Mutation of the proto-oncogene c-*kit* blocks development of interstitial cells and electrical rhythmicity in murine intestine

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1. Interstitial cells of Cajal (ICs) have been proposed as pacemakers in the gastrointestinal tract. We studied the characteristics and distribution of ICs and electrical activity of small intestinal muscles from mice with mutations at the dominant-white spotting/c-*kit* (*W*) locus because the tyrosine kinase function of c-*kit* may be important in the development of the IC network.

2. W/W^v mutants (days 3–30 postpartum) had few ICs in the myenteric plexus region compared with wild type (+/+) siblings. The few ICs present were associated with neural elements and lay between myenteric ganglia and the longitudinal muscle layer.

3. Electrical recordings from intestinal muscle strips showed that electrical slow waves were always present in muscles of +/+ siblings, but were absent in W/W^v mice.

4. Muscles from W/W^v mice responded to stimulation of intrinsic nerves. Neural responses, attributed to the release of acetylcholine, nitric oxide and other unidentified transmitters, were recorded.

5. These findings are consistent with the hypothesis that ICs are a critical element in the generation of electrical rhythmicity in intestinal muscles. The data also show that neural regulation of gastrointestinal muscles can develop independently of the IC network.

6. *W* locus mutants provide a powerful new model for studies of the physiological role of ICs and the significance of electrical rhythmicity to normal gastrointestinal motility.

Interstitial cells of Cajal (ICs) may generate electrical rhythmicity and mediate neural inputs in the gastrointestinal (GI) tract (Thuneberg, 1982; Christensen, 1992; Sanders, 1992; Daniel & Berezin, 1992). This hypothesis has been tested by experiments in which: (i) ICs were removed by dissection (e.g. Suzuki, Prosser & Dahms, 1986; Hara, Kubota & Szurszewski, 1986; Smith, Reed & Sanders, 1987); (ii) chemicals thought to lesion ICs specifically were used to block the function of these cells (Thuneberg, Johanson, Rumenssen & Anderson, 1983; Ward, Burke & Sanders, 1990; Liu, Thuneberg & Huizinga, 1994); and (iii) ICs were isolated by enzymatic dispersion of tissues to study directly their excitability and responses to drugs (Langton, Ward, Carl, Norell & Sanders, 1989; Publicover, Hammond & Sanders, 1993). These studies have supported the proposed role for ICs. However, use of surgical and chemical techniques might be criticized for a lack of specificity, and the role of ICs *in situ* can only be surmised from studies on isolated cells. To understand the physiological role of ICs in GI motility, it is necessary to deactivate selectively or remove these cells without affecting the function of the other cell types (i.e. smooth muscle cells and neurons) responsible for GI motility. One approach to this problem might be to impair selectively the development of the IC network.

We have found that an antibody (ACK2) for the proto-oncogene product, c-*kit* protein, specifically labels interstitial cells in the mouse GI tract (Torihashi, Ward, Nishikawa, Nishi, Kobayashi & Sanders, 1994). Treatment of neonatal animals with ACK2, which blocks the receptor tyrosine kinase function of c-*kit*, caused abnormal intestinal contractions and a distended GI tract (Maeda *et al.* 1992). ACK2 treatment greatly reduced the number of ICs and the postnatal development of the IC network (Torihashi *et al.* 1994). The morphological changes in ACK2-treated animals were paralleled with a loss of electrical rhythmicity (Torihashi *et al.* 1994). These data suggest that the c-*kit* signalling pathway is important in

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the development of ICs, as it is in the development of cell lineages responsible for pigmentation, haematopoiesis and fertility (see Russell, 1979).

IC networks may not develop in animals in which the function of the c-*kit* protein is genetically impaired. There are a variety of mutations of the dominant-white spotting (*W*) locus in mice in which the tyrosine kinase activity of c-*kit* is compromised (Nocka *et al.* 1990). The *W* locus is allelic for c-*kit* (e.g. Geissler, Ryan & Houseman, 1988), and the tyrosine kinase activity attributed to c-*kit* protein is decreased in *W* mutants (Nocka *et al.* 1990). Complete disruption of the tyrosine kinase activity of c-*kit* protein is fatal, and homozygotes (*W*/*W*) usually die *in utero* (Russell, 1979). Other *W* mutants, such as W^V, which is a point mutation in c-*kit* that reduces but does not abolish the tyrosine kinase activity, are viable (Nocka *et al.* 1990). In the present study we have compared the morphology of ICs, spontaneous electrical activity, and responses to nerve stimulation in intestinal muscles of wild type (+/+) animals and W^V mutants.

METHODS

Heterozygotes (*W*/+) and (W^V/+) derived from C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were paired to obtain +/+, *W*/+, W^V/+, *W*/W^V and W^V/W^V offspring. *W* locus homozygotes were clearly distinguishable from their wild type and heterozygote siblings due to their small size, pure white coats, and dark eyes (Russell, 1979). Animals were anaesthesized by chloroform inhalation and exsanguinated by cervical dislocation followed by decapitation between days 3 and 30 postpartum for morphological and electrophysiological studies. The use and treatment of animals was approved by the Institutional Animal Use and Care Committee at the University of Nevada.

Morphological studies

Immunohistochemical studies were performed on tissues that were dehydrated in graded sucrose solutions, embedded in Tissue Tek (Miles, Naperville, IL, USA), and frozen in liquid nitrogen. Cryostat sections were cut at 10 μm thickness, fixed in acetone, and incubated overnight with a monoclonal antibody against the c-*kit* protein (ACK2; 5 μg ml^{-1}; see Nishikawa *et al.* 1991) at 4 °C. Immunoreactivity was detected by an avidin enzyme complex method (Histostain-SP kits; Zymed Labs, San Francisco, CA, USA). The reaction product was dark red. Control tissues were prepared in a similar manner, but the incubation solution did not contain ACK2.

For Methylene Blue staining, whole mounts were incubated in Krebs–Ringer bicarbonate solution (KRB; see below) containing 0·05 mM Methylene Blue bubbled with 97% O_2–3% CO_2 for 30 min in the dark to stain ICs selectively (Thuneberg, 1982). After staining, tissues were washed and examined with bright field illumination. ICs were stained dark blue.

Ultrastructural comparisons were made on tissues fixed with 2·5% glutaraldehyde, 1·25 mM $CaCl_2$ and 3% sucrose in 0·05 M cacodylate buffer (pH 7·4) at 4 °C for 5 h. Tissues were post-fixed with 1% OsO_4, stained *en bloc* with saturated uranyl acetate, dehydrated through ethanol solutions, and embedded in Medcast epoxy resin (Ted Pella Inc., Redding, CA, USA). Ultrathin sections were double stained with uranyl acetate and lead citrate and examined with a transmission electron microscope (Phillips CM10).

Electrophysiological studies

Segments of terminal ileum were isolated and opened along the mesenteric border. Luminal contents were removed with KRB. After removing the mucosa, muscle strips (30 × 6 mm) were cut and pinned to the Sylgard floor of a recording chamber with the mucosal side of the circular muscle facing upward. Parallel platinum electrodes were placed on either side of the muscle strips. Electrical recordings were made in the presence of nifedipine (10^{-6} M) to reduce movement.

Circular muscle cells were impaled with glass microelectrodes, and transmembrane potential was measured (WPI S-7071, Sarasota, FL, USA). Electrical signals were recorded on magnetic tape (Racal 40S, Southampton, UK). Neural responses were elicited by square wave pulses (0·5 ms duration, supramaximal voltage) of electrical field stimulation (EFS; Grass S48, Quincy, MA, USA). Data are expressed as means ± S.E.M. from five +/+ wild type and five *W*/W^V mutant animals (an average of 6 cell recordings were made from each animal). Differences in the data were evaluated using Student's *t* test; *P* values less than 0·05 were taken as a statistically significant difference.

Solutions and drugs

Muscles were maintained in KRB (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; dextrose, 11·5; and bubbled with 97% O_2–3% CO_2. Solutions of nifedipine (10^{-2} M; Sigma Chemical Co., St Louis, MO, USA) prepared in ethanol were diluted to 10^{-6} M in KRB. Atropine sulphate and L-nitroarginine (L-NA; Sigma) were dissolved in distilled water, and diluted in KRB.

RESULTS

ICs in the murine intestine express c-*kit* protein-like immunoreactivity (c-*kit*-LI; Torihashi *et al.* 1994). Intestinal muscles from wild type (+/+) animals showed a population of cells within the myenteric plexus region that were identified as ICs with c-*kit*-LI (IC-MY; Fig. 1*A*). These cells had multiple, long processes extending along the region between muscle layers. Few cells with c-*kit*-LI were observed in cross-sections from *W*/W^V animals (Fig. 1*D*).

Whole-mount preparations were stained with Methylene Blue, which selectively stains ICs in mouse intestine (e.g. Thuneberg, 1982). This reagent stained IC-MY in wild type and *W* locus mutants (days 3–30 postpartum). IC-MY had long, slender processes that appeared to form an extensive network with other IC-MY (Fig. 1*B* and *C*). The number of ICs stained by Methylene Blue in wild type animals averaged 534 ± 45 mm^{-2}. Heterozygotes (W^V/+) contained a similar number of ICs (513 ± 47 mm^{-2}). The number of ICs in homozygotes

(either W/W^{V} or $W^{V}/W^{V)}$ was markedly reduced, averaging 58 ± 8 and 39 ± 11 mm^{-2}, respectively (see Fig. 1*E* and *F*). ICs in *W* mutants displayed long processes, but the network normally formed by these cells appeared to be disrupted as a result of the large reduction in the number of ICs.

The ultrastructure of IC-MYs in +/+ and W/W^{V} animals was compared. IC-MYs in wild type animals were interposed between the circular and longitudinal muscle layers (Fig. 2*A*) and were in close association with myenteric ganglia and neural processes. IC-MY exhibited many mitochondria, well-developed rough endoplasmic reticulum, and free ribosomes and polyribosomes (Fig. 2*B* and inset). Caveolae were also present. Thick filaments, as observed in smooth muscle cells, were not found. The electron density of the nucleus and cytoplasm of IC-MY was greater than that of neighbouring smooth muscle cells.

Few IC-MY were found in W/W^{V} mice. IC-MY were in close association with neural elements (i.e. along the longitudinal muscle aspect of myenteric ganglia). IC-MY had long processes, free and polyribosomes, rough endoplasmic reticulum, caveolae and many mitochondria (Fig. 2*C* and *D*).

Resting membrane potentials recorded from circular smooth muscle cells of +/+ mice averaged -68 ± 4 mV ($n = 5$ animals). Circular muscle cells exhibited spontaneous slow waves, $34{\cdot}1 \pm 3{\cdot}0$ mV in amplitude and $30{\cdot}7 \pm 2{\cdot}0$ cycles min^{-1} (Fig. 3*A*). Circular muscle cells of W/W^{V} mice had resting membrane potentials averaging $-57{\cdot}4 \pm 1{\cdot}8$ mV ($n = 5$ animals; $P < 0{\cdot}05$ when compared with +/+ siblings of the same age). Electrical slow waves were not observed in muscles of W/W^{V} mice (Fig. 3*B*).

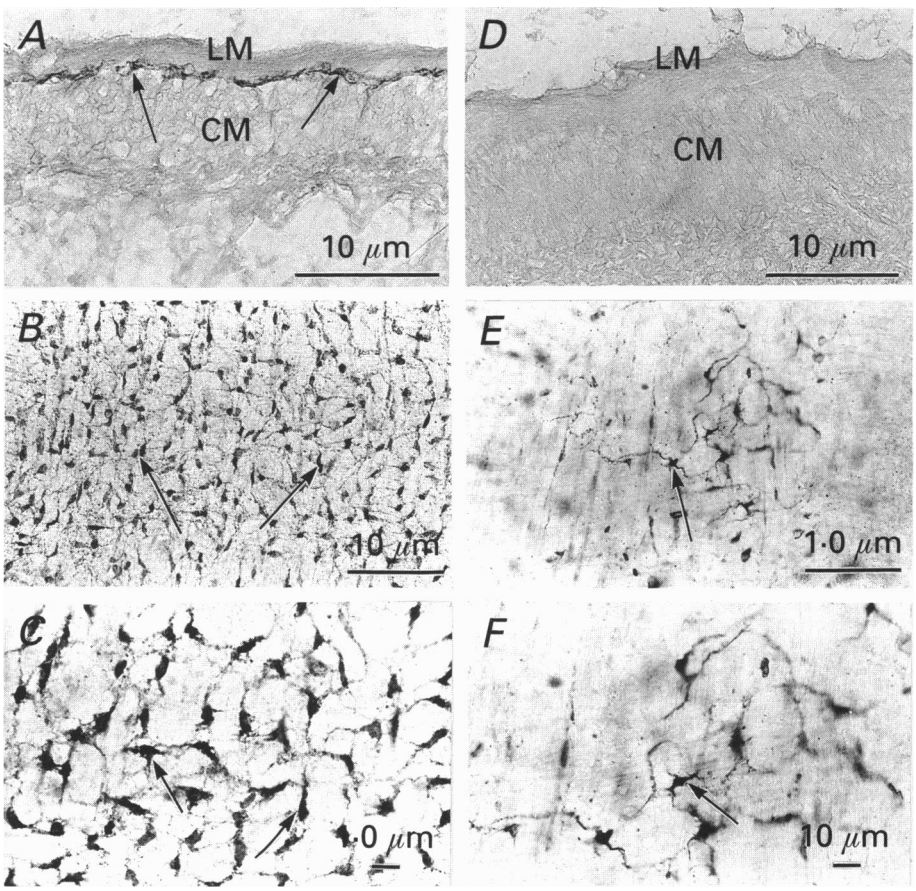

Figure 1.
A shows c-*kit*-like immunoreactivity (c-*kit*-LI) in interstitial cells in the myenteric plexus region (arrows) between longitudinal (LM) and circular (CM) muscle. In cross-sections, few cells with c-*kit*-LI were found in W/W^{V} animals. *B*, *C*, *E* and *F* show Methylene Blue staining of ICs (arrows) in whole-mount preparations. In wild type (+/+) animals a dense, interconnecting network is apparent (*B* and *C*). Cells stained with Methylene Blue were also present in W/W^{V} mutants, but the number of these cells was greatly reduced (arrows in *E* and *F*). *D* shows a typical section in which no c-*kit*-LI could be identified.

The response of +/+ mice to electrical field stimulation (EFS; single pulse, 0·5 ms duration) consisted of hyperpolarization averaging 4·8 ± 1 mV and reduction in slow-wave amplitude (Fig. 4*A*). More sustained stimulation (i.e. 10 Hz for 1 s) caused hyperpolarization averaging 9·8 ± 2·6 mV and attenuation of slow waves. The inhibitory effects of EFS persisted for several slow waves before the resting potential and slow waves returned to control levels. L-Nitroarginine (L-NA; 10^{-4} M for 20 min) had no effect on the response to a single pulse of EFS (Fig. 4*B*, left panel), but the hyperpolarization and reduction in slow-wave amplitude caused by repetitve stimulation (e.g. 10 Hz for 1 s) was blocked by L-NA (Fig. 4*B*), unmasking a sustained depolarization and an enhancement in slow-wave amplitude (Fig. 4*B*, right panel). Further addition of atropine reduced the depolarization unmasked by L-NA. The initial inhibitory response elicited by a single pulse of EFS or by repetitive stimulation was unaffected by L-NA or atropine (Fig. 4*C*).

In W/W^{v} mice a single pulse of EFS produced a triphasic junction potential consisting of an initial fast hyperpolarization (14 ± 1 mV, $n = 9$), partial repolarization, and a slow hyperpolarization (6·9 ± 1 mV, 4·9 ± 0·2 s in duration, $n = 9$). With repetitive stimuli (10 Hz for 1 s) the hyperpolarization phase was enhanced (to 8·2 ± 1 mV, 8·7 ± 0·5 s in duration), and the partial repolarization phase was reduced (Fig. 4*D*). Addition of L-NA (10^{-4} M) to reduce responses dependent upon the synthesis of nitric

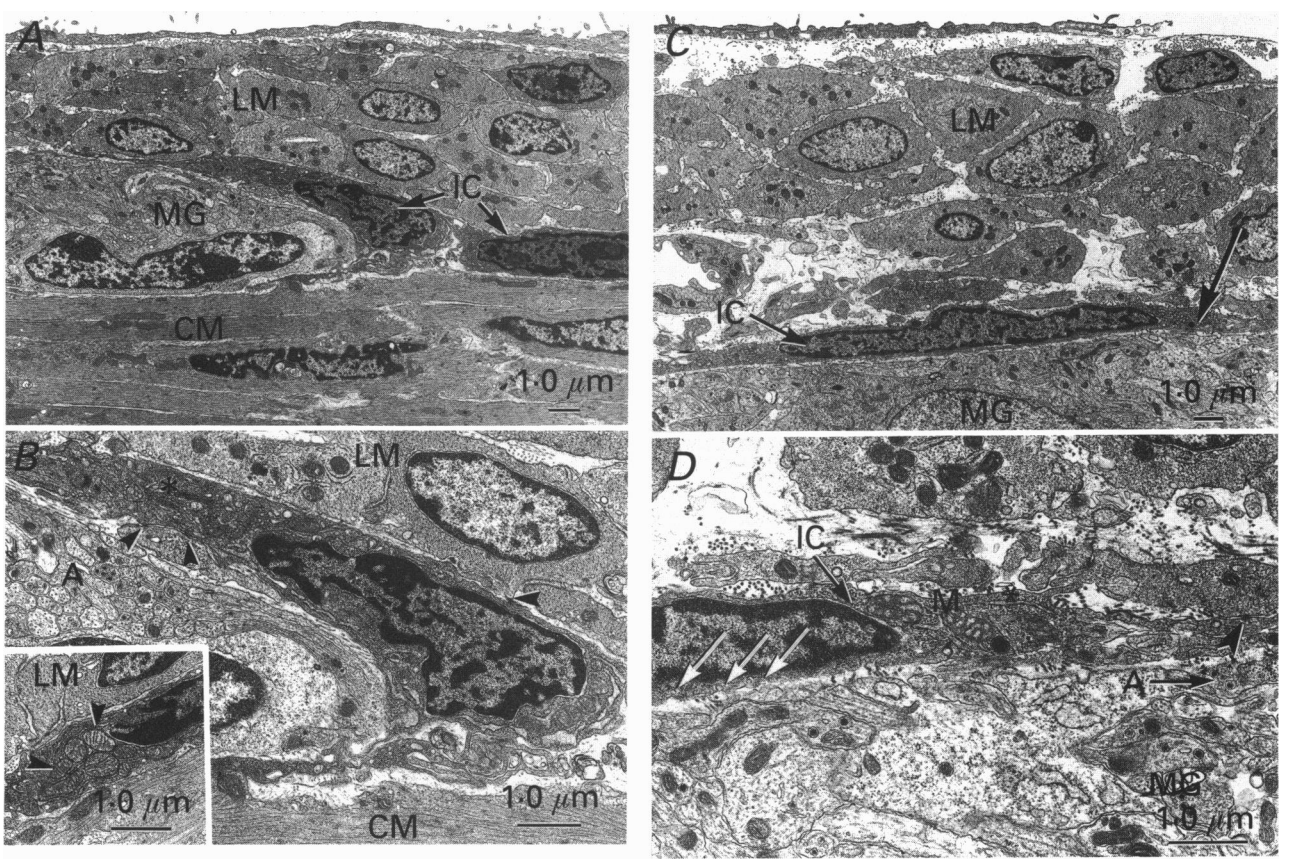

Figure 2. Ultrastructural comparisons of ICs in +/+ and W/W^{v} mice
A and *B* show ICs (arrows) near a myenteric ganglion (MG) between the circular (CM) and longitudinal (LM) muscle layers. *B* shows higher magnification of the IC associated with the myenteric ganglion. Axons and varicosities (A) can be seen in a ganglion. Interstitial cells (IC-MY) had electron-dense cytoplasm and possessed well-developed endoplasmic reticulum (asterisk) and an abundance of mitochondria (inset; arrowheads). IC-MY also formed junctions with smooth muscle cells (arrowheads in *B*). *C* and *D* show IC-MY in tissues from W/W^{v} animals. *D*, a higher magnification micrograph of the area denoted in *C* by the large arrow. ICs (arrows) had an electron-dense cytoplasm and were always associated with myenteric ganglia (MG). The cells had a well-developed endoplasmic reticulum (asterisk in *D*), caveolae (white arrows in *D*), many mitochondria (M), and were often closely associated with axons of a myenteric ganglion (A). Occasional junctions with smooth muscle cells could also be identified (arrowhead).

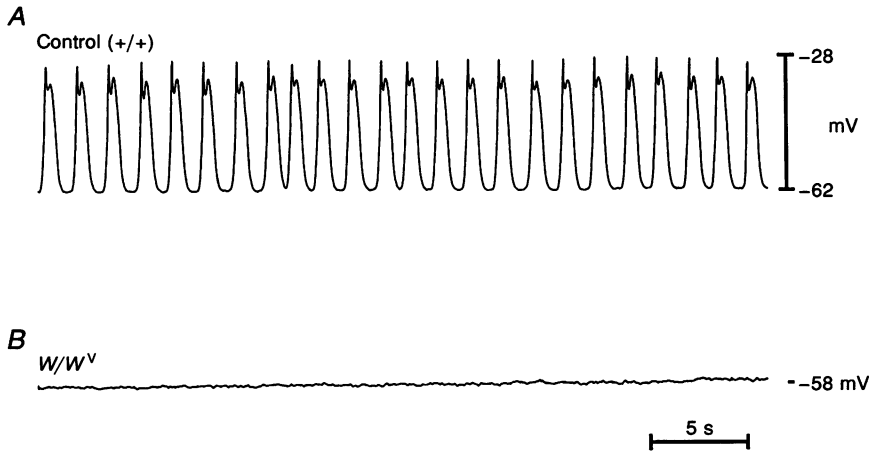

Figure 3. Electrical rhythmicity in wild type and mutant siblings
A shows electrical slow-wave activity recorded from circular muscle of wild type (+/+) mouse small intestine. Slow waves consist of upstroke and plateau components. *B* shows an electrical recording from (W/W^V) sibling. Slow waves were absent in W/W^V mice. Resting membrane potentials were also slightly depolarized in W/W^V mice (see text for details).

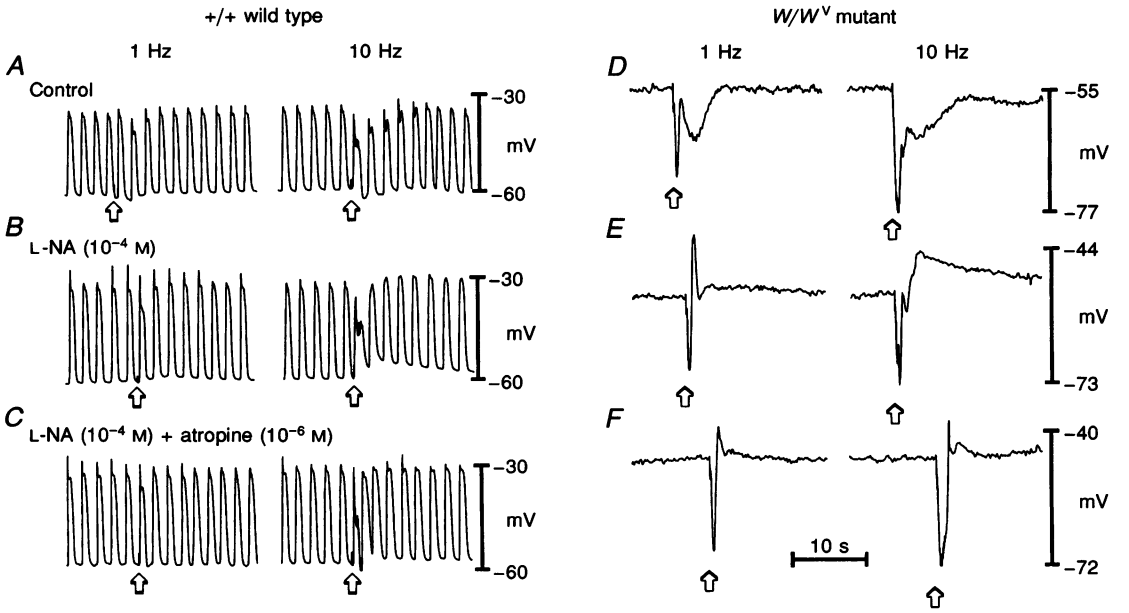

Figure 4. Electrical responses to EFS
Responses in a muscle from a +/+ animal under control conditions (*A*) and after treatment with L-NA (*B*) and L-NA and atropine (*C*). EFS (1 Hz for 1 s) reduced the amplitude of slow waves. At 10 Hz for 1 s a slight hyperpolarization and reduction in slow waves was observed (right-hand trace in *A*). After L-NA, EFS reduced the first slow wave, and at 10 Hz membrane potential depolarized. Membrane depolarization was blocked by atropine (*C*). *D–F* were recorded from a muscle strip from a W/W^V animal. Under control conditions, responses to EFS were triphasic, consisting of initial fast hyperpolarization, partial repolarization, and slow hyperpolarization (*D*). L-NA did not affect the fast hyperpolarization, but abolished the slow hyperpolarization (*E*). An excitatory junction potential (EJP) was revealed when the slow hyperpolarization was blocked. EJPs were blocked by atropine (*F*). These data show that cholinergic and nitric oxide-dependent responses to EFS were retained in W/W^V animals. Points of EFS stimulation are shown by arrows.

oxide, did not affect the initial hyperpolarization, but the slow hyperpolarization was abolished, revealing an excitatory junction potential (EJP; Fig. 4*E*). Repetitive pulses of EFS caused an enhancement in the duration of the EJP. Addition of atropine (10^{-6} M) to block muscarinic responses, reduced the EJP in response to a single or multiple pulses (Fig. 4*F*).

DISCUSSION

Interstitial cells of Cajal (ICs) are found in pacemaker regions in GI muscles (see Christensen, 1992). Some classes of ICs form gap junctions with smooth muscle cells. ICs are also often interposed between varicose nerve fibres and smooth muscle cells. These morphological observations suggest that ICs may function as pacemakers and as mediators of enteric neurotransmission. These hypotheses have been hard to test because of the relatively small size of ICs, their sparse distribution, and the structural complexities of GI muscles. Dissection experiments have shown that electrical rhythmicity is generated in specific regions, usually at the submucosal or myenteric surfaces of the circular muscle layer. Morphological studies show that the pacemaker regions are populated with certain classes of IC (Suzuki *et al.* 1986; Smith *et al.* 1987; Berezin, Huizinga & Daniel, 1988). It has not been possible to remove ICs selectively by dissection, and it is therefore not possible to assign unequivocally a pacemaker function to these cells on the basis of these studies. Others have attempted to remove ICs with cytotoxic chemicals that accumulate in ICs. For example, Methylene Blue and rhodamine 123 label ICs in some species, and both compounds abolish slow waves (Thuneberg *et al.* 1983; Ward *et al.* 1990; Liu *et al.* 1994). It is possible, however, that these compounds could have non-specific effects on smooth muscle cells (e.g. see Sanders, Burke & Stevens, 1989). Freshly dispersed and cultured ICs are excitable, spontaneously rhythmic (Langton, Ward, Carl, Norell & Sanders, 1989) and responsive to neurotransmitters (Publicover *et al.* 1993). Studies on isolated cells support the role of these cells as pacemakers, but it is impossible to determine fully the role of ICs *in situ* from studies on single cells. In the present study we have characterized an animal model in which the IC network fails to develop fully. This model provides an important new means of investigating the role of ICs in GI motility.

ICs are not fully developed at birth in the mouse, and the IC network develops over a period of several days or weeks (Faussone-Pelligrini, 1985). We found that the development of ICs in the myenteric region of the small intestine is impaired in *W* locus mutants. Since the *W* locus is allelic with c-*kit*, our data suggest that the activity of c-*kit* protein, a receptor tyrosine kinase, is required for the normal development of the IC network. This idea is consistent with the known role of c-*kit* as an essential signalling pathway in the development of several cell types (e.g. Russell, 1979; Geissler *et al.* 1988). Although the development of other cells within the muscularis externa could also have been impaired in W/W^{v} mice, in this study and in another (Torihashi *et al.* 1994) we found that anti-c-*kit* monoclonal antibodies selectively labelled ICs. These observations suggest that expression of c-*kit* in the muscularis externa is limited to ICs, and therefore the developmental impact of defective c-*kit* protein may be primarily restricted to ICs. This reasoning does not exclude the possibility, however, that ICs express trophic factors important for the development of other cells in their vicinity and, therefore, impairment of IC development could indirectly affect the development of other cell types. There were, however, no obvious morphological or physiological indications that the development of smooth muscle cells or neurons was compromised in the small intestines of W/W^{v} mice. More precise physiological tests, particularly of smooth muscle cells, will be necessary to investigate rigorously the selectivity of the lesions in *W* mutants.

The electrical activity of intestinal muscles from control animals was characterized by continuous slow-wave activity. Studies on other animal models suggest that slow waves originate from the myenteric plexus region in the small intestine (Suzuki *et al.* 1986; Hara *et al.* 1986), and these authors have speculated that ICs are responsible for the generation of slow waves. As described above, the number of ICs in the myenteric plexus region of W/W^{v} mice was greatly reduced, and slow waves were absent in muscles of these animals. These observations provide another link between ICs and the generation of electrical rhythmicity. Recent studies have also shown that slow waves were absent in animals treated with ACK2, a monoclonal antibody against the c-*kit* protein that restricts the function of the receptor tyrosine kinase (Torihashi *et al.* 1994). Others have described aberrant contractile patterns in muscles from animals treated with ACK2 just after birth (Maeda *et al.* 1992). There is substantial development of the IC network in the myenteric region of the small intestine during the first 1–10 days postpartum, and ACK2 treatments immediately after birth impair this development (Torihashi *et al.* 1994). Taken together these data suggest that the c-*kit* signalling pathway is important for the development of ICs during a window of time shortly after birth, and without normal IC development, electrical rhythmicity does not develop. The use of *W* mutants or anti-c-*kit* antibodies appears to provide a rather selective means of controlling the development of the pacemaker apparatus and electrical rhythmicity in GI muscles.

Based primarily on morphological data, several authors have proposed that neural inputs may be mediated via ICs in GI muscles (e.g. see Daniel & Berezin, 1992). Excitatory and inhibitory neural inputs did not appear to be impaired in W/W^{v} animals; cholinergic and nitric oxide-dependent responses were clearly observed. These data suggest that either compensatory development occurred to strengthen

the associations between nerve terminals and smooth muscle cells, or the role of ICs in neurotransmission has been overestimated.

At present the effects of the impaired pacemaker system on GI motility and intestinal transit are unknown, but the fact that the IC network is underdeveloped and slow waves are absent in these animals provides a unique opportunity to evaluate the importance of pacemaker activity to GI function. Our preliminary studies have shown that tissues in which IC development is impaired are contractile and that they respond to intrinsic nerve stimulation and neurotransmitters. This suggests that reflex activity such as the response to distension may be intact, but segmentation and the migrating myoelectrical complex might be significantly altered in *W* locus mutants.

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