A FUNCTIONAL ANALYSIS OF GASTROINTESTINAL MOTILITY IN THE GUINEA PIG AND HUMAN

A thesis submitted in total fulfilment of the requirements of the degree of doctor of philosophy

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2. CHANGES IN THE PHYSIOLOGY OF SMOOTH MUSCLE CELLS OF THE GUINEA PIG ILEUM AND COLON FOLLOWING DISSECTION

The electrophyisology work in this chapter has been published in the *American Journal of Physiology*: *Gastroenterology and Liver Physiology* (Carbone et al., 2012).

2.1 Introduction

Coordinated contractions and relaxations of the smooth muscle of the gut wall form the motility patterns that are responsible for mixing and propulsion of contents. In most of the gut, the muscularis externa consists of an outermost layer of longitudinal smooth muscle cells and a thicker inner layer of circular smooth muscle cells, which are innervated by separate populations of motor neurons (Brookes et al., 1991, Brookes et al., 1992). Smooth muscle cells have an intrinsic electrical activity called "slow waves". They are initiated by Interstitial Cells of Cajal (ICCs) and spread into smooth muscle cells via gap junctions to activate ion channels and contractile mechanisms (Huizinga and Lammers, 2008, Sanders, 2008). Input from axons of enteric motor neurons is mediated, at least partly, via non-pacemaker types of ICC and possibly other non-muscular cells. Enteric motor input combines with slow waves to generate the complex contractile activity patterns that underlie functional motility.

The neuromuscular apparatus of the mammalian gut expresses a wide range of receptors on nerves, smooth muscle cells and ICCs and has long been used as a versatile preparation for pharmacological studies (Paton, 1955, 1957). It is conventional to allow the preparation an "equilibration period" lasting 30 – 90 minutes before starting recordings. Allowing for this equilibration period is often described in the methods section of papers, the changes that occur have not been systematically studied. During this period, tone increases and responses to electrical stimulation or drugs stabilise. The mechanisms underlying these slow changes are unclear. In this study I have

investigated changes in neuromuscular function in the circular muscle of the guinea pig ileum and colon from the first minutes after the preparation is set up, until consistent responses to electrical stimulation are obtained several hours later. The inhibitory junction potential described by Bülbring and Tomita (1967), and the excitatory junction potential described by Gillespie and Mack (1964) were used as a measure of motor neuron input to smooth muscle (Bülbring and Tomita, 1967, Gillespie and Mack, 1964). I also investigated the changes in spontaneous contractions and muscle tone as preparations recovered from dissection.

2.2 Methods

2.2.1 Dissection

Adult male guinea pigs between 200 and 350g (36) were stunned by a blow to the back of the head and exsanguinated in a manner approved by the Animal Welfare Committee of Flinders University. Specimens of small intestine, more than 10cm proximal to the ileocaecal junction or distal colon were removed, flushed and placed into cooled (~14^0C) modified Krebs solution. For recordings of circular smooth muscle cells, the mucosa and submucosa were usually removed but preparations were not further dissected. For recordings from *S* neurons, the circular muscle layer was also removed. Unless otherwise stated, modified Krebs solution contained mM: NaCl 118; KCl 4.70, $NaH_2PO_4.2H_2O$ 1; $NaHCO_3$ 25; $MgCl_2.6H_2O$ 1.2; D-Glucose 11; $CaCl_2.2H_2O$ 2.5; bubbled with 95%O_2 and 5%CO_2 with 1μM hyoscine and 1μM nicardipine to inhibit excitatory junction potentials and muscle contractions. A segment of tissue approximately10mm long and of full circumference was isolated and pinned, circular muscle uppermost, into a Sylgard-lined (Dow Corning, Midland, MI, USA) recording chamber, with a volume of approximately 1ml, using 50μm tungsten pins (see figure 2.1).

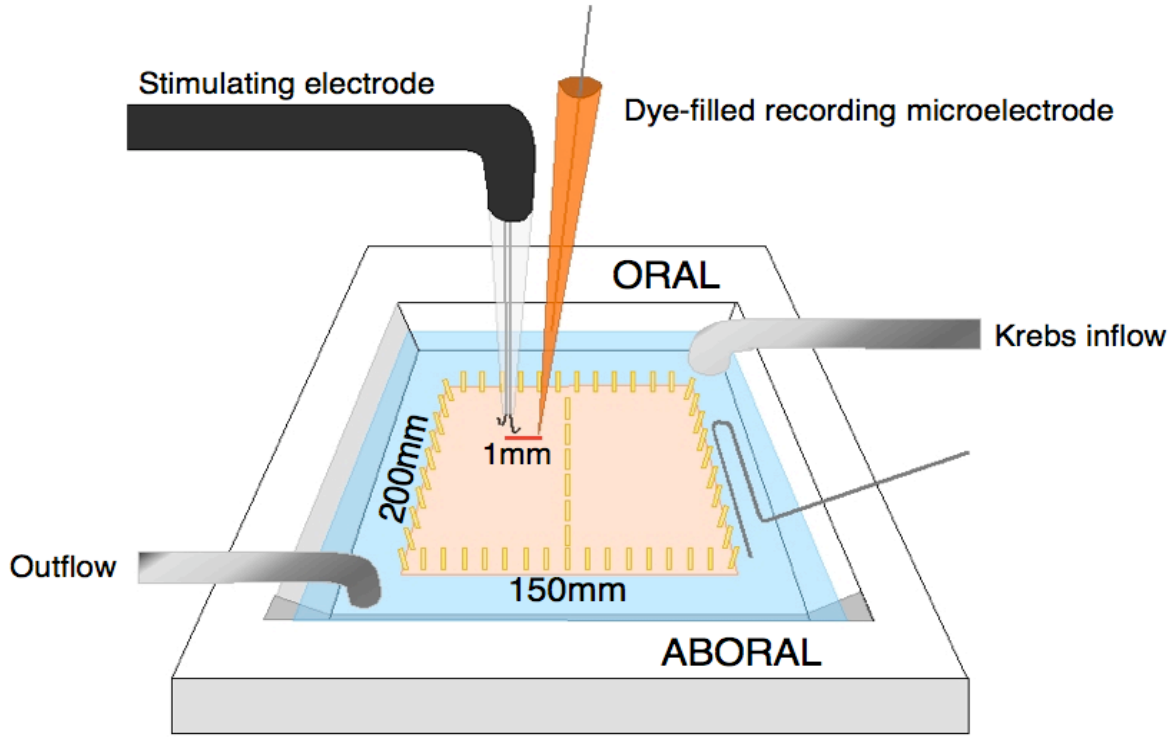

Figure 2.1 Experimental set up for intracellular electrophysiology.

2.2.2 Intracellular recording

The recording chamber was fixed onto the stage of an Olympus LH50A or an Olympus IX71 inverted microscope fitted with fluorescent optics (Japan). Krebs solution at 35^{o}C was constantly superfused, at a rate of 3ml/min. The moment at which warmed Krebs solution first reached the preparation was designated as t=0. From this time point, bath temperature increased to a stable 34-35^{0}C within 5 minutes.

Smooth muscle cells or neurons were impaled using borosilicate glass capillary electrodes (outer diameter 1mm; inner diameter 0.58mm, Harvard Apparatus, UK) filled with 5% 5,6 carboxyfluorescein in 20mM Tris buffer (pH 7.0) in 1M KCl solution. Electrodes had resistances between 50-200ΩM. Recordings of membrane potential were made using an Axoclamp 2A amplifier (Axon instruments, USA) and viewed on an oscilloscope (VP-5220A, Matsushita, Japan) and digitised at 10kHz and stored via an A/D interface (MacLab 8SP, AD Instruments, Sydney, Australia) using Chart 7 software (ADInstruments, Australia). Unless otherwise specified, a single pulse 15V electrical focal stimulus, 0.4ms duration, was applied via paired insulated Pt/Ir wire stimulating electrodes placed 1mm circumferential to the recording electrode. A Grass S48 stimulator and a Grass S1U5 stimulator isolation unit were used to generate the stimulus. Using this stimulus, the large amplitude fast IJP had little contribution by nitric oxide or peptidergic transmitters (Wang et al., 2007, Klemm and Lang, 2002). Input resistance was calculated by injecting current impulses (0.1 – 0.5nA) and measuring related potential changes. At the end of the recording period, cells were labelled with carboxyfluorescein by hyperpolarising current impulses of 0.5nA, with a duration of 0.2s at 2.5Hz (50% duty cycle) for 2 minutes. The electrode was then withdrawn to measure resting membrane potential. Carboxyfluorescein-labelled cells were counted, *in situ*, 1 minute later under a 10x objective using an appropriate dichroic mirror and filter. Neurons were also labelled with carboxyfluorescein to confirm their morphology.

2.2.3 Measuring the force of contractions

Specimens of guinea pig ileum or colon were used (*n*=16). From each specimen, two segments of tissue of similar dimensions to those used for intracellular electrophysiology (10mm long and of full circumference) were isolated and pinned in a dissecting dish. The mucosa was dissected off one specimen so that the recovery of spontaneous contractions could be compared between "partially dissected" (mucosa intact) and "dissected" (mucosa removed) preparations. An array of hooks was connected along the longitudinal axis of each preparation and the opposing axis was pinned with 0.2mm stainless steel pins (Fine Science Tools, USA) down to a Sylgard lined chamber (Dow Corning, Midland, MI, USA). Each hook array connected to an isometric force transducer (model MLT0201/D, ADInstruments, Bella Vista, Australia) via a cotton ligature so that circumferential contractions could be recorded. The chamber, perfused with Krebs solution, was placed on a heat pad so that the temperature within the bath was 34°C. Spontaneous contractions were recorded in control Krebs' solution, which did not contain nicardipine or hyoscine, for more than 120 minutes. Recordings were made under isometric conditions. Transducers were connected to Maclab Bridge Amp, which then connected to a Powerlab (model 4/30, ADInstruments, Bella Vista, Australia). Labchart 6 was used for data capture and analysis (ADInstruments, Australia).

2.2.4 Data Analysis

For intracellular data, results are expressed as means ± standard error of mean. The use of "*n*" refers to the number of preparations. Statistical analysis was performed by Student's two-tailed *t*-test for paired or unpaired samples using Microsoft Excel 2004. Where applicable, X/Y plots were analysed using linear regression (Microsoft Excel), treating the first 120 minutes and time after 120 minutes separately.

For force measurements, recordings were analysed in 20s episodes. Once preparations became active, periodic contractions persisted for the length of the recording. The duration of periodic contractions ranged from around 7 to 13s, therefore 20s provided a sufficient data sample for a complete contraction to be highlighted. Data was analysed

from 450s-7630s (7.5-127.2 minutes) to monitor the recovery from dissection, where time=0 was the point where the preparation was placed in the recording chamber. The first 450s were discarded because of artifacts due to adjusting force transducers and resting length. For each 20s of recording, values were obtained for the minimum force generated, the difference between the maximum and minimum forces for that epoch and the integral from the minimum value. Minimum force demonstrated base smooth muscle tone, the difference between the maximum and minimum force represented the change in amplitude of periodic contractions, and the integral represented area under the curve of that contraction. Data was binned into 60s intervals and the average values for each minute were plotted as scatter plots. Linear regression analysis was used to calculate whether changes in smooth muscle tone or the amplitudes of contractions were dependent on time from dissection. Values are written as mean ± standard error of mean, '*n*' refers to the number of preparations and corresponds with the number of animals.

2.3 Results

2.3.1 Electrophysiological properties of circular smooth muscle cells following dissection.

Single electrical stimuli evoked fast inhibitory junction potentials (fIJPs) that averaged 1.3 ± 1.0 mV in amplitude in the first 30 minutes after setting up preparations (Fig 2.2D*,* 20 cells). However, their amplitude increased significantly over time so that after 120 minutes they averaged -15.8±0.9mV (Fig 2.2D*, n*=12, 48 cells, $P<0.001$). The averaged rate of increase of the fIJP, after commencing superfusion of the preparation with warmed Krebs solution, is plotted in Figure 2.2C. Linear regression over the first 120 minutes revealed a significant time-dependent increase in fIJP amplitude ($P<0.001$, *n*=12, Fig 2.2B) however it did not increase further after 120 minutes.

Resting membrane potential (RMP) depolarised significantly over the first 120 minutes ($P<0.001$) and then did not significantly change after this time ($P>0.05$, *n*=12, Fig 2.3A). In the first 30 minutes, resting membrane potential averaged -58.9 ±

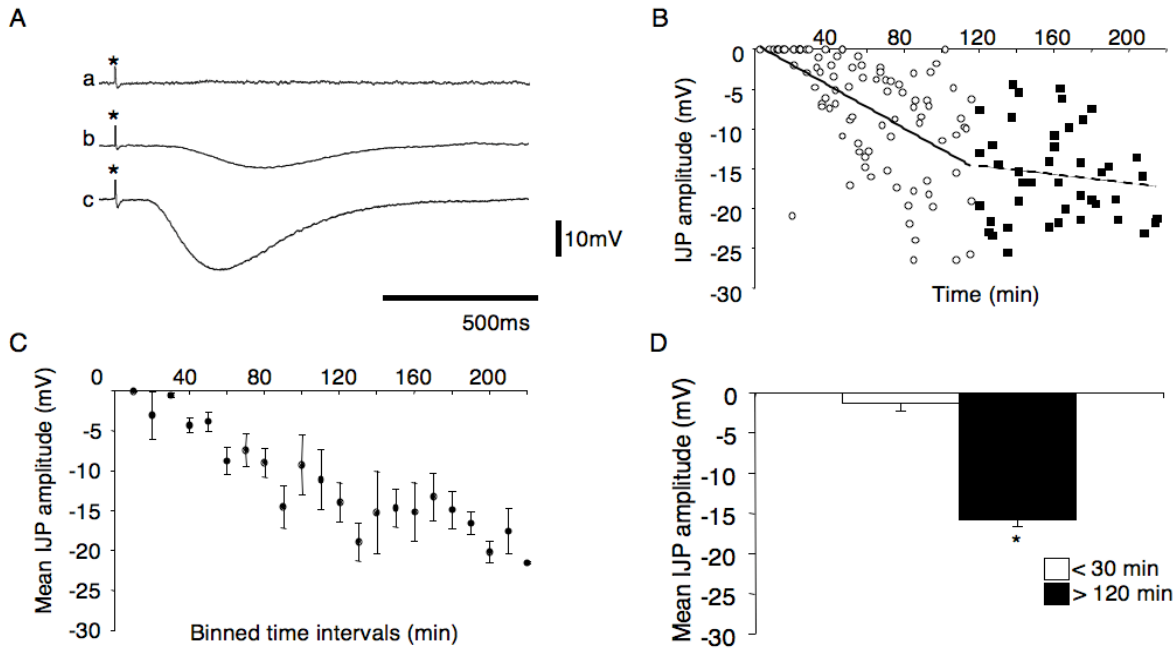

Figure 2.2 Amplitude of evoked IJPs in circular smooth muscle cells increases with recovery from dissection. Recordings from cells were made at different stages of recovery. A single pulse electrical stimulus* was applied 1mm circumferential to the site of impalement evoked a fast IJP (Aa t=9.5 minutes; Ab t=54 minutes; Ac t=137.5 minutes, from the same preparation). Fast IJP amplitude was negligible in cells impaled in the first 20 – 40 minutes of recording and increased over time. B) Scatter plot of fIJPs from 134 cells, (n=12) plotted against time after setting up the preparation. Responses increased significantly within the first 120 minutes (white circles, P<0.001). IJP amplitude plateaued after 120 minutes (black squares, P>0.05). C) Binned data (same data as B) shows mean fIJP amplitude recorded in 10 minute time intervals. D) Mean fIJP amplitude of cells impaled in the first 30 minutes was significantly smaller than cells impaled from 120 minutes onwards (*P<0.001, 20 and 48 cells respectively, n=12).

1.3 mV (20 cells) vs. -47.7 ± 0.6mV after 120 minutes (48 cells, n=12, P<0.001, Fig 2.3C). Apparent input resistance of circular muscle cells was calculated from an I/V curve plotted for 10ms long current pulses (300-500pA) after first balancing the bridge by the method of Martin and Pilar (1963) (Fig 2.4A and 2.4B (Martin et al., 1974)). Measurements showed a decreasing trend over the first 120 minutes, but this did not reach statistical significance. Input resistance then stabilised over the time that followed (P>0.05, n=12, Fig 2.4C). Mean input resistance in the first 30 minutes was significantly greater than cells impaled after 120 minutes (14.4±2.2MΩ (16 cells) vs. 8.4±0.7MΩ (42 cells) P<0.05, n=12, Fig 2.4E).

Impaled muscle cells were filled with 5% carboxyfluorescein in the recording microelectrode using a standardised current injection protocol at the end of the recording. When viewed one minute later, under standard illumination and magnification, the recorded cell could be clearly distinguished from coupled cells by its higher fluorescent intensity. Average dimensions were 476 ±25 µm long, with a maximum diameter that averaged approximate 3µm in the spindle shaped cells. In the first 30 minutes of the recording period, 1-3 dye filled cells were labelled after filling a single smooth muscle cell. The longer after setting up the preparation, the more dye-coupled cells were distinguishable, extending beyond the ends of the impaled cell (Fig 2.5D). Dye coupling increased significantly, in a time-dependent manner within the first 120 minutes (P<0.001, Fig 2.5A) and stabilised beyond this time point. Cells recorded within the first 30 minutes of the recording period had significantly less dye coupling (mean value= 2.0±0.3, 20 cells) than cells recorded from after 120 minutes (mean value= 4.2±0.3, 46 cells, n=12, P<0.001, fig 2.5C). These results suggest that gap junction coupling between circular smooth muscle cells increased significantly over the first 120 minutes after setting up a preparation, consistent with the trend towards decreased input resistance.

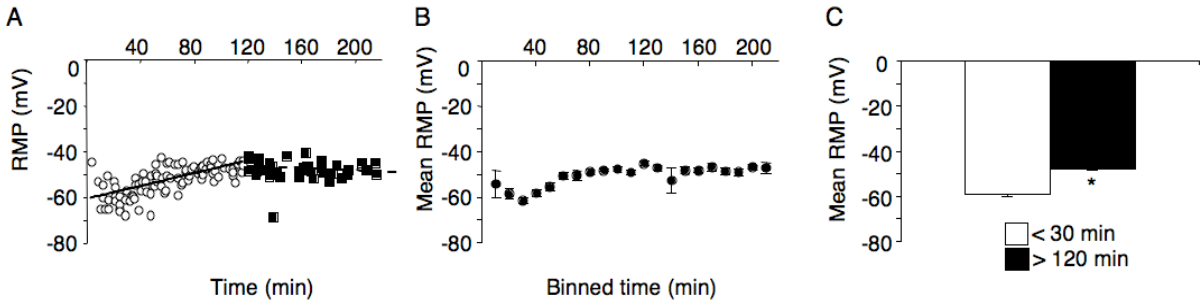

Figure 2.3 Unresponsive cells have a hyperpolarised resting membrane potential. A) RMP for individual cells impaled during the first 120 minutes of the experiment depolarise significantly (white circles, $P<0.001$). No significant difference in RMP of cells impaled after 120 minutes (black squares, $n=12$). B) The same data as A binned into 10 minute intervals highlights the change in RMP during the experiment. C) Cells impaled within the first 30 minutes have significantly more negative RMP than cells impaled from 120 minutes onwards (*$P<0.001$, 20 and 49 cells respectively, $n=12$).

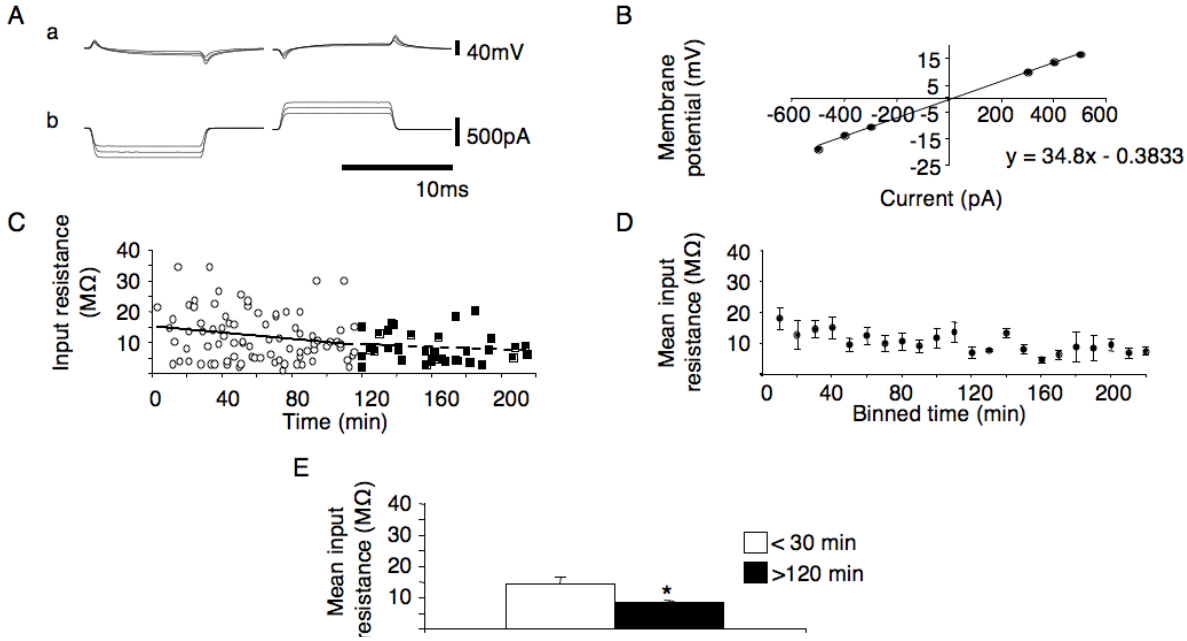

Figure 2.4 Apparent input resistance is greater for unresponsive cells than responsive cells. Injecting hyperpolarising and depolarising pulses of current (A) into cells resulted in small deflections of the membrane potential. Input resistance was calculated from the gradient of the I/V curve (B). C shows the input resistance reduced in the first 120 minutes (white circles, P=0.07) and did not change after this (black squares, n=12*)* with the same data in 10 minute bins shown in D. In E, mean data is shown for apparent input resistance of cells impaled within the first 30 minutes versus cells impaled post 120 minutes. Note that the absolute values of input resistance are very low, probably reflecting a poor seal around the sharp microelectrode. Nevertheless the change in input resistance was highly significant (* P<0.05, cells=16 and 42 respectively, n=12, E).

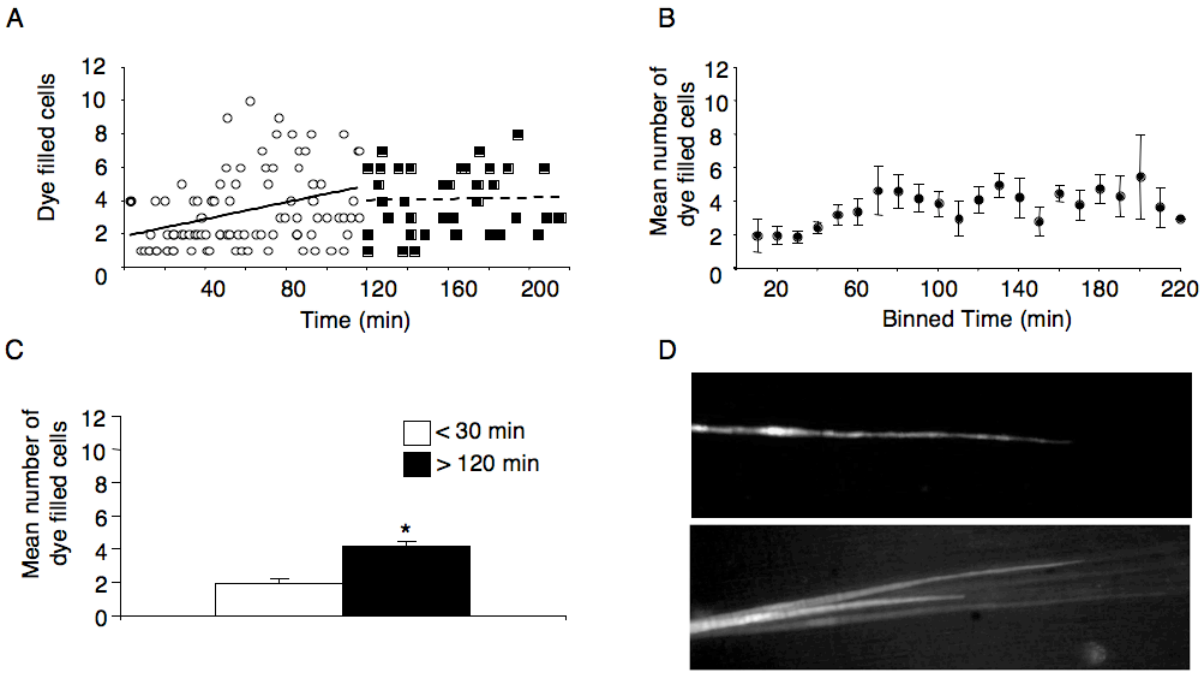

Figure 2.5 Dye coupling between circular smooth muscle cells increases as the preparation recovers. A) Dye coupling of cells impaled and filled with carboxyfluorescein using a standard current injection protocol. Coupling increased significantly during the first 120 minutes (white circles $P<0.001$) but did not significantly change after this (black squares). The same data is presented in B in 10 minute bins. C) On average, cells impaled after 120 minutes had significantly more dye coupling than cells impaled within the first 30 minutes (C * $P<0.001$, 20 and 46 cells respectively, $n=12$). D) *Top* a micrograph of 1 carboxyfluorescein-filled smooth muscle cell. *Bottom* a micrograph shows 3-4 dye coupled cells

2.3.2 The electrophysiological properties of longitudinal muscle cells

Immunohistochemical labelling of connexins shows that they are consistently less abundant in the longitudinal muscle layer than in the circular muscle layer (Daniel and Wang, 1999). To validate my techniques I compared the electrophysiological properties of circular and longitudinal muscle cells, once preparations had recovered from dissection (>120 minutes). Longitudinal muscle had smaller fIJPs (0.4±0.1mV, n=6) than circular muscle cells even after 120 minutes (14.7±0.9mV, P<0.001, n=6, Fig. 2.6A) as previously reported (Bywater and Taylor, 1986, Bywater and Taylor, 1983). This probably reflects a sparser innervation by inhibitory motor neurons (Brookes et al., 1992). There was no difference between the resting membrane potential of longitudinal muscle (-48.9 ± 0.7mV,) and circular muscle cells (-47.5±0.6mV, n=6, Fig 2.6B). The mean input resistance of longitudinal muscle cells was 26.5±2.8MΩ, which was significantly greater than that of circular muscle cells recorded more than 2 hours after setting up (8.9±0.9MΩ, P<0.001, n=5, Fig. 2.6C). Longitudinal muscle cells also showed significantly less dye coupling (1.2± 0.1 cells) than circular muscle cells (4.0±0.3 cells, P<0.001, n=6, Fig. 2.6D). These results confirm previous findings and validate the methods used to measure dye coupling and input resistance.

2.3.3 Effects on colonic circular smooth muscle cells

Similar initial suppression of responses followed by recovery was also observed in colonic circular smooth muscle cells. Fast IJP amplitudes increased significantly with time within the first 120 minutes of recording (P<0.001, 40 cells, n=5). Beyond 120 minutes, responses approached a plateau and did not significantly change (26 cells, n=5). Fast IJP amplitudes within the first 30 minutes following dissection averaged -0.4± 0.2mV and significantly increased to -27.6±2.5mV after 120 minutes (P<0.001, n=5). RMP of cells impaled within 120 minutes displayed time dependent depolarisation (P<0.001, n=5). RMP values stabilised for cells impaled after 120 minutes (n=5). Cells impaled within 30 minutes of dissection had a hyperpolarised RMP compared to cells impaled after 120 minutes (-57.4±3.9mV versus -43.3±0.7mV, P<0.05). Over the 120 minutes following dissection, input resistance

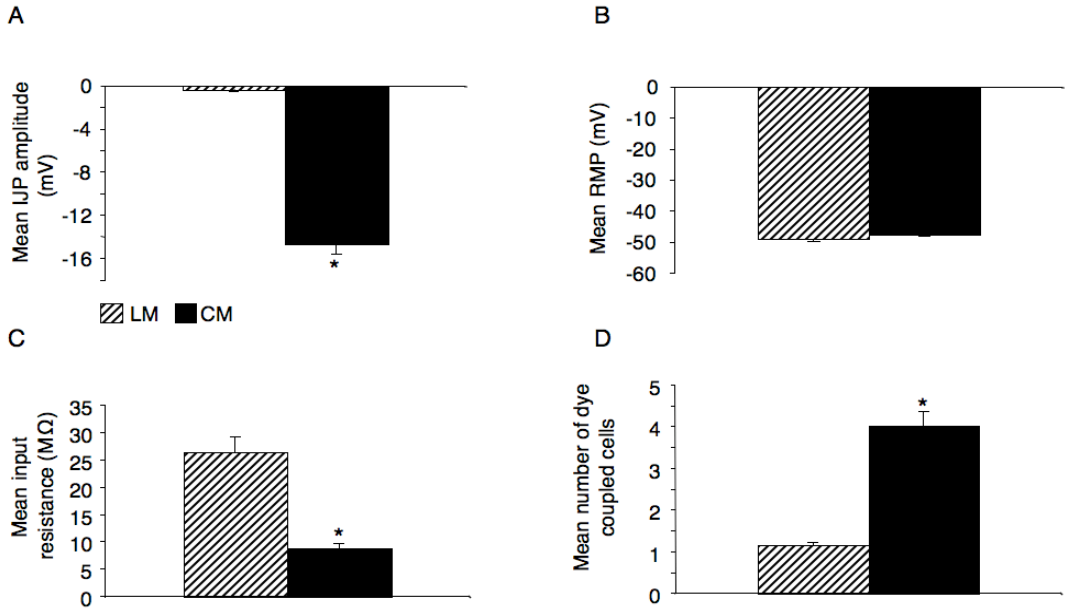

Figure 2.6 Comparison of longitudinal and circular smooth muscle cells. A) The mean fIJP amplitude of longitudinal muscle cells was significantly smaller than that of circular muscle cells (*P<0.001, n=6), however RMP of cells was not significantly different (B). Longitudinal muscle cells had significantly greater input resistance (C * P<0.001, n=5) and significantly fewer dye coupled cells (D * P<0.001, n=6) than circular muscle (C and D)

Table 2.1 Electrophysiological properties of colonic circular smooth muscle cells within 30 minutes or after 120 minutes from dissection.

	Time from dissection	
	<30 minutes	**>120 minutes**
fIJP amplitude, mV	-0.4±0.2	-27.6±2.5 †
RMP, mV	-57.4±3.9	-43.3±0.7 *
Input resistance, MΩ	10.9±1.1	8.6±0.4
Dye coupling	4.5±0.7	6.7±0.6 *

Values are mean± SEM, n=6, *P<0.05, †P<0.001

of colonic circular muscle cells time-dependently decreased ($P<0.05$, $n=5$) and did not change after this. Cells impaled within 30 minutes tended to have a greater input resistance after 120 minutes (10.9±1.1MΩ versus 8.6±0.4MΩ) however this was not significant ($P=0.06$, $n=5$). Dye coupled profiles significantly increased over the first 120 minutes ($P<0.0001$, $n=5$), however there were no significant differences for cells impaled with time that followed. The number of dye filled profiles within 30 minutes of dissection was significantly less than profiles counted after 120 minutes (4.5±0.7 versus 6.7±0.6, $P<0.05$, $n=5$, summarised in table 2.1).

2.3.4 Effects of dissection on nitrergic responses.

The amplitude of slow, nitrergic inhibitory junction potentials (sIJPs) following dissection was measured from ileal smooth muscle cells. In the presence of apamin (0.25μM) fIJPs were inhibited. Hyoscine (1μM) was also added to reduce excitatory junction potentials (EJPs) and nicardipine (1μM) to limit contractions. A higher frequency stimulus (3 pulses, 50ms interval, 0.5ms duration) was used to generate nitergic junction potentials. Slow IJPs were not measurable in smooth muscle cells impaled within the first 30 minutes following dissection, but were detected in cells impaled after 120 minutes (-3.1±0.5mV, $P<0.005$, $n=5$, Fig 2.7C). The amplitude of sIJPs showed a significant time dependent increase over the 120 minutes following dissection ($P<0.01$, $n=5$) and amplitudes continued to increase after 120 minutes ($P<0.05$, $n=5$, Fig 2.7B).

2.3.5 Excitatory junction potentials in the ileum and colon

Excitatory junction potentials (EJPs) were not reliably recorded from circular muscle cells in the guinea pig ileum (Bauer and Kuriyama, 1982b). In colonic preparations, addition of apamin (0.25μM) inhibited fIJPs, and nicardipine (1μM) limited contractions. Under these conditions EJPs could be evoked by electrically stimulating intrinsic excitatory motor neurons with single stimuli applied 1mm aboral to the impaled cell. EJPs had amplitude of 1.8±1.1mV in the first 30 minutes of dissection, but

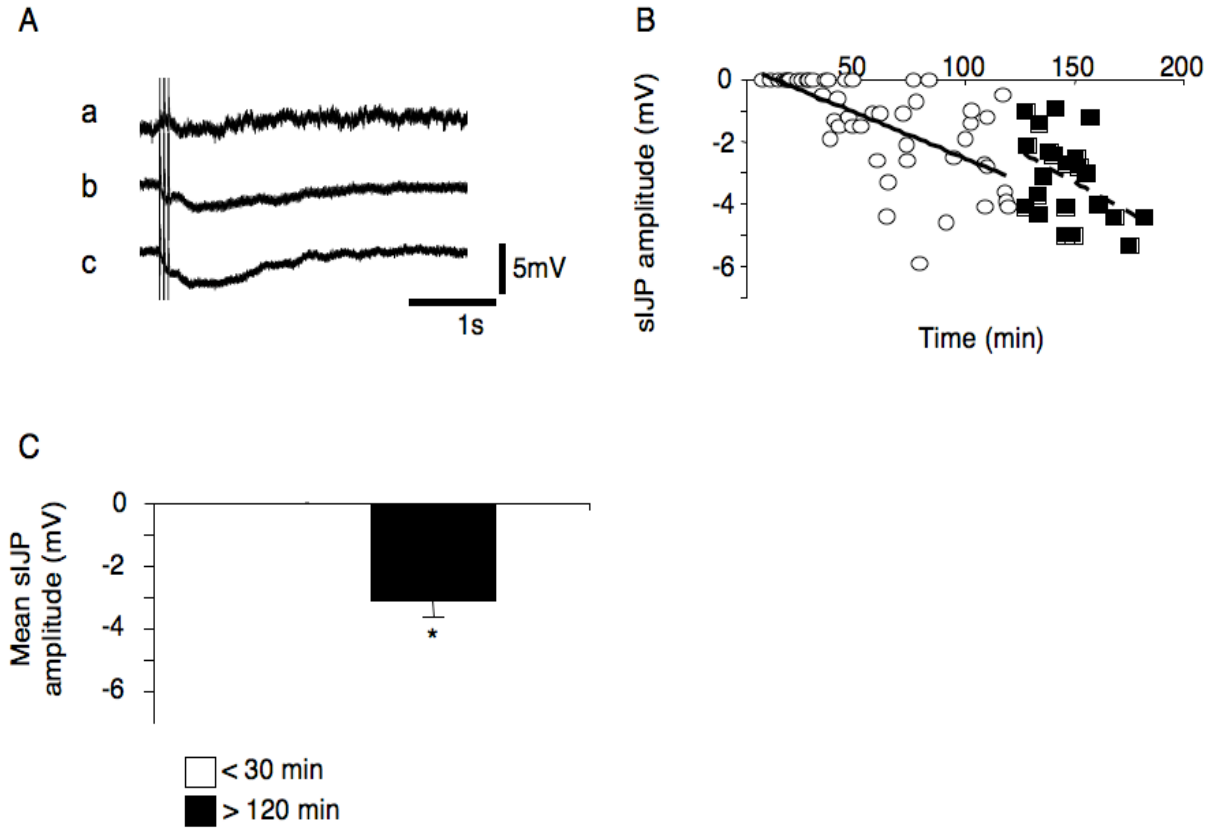

Figure 2.7 Slow IJPs increase in amplitude with time following dissection. A) Slow IJPs from cells impaled at t= 10.5 min (a) 64.5 min (b) and 145.6 min (c). B) Slow IJPs significantly increased in amplitude 120 minutes following dissection (white circles, $P<0.001$, $n=5$). Amplitudes continued to increase after this (black squares, $P<0.005$, $n=5$). C) Measurable sIJPs were not recorded within 30 minutes of dissection and amplitudes were significantly greater after 120 minutes (* $P<0.005$, $n=5$).

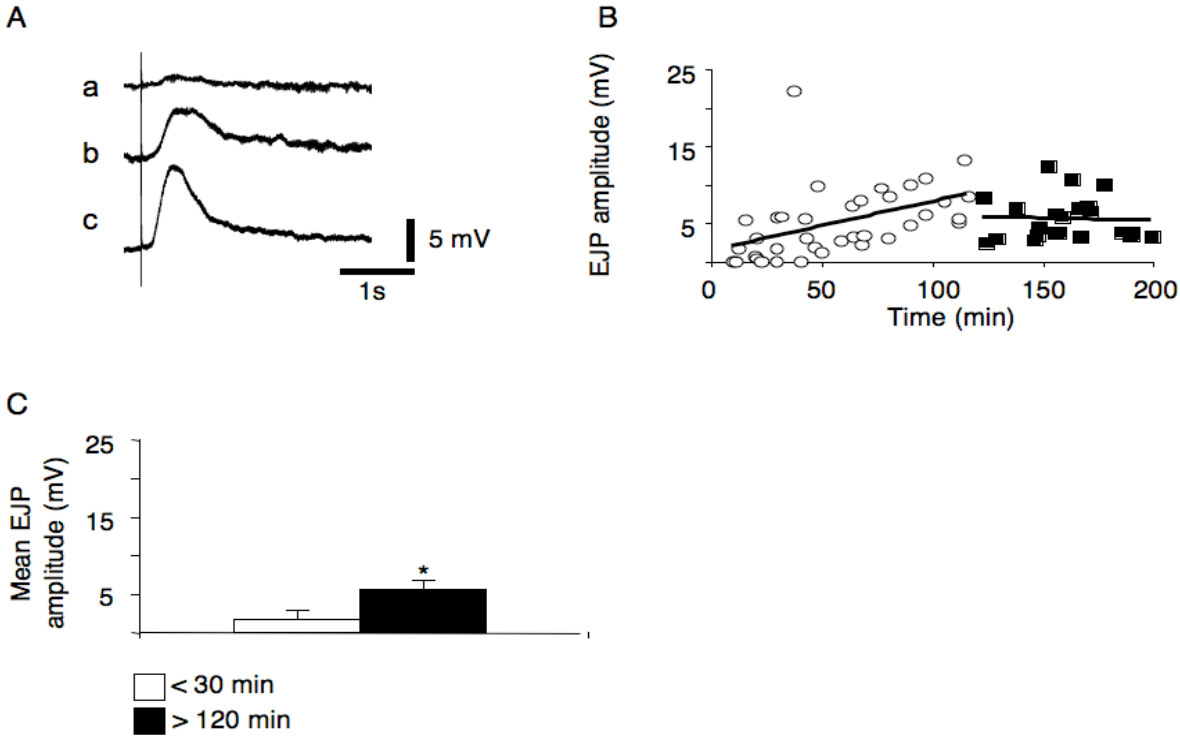

Figure 2.8 EJPs from colonic smooth muscle cells increase in amplitude with time following dissection. A) EJPs from cells impaled at t=12.0 min (a), 63.5 min (b) and 152 min(c). B) EJP amplitudes increased with time for cells impaled within 120 minutes following dissection (white circles, $P<0.005$, $n=5$). EJP amplitudes did not significantly change with time after this. C) Mean EJP amplitudes were significantly smaller in cells impaled in the first 30 minutes following dissection versus cells impaled after 120 minutes (* $P<0.05$, $n=5$.)

this increased to 5.6±1.2mV after 120 minutes (P<0.05, n=5, Fig 2.8C). EJP amplitudes showed a significant time-dependent increase over 120 minutes following dissection (regression analysis, P<0.005, n=5, Fig 2.8B) however there were no further increase after 120 minutes (Fig 2.8B).

2.3.6 Neurotransmission between enteric neurons was not blocked in the first 30 minutes

I tested directly whether neurotransmission was generally suppressed during the first 30 minutes by recording from nerve cell bodies in myenteric ganglia in the presence of nicardipine and hyoscine (both at 1μM). Six S neurons were recorded from 6 preparations within 30 minutes of superfusion with warmed Krebs solution. Mean RMP was -53.1mV±7.4mV. A single electrical stimulus (0.4ms duration) was applied 1mm circumferential to the impaled nerve cell body. Fast excitatory post synaptic potentials (EPSPs) were recorded in 5/6 cells with a mean amplitude (at a holding potential of -90mV) of 9.6±2.6mV (n=5); in the remaining cell the fast EPSP could not be measured due to superimposed action potentials. Injection of constant current to shift membrane potential was used to calculate the reversal potential of fast EPSPs, which ranged from 2-7mV between cells (average: 3.4±1.2mV) (Fig 2.9A). Carboxyfluorescein dye injection revealed that 6/6 cells had Dogiel type I morphology (Fig 2.9B). These results demonstrate that fast cholinergic neurotransmission between enteric neurons is not blocked during the first 30 minutes of recording.

2.3.7 The recovery of spontaneous contractions following dissection

Experiments were first trialled in the guinea pig ileum. Spontaneous contractions in this tissue were typically of very low amplitude and consistent recordings were very difficult to achieve (n=12).

In specimens of guinea pig distal colon, basal tone of preparations with intact mucosa significantly increased during the first 120 minutes (regression analysis, P<0.001, Fig 2.10A, 2.11A).

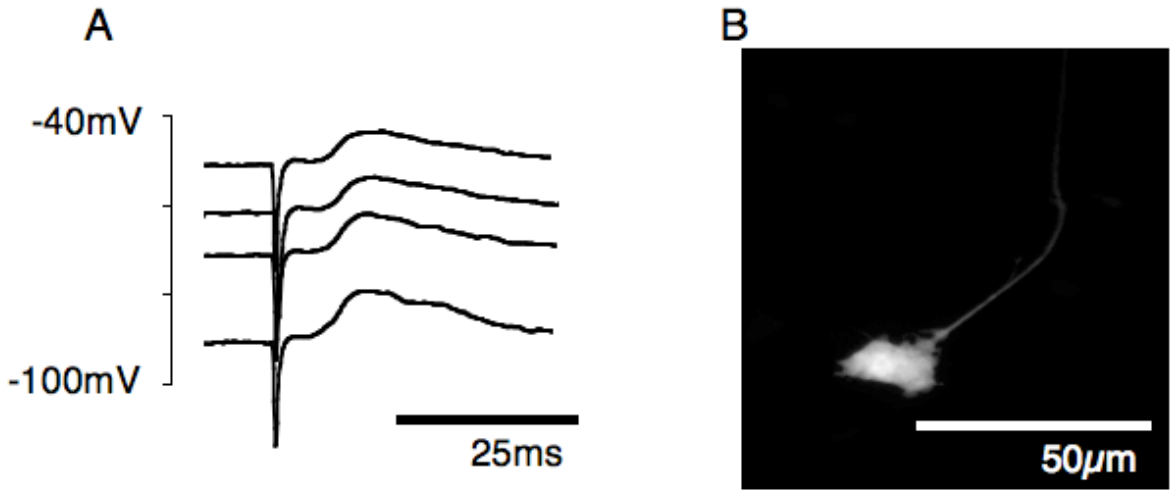

Figure 2.9 Fast excitatory postsynaptic potentials were recorded in *S* neurons impaled within 30 minutes of dissection. A) fESPs were recorded in neurons over a range of membrane potentials. B) Carboxyfluorescein was injected into the impaled cell and visualized *in situ.* 6 of 6 Cells had Dogiel type I morphology.

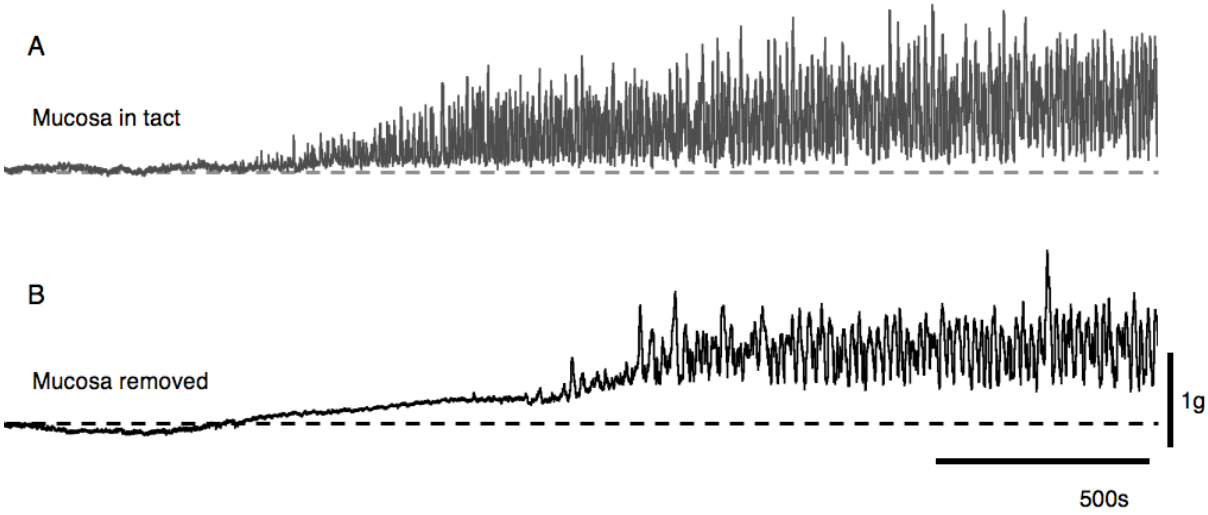

Figure 2.10 Basal tone increase and spontaneous contraction develop as preparations recover from dissection in preparations of colon. A) The recovery of contractions in colon specimen with the mucosa intact. B) The recovery of preparation of colon in a separate segment of colon from the same specimen from which mucosa and submucosa had been removed by sharp dissection.

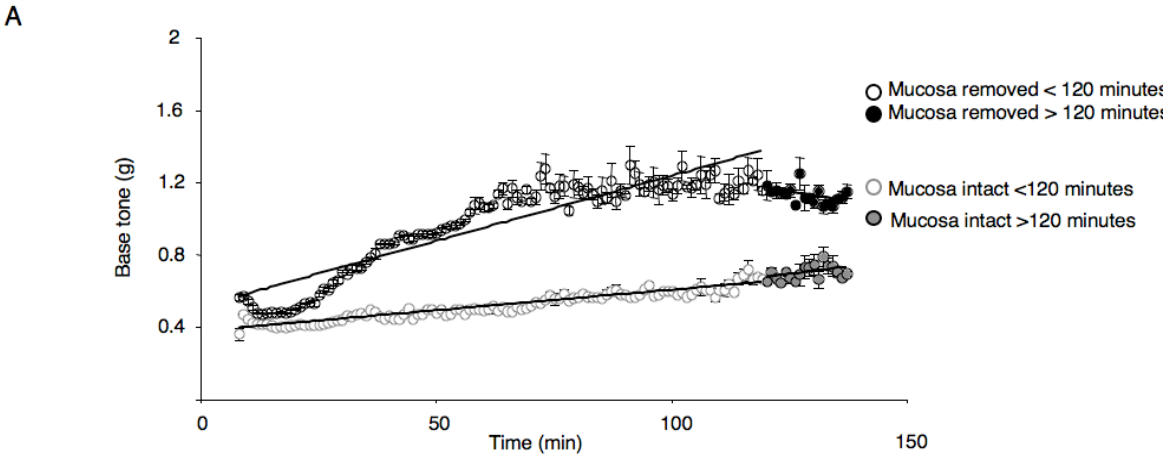

Figure 2.11 Basal tone increased time dependently with recovery from dissection. A) Scatter plot of mean basal tone for data binned in one minute intervals. For preparations with the mucosa intact, basal tone significantly increased during the first 120 minutes after dissection (grey circles, $P<0.001$, $n=4$) then did not change further. Basal tone also significantly increased in the first 120 minutes after dissection in preparations without the mucosa, but did not significantly change after this (black circles, $n=4$, $P<0.001$).

In preparations without the mucosa, tone also significantly increased over the first 120 minutes (P<0.001, n=4) but did not significantly change after this (Fig 2.10B, 2.11A). The maximal basal tone reached after 120 minutes did not differ between preparations with mucosa (0.7±0.2g) compared to preparations without mucosa (1.2±0.3g) (n=4). These results suggest that a period of recovery occurred in both preparations with and without mucosa, but was more marked in the latter.

In preparations with the mucosa attached, the amplitude of spontaneous contractions increased time dependently during the first 120 minutes following dissection (P<0.001, n=4, Fig 2.12A) and amplitudes decreased after this. The amplitude of contractions in preparations without the mucosa also increased significantly during the first 120 minutes (P<0.001, n=4, Fig 2.12B). The mean amplitude of contractions after 120 minutes of equilibration was not different between preparations with and without mucosa (0.6±0.2g versus 1.0±0.3g, n=4).

The area under the curve (AUC), for one minute time increments, increased significantly during the first 120 minutes after dissection in preparations with and without the mucosa (P<0.001, n=4, Fig 2.13A). AUC continued to increase after 120 minutes for preparations with mucosa intact (P<0.005, n=4) but not for those without the mucosa (n=4). Mean AUC was not significantly different at 120 minutes for preparations with (1.5±0.7gs) or without the mucosa (6.0±2.5gs).

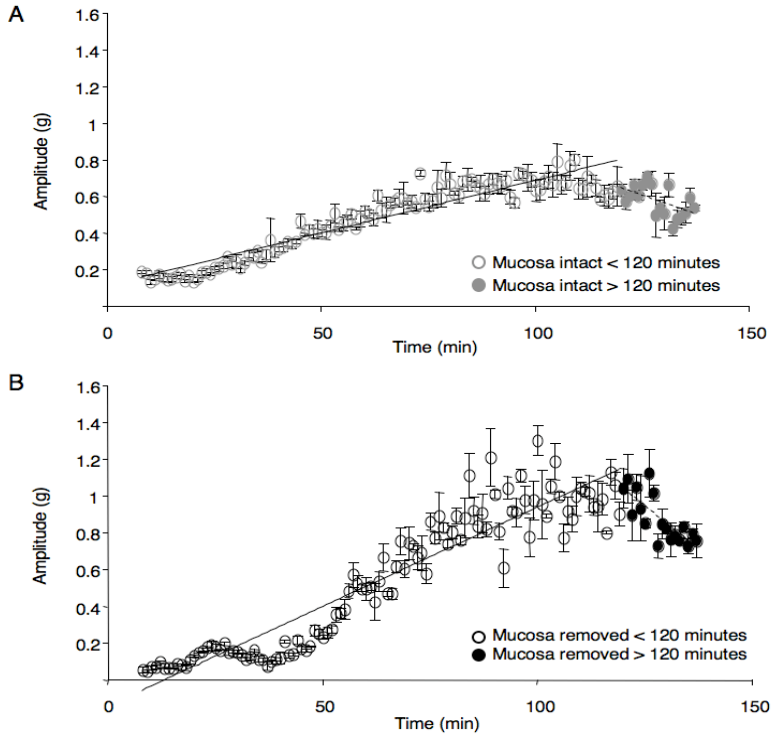

Figure 2.12 The amplitude of spontaneous contractions increased as preparations recovered from dissection. A) For preparations without the mucosa, spontaneous contractions time-dependently increased 120 minutes after dissection ($P<0.001$, $n=4$). After 120 minutes they then decreased ($P<0.05$, $n=4$). B) The amplitude of spontaneous contractions significantly increased 120 minutes after dissection ($P<0.0001$, $n=4$) and decreased with time after this ($P<0.005$, $n=4$).

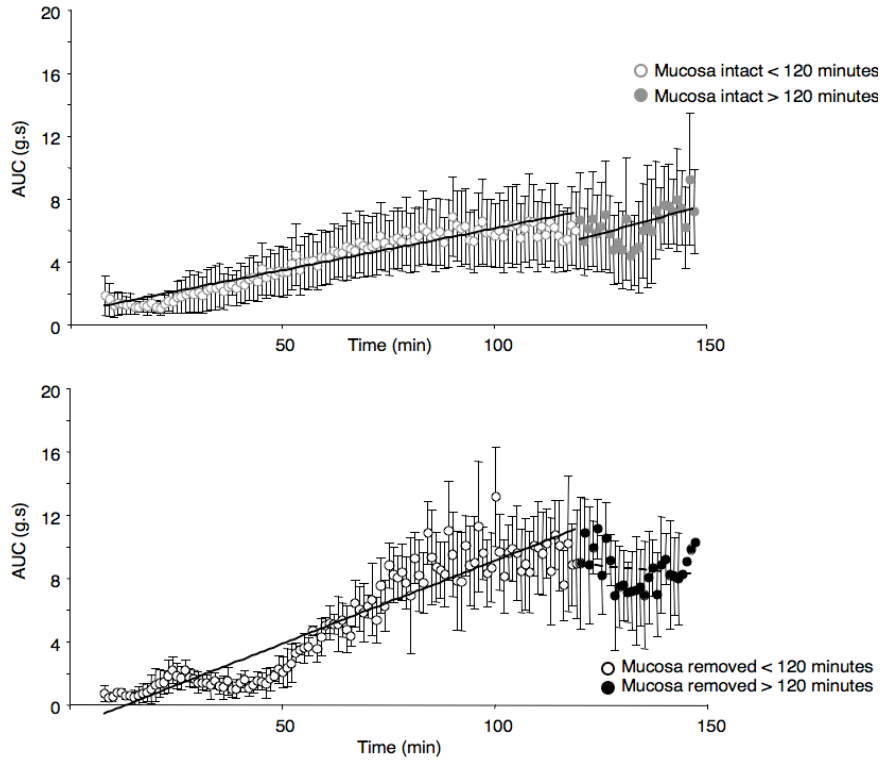

Figure 2.13 AUC (per minute interval) increased as preparations recovered from dissection. A) For preparations with the mucosa attached, spontaneous contractions time-dependently increased in the 120 minutes after dissection (P<0.0001, n=4); this trend continued after 120 minutes (P<0.005, n=4). B) For preparations without the mucosa AUC significantly increased during the first 120 minutes after dissection (P<0.0001, n=4) but did not change significantly after 120 minutes (n=4).

2.4 Discussion

2.4.1 Post dissection 'unresponsiveness'

When preparations of circular smooth muscle from the guinea pig ileum and colon were mounted in a recording chamber, they consistently lacked fast and slow inhibitory junction potentials and excitatory junction potentials, for at least the first 30 minutes. Over the first 120 minutes, responses increased, then remained stable. Since this phenomenon occurred in both the ileum and colon this suggests that this loss of responsiveness is not limited to a particular organ and is likely to occur in other smooth muscle preparations. For colon preparations without the mucosa, this period of unresponsiveness was reflected in negligible spontaneous contractions compared to contractions measured after 120 minutes. It appears that the process of setting up the preparation caused a temporary and reversible loss of neuromuscular transmission and muscle activity. This suppression coincided with an initial loss of gap-junction coupling between smooth muscle cells.

Recovery following dissection varied greatly in time course: in some preparations, full amplitude junction potentials were reached within 40 minutes; in others it took over 120 minutes. However, each preparation showed a similar pattern of recovery: over time cells depolarised from an average resting membrane potential of -59 mV to a stable level of -47mV, apparent input resistance decreased, junction potential amplitudes increased and dye coupling increased significantly. It is not clear whether these four changes are independent or whether some may share common mechanisms. For example, as cells depolarise, the amplitude of the fIJPs would be expected to increase, due to the larger electromotive driving force. However, the reversal potential for fIJPs in the guinea pig ileum is close to -90mV (Bywater et al., 1981). Thus a -16mV IJP measured at a resting membrane potential of -47mV would be expected to decrease to about -12mV in amplitude at a membrane potential of -59mV. This does not come close to accounting for the fIJP amplitude of less than 1mV during the initial equilibration period. The hyperpolarised resting membrane potential can not account for the diminished sIJP and EJP, since the reversal potentials for these has been shown to

be -25mV in the guinea pig ileum (Crist et al., 1991, Bauer and Kuriyama, 1982a). The EJP is mediated by Na^+ ions, which are higher in concentration in the extracellular solution. Since the reversal potential for EJPs is -25mV, at more negative RMP there is a greater driving force on Na^+. Hence, the amplitude of the EJP would be expected to be larger at more negative RMP than smaller. The sIJP is mediated by Cl^- ions, however unlike the previous examples, reduced Cl^- conductance increases the amplitude of the sIJP. The amplitude of the sIJP increases at more negative RMP than -25mV (Crist et al., 1991).

2.4.2 Loss of gap junction coupling

One of the main findings of this study was a major reduction in dye-coupling between circular smooth muscle cells in the first 30 minutes after setting up a preparation for intracellular recording. One would expect then that electrical coupling between smooth muscle cells should be similarly suppressed. This was supported by our observation that apparent input resistance fell significantly over the corresponding period, consistent with resumption of electrical coupling. Generally, in electrophysiology studies input resistance is measured in individual cells and used as a reflection of ion channels opening in that cell. Current injected into a cell leaks out via open channels. Input resistance is calculated from Ohm's law where resistance (R) is inversely proportional to the number of open ion channels, hence V=IR. In my preparation, gap junction coupling between smooth muscle cells form low resistance pathways, therefore current injected into the impaled cell flows into the coupled cells and can exit via open channels on these cells. Input resistance is very low in well coupled cells compared to uncoupled cells. Thus, in my experiments input resistance largely reflects electrical coupling rather than just specific membrane resistance in the uncoupled cell. It should be noted that the absolute values of input resistance were much smaller than those recorded from single isolated smooth muscle cells using patch-clamp techniques (Langton et al., 1989); nevertheless, changes in input resistance were significant and in the anticipated direction. Using the same dye and current-injection protocols in longitudinal muscle, significantly less dye coupling was seen and input resistance (in fully recovered preparations) was significantly higher than for circular muscle cells. This is compatible with previous reports that immunohistochemically characterised connexins and

ultrastructurally identified gap junctions are less abundant in longitudinal than circular smooth muscle (Daniel and Wang, 1999, Gabella and Blundell, 1981, Mikkelsen et al., 1993).

2.4.3 Reduction of neuromuscular transmission

This study has shown that there is an apparent suppression of neurotransmission between the enteric motor neurons and the smooth muscle cells during the period when dye coupling is reduced. In the presence of 1μM hyoscine, electrical stimulation normally evokes prominent fIJPs in circular smooth muscle cells (Bülbring and Tomita, 1967) which include a fast component mediated by a purine (Crist et al., 1992). Depending on the frequency of stimulation, a slower IJP mediated by NO may also be present, and in some preparations an additional peptidergic component, mediated by VIP or related peptide, can be recorded (He and Goyal, 1993, Lyster et al., 1992). In the presence of 0.25μM apamin without hyoscine, electrical stimulation can evoke an excitatory junction potential mediated by either acetylcholine acting on muscarinic receptors (Campbell, 1966, Cousins et al., 1993) or by a non-cholinergic mediator such as a tachykinin (Bauer and Kuriyama, 1982a, Bywater et al., 1981, Bywater and Taylor, 1983). In this study, I found that fast and slow IJPs and EJPs were all profoundly depressed in the first 30 minutes. In many cells, junction potentials were not detectable at all during this period. The observations that action potentials and fast excitatory post-synaptic potentials were robustly present in enteric neurons during the first 30 minutes, discounts the possibility of a generalised impairment of neuronal function during the period when neuromuscular transmission was suppressed. To confirm that a disruption at the post-junctional site accounts for the impaired junction potentials, the responses of the impaled smooth muscle cells to exogenous ATP, sodium nitroprusside (SNP, NO donor) and bethanechol (muscarinic recpetor agonist) were tested (see the next chapter). Such a disruption at the post-junctional site may include a loss of coupling and if this interpretation of the finding is correct, application of gap junction blockers would be predicted to result in diminished junction potentials (see the next chapter.)

2.4.4 Resting membrane potential

The resting membrane potential of circular smooth muscle cells in both the ileum and colon were significantly hyperpolarised in the first 30 minutes of recording. The resting membrane potential of circular smooth muscle varies through the thickenss of the circular muscle layer. In the gastric antrum and the ileum of the dog (Bauer et al., 1985, Bauer and Sanders, 1985), cat (Hara et al., 1986, Suzuki et al., 1986), mouse and human (Sha et al., 2007), cells near the myenteric border are more hyperpolarised than cells at the submucosal border. Heme-oxygenase in ICC-MY has been suggested to produce CO which hyperpolarises the smooth muscle cells close to the myenteric border (Sha et al., 2007). However in the colon of the dog (Smith et al., 1987a, Liu and Huizinga, 1993) and mouse (Sha et al., 2010) the RMP of circular muscle cells at the submucosal border is hyperpolarised compared the myenteric border. This gradient is sensitive to TTX and abolished by removal of the mucosa and submucosa. Production of CO from submucosal neurons has been suggested to hyperpolarise muscle cells in this region (Sha et al., 2010). Whether such a gradient exists in the circular muscle of the guinea pig ileum or colon is uncertain. In my experiments, I could not determine the depth of the impaled circular smooth muscle cell accurately in this relatively thin layer of tissue however it is likely that I mostly recorded cells towards the submucosal surface of the preparation. If so, blocking gap junctions could cause hyperpolarisation if distant cells with a depolarising influence became uncoupled.

2.4.5 Spontaneous contractions

In segments of colon, the amplitude of spontaneous contractions significantly increased in a time dependent manner as preparations recovered from dissection. It is worth noting that the amplitude of contractions recovered more rapidly in preparations where the mucosa was kept intact, compared to preparations where the mucosa was removed. The amplitude of contractions and area under the curve were not significantly different between mucosa intact and mucosa removed once these preparations had recovered. In chapter 4 I investigated which aspects of dissection contributed to the loss of responsiveness of smooth muscle cells, the effects of removing mucosa were considered. For preparations where the mucosa was removed, the amplitude and area under curve of spontaneous contractions was significantly lower within 30 minutes of

dissection versus after 120 minutes (parallelling changes in fIJP amplitude). Smooth muscle cells contract when the membrane potential of that cell depolarises beyond a mechanical threshold. In the GI tract this is brought about by the generation of slow waves by ICC-MY and ICC-SM in the colon (Hulzinga et al., 1995, Dickens et al., 1999, Ward et al., 1994). ICCs coupled to smooth muscle cells via gap junctions (Faussone-Pellegrini and Cortesini, 1985, Gabella and Blundell, 1981). Uncoupling could account for the absence of spontaneous contractions in the first 30 minutes as it would disrupt slow wave propagation from the ICC to the smooth muscles. Basal tone increased after initially setting up preparations; this may be a reflection of the membrane depolarisation that occurs concurrently.

2.5 Conclusions

Reponses of circular smooth muscle cells in the guinea pig ileum and colon to excitatory and inhibitory neurotransmission are suppressed within the first 30 minutes following dissection. Within 120 minutes, post-junctional potentials increase in amplitude and then stabilise. The development of junction potentials is associated with a slight depolarisation of RMP, decreased input resistance and a reduction in dye coupling. This loss of responsiveness is also reflected in decreased spontaneous contractions and tone, which recover over the same time-course. I hypothesise that changes in gap junction coupling may underlie that apparent suppression of post-junctional potentials. This was pharmacologically tested in the next chapter.