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**Ascending excitatory neural pathways modulate slow phasic myogenic contractions in the isolated human colon**

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**Running Head:** Modulation of myogenic contraction in human colon

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## Abstract

**Background:** In animal models, enteric reflex pathways have potent effects on motor activity; their roles have been much less extensively studied in human gut. **Aim:** to determine if ascending excitatory interneuronal pathways can modulate spontaneous phasic contractions in isolated preparations of human colonic circular muscle. **Methods:** Human colonic preparations were cut into T shapes, with vertical bar of the “T” pharmacologically isolated. Electrical stimulation and the nicotinic agonist, DMPP, were applied to the isolated region and circular muscle contractile activity was measured from the cross-bar of the T, more than 10mm orally from the region of stimulation. **Key results:** The predominant form of spontaneous muscle activity consisted of tetrodotoxin-resistant, large amplitude, slow phasic contractions (SPCs), occurring at average intervals of  $124\pm 68$  s. Addition of a high concentration of hexamethonium (1mM) to the superfusing solution significantly increased the interval between SPCs to  $278.1\pm 138.3$ s ( $P<0.005$ ). Focal electrical stimulation more than 10mm aboral to the muscle recording site advanced the onset of the next SPC, and this effect persisted in hexamethonium. However, the effect of electrical stimulation was blocked by tetrodotoxin (TTX,  $1\mu\text{M}$ ). Application of the nicotinic agonist DMPP (1mM) to the aboral chamber often stimulated a premature SPC ( $n=4$ ). **Conclusion & Inferences;** The major form of spontaneous contractility in preparations of human colonic circular muscle are SPCs which are myogenic in origin. Activation of ascending excitatory neural pathways, which involve nicotinic receptors, can modulate the timing of SPCs and thus influence human colonic motility.

## INTRODUCTION

The enteric nervous system is important for many functions of the gastrointestinal tract in mammals. Whilst laboratory animals have long been used to study the enteric neural circuitry in the gut wall, investigations of the functional enteric neural pathways in the human gastrointestinal tract have been less extensive. In the human colon, enteric motoneurons innervating the circular muscle project less than 10mm orally<sup>1</sup>. Retrograde labelling combined with immunohistochemistry showed that ascending motor neurons are likely to be excitatory since they are immunoreactive for excitatory markers choline acetyltransferase (ChAT) and tachykinins (TK). Ascending interneurons have also been characterised in human colon; these are also immunoreactive for ChAT and tachykinins.<sup>2,3</sup> These anatomical studies, suggest that acetylcholine and tachykinins are likely to be neurotransmitters for both neuronal and excitatory neuromuscular transmission in the ascending pathways. On the other hand, aborally projecting motor neurons are probably inhibitory since they are immunoreactive for inhibitory markers such as nitric oxide synthase (NOS) and vasoactive intestinal peptide (VIP)<sup>2,3</sup>. Electrical field stimulation, pharmacological agents and physiological stimuli have all been used to investigate the contribution of neurons to colonic contractility<sup>4-7</sup>. However, we have much less functional understanding about how these anatomically-polarised pathways contribute to the control of contractility in the human gut.

In the colon of most mammals, including humans, two separate pacemaker regions have been identified, which give rise to distinctive components of myogenic contractility<sup>6,8,9</sup>. *In vitro* recordings from small segments of human colon have demonstrated high frequency contractions (8-30 cycles per minute) and intermediate frequency contractions (2-4 cpm), driven by pacemaker networks located at the myenteric and submucosal borders respectively.

They are thought to be initiated by two populations of interstitial cells of Cajal (ICC) in these regions<sup>6, 10, 11</sup>. A third type of spontaneous slower phasic contraction has also been reported, but has received considerably less attention. It occurs at less than 1 cycle per minute in colon from humans and other mammals<sup>6, 8, 9</sup>. In some cases, these slow contractions appear to be myogenic, since they persist in the presence of tetrodotoxin<sup>10</sup>.

In the present study, Slow Phasic contractions (SPCc) were the predominant form of contractility in circular muscle of isolated specimens of human colon. Our aim, therefore, was to determine if activation of enteric neural pathways could modulate SPCs. To preserve the integrity of ascending interneuronal pathways, larger specimens of human colon were used, compared to most previous studies<sup>10-12</sup>.

## **MATERIALS AND METHODS**

Specimens of human colon were obtained, with prior written informed consent from 12 patients (6 male:6 female; mean age  $63.4 \pm 13.3$  years) undergoing partial colectomy for the removal of cancer in the Flinders Medical Centre (approved by the Flinders Clinical Research Ethics Committee now the Southern Adelaide Clinical Research Ethics Committee).

Four of 12 patients were administered Celecoxib, a cyclooxygenase inhibitor, prior to surgery, as part of another study,<sup>13</sup>. Patients who were part of this study were administered either placebo, celecoxib (100 mg) or diclofenac (50 mg) one to two hours prior to surgery.

Unpublished data from a separate series of experiments conducted in our laboratory compared the contractility of tissue obtained from patients with or without Celecoxib and revealed no significant differences in the mean frequency or amplitude of slow phasic or intermediary contractions. For this reason, data from

Immediately following removal of the section of colon, a specimen was removed from the proximal or distal regions of tissue unaffected by the tumour and placed in oxygenated Krebs's solution, at room temperature. Krebs solution contained (in mM:) NaCl 118; KCl 4.70,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  1;  $\text{NaHCO}_3$  25;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  1.2; D-Glucose 11;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  2.5; bubbled with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Preparations were cut open along the longitudinal axis. Fat, mesentery, mucosa and most of the submucosa were removed by sharp dissection leaving the longitudinal and circular muscle layers, and the myenteric plexus intact. Taenia coli were included in some of the specimens, but care was taken to ensure that these were not located at the recording site or the stimulation site.

### **Preparations of colonic strips of different sizes.**

We investigated the effect of the size of muscle strips on the spontaneous contractions. For this, rectangular preparations were set up: (circumferential axis x longitudinal axis) 10x2mm, 20x4mm, 40x8mm and 80x15mm. Arrays of hooks were attached at either circumferential end of each specimen: one was connected to a force transducer via a cotton ligature, the other was fixed in position allowing circumferential contractions to be recorded, against a pre-load of 1-2g resting tension. Each specimen was placed into a 10ml water bath containing warmed Krebs solution ( $\sim 36^\circ\text{C}$ ) bubbled with 95% oxygen, 5% carbon dioxide. Tension recordings were digitised at 200Hz and stored via an A/D interface (MacLab 8SP, AD Instruments, Sydney, Australia) using Chart 6 software (ADInstruments, Australia).

Following an equilibration period of 1-2 hours<sup>5</sup>, control data was collected for 1 hour and the frequency of spontaneous contractions was analyzed. An observer, blinded to test condition and tissue size, analysed patterns of contractile activity. Contractions could be separated based on frequency, duration and amplitude. Dominant frequencies were obtained from power spectra generated from Fast Fourier transforms (FFT) of 600s epochs with 12,000 datapoints.

The FFT software was written in Matlab® (The MathWorks, Natick, Massachusetts, USA). When comparing the amplitude of contractions across preparations of different size, the amplitude of the contraction (measured in gramme-force (g)) was divided by cross sectional of the preparation ( $\text{mm}^2$ ).

### **Activation of ascending pathways**

Of the 12 specimens collected, 8 were dissected into 'T' segments; ascending colon ( $n=1$ ), splenic flexure ( $n=1$ ); descending colon ( $n=5$ ), sigmoid colon ( $n=1$ ). Preparations were cut into a "T" shape where the cross bar of the T was at the oral end of the preparation. Maximal circumferential length was ~80mm and maximal longitudinal length was ~20mm.

Preparations were pinned in Sylgard-lined chamber (Dow Corning, Midland, MI, USA) with the circular muscle layer facing uppermost. An array of hooks attached along the longitudinal axis of the oral segment was connected to a force transducer to measure circumferential isometric contractions. The aboral segment, the vertical bar of the "T", was pharmacologically isolated by a glass coverslip, sealed with a barrier of inert silicon grease (High Vacuum Silicon Grease, Ajax Chemicals, Sydney Australia). Both chambers were superfused with Krebs solution at  $36^{\circ}\text{C}$ . Preparations were left to equilibrate for 1-2 hours against a pre-load of 1-2g resting tension, before control data was collected for 1 hour.

Preparations were stimulated with electrical stimuli of 80V, 0.4ms duration, at 10Hz for 5s, via a Grass SD9 stimulator. Stimulation was applied on the isolated segment more than 10mm away from the recording site, to avoid direct activation of the excitatory enteric motor neurons. Since the cross sectional area of specimens used in these experiments did not differ, the amplitude of contractions did not need to be corrected. The relationship between electrical stimulation and contractions was investigated quantitatively. The delay between an electrical stimulus and the next SPC was measured. This was compared to the delay between a

randomly selected point and the next spontaneous contraction. Five randomly selected points were chosen by an 'observer,' who was unable to see the recording. At least three spontaneous SPCs were allowed to occur before the next bout of electrical stimulation, to minimise long-term interactions.

### **Drugs used**

1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP) was prepared in Krebs solution at 1mM prior to experiments. In control conditions, 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP, 1mM) was applied by pipette directly onto the pharmacologically isolated segment to activate nicotinic receptors on enteric neurons. This was performed once per experiment as the effectiveness of DMPP diminishes with repeated application. Hexamethonium and tetrodotoxin were stored in aqueous solution ( $10^{-2}$ M stock concentration made up in distilled water) and diluted in Krebs solution to the required concentration immediately before use. All drugs were purchased from Sigma Aldrich (Castle Hill, Australia). Antagonists were superfused for 30 minutes, spontaneous data was collected for an additional 30 minutes before electrical stimulation protocols were applied.

### **Data analysis**

Results are expressed as means  $\pm$  standard deviation. *n* refers to the number of patients. Statistical analysis was performed by: student's two-tailed *t*- test for paired samples using Microsoft Excel 2004; and either ANOVA or MANOVA using GraphPad Prism 4, software. One-way ANOVAs were used to analyse whether preparation size affected the frequency of slow events. MANOVA analysis was used to compare the frequency of slow events across preparations of all sizes in control versus TTX. One-way ANOVA was used to compare the intervals between spontaneous contractions before and after electrical stimulation, with



intervals between the evoked contraction and the next spontaneous contraction. One-way ANOVA and MANOVA were followed by Bonferroni post hoc tests.

## RESULTS

### Spontaneous contractions in muscle strips

Patterns of contraction of preparations ranging in size from 2 x 10mm to 15 x 80mm were compared in vitro (transverse colon  $n=2$ ; descending colon  $n=2$ ). Following equilibration, basal tension ranged from 2.5-5.0g. Low frequency, intermediate frequency and high frequency contractions were recorded in most preparations.. Fast Fourier transform (FFT) analysis demonstrated peaks within 3 frequency ranges: 0 -1cpm, 2-6 cpm and 10-25 cycles per minute (Fig 2). These corresponded to Slow Phasic contractions (SPCs), "intermediate phasic contractions" and "high frequency contractions" respectively (0.0-1.3, 1.5-4.0 and 7.0-25.0 cpm). Slow Phasic Contractions, with dominant FFT frequencies between 0-1 cycles per minute were prominent in 13/16 recordings and were typically the largest amplitude events, averaging  $44.2 \pm 30.5 \text{mg} \cdot \text{mm}^{-2}$  ( $n=4$ ) Intermediate phasic contractions, with FFT peaks between 2-6 cycles per minute were recorded in 13/16 recordings and typically had lower amplitudes ( $30.7 \pm 20.1 \text{mg} \cdot \text{mm}^{-2}$ ,  $n=4$ ). They have similar frequencies to the slow waves that have been recorded near the submucosal border in specimens of human colonic circular muscle, in vitro <sup>11</sup>. High frequency contractions, with corresponding FFT peaks at 10-25 cycles per minute were observed in 9/16 recordings and typically had the smallest amplitudes ( $8.9 \pm 6.8 \text{mg} \cdot \text{mm}^{-2}$ ,  $n=4$ ). These had similar frequency characteristics to the myenteric potential oscillations (MPOs) recorded at the myenteric border of human colonic circular muscle in vitro <sup>11</sup>. The frequency of all three types of contractions was similar across preparations ( $n=4$ ). While these three different frequencies of motor activity were present in

most of the preparations, SPCs were the dominant behaviour, with the largest amplitude. These were the focus of the rest of this study.

### **Spontaneous activity in 'T' segments of colon**

The resting tension of the circular muscle averaged  $2.7 \pm 1.9$  g ( $n=8$ ). SPCs occurred at a frequency of  $0.6 \pm 0.3$  cycles per minute (cpm), with mean interval between contractions of  $124.1 \pm 68.4$  s ( $n=6$ ). Hexamethonium (1mM) increased the mean interval to  $278.1 \pm 138.3$  s ( $P < 0.005$ ,  $n=8$ , Fig 2B), however the mean amplitude of SPC was not significantly affected (control:  $6.0 \pm 3.5$  g versus hexamethonium:  $6.5 \pm 4.1$  g,  $n=8$ , Fig 2C). Further addition of  $1 \mu$ M tetrodotoxin significantly affected neither the amplitude of contractions ( $5.3 \pm 2.9$  g,  $n=6$ ) nor the intervals between contractions ( $428.6 \pm 282.3$  s,  $n=6$ ). These results suggest that SPCs must be essentially myogenic in nature, and that their initiation, but not their amplitude, may be modulated by ongoing enteric neuronal activity.

### **Stimulation of ascending interneuronal pathways and its effects on SPCs**

Supramaximal transmural electrical nerve stimulation of the aboral segment of colon (0.4ms, 10Hz, 5s, 80V) was followed by a contraction that resembled a SPC in both amplitude and timecourse, in all preparations. The delay between the electrical stimulus and the peak of the following SPC was significantly shorter than the time between a randomly selected point and the peak of the following SPC ( $10.8 \pm 2.3$  s vs  $152.0 \pm 52.1$  s,  $P < 0.005$ ,  $n=6$ , Fig 3A) indicating that the electrical stimulus evoked a premature SPC. It is interesting to note that the amplitude of evoked SPCs did not significantly differ from spontaneous SPCs ( $4.7 \pm 4.2$  g versus  $6.1 \pm 3.5$  g,  $n=6$ , Fig 3B).

Electrical stimulation also re-set the intervals between slow myogenic spontaneous contractions. The mean interval between the pair of spontaneous contractions that preceded an electrical stimulus was  $200.6 \pm 109.8$  s ( $n=4$ , see: first spontaneous interval, Fig 4). This was significantly shorter than the interval between the spontaneous contraction preceding an electrical stimulus and the next spontaneous contraction (ie: when the intervening, electrically evoked contraction was ignored - which averaged  $329.8 \pm 188.1$  s, second spontaneous interval,  $n=4$ , Fig 4  $P < 0.05$ ). The interval between the pair of contractions preceding an electrical stimulus was not significantly different to the interval between the evoked contraction and the next spontaneous contraction ( $200.6 \pm 109.8$  s vs  $241.1 \pm 127.5$  s, evoked interval, Fig 4). Thus activation of ascending neural pathways resets the pacemaker system responsible for the spontaneous myogenic slow myogenic contractions.

#### **Inhibiting nicotinic pathways: effects on evoked contractions.**

To test the role of nicotinic pathways in the initiation of premature SPCs, a high concentration of hexamethonium (1mM) was added to both compartments of the organ bath. Electrically evoked premature SPCs persisted in hexamethonium (Fig 5A) and there was no detectable change in the time taken for the response to occur after the stimulus. As in control Krebs solution, the delay between the electrical stimulus and the peak of the following SPC was still significantly shorter (in the presence of hexamethonium) than the time between a random point and the next SPC ( $11.8 \pm 4.9$  versus  $206.8 \pm 138.8$  s,  $P < 0.05$ ).

The delay between the electrical stimulus and the contraction it evoked ( $11.8 \pm 4.9$  s) was similar in hexamethonium to that in control Krebs solution ( $10.8 \pm 2.3$  s). The amplitudes of evoked contractions were not significantly affected by the addition of hexamethonium ( $4.0 \pm 3.8$  g versus  $4.3 \pm 5.1$  g,  $n=5$ , Fig 5B). Thus, nicotinic transmission is not required for

nerve-dependent triggering of SPCs (Fig 5B). Since evoked contractions persisted in hexamethonium, we tested whether the initiation of the contraction was delayed by inhibiting nicotinic pathways. The time from the end of the train of electrical stimuli to 10% amplitude of the contraction was compared in control with hexamethonium. Application of hexamethonium did not significantly affect this latency ( $4.7 \pm 1.2$ s to  $3.8 \pm 0.9$ s Fig 5C). Application of the nicotinic agonists DMPP (1mM) to the aboral chamber typically evoked a contraction (Fig 6A) in the oral segment with a delay of  $26.3 \pm 4.7$ s. However, when compared with the time between random points and the peak of the following contraction ( $74.6 \pm 37.5$ s), this did not reach significance ( $P=0.07$ ,  $n=4$ , Fig 6B). These results suggests there are probably nicotinic receptors in ascending neural pathways, but that their contribution may be relatively small.

## DISCUSSION

The aim of the current study was to characterize the myogenic patterns of mechanical activity in the circular muscle layer of isolated segments of human colon and determine the functional role of ascending excitatory nerve pathways in the control of these motor patterns. One very striking finding was that the spontaneous contractility of circular muscle in vitro is dominated, not by slow wave-driven patterns, nor by myenteric potential oscillations, but by high amplitude, slow phasic contractions that typically occurred at intervals of 1- 4 minutes. Activation of ascending excitatory neural pathways evoked premature SPCs with very similar characteristics to spontaneous SPCs. Activation of a premature contraction also re-set the timing of subsequent SPCs. It is worth noting that the amplitude of the spontaneous and neurally-evoked contractions were very similar. This suggests that neural inputs sum with the

myogenic pacemaker to bring the system to threshold, and then endogenous mechanisms take over to produce the full mechanical event, and then reset the cycle.

### **Ascending neural pathways**

The ascending motoneurons innervating the circular muscle layer of the human colon have a maximal projection up the gut of 10mm<sup>1</sup>. Thus, electrical stimulation applied more than 10mm from the partition in the current study cannot have activated excitatory motor neurons directly. There are two possible explanations for the effects seen. The first is that electrical stimulation activated ascending interneurons, which form functional chains running up the gut<sup>14</sup>, which then excited excitatory motor neurons. The alternative is that electrical stimulation antidromically activated the axons of long descending interneurons, which had upstream collateral contacts onto excitatory circular muscle motor neurons. In studies combining retrograde labelling and immunohistochemistry in human colon, 23% of orally projecting interneurons were immunoreactive for the cholinergic marker, ChAT and for tachykinins<sup>3</sup>. It is possible that the effects of DMPP in the present study were due to the activation of these ascending interneuronal pathways. Tachykinins are responsible for some non-cholinergic neuron-to-neuron transmission in the human ileum<sup>15</sup> and are also involved in neuro-muscular transmission in human gut<sup>16,17</sup>. In addition, NK2-receptor-mediated excitation has been reported in human colon<sup>20</sup>. Hexamethonium-resistant colonic motor patterns have also been reported in some experimental animals<sup>18,19</sup>. We speculate that tachykinins may be involved in some of the hexamethonium-resistant effects of neural stimulation in the present study, but emphasise that this remains to be tested directly.

### **Permissive role of nerves and conditional pacemakers**

The current study suggests the existence of limited but specific interactions between neural and myogenic mechanisms. It has previously been reported that activation of cholinergic muscarinic receptors by carbachol initiates SPCs, via muscarinic receptors<sup>10</sup>. Thus, muscle and pacemaker cells were influenced by a continuously superfused cholinergic agonist to produce phasic activity, even in the presence of tetrodotoxin. This latter observation indicates that the phasic contractions are not due to burst firing by motor neurons, but must be an intrinsic property of the muscular apparatus. Enteric excitatory motor neurons are an endogenous source of acetylcholine in the circular muscle of the colon. We speculate that ongoing release of small amounts of acetylcholine from motor neuron axons tonically excites the pacemaker system for SPCs, increasing their frequency above the basal rate. This would explain why hexamethonium reduces SPC frequency. Thus motor neurons modulate the excitability of the pacemaker system but do not drive it. This is consistent with the long latency (>4s) of SPCs after electrical stimulation of ascending interneuronal pathways. The identity of the pacemaker cells in the colon, which are the target of endogenous acetylcholine, remains to be determined. It is worth noting that cholinergic agonists have been shown to increase the frequency of slow waves in the murine gastric antrum<sup>21, 22</sup>. In the rat colon, contractions occurring at similar frequencies to SPCs, termed "rhythmic propulsive motor complexes", were blocked by inhibiting neural activity and then restored following addition of carbachol<sup>12</sup>. In the present study, hexamethonium and tetrodotoxin did not affect the amplitude of SPCs. This suggests that once a threshold has been reached, SPCs are generated in an all-or-nothing fashion.

### **Mechanisms underlying slow phasic myogenic activity?**

Intracellular recordings from circular smooth muscle cells near the myenteric border of human colonic circular muscle have slow waves occurring at about 3 cpm, often with

superimposed action potentials. There is also a pattern of slower contractions, probably corresponding to SPCs, at 0.3 - 0.6 cpm, which often had superimposed smaller contractions at up to 18cpm. The faster small contractions appeared to be related to myogenic potential oscillations (MPOs) which occurred at 17-18 per minute and originated at the myenteric border<sup>11</sup>. Huizinga and Waterfall (1988) observed that during slow contractions, myenteric potential oscillations often had superimposed action potentials, which were largely absent in the quiescent phase<sup>10</sup>. Thus SPCs may be associated with cyclic changes in the ability of MPOs to trigger smooth muscle action potentials. Interstitial cells of Cajal at the myenteric border (ICC-MY) are the likely pacemakers for MPOs - but whether they also contribute to SPCs remains to be established.

In the canine colon, slow phasic contractility may be due to bursts of fast MPO oscillations with superimposed action potentials, which result in fused slow contractions<sup>23</sup>. The intermediate frequency slow waves (2-6 cpm) that originate in ICC-SMP at the submucosal border of the circular muscle, sometimes also contributed to slow contractions. These canine SPCs were not blocked by TTX, but were activated by carbachol and by endogenous acetylcholine in the presence of a cholinesterase blocker such as neostigmine.

It is unclear how far SPCs propagate along the colon. To date, very few experiments have been performed on intact long tubular preparations of human colon<sup>24, 25</sup>. In a recent study<sup>25</sup> slow spontaneous contractions with striking similarity to SPCs, were recorded at about 4 minute intervals in intact tubular specimens of human colon, *in vitro*. Some of these large slow contractions occurred almost simultaneously over a 40-50cm length of the preparation, while others exhibited different times of onset, suggesting propagation over several centimeters. Similar time-locked contractions occurred at the site of electrical stimulation and

these premature contractions reset the time of the subsequent slow contractions in 4 of 6 preparations<sup>25</sup>. Interestingly, manometric recordings of human colonic motility have not reported regular large events at 1-4 minute intervals in ascending, transverse or distal colon<sup>26</sup>. This suggests that this pattern of activity may be tonically suppressed *in vivo*; the mechanisms of this suppression are not clear.

## **Conclusions**

This work demonstrates that in the human colon, myogenic slow phasic contractions recorded *in vitro* can be modulated by ascending excitatory enteric motor nerve pathways. Activation of ascending excitatory pathways for short periods can reset the timing of SPCs. It is likely that ongoing release of the excitatory transmitter acetylcholine contributes to the repetitive cyclical activation of these contractions. The role of SPCs in colonic motility *in vivo* remains to be established as none of the motor patterns recorded so far in patients show convincingly similar features.

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**Competing Interests:** the authors have no competing interests



### **Authors' contributions**

Experiments were primarily designed by SEC, NJS, DAW and SJHB. DAW conducted the surgeries in the Flinders Medical Centre and Flinders Private Hospital, from which the tissue was sourced. Experiments were conducted by SEC at Flinders University, South Australia. All authors were involved in the analysis and interpretation of data. The paper was written by SEC with input from all authors. All authors approve the final manuscript.

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### **Figures**

**Figure 1:** Preparations of human colon displayed various spontaneous motor patterns including: A) high frequency contractions, occurring at about 20 per minute B) intermediate frequency contractions at about 3.8 per minute Ca) Regular slow phasic contractions (SPCs) occurring regularly at about 1 per minute Cb) SPCs occurring as part of a more complex, irregular activity

**Figure 2:** The frequency of SPCs was reduced in the presence of 1mM hexamethonium. A) A clear decrease in the frequency of large SPCs occurs within 1 minute of the addition of 1mM hexamethonium B&C) Mean interval between SPCs and mean amplitude of SPCs in control solution and 1mM hexamethonium showing a significant increase in interval ( \*  $P < 0.005$ ,  $n=8$ ) but non-significant change in amplitude.

**Figure 3:** Aboral electrical stimulation resulted in a premature slow phasic contraction (SPC) and reset the rhythm of SPCs. Aa) The time between the electrical stimulus (grey open arrow; "stim.") and the peak of the next contraction was compared to the time from a randomly

selected point (dashed line) and the peak of the following SPCs. Ab) The time of the contraction following an electrical stimulus was significantly shorter than that following a randomly selected point (\*  $P < 0.005$ ,  $n = 6$ ). Ba) The amplitude of the contraction following an electrical stimulus (grey open arrow) was compared to the amplitude of a SPC following a randomly selected point. Bb) There was no significant difference in the amplitude of contractions.

**Figure 4:** The interval between SPCs was reset by the evoked contraction. A) The interval between SPC preceding the electrical stimulus (1<sup>st</sup> spontaneous interval) was compared to the interval between the next pair of spontaneous contractions (2<sup>nd</sup> spontaneous interval) and the interval between the evoked contraction and the next spontaneous contraction. B) The 2<sup>nd</sup> spontaneous interval interrupted by the electrical stimulus was significantly longer than the 1<sup>st</sup> spontaneous interval ( $P < 0.05$ ,  $n = 4$ .)

**Figure 5:** Premature SPC evoked by electrical stimulation persisted in the presence of hexamethonium. A) Grey arrows ("stim.") indicate aboral electrical stimulation. Stimuli continued to trigger a premature contraction in the presence of 1mM hexamethonium. B) The amplitude of premature contractions was not significantly different in control solution and in hexamethonium ( $n = 5$ ). Ca) The time from the application of the stimulus (open arrow) and 10% amplitude of the contraction (dotted line) was compared to see if nicotinic mechanisms contributed to the rate of onset of evoked contractions. Cb) This was not significantly reduced in hexamethonium ( $n = 4$ ).

**Figure 6:** Pharmacological activation of ascending nicotinic pathways can generate an premature SPC orally. A) Application of 1mM DMPP (grey arrow) to the pharmacologically

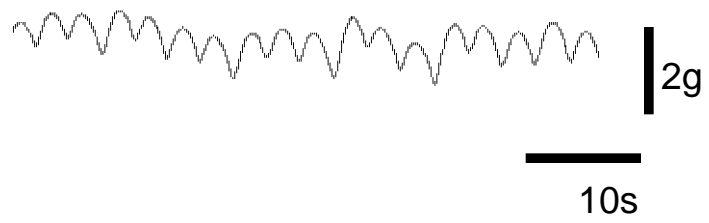
isolated aboral segment evoked a premature SPC with short latency. B) The mean time DMPP application and the peak of the next SPC was shorter than the delay between random points and the peak of the next SPC, however this was not significant with the small numbers available ( $n=4$ )

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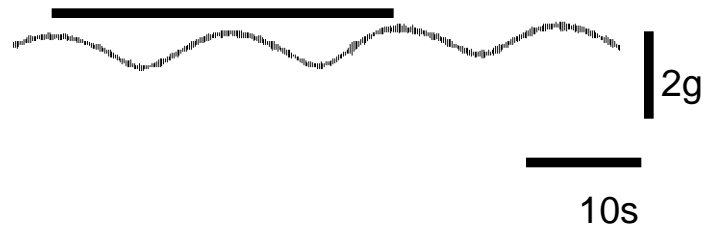
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A



B



C

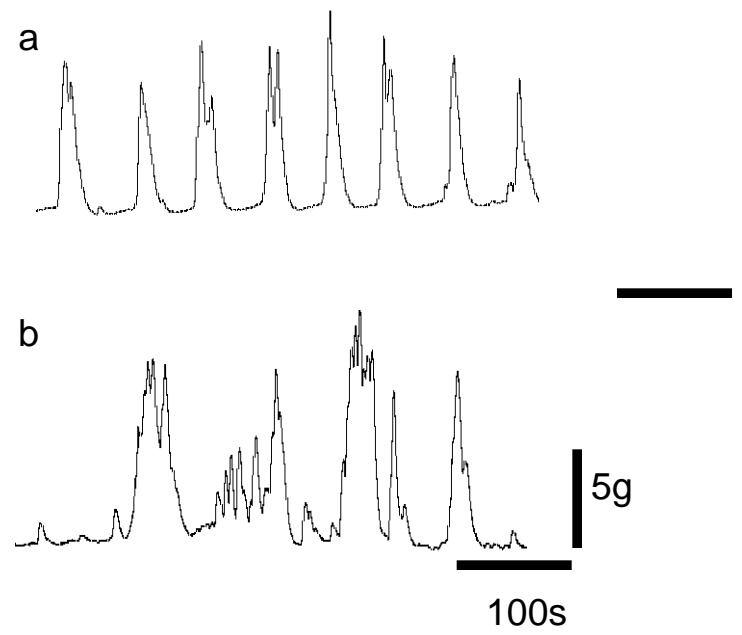


Fig 1

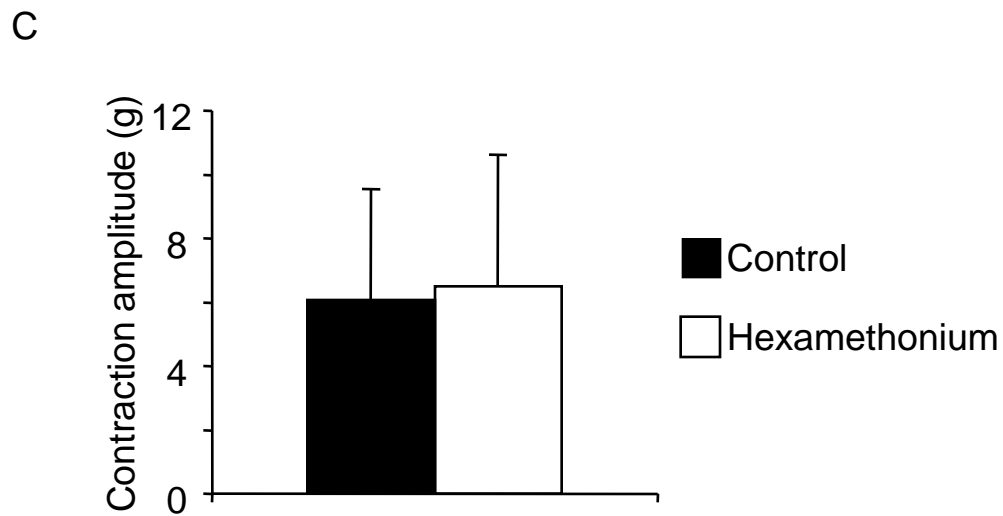
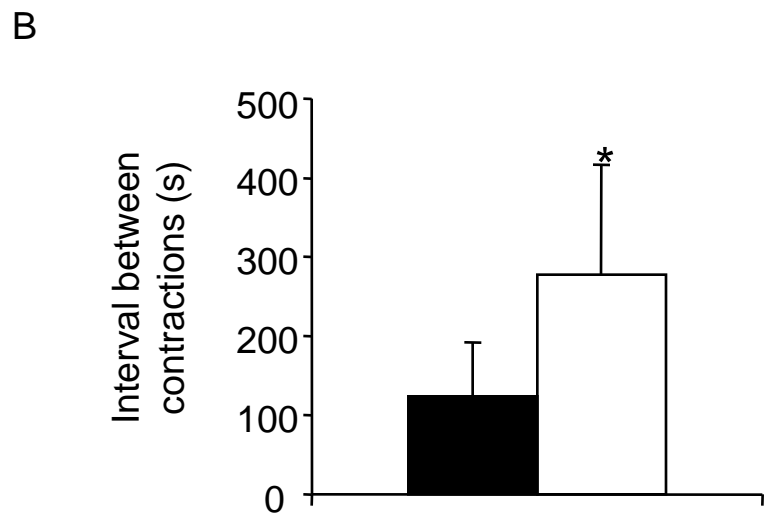
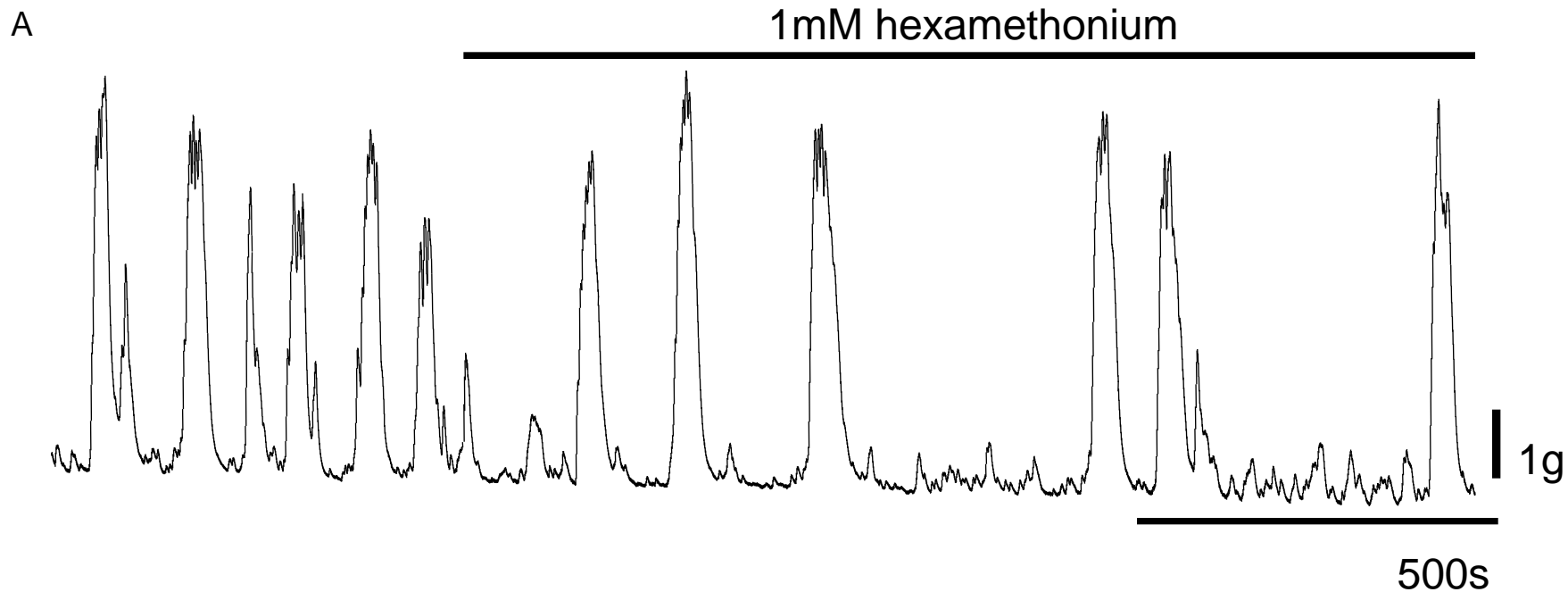
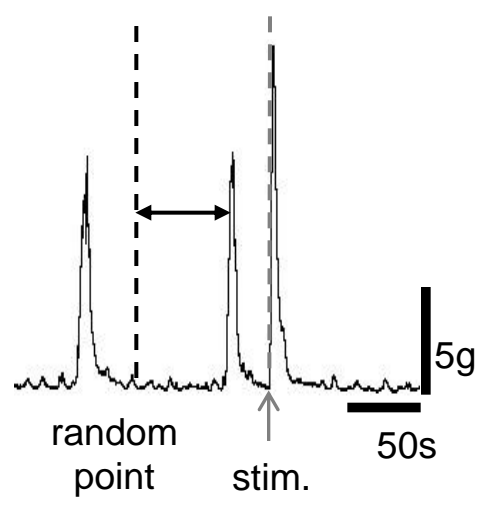


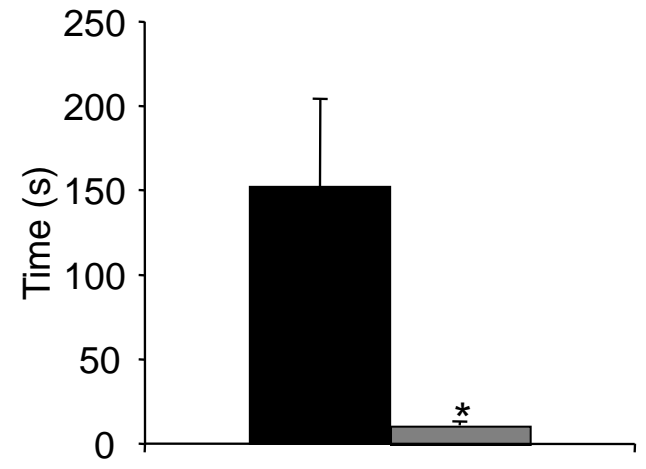
Fig 3

A

a

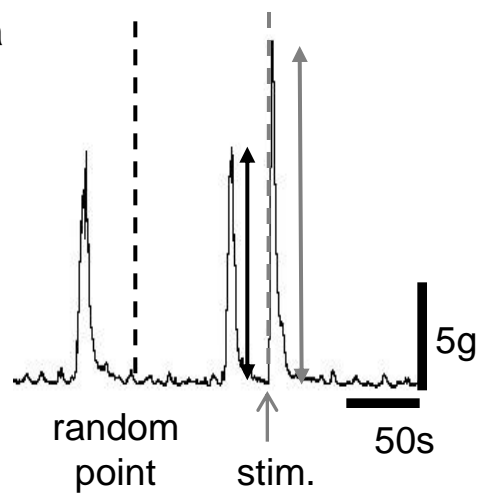


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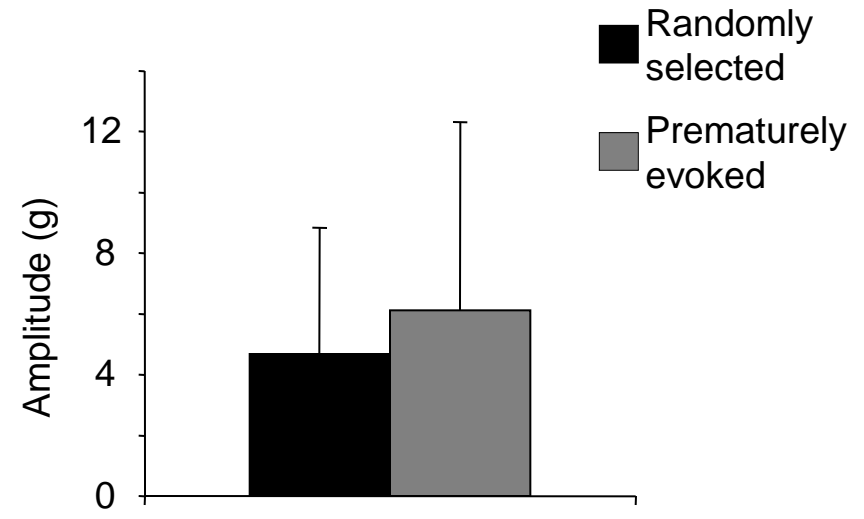


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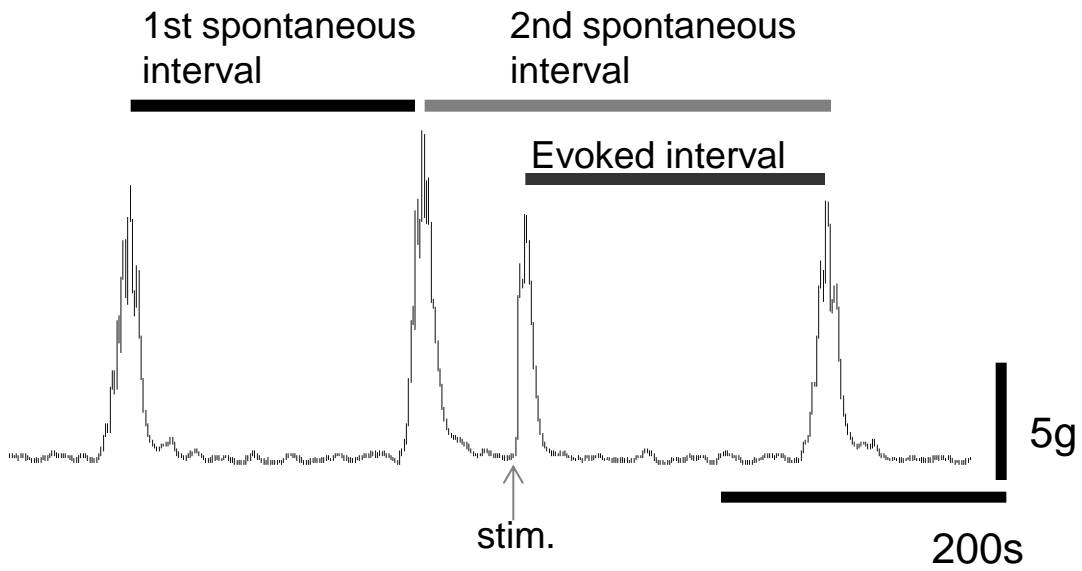


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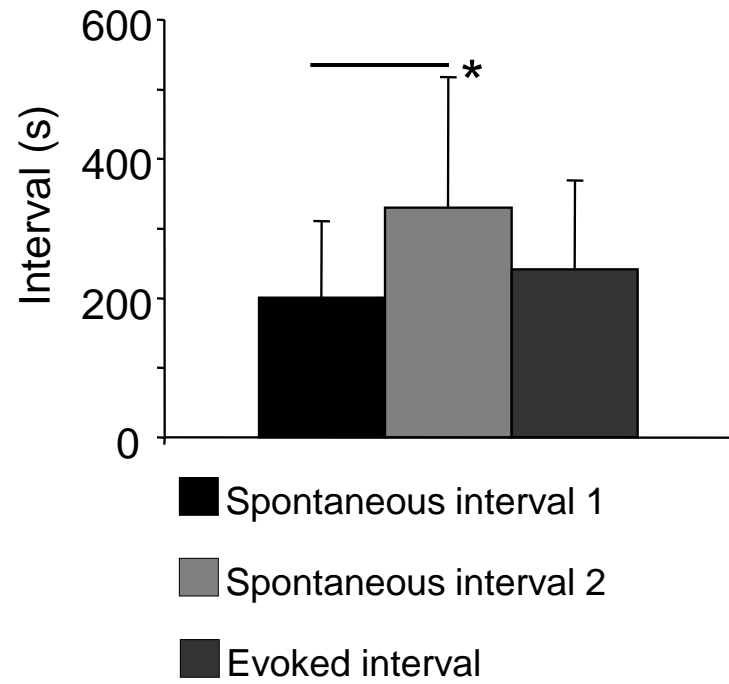
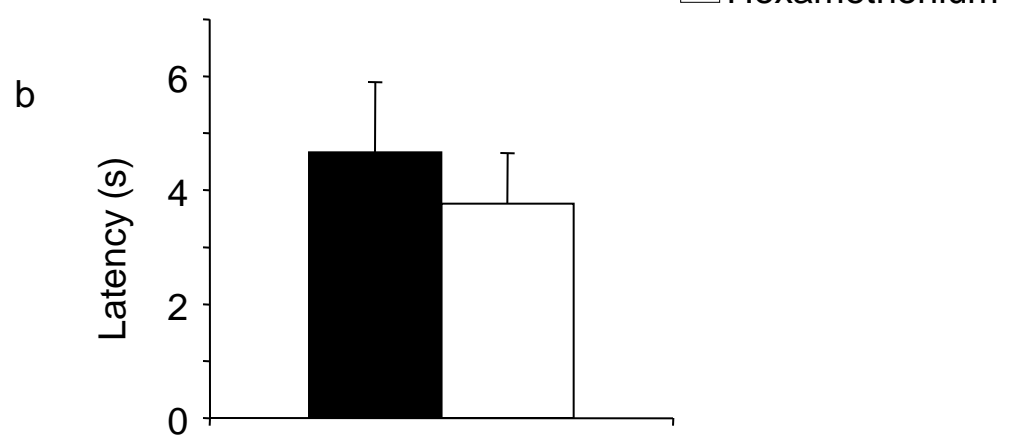
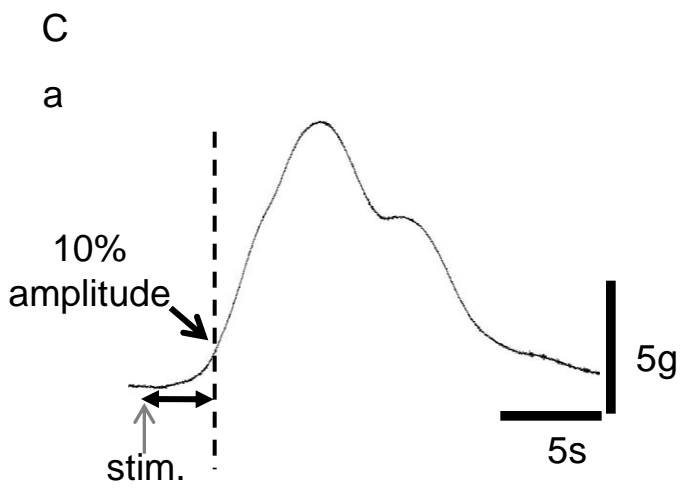
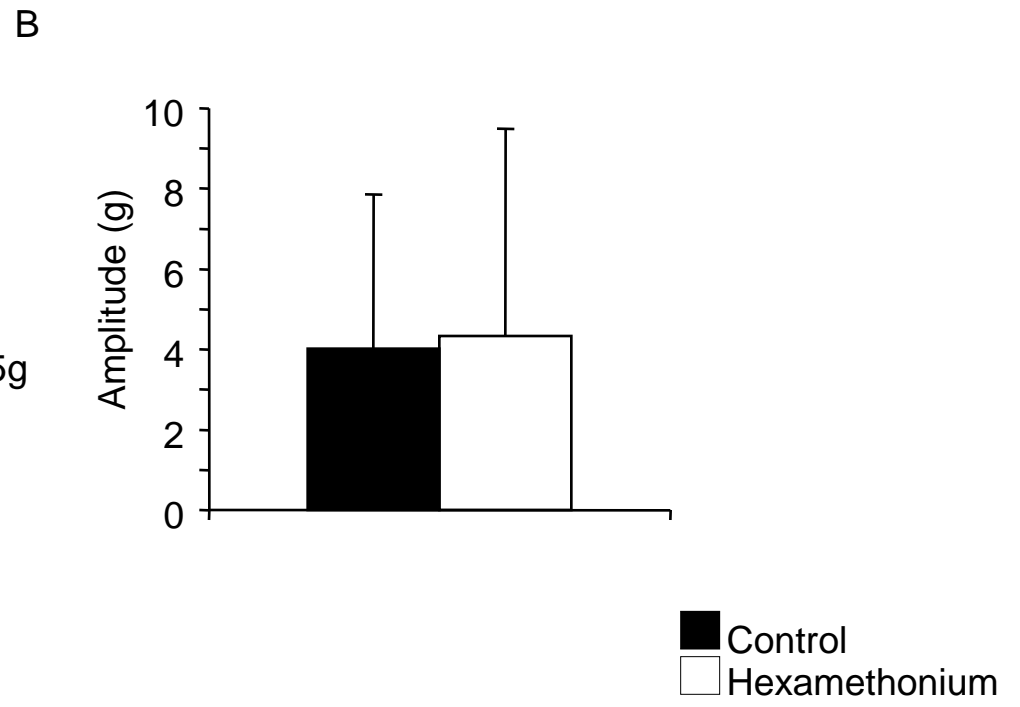
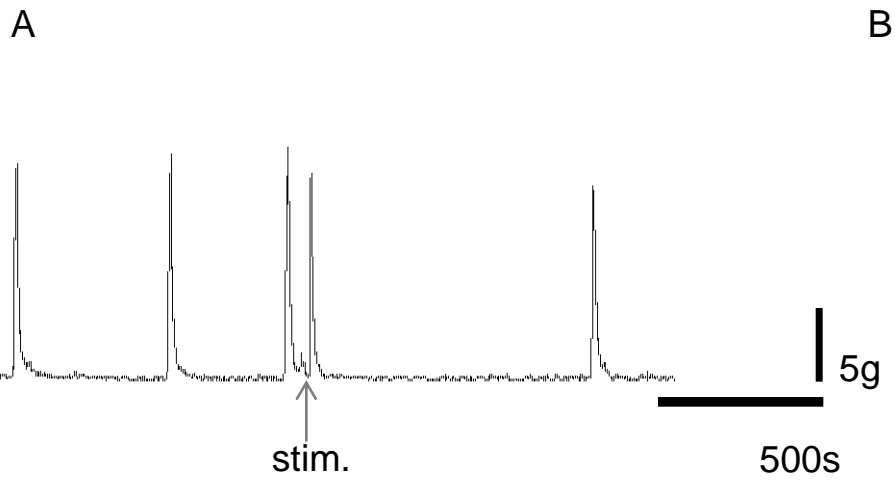
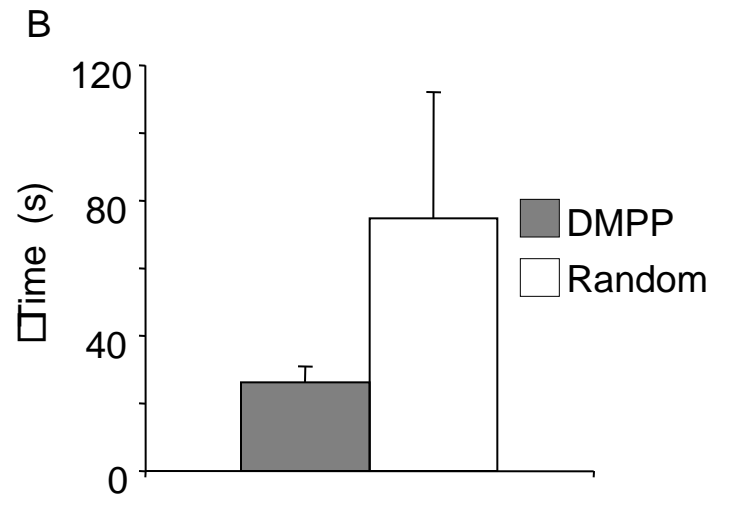
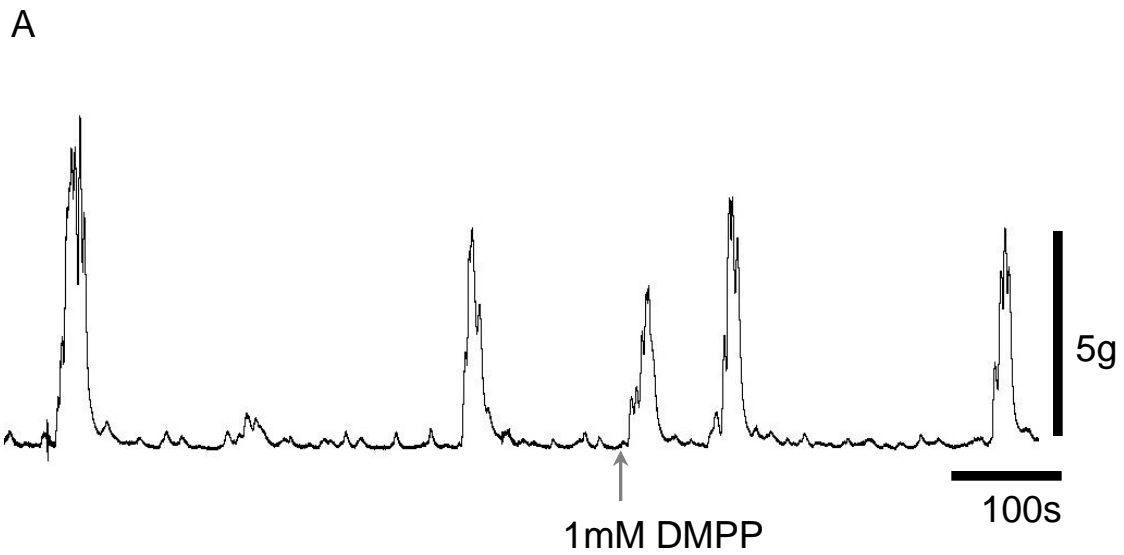


Fig 5



**Fig 6**



**Fig 7**

	<b>Size of tissue (mmxmm)</b>			
	<b>10x2</b>	<b>20x4</b>	<b>40x8</b>	<b>80x15</b>
<b>Slow phasic ± SD</b> cycles per minute	0.5±0.3	0.6±0.5	0.4±0.2	0.5±0.2
<b>Intermediate ± SD</b> cycles per minute	3.3±0.3	1.7±0.3	2.9±1.4	2.0±0.5
<b>High frequency ± SD</b> cycles per minute	15.0±2.0	17.6±4.5	12±7.0	11.7±1.5

**Table 1**