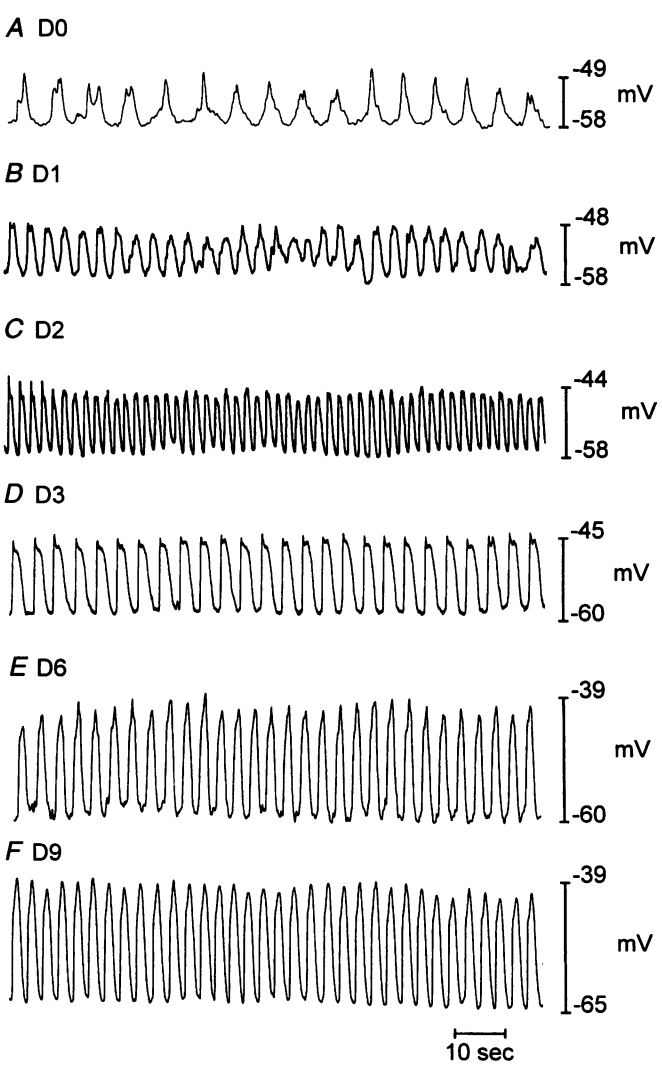

Figure 4. Development of electrical activity in the circular muscle of the murine jejunum

Examples of electrical activities recorded from muscles of D0 to D9 neonates are shown (*A*–*F*). Spontaneous slow wave activity was recorded within the circular muscle layer at birth. Resting membrane potential and slow wave amplitude and frequency increased after birth such that mature activity was recorded by D9.

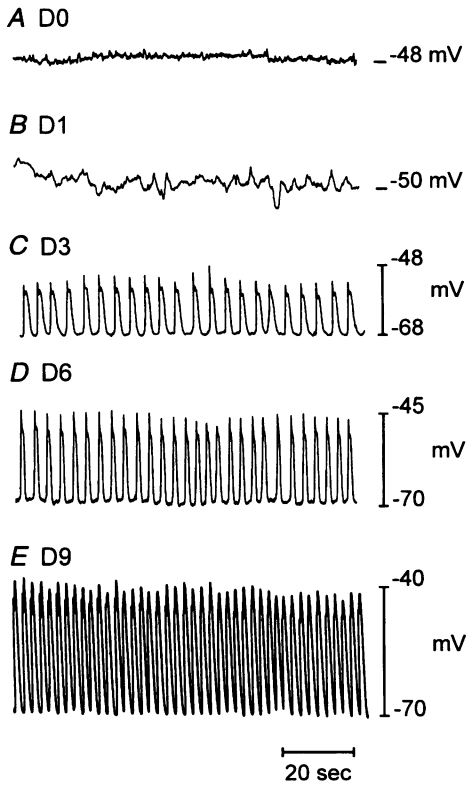

Figure 5. Development of electrical activity in the circular muscle of the murine ileum

Examples of electrical activities recorded from muscles of D0–D9 neonates are shown. Spontaneous slow wave activity in the circular muscle layer was absent at birth and for 1–2 days after birth (*A* and *B*), but slow waves developed by D3 (*C*). Slow waves increased in frequency and amplitude during the first week. Concurrent with the onset and development of slow waves, V_{rest} became more polarized, such that by D3 V_{rest} was similar to that recorded in mature animals (Ward *et al.* 1994, 1995). Slow waves by D9 were similar in frequency and amplitude to adult activity (e.g. Ward *et al.* 1994).

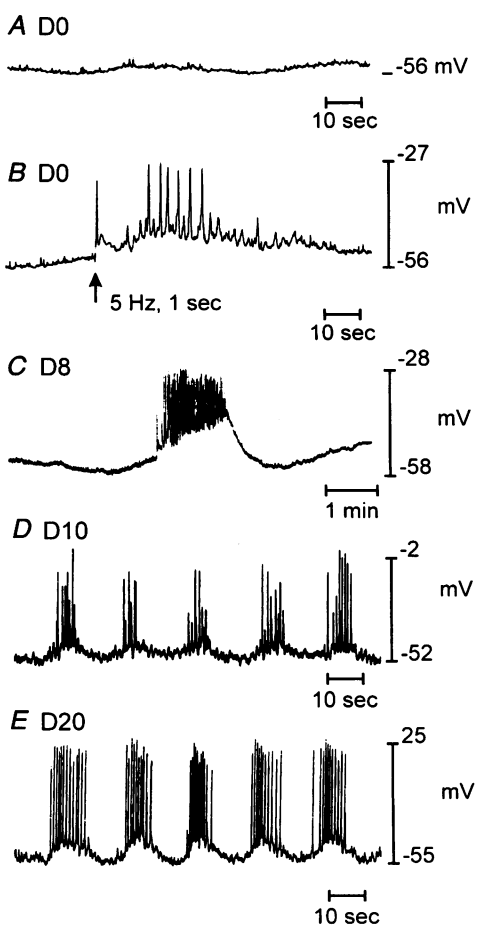

Figure 6. Electrical activity of the circular muscle of the proximal colon

A, electrical activity at birth (D0). Muscles were not spontaneously active, except for small, irregular fluctuations in membrane potential. *B*, spike complexes similar to those observed spontaneously in mature tissues could be elicited at D0 when intramural nerves were activated with electrical field stimulation (5 Hz, 1 s; 0·5 ms pulses). *C*, a typical spike complex that could first be observed between D6 and D8. These events were characterized by multiple spikes superimposed upon a sustained period of depolarization. The spike complexes occurred infrequently with long periods (several minutes) of quiescence occurring between complexes. The trace in this panel was taken from an animal at D8. *D*, activity recorded from a strip of muscle at D10. *E*, activity from an animal at D20. Regular spike complexes were observed as is typical of the adult pattern (Bywater *et al.* 1989).

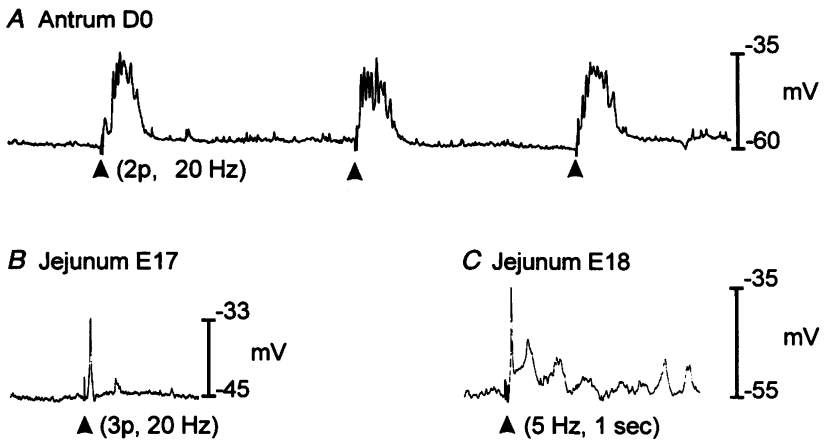

Figure 7. Nerves evoked excitatory responses in stomach and small bowel muscles before the onset of normal electrical rhythmicity

A, slow wave-like events could be evoked by electrical field stimulation of transmural nerves (2 pulses at 20 Hz). *B* and *C*, nerve-evoked excitatory junction potentials (EJPs) could be elicited as early as E17 (*B*; arrow, 3 pulses at 20 Hz, 0·5 ms duration), and at E18 (*C*), multiple pulses (arrow) caused an EJP and initiated a series of low amplitude oscillations for a period of at least 1 min.

periods of EFS were followed by a sustained period of electrical oscillations in membrane potential (averaging 5·0 mV amplitude, at a frequency of 12 min^{-1}) for 1–2 min. At D0 regular slow wave activity was observed, although these events were attenuated in amplitude compared with the adult animal, as described above. Responses to EFS were predominantly excitatory, although 40% of preparations exhibited complex neural responses consisting of an EJP followed by inhibitory junction potentials (IJPs). EJPs averaged 11·5 ± 4·4 mV and IJPs were 6·0 ± 1·4 mV in amplitude. EFS evoked excitatory responses in two of six ileal muscles tested, and inhibitory responses in one of six muscles tested. The excitatory responses consisted of an EJP followed by a period of electrical oscillations. These

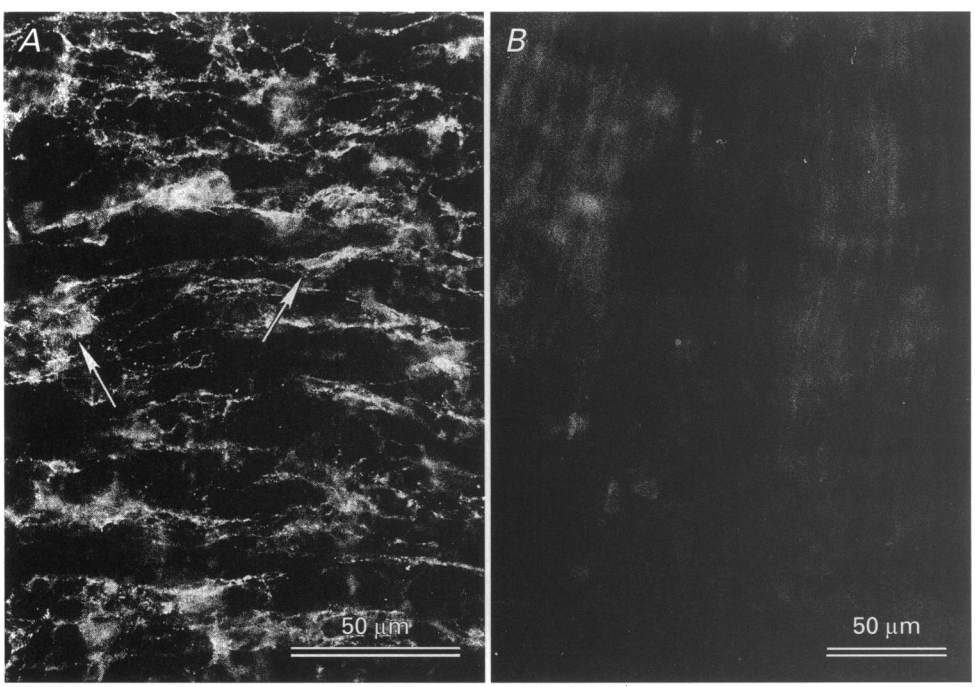

Figure 8. c-Kit-immunopositive cells were maintained in organ culture

A, c-Kit immunoreactivity after culturing a strip of ileal muscle from birth for 10 days (arrows). *B*, the addition of the c-Kit neutralizing antibody ACK2 (5 $\mu g\ ml^{-1}$) reduced c-Kit immunoreactivity in cultured tissues.

events were similar to slow waves, and they lasted from several seconds to several minutes following EFS before the tissues returned to a quiescent state. Rhythmic activity could also be evoked in the proximal colon prior to the development of spontaneous activity. At D0 the circular muscle layer was electrically quiescent, but EFS evoked EJPs which were often followed by periods of spiking superimposed upon a slow membrane depolarization. These spike complexes were similar to the type of electrical activity recorded from adult animals (Fig. 6*C*).

Development of ICCs and rhythmicity in organ culture

Immunohistochemical experiments were performed to characterize the distribution of c-Kit-LI in cultured muscle strips. Normal-appearing ICC networks were observed in small intestinal and gastric muscle strips after 10 days in culture (e.g. Fig. 8*A*). As observed *in situ*, the frequency and amplitude of gastric slow waves increased in muscles cultured from birth for 10 days (Fig. 9*A* and *B*). In the small intestine a gradient was noted in the development of electrical rhythmicity. Slow waves developed in the proximal small bowel before this activity could be recorded in the ileum (see Fig. 9*C* and *E*). With small intestinal muscles from ten animals, we tested whether rhythmicity, already established at birth in jejunal muscles, continued to develop in organ culture, and whether rhythmicity, not yet established in ileal muscles, was initiated. Muscles of each region were cultured from D0 for 7–10 days before electrophysiological experiments were performed. At the end of the culture period the tissues were normal in gross appearance, and spontaneous phasic contractions were noted by eye under the dissecting microscope. Slow wave activity continued to develop in jejunal cultures (Fig. 9*D*), such that activity recorded from cultured muscle strips was similar to that observed in tissues from D10 animals. For example, the V_{rest} of circular muscle strips of jejunum cultured for 10 days averaged -65 ± 2 mV (-67 ± 1 mV in fresh D10 muscles). Slow waves averaged $29{\cdot}1 \pm 3{\cdot}0$ mV in amplitude and $2{\cdot}3 \pm 0{\cdot}2$ s in duration and occurred at a frequency of 22·5 cycles min^{-1} in cultured muscles ($n = 58$ from 10 animals). In fresh D10 muscles slow waves averaged $23{\cdot}0 \pm 1{\cdot}4$ mV in amplitude and $1{\cdot}9 \pm 0{\cdot}04$ s in duration, and occurred at a frequency of $25{\cdot}0 \pm 1{\cdot}1$ cycles min^{-1} (see Fig. 4; $n = 50$ from 10 animals).

Studies were also performed to study postnatal development of ICCs in organ culture. Ileal muscles, which were electrically quiescent at D0, developed slow wave activity in essentially a normal manner in culture (Fig. 9*E* and *F*). After 10 days in culture V_{rest} averaged -63 ± 3 mV and slow waves averaged $32{\cdot}0 \pm 3{\cdot}8$ mV in amplitude and $2{\cdot}5 \pm 0{\cdot}2$ s in duration and occurred at a frequency of $20{\cdot}9 \pm 1{\cdot}3$ cycles min^{-1} ($n = 48$ from 10 animals). This activity was similar to the activity recorded from ileal tissues of mature animals (Fig. 5), but the resting membrane

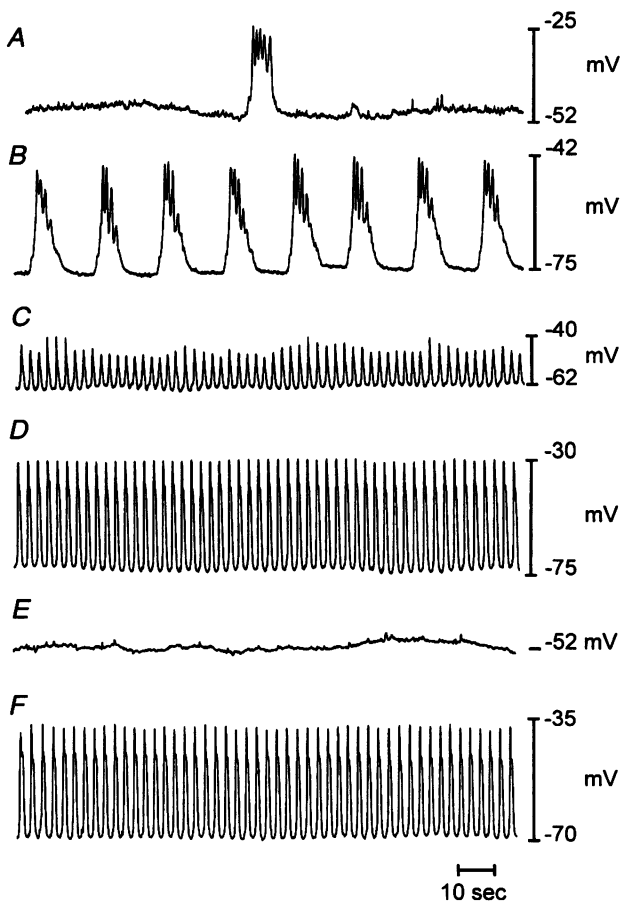

Figure 9. Electrical slow waves develop in organ culture

A, slow waves recorded from the circular muscle of the gastric antrum at D0 (top trace). Slow wave activity developed in organ culture in a manner similar to the development observed *in vivo*. *B*, after 7 days in culture resting membrane potential and frequency of slow waves increased. *C*, jejunal slow wave activity on D0. *D*, resting membrane potential and slow wave amplitude increased during 10 days in organ culture. *E* and *F*, typical examples of electrical activities recorded in the circular muscle layer from the ileum of a D0 animal (*E*) and the ileum of a D0 animal placed in organ culture for 10 days. Slow wave activity was absent at D0 and developed into activity similar to that recorded from tissues that developed *in vivo* (see Fig. 5).

potentials were somewhat depolarized from values recorded from ileal muscles of adult animals (i.e. control values were -68 ± 4 mV; $P < 0{\cdot}05$; control data taken from Ward *et al.* 1994).

Cells positive for c-Kit-like immunoreactivity appear in the GI tracts of embryos by E12 (Torihashi *et al.* 1997). By E15 c-Kit-positive cells can be observed around the periphery of the gut, but adult-like networks have not yet formed. We tested whether the final stages of development that occur during the late embryonic period continue in organ culture and whether incubation with neutralizing anti-c-Kit antibodies would disrupt the development of ICCs. Segments of the small intestine were removed from ten animals at E15 and cultured for 5 days with and without anti-c-Kit antibodies (5 μg ml^{-1}). In control tissues normal appearing networks of ICCs developed, but c-Kit-positive cells disappeared from tissues treated with the antibody (Fig. 10).

The effects of neutralizing antibodies were also studied on muscles taken from newborn animals in which networks of IC-MY were already established. Incubation of these tissues with anti-c-Kit antibodies (5 μg ml^{-1}) resulted in the disappearance of IC-MY from small intestinal muscle strips (Fig. 8*B*). In control experiments in which the incubation media contained non-immune serum (up to 10%), ICCs were not affected. Loss of IC-MY reversed the development of slow waves in jejunal muscle strips (i.e. slow waves were present at birth but not observed after incubation with anti-c-Kit antibody) and blocked the development of slow waves in ileal muscles. Slow waves were not affected in control

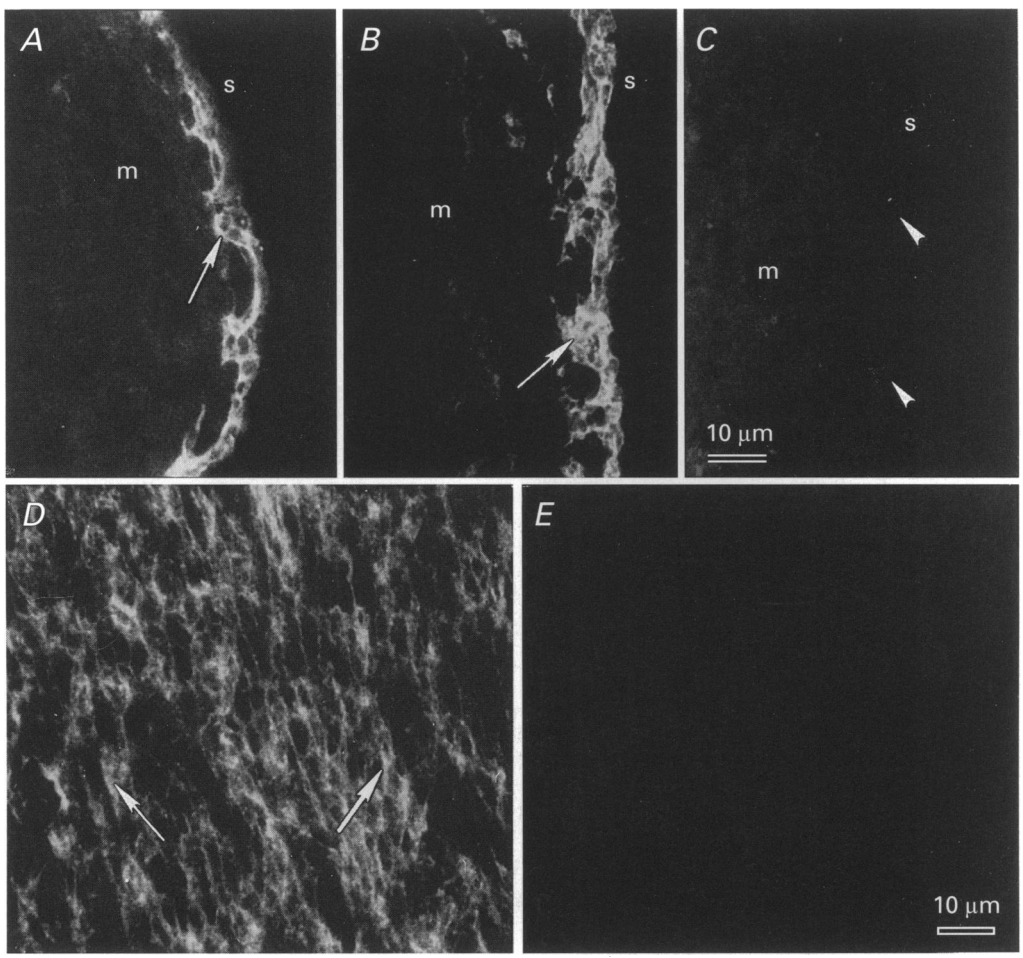

Figure 10. Development of ICCs in organ culture

A, cryostat cross-section of jejunum taken from an animal at E15. Note c-Kit-positive cells distributed along the serosal (s) surface of the bowel (arrow). The circular muscle and mucosa (m) are negative for c-Kit. *B*, a cross-section and *D*, a whole mount of the bowel after 5 days in organ culture. c-Kit-positive cells are identified by arrows. Cells with c-Kit-like immunoreactivity continued to develop and formed a network of ICCs in the myenteric region (*D*; arrows). Other segments of jejunum removed at E15 were cultured with anti-c-Kit antibody. After 5 days in culture, cells positive for c-Kit were absent, as shown in cross-sections (*C*) and whole mounts (*E*). In *C* the position of the myenteric plexus region, running from top to bottom of the micrograph, is denoted by arrowheads. Scale bar in *C* applies to *A*, *B* and *C*; scale bar in *E* applies to *D* and *E*.

Table 1. Loss of slow wave activity in tissues cultured with anti-c-Kit antibody

Time in culture (h)	Organ culture (control) V_{rest} ($n = 68$) (mV)	Slow wave amplitude (mV)	Slow wave frequency (cycles min^{-1})	Organ culture with c-Kit antibody (5 μg ml^{-1}) V_{rest} ($n = 50$) (mV)	Slow wave amplitude (mV)	Slow wave frequency (cycles min^{-1})
0	−59 ± 1	12·3 ± 1·0	19·6 ± 1·0	—	—	—
12	−60 ± 3	22·0 ± 2·4	21·9 ± 1·4	−66 ± 1	12·5 ± 4·2	10·3 ± 4·2
24	−63 ± 3	19·6 ± 2·7	20·7 ± 2·3	−61 ± 3	9·7 ± 7·6	0·9 ± 1·0
48	−67 ± 2	25·4 ± 3·2	21·9 ± 2·1	−68 ± 1	0	0
72	−69 ± 2	24·2 ± 2·7	20·6 ± 2·1	−66 ± 2	0	0

Numbers in parentheses indicate number of experiments from which mean values were calculated.

experiments in which non-immune serum was added to the culture medium. Culturing in the presence of anti-c-Kit antibody caused the V_{rest} of muscle cells to depolarize in comparison with control cultured muscles (i.e. to −55 ± 2 mV in the jejunal muscles and to −57 ± 5 mV in ileal muscles; Fig. 11). Similar depolarization has previously been observed in muscles of animals in which ICCs were removed or developmentally impaired (Ward *et al.* 1994; Ward *et al.* 1995; Torihashi *et al.* 1995). This degree of depolarization has been shown to be insufficient *per se* to block slow waves in the murine small intestine (Ward *et al.* 1994).

The time course of the effects of anti-c-Kit antibodies was studied in jejunal tissues of twelve animals taken immediately after birth and placed in culture for 12–72 h. Control muscles developed normal slow wave activity in culture (Table 1). Muscles exposed to antibody for as little as 12 h showed a reduction in slow wave frequency and amplitude, and after 1 day slow waves became irregular. A few of the remaining slow waves were of sufficient amplitude to cause generation of action potentials during the first day in culture. Two days of exposure to anti-c-Kit antibodies resulted in a loss of slow wave activity (Fig. 12; Table 1). Immunohistochemical analysis showed concomitant disruption of the IC-MY networks and disappearance of IC-MY (Fig. 13).

We also tested the effects of anti-c-Kit antibodies on small intestinal muscles removed from animals aged D0, D10, D15 and D20. Muscle strips were cultured for 5 days with or without anti-c-Kit antibodies (5 μg ml^{-1}). Only muscles put into culture from D0 animals responded to anti-c-Kit antibodies with a loss of ICCs and electrical slow waves (Fig. 14*A* and *B*). Tissues from D10, D15 and D20 animals were not affected by c-Kit antibodies (Fig. 14*C–E*).

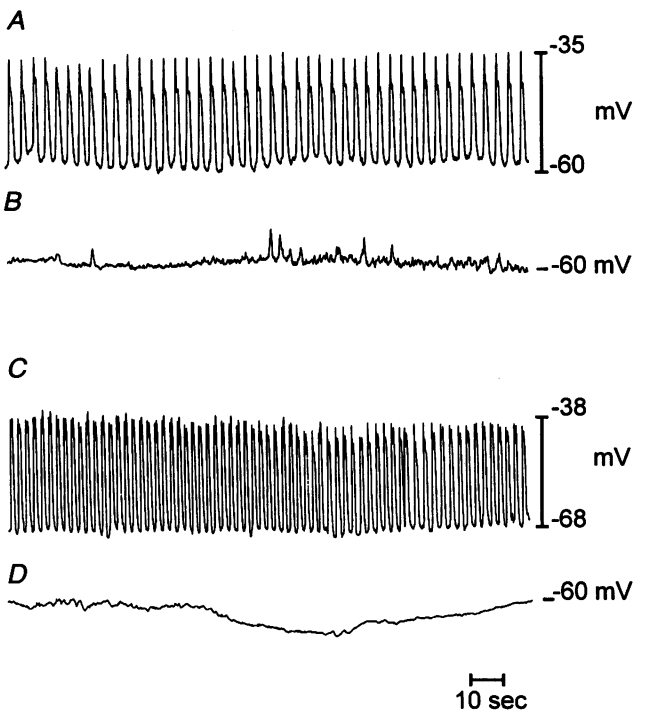

Figure 11. The development of slow wave activity from birth could be blocked by the addition of a c-Kit neutralizing antibody (ACK2) to the culture media
A, slow wave activity recorded from the circular muscle layer of the murine jejunum cultured from birth for 7 days. *B*, a recording from a second muscle strip from the same animal that was cultured for the same period in the presence of ACK2 (5 μg ml^{-1}). ACK2 abolished the slow wave activity in jejunal muscles present at birth. *C*, typical slow waves from a strip of ileal muscle that was cultured from birth for 7 days. *D*, ACK2 blocked the development of slow wave activity in the ileum.

DISCUSSION

The present study provides additional support for the pacemaker role proposed for ICCs by documenting the relationship between the development of ICCs and the development of electrical rhythmicity in several regions of the murine GI tract. Our experiments show that: (i) rhythmicity develops after formation of the ICC networks in the stomach, small bowel and colon; and (ii) disruption of ICC networks by blocking the function of c-Kit in organ culture causes loss of rhythmicity.

Recent developmental studies have supported the view that ICCs are of non-neural origin and derived from the mesenchyme of the gut. Cells with c-Kit-LI appear in segments of avian and mammalian GI tracts that are uncolonized by enteric neurons (Lecoin, Gabella & Le Douarin, 1996; Young, Ciampoli, Southwell & Newgreen, 1996), and c-Kit-positive cells do not express markers characteristic of neural crest derivatives (Torihashi *et al.* 1997). It is as yet unclear whether the full functional phenotype of ICCs develops in the absence of enteric neurons, because these cells appear to be a major source of stem cell factor (SCF; see Torihashi, Yoshida, Nishikawa, Kunisada & Sanders, 1996), the natural ligand for c-Kit (e.g. Williams *et al.* 1990). Other cells in the tunica muscularis, however, may also express SCF (Lecoin *et al.* 1996). Cells with c-Kit-like immunoreactivity emerge within the gut wall well before rhythmicity is initiated (Torihashi *et al.* 1997; present study), and several days of embryonic development, in which signalling via c-Kit is obligatory, pass before pacemaking commences.

Previous developmental studies using ultrastructural criteria have concluded that ICCs develop much later than observed in the present study (e.g. Faussone-Pellegrini, 1984, 1985). Cells thought to be 'ICC-blasts' were apparent in unfed neonates and suckling animals from 3–14 days after birth in the myenteric plexus region of the mouse intestine, but positive identification of ICCs was not possible until weaning (i.e. at 17–21 days after birth). The developmental time course revealed by ultrastructural analysis is not consistent with the role of ICCs as

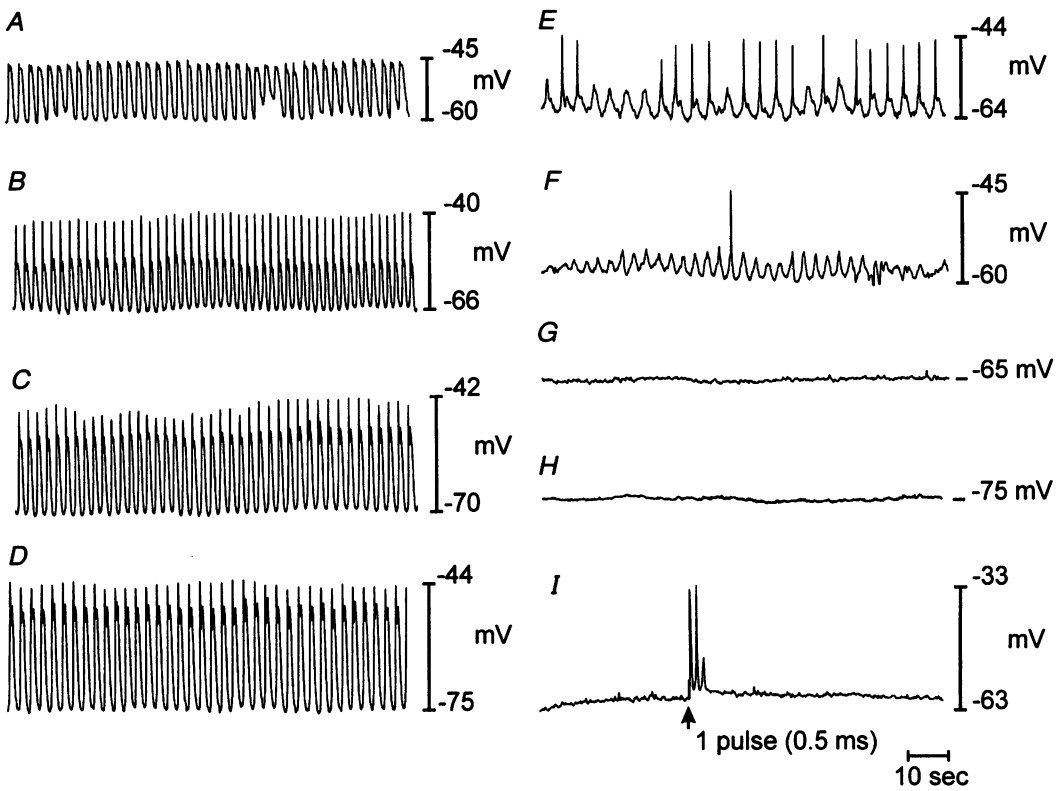

Figure 12. Time course of the effects of anti-c-Kit antibodies on jejunal electrical activity

Muscles were cultured from D0 for 3 days. In control muscles slow wave activity and resting membrane potential developed normally from D0 (*A*). *B*, a recording from a muscle after 1 day in culture; *C* and *D*, recordings from muscles after 2 and 3 days in culture, respectively. *E–I*, electrical activity of tissues cultured in the presence of anti-c-Kit antibody. *E* was recorded after 12 h in culture; *F* is after 1 day in culture; *G* is after 2 days; and *H* is after 3 days. In *I* (taken after 2 days in culture), electrical field stimulation (1 pulse, 0·5 ms duration) activated multiple spike-like depolarizations, confirming the sustained excitability of the muscle and integrity of intrinsic neural inputs despite the loss of spontaneous rhythmicity. *A–I* are recordings from different muscles, but all tissues came from sibling animals.

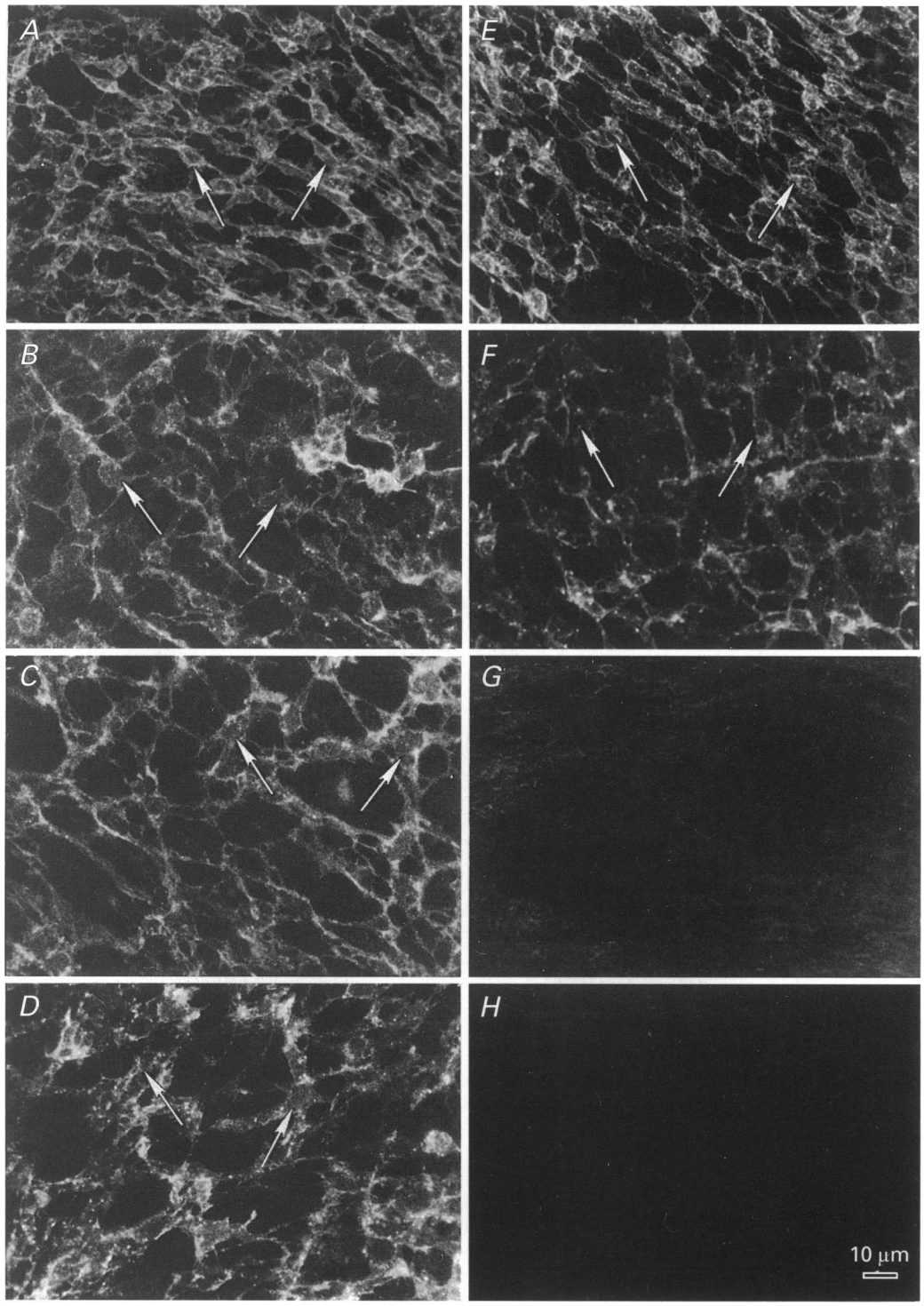

Figure 13. Time course of effects of anti-c-Kit antibodies on ICC networks

Jejunal muscles were placed into culture at birth (D0). *A–D*, ICC networks 2, 12, 24 and 48 h after the muscles were removed from the animals, respectively (arrows indentify examples of c-Kit-positive cells in each micrograph). Normal distributions of ICCs and ICC networks were found at all time periods. *E–H*, ICCs in muscles cultured for the same time periods, but these muscles were exposed to anti-c-Kit antibody (5 μg ml^{-1}) during the culture period. Note that c-Kit-positive cells were reduced in these tissues after 12 h of antibody exposure, and after 24 h and 48 h ICCs were not resolvable by c-Kit immunofluorescence. Scale bar in H applies to all panels.

pacemakers since rhythmicity is present at birth or soon after in all the normally rhythmic regions of the GI tract. Immunological identification of ICCs depicts a much earlier developmental sequence that corresponds more closely with the initiation of rhythmicity. Formation of ICC networks may be the structural step necessary for functional pacemaker activity, and expression of ion channels responsible for electrical coupling and/or excitability may be the molecular events that immediately precede pacemaking. The ultrastucture common to adult ICC develops well after birth, perhaps as a result of maturation.

The ICC networks likely to be responsible for pacemaker activity were well formed in each region of the GI tract at birth. Electrical rhythmicity was clearly present in the stomach and proximal small intestine before birth, but rhythmicity developed soon after birth in the ileum and after several days in the colon. This proximal to distal developmental gradient is difficult to explain on the basis of light microscopic examination of the ICC networks because these structures appeared to be well developed in all regions at birth. Subtle differences in the timing of expression of ionic conductances may be responsible for the gradient in the development of pacemaker activity. During the postnatal period, slow waves grew in amplitude, suggesting that the processes underlying pacemaking and/or amplification of spontaneous rhythmicity were reinforced. It is also possible that tonic inhibitory factors, neural or humoral, delay the onset of rhythmicity. Similar postnatal enhancements in slow waves have previously been documented in studies of the canine proximal colon (Ward, 1996). Understanding the sequence of development and the timing of the onset of rhythmicity down to a period of a few days allows future studies to focus on the steps immediately preceding the initiation of pacemaker activity. Knowledge of the factors that regulate the development and plasticity of the ICC phenotype may be clinically relevant because others have shown defects in ICC networks to be associated with some motility disorders. For example, ICCs were reduced in number in the pyloric sphincter region in infantile hypertrophic pyloric stenosis (Vanderwinden, Liu, De Laet & Vanderhaeghen, 1996), and a loss of ICCs occurs in chronic ideopathic intestinal pseudo-obstruction (Isozaki *et al.* 1996) and achalasia (Faussone-Pellegrini & Cortesini, 1985).

Previous studies have documented the presence of ICCs at the submucosal surface of the circular muscle layer in the murine proximal colon (Torihashi *et al.* 1995), and this population of ICCs has been associated with generation of slow waves in canine and feline colons (Smith *et al.* 1987*a*; Conklin & Du, 1990). In the present study we confirmed the existence of IC-SM in the murine proximal colon using immunohistochemical techniques on cryostat sections and whole mounts. These cells were more sparse in the mouse

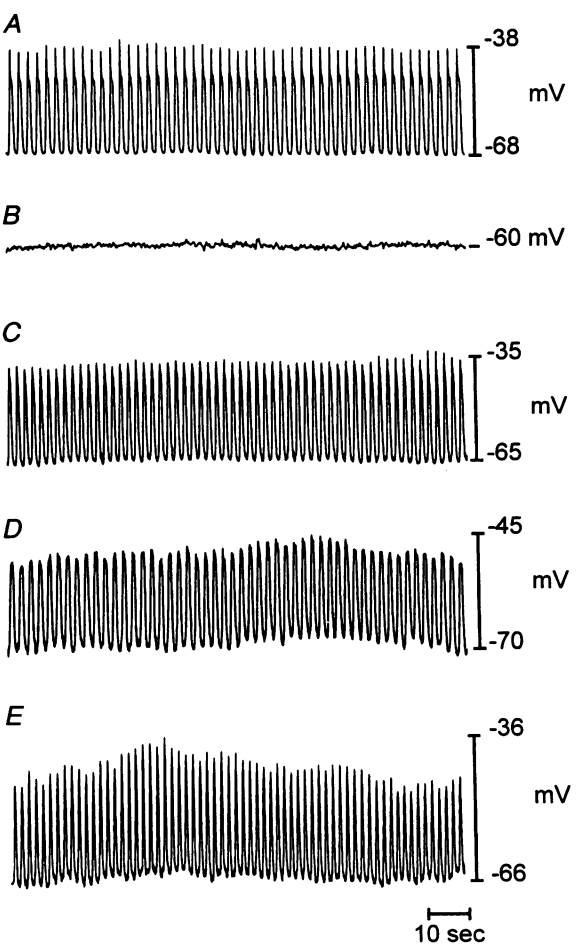

Figure 14. Electrical activity recorded from muscles placed in organ cultures at various times after birth

A, typical slow wave activity recorded from a jejunal muscle strip after 5 days in culture. *B*, electrical activity from a muscle strip taken at birth (D0) and cultured with anti-c-Kit antibody (5 μg ml^{-1}). Slow waves were abolished. *C–E*, slow wave activities in muscle strips taken from animals on D10, D15 and D20, respectively, and cultured with anti-c-Kit antibody (5 μg ml^{-1}) for 5 days. Antibody treatment was not able to block electrical activity or disrupt the ICC networks (data not shown) in these older strips of muscle.

than in the canine colon. The role of IC-SM is unclear because we have never recorded slow wave activity in the mouse colon. IC-MY were present at birth in the colon, but IC-SM develop at about D5 (Torihashi *et al.* 1995). Electrical rhythmicity (i.e. spike complexes) was not observed before D5 but became quite a prominent feature of electrical activity between D5 and D10. Therefore, it is possible that IC-SM could contribute to pacemaker activity or help reinforce pacemaker activity generated by IC-MY.

The sequence of the development of the resting membrane potential should be noted. In most regions studied (and, in fact, possibly in all regions because we were unable to study adequately embryonic colonic muscles), membrane potential became more negative as the ICC network developed and rhythmicity was initiated. The increase in membrane potential could be a consequence of the development of ICCs or coupling of ICCs to smooth muscle cells because several studies have noted that resting potentials were reduced when ICCs are damaged (Thuneberg *et al.* 1983; Ward *et al.* 1990; Liu *et al.* 1994), removed by dissection (e.g. Smith *et al.* 1987*a*, *b*), or developmentally impaired (Ward *et al.* 1994, 1995; Huizinga *et al.* 1995; Torihashi *et al.* 1995).

It has been recognized for several years that ICCs are in close association with enteric motor neurons, and often appear to be intercalated between varicosities and smooth muscle cells (e.g. Daniel & Posey-Daniel, 1984). We have shown that certain classes of ICC in the dog and mouse are responsive to neurotransmitters (Publicover, Horowitz & Sanders, 1992; Publicover *et al.* 1993), functionally innervated by inhibitory motor neurons (Shuttleworth *et al.* 1993), and necessary for nitric oxide-dependent neurotransmission (Burns *et al.* 1996). The present study was primarily concentrated on the development of ICCs within pacemaker regions, and the majority of neural inputs mediated via ICCs may occur through different types of ICC from the cells that initiate rhythmicity (see Burns *et al.* 1996). For example, ICCs in the pacemaker region of the small intestine do not appear to be critical for neurotransmission because responses attributed to activation of excitatory and inhibitory neurons could be recorded in W/W^V and Sl/Sl^d mutants in which IC-MY were largely absent (Ward *et al.* 1994, 1995). We noted excitatory responses in each region of the GI tract in the present study before or near the time of the onset of rhythmicity, suggesting that the receptors and cellular transduction mechanisms necessary for eliciting responses were in place at birth. In many cases activation of excitatory neural inputs tended to reinforce or unmask an underlying tendency towards electrical rhythmicity. This suggests that prior to the onset of electrical rhythmicity the pacemaker cells may be capable of generating pacemaker current, but the current density may not be sufficient to drive the smooth muscle syncytium. This could result from too little current generation by pacemaker cells, inadequate coupling between pacemaker cells and smooth muscle cells, or too much resting conductance in smooth muscle cells. Any or all of these factors could be influenced by neurotransmitters to facilitate the oscillatory activity that was often seen in response to electrical field stimulation.

Our observations that slow waves develop under organ culture conditions is useful since these preparations are much easier and cheaper to manipulate in chronic experiments than animals. These experiments demonstrate for the first time that functional development of ICCs and pacemaker activity are intrinsic to the tunica muscularis and not dependent upon postnatal factors such as suckling or weaning (Faussone-Pelegrini, 1985), bacterial colonization, the presence of a mucosa, afferent sensory neural inputs, or extrinsic, blood-born factors. We also demonstrated that ICCs and slow waves can be abolished in organ culture with neutralizing antibodies to c-Kit, as has been demonstrated previously *in vivo* (see Torihashi *et al.* 1995). Although networks of ICCs were present at birth, treatment with neutralizing c-Kit antibodies *in vitro* caused these networks to disappear after a few days in culture. This observation further reinforces the concept that c-Kit signalling is important not only for ICC development, but for the maintenance of the ICC phenotype through a critical period of development (see Torihashi *et al.* 1995). An examination of the time course of the effects of neutralizing anti-c-Kit antibodies revealed that c-Kit-positive cells decreased dramatically within 2–3 days. During this same period slow wave activity became irregular and then ceased. Immunofluorescence for c-Kit disappeared before all oscillatory activity was lost. Thus, downregulation of c-Kit receptors may slightly precede loss of ICC function. The exact sequence of events elicited by blocking c-Kit signalling will need to be examined carefully with immunocytochemical techniques. We also tested the effects of anti-c-Kit antibodies on muscles taken for organ culture several days after birth and found no effect on ICCs and slow waves. These observations suggest that once ICC networks are established and the cells mature to a certain point after birth, c-Kit signalling may decrease in importance in terms of maintenance of the ICC phenotype. Organ cultures may provide a useful new experimental means of studying other factors that affect the development and plasticity of ICCs.

Some confusion appears to have developed in the literature about the importance of c-Kit in the development of the various classes of ICC. We reported that IC-DMP in the small intestine and IC-MY in the stomach and colon are not affected in animals with particular stem cell factor (i.e. *steel*; Sl/Sl^d) and c-*kit* mutations (i.e. W/W^V; see Ward *et al.* 1995; Burns *et al.* 1995) suggesting that either c-Kit is not a critical factor in development of these ICCs or the lesions produced in W/W^V and Sl/Sl^d mutations are insufficient to hamper the development of some types of ICC. Others have confirmed that compromised c-Kit signalling does not affect IC-DMP of the small intestine (Malysz, Thuneberg, Mikkelsen & Huizinga, 1996), and these authors concluded

that IC-DMP do not need c-Kit protein for normal development. Caution must be applied to this conclusion, however, because of the non-totality of the lesion in W/W^V and Sl/Sl^d mutations (the function of c-Kit and stem cell factor, respectively, is not totally blocked in these compound heterozygotes). Other studies have shown that when the function of c-Kit was blocked with neutralizing antibodies, all classes of c-Kit-positive cells in the stomach, small bowel and colon were reduced in number (Maeda *et al.* 1992; Torihashi *et al.* 1995). We have reinforced these observations in the present study by showing that block of c-Kit immediately after birth can disrupt development and maintenance of the ICC phenotype in organ culture. Therefore, we would suggest that the general expression of c-Kit by ICCs is matched by its importance in the development of each class of ICC. The significance of c-Kit signalling can be further resolved by organ culture experiments designed to characterize the development and functions attributed to specific classes of ICC.

The timing of c-Kit signalling is an extremely important question in the development of ICCs. A developmental window exists between first expression of c-*kit* and maturity in which c-Kit signalling is necessary for establishment and maintenance of the ICC phenotype (Maeda *et al.* 1992; Torihashi *et al.* 1995). There is some controversy about the duration of this developmental window. A recent study concluded that while c-Kit is important for ICC development after birth, the embryonic stage of ICC development was independent of functional c-Kit (Bernax, De Sepulveda, Kress, Elbaz, Delouis & Panthier, 1996). This study, using transgenic mice in which the wild-type c-*kit* gene was replaced with a c-*kit*–*lacZ* construct that rendered the gene product of c-*kit* non-functional, reported that cells with c-*kit*-like expression (observed with β-galactosidase histochemistry) were present in gastrointestinal tissues throughout the embryonic period. The authors suggested that functional c-Kit protein and, therefore, signalling via the stem cell factor–c-Kit pathway, is not necessary for the migration, proliferation and/or survival of ICCs during embryogensis. These conclusions seem to contrast, however, with earlier reports that ICCs are greatly reduced in number near the time of birth in specific regions of the gastrointestinal tract in *W* (c-*kit*) mutants (Ward *et al.* 1994). In the present study we have directly tested this hypothesis by applying neutralizing c-Kit antibodies to tissues during the final 25% of the embryonic period. Control tissues not exposed to c-Kit antibody developed ICC networks, but ICCs disappeared from tissues incubated with antibody. These data suggest that the critical period for c-Kit signalling begins well before birth. We have also confirmed previously conclusions that once ICCs are established, structurally and functionally, c-Kit signalling appears to lose importance in maintaining the phenotypes of ICC (Torihashi *et al.* 1995). Treating muscles taken from 10- to 20-day-old animals with anti-c-Kit antibodies failed to affect ICC and electrical rhythmicity. Based on current and previous observations it appears that a developmental window exists during which c-Kit is crucial for ICC development. This window extends from the mid to late embryonic period (at least from E15) through the early postnatal period.

In conclusion, the time course of development of ICC networks and electrical rhythmicity supports the hypothesis that ICCs are involved in pacemaking. In each of the phasically active regions of the GI tract, well-formed ICC networks were in place before the onset of electrical rhythmicity. Over the final few days before birth and during the first 7–10 days following birth, electrical rhythmicity became more robust. During a critical period from mid to late gestation through birth, the ICC phenotype is not firmly established, and blocking c-Kit signalling results in the disruption of ICC networks, reduction in the numbers of ICCs, and loss of electrical rhythmicity.

Bernax, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C. & Panthier, J.-J. (1996). Spatial and temporal patterns of c-kit-expressing cells in $W^{lacZ}/+$ and W^{lacZ}/W^{lacZ} mouse embryos. *Development* **122**, 3023–3033.

Burns, A. J., Lomax, A. E. J., Torihashi, S., Sanders, K. M. & Ward, S. M. (1996). Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach. *Proceedings of the National Academy of Sciences of the USA* **93**, 12008–12013.

Burns, A. J., Torihashi, S., Harney, S. C., Sanders, K. M. & Ward, S. M. (1995). The effect of c-*kit* mutation on development of the interstitial cell network in the murine stomach and colon. *Neurogastroenterology and Motility* **7**, 249 (abstract).

Bywater, R. A. R., Small, R. C. & Taylor, G. S. (1989). Neurogenic slow depolarizations and rapid oscillations in membrane potential of circular muscle of mouse colon. *Journal of Physiology* **413**, 505–519.

Christensen, J. (1992). A commentary on the morphological identification of interstitial cells of Cajal in the gut. *Journal of the Autonomic Nervous System* **37**, 75–78.

Conklin, J. L. & Du, C. (1990). Pathways of slow-wave propagation in proximal colon of cats. *American Journal of Physiology* **258**, G894–903.

Daniel, E. E. & Berezin, I. (1992). Interstitial cells of Cajal: are they major players in control of gastrointestinal motility? *Journal of Gastrointestinal Motility* **4**, 1–24.

Daniel, E. E. & Posey-Daniel, V. (1984). Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *American Journal of Physiology* **246**, G305–315.

Faussone-Pelegrini, M. S. (1984). Morphogenesis of the special circular muscle layer and of the interstitial cells of Cajal related to the plexus muscularis profundus of mouse intestinal muscle coat. An E.M. study. *Anatomy and Embryology* **169**, 151–158.

Faussone-Pellegrini, M. S. (1985). Cytodifferentiation of the interstitial cells of Cajal related to the myenteric plexus of mouse intestinal muscle coat. *Anatomy and Embryology* **171**, 163–169.

Faussone-Pellegrini, M. S. & Cortesini, C. (1985). The muscle coat of the lower esophageal sphincter in patients with achalasia and hypertensive sphincter. An electron microscopic study. *Journal of Submicroscopy and Cytology* **17**, 673–685.

HARA, Y., KUBOTA, M. & SZURSZEWSKI, J. H. (1986). Electrophysiology of smooth muscle of the small intestine of some mammals. *Journal of Physiology* **372**, 501–520.

HUIZINGA, J. D., THUNEBERG, L., KLUPPEL, M., MALYSZ, J., MIKKELSEN, H. B. & BERNSTEIN, A. (1995). W/kit gene required for intestinal pacemaker activity. *Nature* **373**, 347–349.

ISOZAKI, K., SHINOMURA, Y., MIYAGAWA, J.-I., KANAYAMA, S., MINAMI, T., TANIGUCHI, M., HIROTA, S. & MATSUZAWA, Y. (1996). Deficiency of c-kit-expressing cells in the gastrointestinal tract of patients with chronic idiopathic intestinal pseudo-obstruction. *Gastroenterology* **110**, A685 (abstract).

LANGTON, P., WARD, S. M., CARL, A., NORELL, M. A. & SANDERS, K. M. (1989). Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. *Proceedings of the National Academy of Sciences of the USA* **86**, 7280–7284.

LECOIN, L., GABELLA, G. & LE DOUARIN, N. (1996). Origin of the c-kit-positive cells in the avian bowel. *Development* **122**, 725–733.

LEE, H. K. & SANDERS, K. M. (1993). Comparison of ionic currents from interstitial cell and smooth muscle cells of canine colon. *Journal of Physiology* **460**, 135–152.

LIU, L. W. C., THUNEBERG, L. & HUIZINGA, J. D. (1994). Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. *American Journal of Physiology* **266**, G485–496.

MAEDA, H., YAMAGATA, A., NISHIKAWA, S., YOSHINAGA, K., KOBAYASHI, S., NISHI, K. & NISHIKAWA, S. (1992). Requirement of c-kit for development of intestinal pacemaker system. *Development* **116**, 369–375.

MALYSZ, J., THUNEBERG, L., MIKKELSEN, H. B. & HUIZINGA, J. D. (1996). Action potential generation in the small intestine of W mutant mice that lack interstitial cells of Cajal. *American Journal of Physiology* **271**, G387–399.

PUBLICOVER, N. G., HAMMOND, E. M. & SANDERS, K. M. (1993). Amplification of nitric oxide signaling by interstitial cells. *Proceedings of the National Academy of Sciences of the USA* **90**, 2087–2091.

PUBLICOVER, N. G., HOROWITZ, N. N. & SANDERS, K. M. (1992). Calcium oscillations in freshly dispersed and cultured interstitial cells from canine colon. *American Journal of Physiology* **262**, C589–597.

SANDERS, K. M. (1996). A case for interstitial cells of Cajal as pacemakers and mediators of neurotransmission in the gastrointestinal tract. *Gastroenterology* **111**, 492–515.

SHUTTLEWORTH, C. W., XUE, C., WARD, S. M., DE VENTE, J. & SANDERS, K. M. (1993). Immunohistochemical localization of 3′,5′-cyclic guanosine monophosphate in the canine proximal colon: Responses to nitric oxide and electrical stimulation of enteric inhibitory neurons. *Neuroscience* **56**, 513–522.

SMITH, T. K., REED, J. B. & SANDERS, K. M. (1987*a*). Origin and propagation of electrical slow waves in circular muscle of canine proximal colon. *American Journal of Physiology* **252**, C215–224.

SMITH, T. K., REED, J. B. & SANDERS, K. M. (1987*b*). Interaction of two electrical pacemakers in the muscularis of the canine proximal colon. *American Journal of Physiology* **252**, C290–299.

SUZUKI, N., PROSSER, C. L. & DAHMS, V. (1986). Boundary cells between longitudinal and circular layers: essential for electrical slow waves in cat intestine. *American Journal of Physiology* **250**, G287–294.

THUNEBERG, L. (1982). Interstitial cells of Cajal: Intestinal pacemakers? *Advances in Anatomy, Embryology and Cell Biology* **71**, 1–130.

THUNEBERG, L., JOHANSON, V., RUMENSSEN, J. J. & ANDERSON, B. G. (1983). Interstitial cells of Cajal (ICC): selective uptake of methylene blue inhibits slow wave activity. In *Gastrointestinal Motility*, ed. ROMAN, C., pp. 495–502. MTP, Lancaster, UK.

TORIHASHI, S., WARD, S. M., NISHIKAWA, S.-I., NISHI, K., KOBAYASHI, S. & SANDERS, K. M. (1995). c-Kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. *Cell and Tissue Research* **280**, 97–111.

TORIHASHI, S., WARD, S. M. & SANDERS, K. M. (1997). Development of c-Kit positive cells and the onset of electrical rhythmicity in murine small intestine. *Gastroenterology* **112**, 144–155.

TORIHASHI, S., YOSHIDA, H., NISHIKAWA, S. I., KUNISADA, T. & SANDERS K. M. (1996). Enteric neurons express steel factor-lacZ transgene in the murine gastrointestinal tract. *Brain Research* **738**, 323–328.

VANDERWINDEN, J.-M., LIU, H., DE LAET, M.-H. & VANDERHAEGHEN, J.-J. (1996). Study of the interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. *Gastroenterology* **111**, 279–288.

WARD, S. M. (1996). Changes in electrical and mechanical activity during ontogeny of the canine proximal colon. *American Journal of Physiology* **271**, G184–191.

WARD, S. M., BURKE, E. P. & SANDERS, K. M. (1990). Use of rhodamine 123 to label and lesion interstitial cells of Cajal in canine colonic circular muscle. *Anatomy and Embryology* **182**, 215–224.

WARD, S. M., BURNS, A. J., TORIHASHI, S., HARNEY, S. C. & SANDERS, K. M. (1995). Impaired development of interstitial cells and intestinal electrical rhythmicity in steel mutants. *American Journal of Physiology* **269**, C1577–1585.

WARD, S. M., BURNS, A. J., TORIHASHI, S. & SANDERS, K. M. (1994). Mutation of the proto-oncogene c-kit blocks development of interstitial cells and electrical rhythmicity in murine intestine. *Journal of Physiology* **480**, 91–97.

WILLIAMS, D. E., EISENMAN, J., BAIRD, A., RAUCH, C., VAN NESS, K., MARCH, C. F., PARK, L. S., MARTIN, U., MOCHIZUKI, D. Y., BOSWELL, H. S., BURGESS, G. S., COSMAN, D. & LYMAN S. D. (1990). Identification of a ligand for the c-kit proto-oncogene. *Cell* **63**, 167–174.

YOUNG, H. M., CIAMPOLI, D., SOUTHWELL, B. R. & NEWGREEN, D. F. (1996). Origin of interstitial cells of Cajal in the mouse intestine. *Developmental Biology* **180**, 97–107.

YOUNG, H. M., MCCONALOGUE, K., FURNESS, J. B. & DE VENTE, J. (1993). Nitric oxide targets in the guinea-pig intestine identified by induction of cyclic GMP immunoreactivity. *Neuroscience* **55**, 583–596.

Acknowledgements

The authors are grateful to Gerard Morris for technical assistance. This work was supported by DK40569. The Morphology Core Laboratory supported by our Program Project Grant, DK41315, was used for the immunohistochemical studies.

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Received 19 December 1996; accepted 6 August 1997.