Evolving new drug treatments for Faecal Incontinence

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Abstract

Faecal incontinence is an embarrassing and socially debilitating condition which is primarily acquired and increases in prevalence with age. Current conservative measures are aimed at dietary modification and changing the consistency of stool with no targeted treatments available to address the underlying cause of incontinence. Surgical treatments are either unsatisfactory or carry significant morbidity.

There is currently increasing interest in the use of α adrenoceptor agonists to increase the tone of the anus and thereby improve continence. One of the potential drugs, L-erythro-methoxamine, has been shown to increase mean anal resting tone in healthy volunteers and is well tolerated as suppositories.

Extensive data exists on the neuromyogenic properties of the human internal anal sphincter (IAS) and its response to various drugs, particularly α adrenoceptor agonists. Little data exists on the response of the rectum to α agonists. The ideal drug treatment for incontinence would cause a contraction in the IAS and a relaxation in the rectum – increasing the reservoir of stool while augmenting the sphincter to aid continence.

We performed *in vitro* experiments on sheep internal anal sphincter (IAS) using an organ bath method and subjected the tissues to electrical field stimulation to mimic nerve stimulation. Our results were comparable to results of previous authors who also examined the sheep IAS. Using this validated protocol, we investigated the sheep rectum to identify the neuronal mediators of the EFS response and to investigate the effect of α_1 adrenoceptor agonists. Our results showed that sheep

rectum relaxes in response to nerve stimulation and this relaxation is the result of the release of nitric oxide. Contraction in response to nerve stimulation is primarily mediated by acetylcholine acting on muscarinic receptors. Methoxamine caused a contraction in the sheep rectum.

We also examined the pig IAS and rectum *in vitro*. An identical organ bath technique was used with Electrical Field Stimulation to mimic nerve stimulation. For both IAS and rectum in the pig, nerve stimulation caused a relaxation via nitric oxide and a contraction mediated primarily by noradrenaline acting on α_1 adrenoceptors with a small component mediated by acetylcholine acting via muscarinic receptors. Methoxamine caused a contraction in both IAS and rectal tissue with similar potency in each.

We were part of an industry-sponsored multi-centre randomised placebo-controlled clinical trial investigating the safety and efficacy of L-erythro-methoxamine on patients with faecal incontinence. The nine patients recruited from our centre showed no significant improvement in the number of episodes of incontinence or in the questionnaire scores measuring the impact of incontinence on quality of life after eight weeks of daily suppositories. The drug was well tolerated with few adverse events. There were no significant safety concerns although there was a prolongation of the PR interval in post-treatment ECGs and a positive correlation between the QT intervals on ECGs with serum concentrations of the drug. Our results were typical of those obtained in the other centres. The overall result of the trial was that there was no improvement in episodes of faecal incontinence following treatment with L-erythro-methoxamine at the chosen doses.

The results from our *in vitro* experiments suggest that α adrenoceptor agonists may not be the best methods of treating faecal incontinence. The results from the clinical study support this finding. We believe that more *in vitro* studies need to be performed on human rectal tissue to confirm our findings that α adrenoceptor agonists cause a contraction in the rectum.

Statement of originality

I hereby declare that this thesis and the work reported herein was composed by and originated entirely from me. Information derived from the published and unpublished work of others has been acknowledged in the text and references are given in the list of sources.

The data described in the experimental chapters is entirely my own work with guidance from my supervisors. The data was analysed by me and the conclusions are my own interpretation of the data. At our centre, previous researchers had used electric field stimulation on sheep internal anal sphincter tissue and therefore the experimental setup for electrical field stimulation already existed. The setup was modified to the needs of the current project with consultation with my supervisor (Dr V Wilson). The experiments were designed by me after consultation with my supervisor.

The clinical study was part of an industry-sponsored Europe-wide randomised control trial which had already received ethical approval in the UK. The study design had already been approved by the ethics committee and I was not involved in the design of this study. My role involved setting up the trial at our centre including negotiating a contract between the Research and Development department of the Trust and the pharmaceutical company (Norgine Ltd). I screened and recruited all the patients from colorectal clinics after informing the consultants about the study. I also recruited patients from a list of patients who had taken part in previous studies. If patients required an endo-anal ultrasound, this was performed by one of the specialist nurses and I reviewed the images with her to confirm inclusion/exclusion.

I performed every study visit for the patients and performed all the monitoring of physiological parameters including the ECGs and the venesections. I interpreted all the ECGs also compared them to a formal report issued by CardioAnalytics (a private firm contracted by Norgine for the conduct of the study). The holter data was analysed directly by CardioAnalytics and a report was supplied. For each study visit, I entered the data on the paper and electronic Case Report Forms (CRFs). The routine blood tests were analysed by the hospital laboratory, the serum samples for LEM were processed by me (centrifuged, supernatant drained and sample frozen) and they were then transported to a private firm for analysis. During the study, the Medicine and Healthcare Regulatory Agency (MHRA) selected our site for a routine inspection and I led the team in this inspection. While some minor errors were identified, the inspection passed without serious issues. Two errors highlighted were that one subject had been randomised one day before the holter data was available and that the serum samples were not held in a continuously monitored freezer.

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I am immensely grateful to my supervisors for their support, guidance and criticisms during the course of this thesis. Dr Wilson provided me with expert guidance in the design and conduct of the experimental sections of this project and provided valuable insights into previous evidence on the sphincter and rectum. Mr Maxwell-Armstrong was the principle investigator for the clinical trial and helped me set up the trial at our centre and was always ready with advice and support – be it in recruiting patients or negotiating with the R&D department. Professor Scholefield allowed me the opportunity to enrol and complete the project and directed me towards its successful conclusion.

Special thanks is due to Mr Maxwell-Armstrong for his unstinting support and impartial advice as without him this project would have been stillborn.

I would also like to acknowledge Mr Dave Walker of Norgine Ltd who allowed me access to the unblinded data from the patients in our centre where the data had been generated by me.

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1 Chapter 1 – Introduction

Faecal incontinence is an embarrassing and potentially debilitating condition. Recently, epidemiological studies have demonstrated a significantly wider prevalence of this problem than previously anticipated. The embarrassment caused as a result often means that patients become house-bound with a severe impact on their quality of life. It is also a significant contributing factor leading to institutionalising of older people. As the condition is significantly commoner in the older age groups, with an aging population the problem of dealing with this complex condition is one that requires urgent attention.

Treatments for faecal incontinence are often difficult with medical treatment less than effective and surgical treatment associated with a high morbidity. A greater understanding of the pathophysiology of faecal incontinence along with an understanding of the normal physiology of the ano-rectum is essential for the development of new treatment strategies for faecal incontinence.

1.1 Definition of faecal incontinence (FI)

There is no agreed threshold for defining FI [1]. Definitions can vary from being *any* loss of continence of faeces to a weekly leakage of faeces – any leakage [2, 3], yearly [4, 5], monthly [6], weekly [7, 8]. Various distinctions have been made in order to quantify the amount of incontinence from frequency to the degree of soiling – from soiling of underwear to staining of underwear [1].

Broadly, it is the loss of control of bowels and leakage or seepage of faecal material.

1.2 Prevalence

Prevalence of FI increases with age [1]. While it is uncommon among people aged less than 65 years it is relatively common in people older than 65 years [6]. Estimates of FI vary significantly in different studies partly due to a lack of consensus in definitions studied. In a recent systematic review, Merrie *et al.* (2004) noted that the rates of incontinence reported in studies varies from 2 to 24% (including flatus incontinence) and between 11 to 15% (excluding flatus incontinence) [9]. They concluded that a consensus definition needs to be adhered to for all studies. The International Continence Society defines FI as any loss of control of flatus, liquid or solid stools that is a social or hygiene problem [10].

Incontinence is commoner in older people. FI is quoted as having a prevalence of 1% among all people aged over 60 years living in the community [11] in 1985. Prevalence is significantly higher among the institutionalised elderly. In a systematic review, Macmillan *et al.* (2004) found a prevalence of incontinence among 50% of the institutionalised elderly [12]. One French study in 1999 [13] investigated the incidence of FI in patients over the age of 60 living in long-term residential care homes. After excluding patients with known FI (following a 2-month period of observation), they found that over the course of the study (296 days), 20% of subjects developed FI. In this study, FI was defined as at least one involuntary loss of faeces. Some studies report higher prevalence among women (63% vs. 47% [10]) whereas other studies report no sex differences [1].

Overall, prevalence varies between studies. The largest study on FI in the UK was performed by Perry *et al.* in 2002. Of 15,904 patients surveyed via postal survey,

10,116 people responded. They found an overall monthly reported leakage rate of 3.3% and soiling rate of 2.7%. Surprisingly, they found no difference in the prevalence of incontinence between men and women [1]. In a survey of 10,000 people in France, Siproudhis *et al.* (1999) found a prevalence of FI in 16.8% (yearly, not monthly) of people surveyed [14]. This study did note a significant female bias (63.3% women). A smaller study conducted in the Rhone region of the Alps found an overall prevalence of 5.1% among 2800 people with again a significant female bias (7.5% among women compared to 2.4% among men) [15]. Nelson *et al.* (1995) found a prevalence of FI in 2.2% of the population in a study of 6,959 individuals in with a 63% female preponderance [4]. In a later paper, Nelson *et al.* (1998) found a prevalence of 46% among 18,224 nursing home residents in Wisonsin, USA [16].

1.3 Socioeconomic impact of FI

The economic impact of FI is difficult to gauge due to multifactorial costs involved. As different studies quote costs in different currencies at different times, all values are expressed in terms of US dollars in 2010. Calculations were performed by converting the currency to US dollars using the respective years' exchange rate and expressing the result in dollars in 2010 by adjusting for inflation.

The direct costs in buying protective pads has been estimated at $563 million ($400 million in 1996) [17]. This would equate to $1.81 per capita cost. Costs of treatment vary according to the treatments provided. With more surgical treatments available for FI, the cost of managing FI has increased from $34 million in 1998 ($45.5 million in 2010 USD - $13,292 per patient) to $57.5 million in 2003 ($68.7 million in 2010 USD – $19,578 per patient) [18]. In 1998, Mellgren *et al.* [19] investigated the long

term costs to women suffering from FI secondary to childbirth treated either conservatively, with biofeedback or surgery or both and found an average per patient cost of $17,166 ($22,981 in 2010 USD). For patients in long-term care, Borrie *et al.* (1992) found an annual cost of Can$9,771 per patient per year ($11,769 in 2010 USD) [20]. Costs of conservative treatment have been calculated at €2,169 per patient per year in the Netherlands in 2005 ($1,892 in 2010 USD) [21] of which more than half the costs were associated with loss of productivity due to absence from work. Indirect costs have also been studied in the US – Drossman *et al.* (1993) surveyed 5400 subjects above the age of 15 years and found that 13.4% of those with any faecal incontinence and 29.4% of those with large volume FI described themselves as too ill to go to work or school. An average of 50 days were missed from work or school in adults with FI compared to 4.9 days in healthy individuals [22].

The social impact of FI is significant. People with FI often report depression, anxiety and shame and isolate themselves from friends and society [23]. In a sample of 3000 older people living at home in the UK in 2001, Edwards and Jones found a significant association between FI and anxiety, depression and disability [24]. In a survey of 3345 people in the Netherlands, Verhagen *et al.* (2001) found a high proportion of anxiety, shame and frustration in people suffering from FI [25]. The quality of life (QoL) of people with FI is often measured using the QoL questionnaires – see section 1.6.1 (Grading of FI).

1.4 The physiology of continence

Continence is maintained by the following mechanisms:

- Anatomical features
    - The anorectal angle and the puborectalis sling
    - The internal anal sphincter
- Rectal compliance
- Anorectal sensation
- Consistency of stool

A disorder in one or any of these can lead to incontinence of faeces.

1.4.1 Anatomical features

1.4.1.1 The anorectal angle and the puborectalis sling

The gastrointestinal (GI) tract begins from the mouth and ends in the anus. The rectum and the anus are the terminal portions of the GI tract. The rectum begins where the sigmoid colon stops having a mesentry at the third part of the sacrum and is about 12 cm long. It continues beyond the end of the coccyx and makes a right-angle at the apex of the prostate formed by the levator ani muscles. This angle formed by the levator ani is crucial in the maintenance of continence [26]. This is called the puborectalis sling.

There have been different theories regarding the role of the puborectalis sling. Phillips and Edwards *et al.* (1965) postulated that a 'flutter valve' mechanism existed mediated by the puborectalis sling which was responsible for gross continence [27]. Parks *et al.* (1966) agreed with the findings of Phillips and Edwards and postulated

that the puborectalis contracted with increasing intra-abominal pressure, causing a 'descending perineum' which caused a mechanical occlusion to the passage of stool [28]. However, these findings were contradicted by Bartolo *et al.* (1986) [29]who found tonic activity in the puborectalis muscle akin to sphincter contractions and Bannister *et al.* (1987) [30]demonstrated higher pressures in the anal canal as opposed to the rectum which argued against the postulate of a valve mechanism. They concluded that rising intra-abdominal pressure caused a reflex contraction in the anal sphincter complex which in turn prevented incontinence.

The denervation of the puborectalis is also thought to play a role in idiopathic FI. Neill *et al.* (1981) [31] performed electromyographic studies on the puborectalis (as well as the external anal sphincter) and found significantly reduced action potentials in the puborectalis in patients with FI compared to controls. Beersiek *et al.* (1979) [32] confirmed histopathological features of denervation in the puborectalis of patients with FI compared to controls. It is postulated that traction neuropathy of the motor nerves as a result of straining eventually causes denervation and hypertrophy of the puborectalis in patients with FI (Wexner, 1993).

1.4.1.2 The internal and external anal sphincters

Intrinsic tone of the anus is maintained by three elements: the internal anal sphincter (IAS), the external anal sphincter (EAS) and the anal cushions.

The internal anal sphincter is a condensation of the circular smooth muscle fibres of the distal rectum [33]. Intrinsic tonic contractions of the IAS maintain a significant tone in the anal canal [34]. The IAS also receives contractile neuronal impulses which assist in maintaining tone [35]. 10% of anal tone is maintained by the intrinsic tone

of the IAS, 45% due to nerve-induced IAS tone, 30% due to EAS tone and 15% via expansion of the haemorrhoidal plexus in the anal cushions [35].

The EAS is a sheet-like striated muscle which is intimately related to the puborectalis [33]. It is under control of the pudendal nerves, but unlike other striated muscles, it maintains a degree of baseline tone under resting conditions [36, 37]. With a rise in intra-abdominal pressure (for example on standing), there is a reflex contraction of both the puborectalis and the EAS causing a transient increase in anal pressure in response to intra-abdominal pressure [36, 37]. This reflex is mediated by a spinal arc and is partially preserved in individuals with spinal cord transection [37]. This increase in tone via spinal reflex can only be maintained for 40-60 seconds at a time and is crucial to maintaining continence in the presence of increased intra-abdominal pressures [33].

Injury to the IAS/EAS complex is one of the common reasons for FI. The EAS may be damaged as a result of anal fistula surgery which has been noted as causing incontinence in up to 34% of patients [38]. Stretching of the anal canal, for example in Lord's procedure for the treatment of anal fissures, can cause significant injury to the IAS demonstrated sonographically [39]. This results in a degree of incontinence in a significant proportion of patients [40]. Injury to the sphincter complex [41] and to the pelvic nerves [42] following vaginal delivery is a frequent cause of FI in women. Haemorrhoidal surgery is also implicated in the development of FI [43] and this is primarily a result of a loss of function of the anal cushions rather than a change in rectal sensation.

1.4.2 Rectal compliance

The rectum is a muscular tube with significant elastic properties which allow it to maintain low pressures despite significant increases in volume [33]. In the normal physiological state, the rectum functions as a reservoir of stool. The rectum contracts in response to significant distension and initiates defaecation. The external anal sphincter is a striated muscle and as such cannot sustain voluntary tone for any length of time, therefore the rectum allows for stool storage until such time as it is socially acceptable to defecate. The role of rectal compliance in FI is the subject of debate [44]. While some studies have shown significantly reduced rectal compliance in patients with FI [45, 46] in 30-47% of patients, other studies have disputed these findings [47, 48]. However, anorectal anastomosis after anterior resection has been demonstrated as having poorer continence with reduced rectal compliance compared to patients with good rectal compliance [49]. Recently, rectal hypersensitivity has been identified as a significant exacerbating factor in urge FI [50, 51].

Rectal compliance is classically reduced in operations where the rectum is removed surgically (in anterior resections) or where the rectum has been subjected to radiotherapy as a treatment for rectal cancer. As previously mentioned, there is a significant association in these groups of patients between rectal compliance and urge FI.

In the management of FI, it would be logical to assume that an increase in rectal compliance would aid in continence.

1.4.3 Ano-rectal sensation

The recto-anal inhibitory response (RAIR) was first described by Gowers in 1877 and further categorised by Robertson in 1935 [33]. Distension of the rectum with faecal material causes a transient relaxation of the EAS and the IAS. This causes a shortening of the functional sphincter of the anus and allows rectal contents to come into contact with the sensory zone of the anus [52]. This area is rich in nerve endings and is extremely sensitive to pain, touch and temperature sensation [53]. This allows for 'sampling' of the rectal contents and fine-tunes the distinction between liquid and gaseous contents in the rectum. Miller *et al.* (1988) noted a significant deficiency in sampling in patients with FI compared to normal individuals highlighting the importance of RAIR and sampling in maintaining continence [54]. These findings were confirmed by Bielefeldt *et al.* (1990) who also found significant impairment in anorectal sensation in patients with FI [55].

The rectum itself does not have any sensory innervation [53], but rather relies on the puborectalis muscle and the anal sphincters for proprioceptive sensation. Impaired rectal sensation has been noted in both idiopathic FI [56, 57] and in patients with neurological disorders such as diabetes (with peripheral neuropathy), spinal trauma or surgery or central nervous system disorders [56]. In these cases, the RAIR was not impaired. As a result, high rectal pressures caused initiation of RAIR without a sensation of rectal fullness. This resulted in FI as the patient did not voluntarily contract the EAS.

Impaired anorectal sensation can be a result of neurological disorders (diabetes with peripheral neuropathy, CNS disorders, spinal trauma), or the result of transanal surgery, as a result of rectal prolapse, or idiopathic [33].

1.4.4 Stool consistency

The colon absorbs the majority of the water from the stool produced by the small intestine reducing the volume of faeces from 1-1.5l to 100-150 mls per day. Diarrhoea results in FI in otherwise normal individuals when the volume of liquid stool puts excessive stress on the sphincter mechanisms responsible for maintaining continence [33].

Chronic diarrhoeal diseases result in FI by overwhelming the normal mechanisms of continence and may occur with completely normal anatomical and physiological conditions of the rectum and anus.

1.4.5 Measurement of anorectal function

Rao *et al.* (1999) [58] noted that there was considerable heterogeneity in the measurement of anorectal function in published studies and aimed to standardise the technique of anorectal manometry and report normal values in healthy individuals. Anorectal manometry includes anal sphincter function, rectoanal reflexes and rectal sensation and compliance testing. Although techniques and equipment differ to a certain degree, most anorectal manometry is performed along the techniques described by Rao *et al.* (1999). The patient is placed in the left lateral position and a probe is inserted into the anus. The probe contains microtransducers (usually about 6) along its length and is also fitted with an inflatable balloon. The probe is placed in the rectum with the transducers at variable lengths from the anal

verge (1, 2, 3, 5, 9 and 14 cm in the paper described). Constant measurement of anorectal pressures via an amplifier is recorded at baseline and after various manoeuvres/drug additions. The inflatable balloon is inflated to various volumes and subjects are asked to report the following sensations: first sensation, constant sensation of fullness, a desire to defecate and an urge to defecate. Rectal compliance was indicated by the volume of distension of the rectal balloon which caused the patient to have the urge to defecate – a lower volume causing an urge to defecate indicated a lower rectal compliance.

Rao *et al.* (1999) reported greater resting sphincter pressures in men (70 mm Hg) compared to women (65 mm Hg) and significantly increased duration and magnitude of maximal squeeze pressures in men compared to women. They also noted significantly greater pressures in nulliparous women compared to multiparous women. No significant differences in rectal sensation or recto-anal inhibitory reflex were noted.

A similar technique for anorectal manometry was used by Thekkinkattil *et al.* (2008) [59] who used rectal distension (volume of gas in the rectal balloon) and rectal pressure at each distension level to calculate compliance (compliance = distension volume / rectal pressure in mmHg). They investigated healthy volunteers and found greater resting sphincter pressures in men compared to women, no significant difference between men and women with respect to rectal sensation or rectal compliance. They also found no significant difference between the measurements in subjects older than 50 years and those younger than 50.

1.5 Aetiology of FI

The majority of causes of FI are acquired. Among the congenital causes, rare anatomical defects such as imperforate anus, rectal agenesis, and cloacal defect all can cause faecal incontinence. The degree and severity of FI depends on the degree of dysgenesis of the pelvic floor musculature and nerves. Congenital neurological causes such as spina bifida can also cause faecal incontinence [60].

Causes of acquired FI can be divided into anatomical disruption, neurological causes and age-related causes.

1.5.1 Anatomical disruption

Disruption of the sphincter complex secondary to vaginal delivery is a common cause of FI in women [61]. Recognised obstetric anal sphincter injuries (OASIS) occur in 0.4–19% of vaginal deliveries in centres practising mediolateral and midline episiotomies, respectively [62]. However, with the advent of endo-anal ultrasound, previously unrecognised sphincter injuries are being identified. Andrews *et al.* (2006) found that the rate of sphincter injury was 24.5% in vaginal deliveries – increased from 11% initially identified due to clinical and endo-anal ultrasound performed by an experienced research fellow. The phenomenon of 'occult' sphincter injury following vaginal delivery is being recognised as a major cause of FI in women following vaginal delivery [62]. A systematic review of third degree tears confirmed the following causes as significant risk factors of sphincter disruption: birth weight > 4 kg, persistent occipitoposterior position, nulliparity, induction of labour, epidural anaesthesia, second stage of labour longer than 1 hour, instrumental delivery, midline episiotomy [61]. Reports of FI as a result of vaginal delivery ranged from 15%

[63]to 23% [64]. A recent meta-analysis of 717 vaginal deliveries found a rate of sphincter injury in 26.9% of primiparous women and new sphincter defects in 8.5% of multiparous women. Although 29.7% of patients with identifiable defects were symptomatic, it revealed a probability of incontinence in 77-83% of cases [65] by using a Bayesian calculation (the mathematical method of predicting an event in the presence of uncertainty using all known variables and experience of past events).

Traction neuropathy of the pudendal nerve during vaginal delivery further contributes to FI [66]. Traction neuropathy of the pudendal nerve is also postulated as the cause of FI in patients with perineal descent syndrome. Perineal descent was first observed by Porter *et al.* in 1962 [67]and confirmed by Parks *et al.* in 1966 [28]. Excessive and repeated straining causes a prolapse of the rectal wall into the anal canal with further feeling of incomplete emptying and further straining [68]. A number of studies have confirmed pudendal neuropathy in patients with perineal descent and have identified this as the cause of FI in these patients [68, 69]. Sphincter denervation is one of the causes of FI in patients with rectal prolapse by a mechanism similar to that of perineal descent [70]. In rectal prolapse, neuropathy of the sphincter complex is further complicated by impairment of rectal sensation and anal sampling with an inability to distinguish solid or liquid from gaseous contents [71].

Injury to the sphincter complex following surgery is well recognised. Earlier techniques of fistulotomy and fistulectomy have been abandoned in favour of sphincter-preserving procedures such as cutting seton treatment because of the lower rate of incontinence among patients treated with this procedure [72].

However, recent evidence suggests that seton treatment also has a significant risk of causing incontinence with a total incontinence rate among patients of 12% [72].

Haemorrhoidectomy is a significant cause of long-term FI (upto 30%) [73] but the mechanism is probably due to loss of anal pressure due to the excision of the anal cushions rather than due to a disturbance of ano-rectal sensation [43]. Newer techniques which do not involve excision of the anal cushions have better results. Doppler-guided haemorrhoidal artery ligation surgery is associated with no difference in ano-rectal functional tests at 1-year follow-up [74]. Giordano *et al.* (2009) [75] in their systematic review of the procedure report one year follow-up from six studies (850 patients) with bleeding, pain on defaecation and prolapse in 10%, 9% and 11% of patients post procedure. The National Institute of Health and Care Excellence (NICE) published guidelines in 2010 endorsing haemorrhoid artery ligation as an effective alternative to formal haemorrhoidectomy [76].

1.5.2 Neurological causes of FI

Inherited and congenital causes of pelvic floor denervation are well recognised causes of FI. Congenital causes such as spina bifida, meningocoele and myelomeningocoele are all associated with FI. Acquired injury to the spinal cord, multiple sclerosis, Parkinson's disease and stroke are all recognised as causing FI.

Neurological injury causing FI can be divided anatomically according to the level of the lesion. Lesions can be divided as at the cauda equinis or above the cauda equinis. Colonic transport of stool is primarily under the control of the enteric nervous system but receives stimulatory impulses via the vagus nerve to the splenic flexure and inhibitory sympathetic impulses from the ninth thoracic to the second lumbar

segments of the spinal cord. Generally, parasympathetic denervation has greater effect on functional capacity than sympathetic denervation [77].

Neurogenic FI is a significant health burden. Each year, approximately 10,000 individuals in the EU suffer spinal cord injury. FI is reported in 5% of individuals as daily, and monthly in 20%. Multiple sclerosis has a prevalence of approximately 1 per 1000 of the population. 20-30% of patients suffer from incontinence at least once a week. Parkinson's disease has a stronger association with constipation than incontinence with 37% of patients reporting unsuccessful attempts at defecation. A proportion of these patients also have incontinence. Stroke has an incidence of 2 per 1000 in the developed world. 30-40% of patients suffer from incontinence in the initial stages with a 9-15% prevalence of incontinence long-term [77].

The pathophysiology of FI in patients with spinal cord lesions, myelomeningocoele and multiple sclerosis is similar. The lesion in all these diseases is commonly at the cauda equinis and causes a disruption of the reflex arc between the left colorectum and the spinal cord. This leads to a hypotonic, hyporeflexic left colon and rectum with an impaired Recto-Anal Inhibitory Response (RAIR). This leads to difficult evacuation, rectal distension, constipation and FI [77]. Chronic constipation causes a build-up of large volumes of stool in the rectum which hardens over time due to continued absorption of water by the colonic mucosa. Following this, mucous secretion from the colonic wall causes partial liquefaction of the stool on the surface of the globus and causes leakage of faeculent fluid. This is known as overflow incontinence. This is further compounded by loss control and sensation of the sphincter complex with impaired anal sensation.

Lesions superior to the cauda equinis, for example in some cases of stroke or multiple sclerosis, there is a loss of the inhibitory sympathetic tone to the left colon leading to a hyper-reactive left colon and rectum and an enhanced RAIR. This can lead to significant urge incontinence [77].

Loss of ano-rectal sensation and peripheral neuropathy is a cause of FI in patients with diabetes. Furthermore, patients with dementia often lack interest in bowel function and can develop both constipation and FI as a result [60].

1.5.3 Ageing

Older age is well recognised as one of the risk factors for FI [6]. The cause of idiopathic FI in older people cannot be classified as anatomical or neurological. Although the anatomy of the sphincter complex is preserved, older people demonstrate a significantly poorer anorectal function compared to younger counterparts [78, 79]. Studies have demonstrated increased anal canal pressures and reduced rectal compliance in older subjects with no identifiable pelvic disorders. Sclerosis and thickening of the IAS has been demonstrated by endo-anal ultrasound in subjects with no pelvic floor disorders. This difference was significant for subjects over the age of 55 compared to subjects younger than 55 years [80]. Klosterhalfen *et al.* (1990) demonstrated a significant correlation between sclerosis of the IAS and age with 4 times as much sclerosis in subjects over the age of 80 compared to 20 year old subjects [81]. Sclerosis and thickening of the IAS may be a normal feature of ageing and can contribute significantly with the risk of FI in older life [33]. Percy *et al.* (1982) found markedly increased fibre density in older patients without

incontinence. This difference was more pronounced in patients who had incontinence [82].

FI in older people may be a function purely of the ageing process with denervation and sclerosis of the IAS and hypertrophy of the EAS. However, in many cases, it may a contributory factor in FI in patients already susceptible to FI due to the loss of continence mechanisms as a function of age [82].

1.6 Treatment of FI

Management of FI should always be preceded by accurate assessment of symptoms and pathology. Questionnaire evaluations, bowel diary and endo-anal ultrasound all play a part in the assessment of continence. It is important to exclude malignancy or inflammatory bowel disease in the first instance, therefore a colonoscopy is usually performed. Treatment starts with the least invasive procedures and moves to more invasive surgery [10].

1.6.1 Grading of FI

Over the years, a number of methods have been used to quantify the degree of incontinence. While some authors have used simple 'soiling' or 'staining' of undergarments as the measure [1], other have used various scoring questionnaires to evaluate the degree of FI. Multiple authors have shown a significantly lower QoL as measured on the SF-36 standardised health questionnaire in people with FI compared to healthy individuals [8, 83, 84].

Quantifying the degree of FI is important to assess the outcomes of various treatments for FI. The challenge is to develop a questionnaire that is both easily understood and also adequately assesses the degree of this complex problem. Unlike urinary incontinence, FI can be solid, liquid or gas and the impact on (QoL) can vary greatly between patients.

The Wexner grading system [33] has been widely used to grade FI – it is simple and easily understood by patients. However, it has come under criticism due to the lack of any assessment of faecal urgency and the same weightage that is given to both wearing of pads and overt incontinence [85]. Rothbart *et al.* (2001) found a

significantly reduced Gastrointestinal QoL score in patients with a Wexner score of 9 or greater compared to the general population [86]. The Wexner score has been validated for use in patients with FI and has been found to correlate well with more extensive QoL questionnaires. Rothbart *et al.* [86] investigated 35 women with obstetric injury to the external anal sphincters causing incontinence and had had sphincter repair surgery. They used the gastrointestinal quality of life index (GIQLI) and compared it to the Wexner score in these patients. The GIQLI is a validated score for measuring quality of life in patients with gastrointestinal disorders with a score of less than 105 indicating patients who are house-bound as a result of their symptoms. On performing a correlation between the GIQLI sore and the Wexner scores in each patient and also performing a regression analysis on the GIQLI scores, they found that a Wexner score of greater than 9 corresponded to a GIQLI of less than 105 in patients with FI.

The Vaizey scoring system improves upon the Wexner score and has been validated as having excellent correlation with clinical assessment and incontinence diary assessment of incontinence [85]. However, the Vaizey score does not identify the impact on the QoL of patients with FI. The Faecal Incontinence Quality of Life questionnaire (FIQOL) has 29 items – significantly more than the Vaizey score – but has good correlation with psychometric evaluation of symptom impact [87]. The disadvantage is that it is significantly more complicated and time-consuming.

1.6.2 Dietary modification

Counselling should not be overlooked as part of the treatment of FI. Patients are often distressed and unsure and should be supported psychologically as well as

medically. Simple gut stimulants such as caffeine and nicotine should be avoided from the diet and may improve symptoms for a number of patients with minor seepage. Advice on fluid intake and fibre should be given and can significantly improve incontinence if it is associated with constipation and overflow. Barrier creams should be used on the perineum to prevent skin irritation and excoriation [10].

1.6.3 Biofeedback

Biofeedback for FI is based on the principle of Operant Conditioning first described by Engel *et al.* in 1974 [88]. It has been extensively used as a treatment for FI with mixed results. Three methods for biofeedback have been used – rectal sensitivity training, strength training and co-ordination training. In rectal sensitivity training, a rectal balloon is distended until the patient first reports the sensation of rectal distension. By varying the rectal distension volume, patients can be trained to recognise lower volumes of distension while being able to tolerate higher volumes. Strength training techniques measure anal sphincter pressures and assist patients in sphincter exercises while giving them feedback on the sphincter pressures achieved. Co-ordination training uses three balloons to measure the RAIR and feedback to the patients the threshold at which this is achieved. The patients can then counteract the reduced pressure in the IAS by voluntarily contracting the EAS [89].

Clinically, biofeedback is often the first treatment modality following dietary advice. It is often used in conjunction with constipating medication for the treatment of FI. Treatment regimes can include all or one of the three modalities mentioned.

Norton *et al.* (2003) performed a randomised control trial with patients grouped into 4 groups – conservative care with advice only, advice and instructions and sphincter exercises, hospital based computer assisted sphincter exercises and hospital biofeedback along with home electromyelogram biofeedback device – and found no significant differences in any of the groups [90]. In their study, patients received nine treatment sessions, each session lasting 40-60 minutes, conducted over a course of 3-6 months.

Norton *et al.* (2001), in their systematic review of the literature pooling 1364 patients found a cure rate of 49% with 72% of patients reporting that they were either cured or improved [91]. However, they did note that there was significant heterogeneity between the studies with different techniques employed and only 8 out of the 46 studies had a control arm.

Following this, a meta-analysis of 8 randomised trials found that the evidence for biofeedback as a treatment for FI remains doubtful. Counselling patients may have the same impact as biofeedback techniques. Further good quality studies were suggested before treatment effect could be established [89].

1.6.4 Drug treatments

1.6.4.1 Opiates

Loperamide is a synthetic opioid with μ-receptor agonist properties. When used orally, it increases whole gut transit time and reduces stool volume. It also increases the tone of the IAS [92]. In addition to μ-opioid receptor activity, loperamide also inhibits the calcium-calmodulin complex, behaving as a calcium antagonist. This

causes increased gut transit time, increase contact of faeces with gut mucosa and consequent reduction in stool volume and increase in stool density. It is more effective that other opiates which have little effect on gut secretory function [93]. Loperamide is also noted to increase the maximum tolerated rectal volume in patients with FI by increasing sphincter tone, reducing the time to recovery from RAIR and increasing rectal tone while not impairing rectal compliance [94].

Loperamide is often used as the first line drug treatment for patients with FI. It is available in liquid form and can be titrated to the required dose. Tolerance does not develop and it has few side-effects [10].

Dyphenoxylate is a natural opioid and is used in the treatment of chronic diarrhoea and FI. It is often combined with Atropine to further reduce gut motility. It can cross the blood-brain barrier and cause central nervous system side-effects thereby limiting its use. Atropine can cause anti-cholinergic side-effects [10] – these drugs are rarely if ever used in clinical practice.

Codeine phosphate is an opiate derivative. Side-effects include nausea and tolerance. It is also used in the treatment of FI but tolerance and nausea may limit its use [10].

1.6.4.1.1 Guidance on the use of anti-diarrhoeal drugs

NICE guidelines on the management of FI [95] suggest the use of loperamide as first line drug therapy in the treatment of FI following dietary advice and modification. In a systematic review on the drug treatment of FI, Omar *et al.* (2013) [96] concluded that anti-diarrhoeal medication had advantages over placebo in the treatment of FI

in patients at the cost of side effects (abdominal pain, bloating). One trial investigating the three anti-diarrhoeal medications found that loperamide was the most effective in treating FI, followed by codeine and finally by dyphenoxylate [97].

1.6.4.2 Amitriptyline

Amitriptyline is a tricyclic antidepressant with anticholinergic properties. When used at low doses, it has been shown to reduce the number of episodes of incontinence in patients with FI. Santoro *et al.* (2000) found an increase in gut transit time with amitriptyline and an improvement in continence scores in patients treated with 20mg per day. They attributed this primarily to the reduction in the frequency and amplitude of rectal motor complexes [98].

1.6.4.3 Evolving drug treatments for FI

These are discussed in section 1.11

1.6.5 Surgical management of FI

An overview of the surgical treatments for FI have been listed and the current guidance on these treatments has been discussed in 1.6.5.5.

1.6.5.1 Discontinued treatments

1.6.5.1.1 Post-anal repair

Previous management of FI focussed on recreating the anorectal angle by posterior plication of the puborectalis, the levator ani and the EAS. In most series, less than 50% of patients have any significant improvement in function and radiological studies have failed to demonstrate any relationship between restoration of the anorectal angle and improvement in symptoms [99]. Furthermore, long-term results

demonstrate that less than a quarter of patients are continent at a median follow-up of 6 years [100]. The operation has therefore gradually fallen into disfavour and is rarely performed [10].

1.6.5.2 Current treatments

1.6.5.2.1 Sphincteroplasty

Sphincteroplasty is a common surgical procedure performed for FI. It involves isolating the external anal sphincter and 'tightening' it by overlapping one part of the muscle with the other. However, it is limited to people who have suffered a disruption of the EAS. As mentioned previously, vaginal delivery is a significant risk factor for FI due to traumatic injury of the sphincter complex. Also, anal fistula surgery can leave patients with significant defects in the EAS.

Initial outcomes from overlapping sphincteroplasty were encouraging with over 76% of patients reporting excellent functional outcomes with significantly increased anal squeeze pressures [101]. However, long-term results following sphincteroplasty show disappointing results with less than 25% of patients completely continent [102-104].

This procedure has largely been superseded by sacral neuromodulation which has been shown to be effective in patients with sphincter defects as well [105].

1.6.5.2.2 Dynamic gracilloplasty

This is an invasive surgical procedure reserved for patients who have failed conservative management and do not have repairable sphincter defects. It involves mobilising the gracilis muscle, either unilaterally or bilaterally, and wrapping it

around the native sphincter and attaching it to the contralateral ischial tuberosity. The muscle can remain unstimulated or under continuous low-voltage stimulation which converts a fast-twitch fatiguable muscle into a slow-twitch sphincter-like muscle [106]. Results are varied with reported success rates between 42% and 85% of patients [107]. However, the procedure carries a significant morbidity risk of greater than 50% and a mortality rate of between 0 and 13% [106]. Boyle *et al.* (105) performed a study investigating long-term outcomes in patients with dynamic gracilloplasty over an average 13 years [108]. Their group of patients included patients with obstetric injury, anal surgery, atresia, idiopathic incontinence and ileoanal pouch surgery. They found no overall improvement in continence scores but a significant improvement in continence scores in the patients in the obstetric injury group and the anal surgery group. They concluded that dynamic gracilloplasty was an effective long-term treatment in a select group of patients.

1.6.5.2.3 Artificial bowel sphincter

This procedure is also reserved as an end-stage procedure when other procedures have failed. It involves the placement of an implant around the anal canal with a reservoir in the retropubic region. The reservoir is controlled by a cuff placed in the scrotum or the labia majora. The cuff may be used to inflate the implant with fluid and thereby act as a neosphincter [106]. Results are variable and overall 87% of patients are reported to have device-related complications with up to 58% infections, up to 25% erosions, up to 85% faecal impaction and up to 17% chronic pain [10].

1.6.5.2.4 Stoma

Creation of a stoma involves surgery where the colon, usually the last part (sigmoid colon) is disconnected from the rectum and brought out to the skin leaving a blind-ending rectum connected to the anus. This allows the bowels to empty into a stoma bag which can be emptied at the patient's convenience. This is often viewed as the last resort in the management of FI. It allows the patient to be fully continent and restores normal social function. However, it is invasive and often considered by patients to be unacceptable. More recent evidence suggests that stoma may be a viable option for many patients with good quality of life following the procedure. Norton *et al.* (2005) found that 83% of patients were not affected by the stoma and 84% would choose to have a stoma again [109].

1.6.5.3 Evolving treatments

1.6.5.3.1 SECCA procedure

This procedure involves the application of temperature-controlled radiofrequency to the anal canal and was first trialled in Mexico in 1999. While the mechanisms of action are not well understood, it is postulated that the effects may be due to increased sensitivity of the ano-rectal area and an improvement in sphincter function. In a recent review of the literature, Frascio *et al.* (2014) identified 10 clinical studies between 2002 and 2011 with a total 220 patients treated. At six months and 12 months, there was a significant improvement in symptoms in patients with incontinence, however only one study reported results at 5 years with some residual benefit in symptoms [110]. While long-term results for the SECCA procedure are still awaited, it shows promise as a further treatment option for FI.

1.6.5.3.2 FENIX device

The FENIX is a new magnetic sphincter augmentation system for the treatment of FI which has been shown to be effective in non-randomised studies and has been favourably compared to SNS in a cohort study [111] with similar efficacy and morbidity to SNS. A formal study comparing both SNS and the FENIX system is underway with results not yet published [112].

1.6.5.4 Treatments with questionable benefits

1.6.5.4.1 Injectable bulking agents

Shafik *et al.* (1992) first described the use of injectable bulking agents for the treatment of FI in 1992 using Teflon injected into the submucosa of the anus [113]. Since then a number of different compounds have been used with varying degrees of success [106]. Autologous fat from the abdominal wall has shown good short-term results but long-term results have been disappointing [114]. Silicone implants have been used with more success. In a study of 82 patients, Tjandra *et al.* (2004) found a significant improvement in 55% of patients who had a greater than 50% improvement in symptoms after 6 months [115]. Carbon-coated beads have also been used and have been shown to be effective in treating FI however, a recent randomised trial found that injectable silicone biomaterial (PTQ) was more effective than carbon-coated beads (Durasphere) [116].

NICE guidance on injectable bulking agents (2007) [117] does not support the use of injectable bulking agents for the treatment of FI unless in the context of further research – “Current evidence on the safety and efficacy of injectable bulking agents for faecal incontinence does not appear adequate for this procedure to be used

without special arrangements for consent and for audit or research, which should take place in the context of a clinical trial or formal audit protocol that includes information on well-defined patient groups".

1.6.5.5 Conclusion of surgical management

Dynamic graciloplasty (DC), artificial bowel sphincter (ABS) and end stoma (ES) are all viewed as 'last line' procedures for FI. A recent comparison of the three treatments found ES to be the most cost-effective at 5 years with ABS most cost-effective at 10 years. However, all these treatments come with significant morbidity and DC and ES have a significant mortality as well.

The NICE guidance on surgical management of FI [95] suggests sphincteroplasty as the first line surgical treatment in patients with a greater than 90^{o} disruption of the EAS. Concomitant IAS disruption significantly reduces the success rate from this procedure. In patients where sphincteroplasty fails or in patients unsuitable for sphicnteroplasty (e.g. where there is an intact sphincter), sacral nerve stimulation (SNS) should be considered (see section 1.6.6.1 Sacral nerve stimulation (SNS)). If SNS is unsuccessful, patients could be offered regarding neo-sphincter procedures – dynamic gracilloplasty or artificial bowel sphincter – after careful counselling regarding the risks and benefits of the procedure. NICE guidance on the SECCA device (2016) [118] lists the cost of the single-use handpiece at £1,495 and the re-usable radiofrequency generator at £25,000. There is no guidance on the use of the device given the paucity of evidence supporting it save that "the procedure is only carried out in units specialising in assessing and treating faecal incontinence, as one of a range of treatment options, and that further research is done on the procedure".

No satisfactory surgical management of FI currently exists with sphincteroplasty (where there is an identifiable sphincter defect) and injectable agents providing the best outcome on review. Newer treatments are still being investigated although do show promise.

1.6.6 Neuromodulation

1.6.6.1 Sacral nerve stimulation (SNS)

Sacral nerve stimulation provides an attractive alternative to invasive surgery. SNS was first used in for the treatment of urinary incontinence in 1981 [119] and was subsequently used in the treatment of faecal incontinence in 1995 [120]. Since then, it has been shown to be effective in both short-term and long-term improvement of symptoms [121]. Matzel *et al.* (2009) [121] demonstrated persistent improvement in symptoms at mean follow-up of 9.8 years in their cohort of 9 patients. In a recent meta-analysis of SNS [122], Tan *et al.* (2011) reported a significant improvement in incontinence outcomes, anal resting and squeeze pressure (but not rectal sensitivity), and quality of life. Sacral nerve stimulation requires two surgical procedures – the first to implant a temporary pacing wire, the second to implant a permanent electrode if temporary stimulation is effective – and incurs a cost of €6,581 [123]. SNS is usually well tolerated with the commonest cause of morbidity being pain either at the site of electrode placement or at the perineum [124].

The mechanism of action remains unclear. SNS is hypothesised to modulate autonomic and somatic afferent and efferent impulses from the anus and the rectum to improve rectal compliance and increase anal resting pressure [125].

The fourth International Conference on Incontinence recommended the use of SNS after failure of medical and conservative therapy in patients with intact external anal sphincters (Grade B) and the use of SNS in patients with external sphincter disruption after surgical correction of sphincter defects (Grade C) [126].

1.6.6.2 Posterior tibial nerve stimulation (PTNS)

Peripheral neuromodulation was first described by McGuire *et al.* in 1983 [127] as a treatment for detrusor instability and was first described by Shafik *et al.* in 2003 as a method of treating faecal incontinence [128]. As with SNS, the mechanism of action remains unclear. The posterior tibial nerve arises from the ventral branches of the ventral rami of the fourth and fifth lumbar and the first to the third sacral nerves. Stimulation of the posterior tibial nerve at the ankle is postulated to send retrograde stimulation to the sacral nerve thereby mimicking the action of SNS [129].

Two different methods of stimulation include percutaneous and transcutaneous stimulation of the posterior tibial nerve. Adequate placement of the needle or transcutaneous electrode is achieved by observing a flexion response in the toes or paraesthesia in the foot or the toes. Queralto *et al.* (2006) demonstrated significant improvement in symptoms using a transcutaneous technique [130] while Shafiq *et al.* (2003) [128] first demonstrated PTNS using a percutaneous technique. In a recent study, George *et al.* (2013) demonstrated a greater improvement in the percutaneous group compared to the transcutaneous group [131].

At this time, there is no consensus on the optimum treatment protocol and various stimulation protocols have been established. While Shafiq *et al.* (2003) initially used daily stimulation for 4 weeks [128], more recent studies have used either once

weekly [132, 133] or twice weekly treatments [131, 134] for a total of 12 treatments. No clear evidence exists to support one protocol over the other. Stimulation current is increased till the maximal tolerable threshold is reached and then reduced slightly. Continuous stimulation is delivered for 30 minutes per treatment session [135]. The treatment is well tolerated with few reported complications including gastrodinia, paraesthesia and pain at the needle insertion site and bleeding from the needle site.

A recent multicentre study investigating PTNS versus sham stimulation in patients with FI found that 38% of patients in the treatment group and 31% of patients in the sham stimulation group reported greater than 50% improvement in the number of incontinence episodes [136]. I ran this study at the Nottingham site. Sham stimulation was given by placing the electrode just deep to the skin whereas the treatment required inserting the stimulating electrode deeper in the subcutaneous plane. As patients had no previous experience of PTNS, they did not know if they were receiving sham or true treatment. This study concluded that there was no demonstrable improvement with PTNS over placebo and highlighted the high placebo effect in treating FI.

1.7 Smooth muscle function

Both the IAS and the rectal muscles are smooth muscle. An understanding of the neuromyogenic properties of the IAS and rectum is essential to developing new locally-active drug treatments for FI.

1.7.1 An overview of smooth muscle contraction

Striated muscle and smooth muscle have different controls involving the initiation and regulation of contraction, but the underlying mechanism of contraction is similar – myosin 'light' chain moving along the actin 'heavy' chain.

In smooth muscle, the myosin binding site on actin is inactivated by the presence of myosin light chain (MLC) in the resting state. MLC is phosphorylated by MLC kinase (MLK) which causes a configuration change and exposes the binding site for myosin with actin. Inactivation of MLC via dephosphorylation is mediated by myosin phosphatase and the muscle returns to resting (relaxed) state. MLK is activated by the binding of calcium-calmodulin – calmodulin is a ubiquitous intracellular protein and is primarily a carrier for intra-cellular calcium ($[Ca^{2+}]_i$) and is the effector molecule for the effects of calcium. Therefore, an increase in $[Ca^{2+}]_I$ causes activation of MLK and thereby activation of MLC and exposing the binding site for actin, causing contraction. MLK and myosin phosphatase activity is also regulated by the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). Intracellular calcium ($[Ca^{2+}]_i$) is a crucial step in initiation of contraction as well as the activity of both cAMP and cGMP – both are regulated by the action of G proteins.

1.7.1.1 The role of calcium

As mentioned previously, $[Ca^{2+}]$i increase is a key process in the initiation of contraction in smooth muscle. Calcium ingress into the cell is mediated in smooth muscle primarily by L-type calcium channels in response to agonist stimulation of membrane receptors. The other major method for increasing $[Ca^{2+}]$i is the release of

calcium from intracellular stores from the endoplasmic reticulum. This, in turn, is mediated by the second messenger inositol triphosphate (IP_3) produced by the cleavage of phosphatidyl inositol diphosphate (PIP_2) by G proteins following agonist stimulation at the plasma membrane. Calcium is removed from the cytoplasm of the cell by Ca^{2+}-ATPase via an energy-dependent process and also by Na^{+}-Ca^{2+} exchangers.

1.7.1.2 G proteins and G protein-coupled receptors (GPCR)

GPCRs are a large family of transmembrane receptors. As their name suggests, GPCRs are coupled to G-proteins with each receptor specific to each G protein. G proteins are a family of membrane-resident proteins which consist of three subunits – α, β and γ. The α subunit has a binding site with a resident GDP molecule. On activation by ligand, GPCR acquires a high affinity for αβγ and consequently, the GDP is replaced by GTP due to conformational change in the G-protein. This causes the α subunit to dissociate from βγ. Both α and βγ subunits then act on their respective targets to produce second messengers. Following a variable period (determined by the particular G protein activated), the ATPase activity of the α subunit hydrolyses GTP to GDP. This causes the α subunit to dissociate from the target enzyme and re-joining with the βγ subunit.

While G-proteins have myriad functions, of particular interest in the context of smooth muscle stimulation is its interaction with adenylyl cyclase – the enzyme that catalyses the production of cAMP from cytosolic ATP. In smooth muscle, cAMP-dependent protein kinase phosphorylates (thereby inactivating) MLK thereby causing a relaxation.

1.7.1.3 Role of Nitric Oxide

Nitric oxide is now recognised as an important mediator in smooth muscle function. It is synthesised from molecular oxygen and L-arginine by the action of NO synthase enzyme (NOS). NO is an activator of soluble guanylyl cyclase within the cell which causes increased synthesis of cGMP from GTP. cGMP inhibits $[Ca^{2+}]_i$-induced smooth muscle contraction. Three isoforms of NOS exist – two 'constitutive' isoforms (eNOS and nNOS) which are present in cells in the resting state, and one 'inducible' isoform (iNOS) which is activated in response to various stimuli. Certain arginine analogues N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methyl ester (L-NAME) are recognised inhibitors of NOS via competitive inhibition with arginine for the binding site.

1.7.1.4 Role of purines and purinergic receptors

Purines (adenosine and uracil) have recently been discovered as being yet another family of neurotransmitters. Their role in physiology is still being fully categorised. Purine receptors respond to either ATP, ADP, UTP or UDP to exert their actions. Three broad families of purine receptors are known – adenosine receptors (also called P_1 receptors) which act through GPCRs to modulate the concentration of cAMP; P2Y receptors which also act through GPCRs and effect their actions through cAMP and phospholipase C; and P2X ionotropic receptors which are multimeric ATP-gated cation channels. P2Y receptors have been identified in rat IAS.

1.7.1.5 Role of hydrogen sulphide (H_2S)

Hydrogen sulphide (H_2S) has been identified as the third gaseous mediator in the human body after NO and carbon monoxide (CO) [137]. H_2S is produced in the body

by reduction of cysteine via two enzyme – cystathione β-synthase (CBS) (present in brain and peripheral tissues including the gut) and cystathionine γ-Lyase (CSE) (found in peripheral tissues). Both CBS and CSE are present in the human enteric nerves [138] and it has been shown to have a pro-secretory effect on the human colon. H_2S has also been shown to inhibit *in vitro* intestinal motor patterns [137]. Aminooxyacetic acid (AOAA) is an inhibitor of CBS and DL-propargylglycine (PPG) inhibits CSE and have been used to study the effects of H_2S in the human gut [139]. L-cysteine is an exogenous substrate for H_2S.

1.8 Neuromyogenic properties of the internal anal sphincter (IAS)

The internal anal sphincter is a condensation of the circular smooth muscle of the distal rectum [140]. It is distinct from the rectal smooth muscle and has neuromyogenic properties which have been investigated both *in vivo* and *in vitro*. As the IAS provides a significant component of the resting anal pressure, it is an attractive site for potential drug treatments for FI. Any potential treatment that increases IAS tone and pressure would increase resting pressure of the anal canal and would potentially alleviate the symptoms of FI. And understanding of the neuromyogenic properties of the IAS is therefore essential to developing treatments for FI.

1.8.1 Myogenic properties of the IAS

The IAS maintains a normal continuous resting tone. This is punctuated by slow-wave contractions and ultra-slow wave contractions [141]. The normal tone of the IAS and the slow-wave activity is present even in anaesthetised patients with a paralysed EAS suggesting that this activity is independent of neuronal stimulation and respiration. Slow-wave frequency is highest at the distal anal canal suggesting that this may be a method of keeping the distal anal canal empty and thereby not fatigue the sensory neurones of the distal anus [142]. Ultra-slow waves are a feature observed with varying frequency in normal subjects and do not vary according to the level of the anus. This indicates that they may represent a synchronous contraction of the entire IAS [141]. Ultra-slow waves are more frequently seen in patients with haemorrhoids and are abolished in patients following haemorrhoidectomy suggesting that they may originate from the anal cushions. Sørensen *et al.* (1989) found a synchronous

contraction of the rectum and the IAS. They postulated that slow-wave IAS contractions prevents FI when rectal spontaneous activity exceeds resting anal tone [143]. Slow waves are present after curarisation and in isolated IAS strips indicating that these waves originate in the IAS [144, 145]. Ultra-slow waves and their clinical significance is yet to be categorised.

Slow-wave contractions have been shown to be dependent on extracellular calcium [146, 147]. Cook *et al.* (1999) demonstrated that spontaneous activity in the IAS and baseline tone was inhibited in calcium poor solutions *in vitro* and that noradrenaline-induced contractions were also abolished in calcium poor solutions. Nifedipine (a Ca^{2+} channel blocker) abolished tone and spontaneous activity but only mildly attenuated contractions to NA [146] – they concluded that tone and spontaneous activity were primarily due to ingress of extracellular Ca^{2+} whereas NA-induced contractions in the IAS are primarily due to release of Ca^{2+} from the sarcoplasmic reticulum. They confirmed their findings in a different set of experiments on pig IAS where the same results were obtained [147]. These results confirm that the IAS maintains slow-wave contractions independent of neuronal stimulation mediated by extracellular calcium.

1.8.2 Neuronal control of the IAS

Muscle innervation is either by somatic or by the autonomic nervous system. Smooth muscle receives only autonomic input. The autonomic nervous system can be divided into the parasympathetic and the sympathetic systems. In the parasympathetic system, the neurotransmitter is predominantly acetylcholine, in both pre- and post-ganglionic neurones. In the sympathetic system, the pre-

ganglionic neurotransmitter is acetylcholine with noradrenaline released from most post-ganglionic neurones (with few exceptions, e.g. the sweat glands where the neurotransmitter is acetylcholine).

Two broad categories of receptors have been identified which respond to acetylcholine – nicotinic and muscarinic. Nicotinic receptors are primarily pre-ganglionic and are excitatory. Muscarinic receptors are post-ganglionic and are responsible for mediating the effects of parasympathetic stimulation. Two distinct groups of receptors for noradrenaline are α and β receptors. In smooth muscle, α-adrenoceptors are primarily excitatory while β-adrenoceptors are primarily inhibitory.

The IAS receives sympathetic stimulation from the hypogastric plexus and parasympathetic stimulation from the first to third sacral nerves via the pelvic plexus. It is further supplied by the enteric nervous system. The enteric nervous system was first described by Langley in 1921 [148]. Two major plexuses exist – the myenteric (Auerbach's plexus) and the submucous (Meissner's)plexus. There are extensive connections between the two plexuses and together they are responsible for spontaneous gut peristalsis in the absence of autonomic stimulation.

Both *in vitro* and *in vivo* studies have been carried out to extensively investigate the IAS response to neurogenic stimulation in both humans and in other animal species.

1.8.2.1 In vivo studies

Incoming post-ganglionic sympathetic nerves to the GI tract are primarily inhibitory whereas the parasympathetic nerves are primarily excitatory [149]. Gaskell (1920)

proposed that parasympathetic stimulation causes relaxation whereas sympathetic stimulation causes contraction in the IAS [150] which contradicts the rest of the GI tract. Rankin *et al.* (1930) demonstrated contraction of the anal sphincter in response to stimulation of the presacral (sympathetic) nerve in an anaesthetised woman undergoing a sympathectomy for Hirschprung's disease [151] confirming that sympathetic innervation is excitatory to the IAS. These findings were challenged by Shepherd *et al.* (1968) who stimulated the presacral nerve in 14 subjects and found no contractions of the IAS. They report a 'clear inhibition' of the sphincter with a rebound contraction [152]. However, they acknowledge that stimulation of a whole nerve cluster, which may contain both sympathetic and parasympathetic elements, yields mixed results that are difficult to interpret. Carlstedt *et al.* (1988) performed stimulation of both the presacral hypogastric nerve and the periarterial lumbar colonic nerves in anaesthetised patients. They noted that epidural anaesthesia caused a reduction in anal canal pressures in all patients while stimulation of the nerves caused a contraction in the majority of patients. They concluded that the IAS received sympathetic excitatory stimulation from the presacral and the periarterial nerves [153]. These findings are contradicted by Lubowski *et al.* (1987) who found a decrease in anal pressure on stimulation of the presacral hypogastric nerves [154]. The reason for this difference is not obvious but may reflect differences in stimulation parameters and background sympathetic activity [155].

Frenckner *et al.* (1976) observed anal canal pressures in patients with either high spinal anaesthesia (blocking both sympathetic and parasympathetic outflow) and low

spinal anaesthesia (blocking only parasympathetic stimulation) and found that there was a significantly greater decrease in anal canal pressures with high spinal anaesthesia. They concluded that the IAS receives tonic excitatory stimulation from sympathetic nerves with no background stimulation from parasympathetic nerves [156].

Infusion of phentolamine, an α adrenoceptor antagonist has been shown to reduce the anal canal pressure indicating that noradrenaline acts via α-adrenoceptors to cause an excitation and contraction in the IAS [157].

A table summarising these contradictory findings is provided.

Study	Sympathetic contraction	Sympathetic relaxation
Gaskell [150]	Yes	
Rankin *et al* [151]	Yes	
Shepherd *et al* [152]		Yes
Carlstead *et al* [153]	Yes	
Lubowski *et al* [154]		Yes
Freckner *et al* [156]	Yes	

Table 1 – Summary of in vivo studies on human IAS

Four out of the six suggest that sympathetic stimulation causes contraction of the IAS and parasympathetic stimulation causes relaxation of the IAS. Of two studies which suggest relaxation in response to sympathetic stimulation, one of them (Shepherd *et*

al), stimulated the whole nerve plexus and may well have caused both sympathetic and parasympathetic stimulation.

The available evidence suggests that sympathetic stimulation causes contraction in the IAS and parasympathetic stimulation causes relaxation of the IAS.

1.8.2.2 In vitro studies

In vitro studies of the IAS are more numerous than *in vivo* studies. Adrenergic, cholinergic and non-adrenergic-non-cholinergic (NANC) neurotransmitters have been investigated in human and animal specimens. Animal studies are discussed later and the human *in vitro* studies are described here.

Parks *et al.* (1969) [158]investigated the response of isolated IAS strips from 87 subjects. They found that the IAS developed a steady baseline tone which was unaffected by hexamethonium, an antagonist of nicotinic receptors in autonomic ganglia which effectively inhibits all neuronal transmission in post-ganglionic neurones. This suggested that the baseline tone was independent of neuronal stimulation. In their experiments, they found that noradrenaline (NA) caused a contraction in the IAS which could be antagonised by phenoxybenzamine, a selective antagonist of α-adrenoceptors. In the presence of phenoxybenzamine, NA caused a relaxation which was sensitive to pronethalol, a non-selective β-adrenoceptor antagonist. Their results indicate that NA released by sympathetic nerves causes a contraction mediated by α adrenoceptors and a relaxation mediated by β adrenoceptors. These results were confirmed by O'Kelly *et al* (1993) who also describe contraction of the IAS in response to α-adrenoceptor stimulation and relaxation in response to β-adrenoceptor stimulation [159].

Burleigh *et al.* (1979) [160] used electrical field stimulation (EFS) to further investigate the neurotransmitters involved in the IAS response to nerve stimulation. EFS caused a relaxation in IAS strips which was abolished by the presence of tetrodotoxin, an inhibitor of nerve impulses by binding to voltage gated sodium channels on nerves. This confirmed that the relaxations were due to nerve stimulation of the IAS. The relaxations were not abolished by blocking adrenoceptors and cholinergic receptors indicating a NANC neurotransmitter. Burleigh *et al* (1979) also discounted 5HT, histamine, prostaglandin and dopamine as possible NANC transmitters and suggested vasoactive instestinal peptide (VIP) and adenosine triphosphate (ATP) as potential transmitters although the evidence for ATP as the neurotransmitter was not clear.

O'Kelly *et al.* (1993) [161] identified nitric oxide as the primary neurotransmitter responsible for the relaxations observed in response to EFS. In their experiments, EFS produced relaxation in the IAS which was abolished by tetrodotoxin, confirming that the response was neuronally mediated. The EFS induced relaxations were not abolished in the presence of atropine (a muscarinic antagonist) and guanethidine (an antagonist to NA which prevents the release of NA from nerves) indicating that neither NA nor acetylcholine (Ach) were responsible for the relaxation. These relaxations were abolished by antagonists to nitric oxide synthase. The relaxations were restored by the addition of L-arginine, a nitric oxide donor. This indicates that nitric oxide is the NANC neurotransmitter involved in the relaxation produced by nerve stimulation in the IAS observed by Burleigh *et al.* (1979) [160].

The findings of O'Kelly *et al* (1993) were confirmed by Glavind *et al.* (1997) who conducted experiments on IAS strips and subjected them to EFS. They found that the relaxation to EFS was abolished by the addition of N-nitro-L-arginine (L-NNA), an inhibitor of nitric oxide synthase, and a contraction to EFS was revealed in half the preparations. This contraction was abolished by the addition of phentolamine. They concluded that the relaxatory neurotransmitter in the IAS is nitric oxide and the excitatory transmitter is NA acting via α adrenoceptors [162].

The role of mAchR is more difficult to establish from the published studies. Parks *et al.* (1969) [158] stimulated IAS strips with both acetylcholine and nicotine. They found that acetylcholine caused a contraction in the majority of tissues but also caused a relaxation in some tissues. Nicotine caused a relaxation only. Burleigh *et al.* (1979) [160] found that IAS relaxed in response to Ach stimulation which was antagonised by Hyoscine, which would indicate that the relaxation was due to mAchR stimulation. However, when tetrodotoxin (an inhibitor of nerve action potentials) was added, it abolished the relaxation noted to Ach and to bethanechol (a stimulator of mAchR). This would indicate that the relaxations noted were due to stimulation of post-ganglionic nerves which caused a release of a second mediator which caused the relaxation noted. O'Kelly *et al.* (1993) [159] found that carbachol (a cholinergic agonist) caused a relaxation in the IAS which was antagonised by atropine (an antagonist of mAchR) which provides more convincing evidence of the relaxatory effect of mAchR stimulation.

A table summarising these findings is provided.

Study	Tone	α adreno-receptors	β adreno-receptors	NO	mAchR	EFS
Parks *et al* [158]	Yes	Contraction	Relaxation		Contraction	
O'Kelly *et al* [159]	Yes	Contraction	Relaxation		Relaxation	
Burleigh *et al* [160]	Yes		Relaxation		?Relaxation	Relaxation
O'Kelly *et al* [161]	Yes			Relaxation		Relaxation
Glavind *et a-* [162]	Yes	Contraction		Relaxation		Relaxation

Table 2 – Summary of in vitro studies on human IAS

Abbreviations – NO nitric oxide; mAchR muscarinic acetylcholine receptors; EFS electrical field stimulation.

In summary, there is more agreement in the *in vitro* studies compared to the *in vivo* studies. In all the studies, myogenic tension produced stable baseline tone in tissues. The studies investigating α and β-adrenoceptors all report a contraction mediated by α-adrenoceptors and a relaxation mediated by β-adrenorecptors. Stimulation of mAchR produced relaxation in two studies. In the three studies investigating the effects of EFS, all three report a relaxation. This correlates well with *in vivo* findings where a majority of studies found a contraction in response to sympathetic

stimulation and all studies report a relaxation in response to parasympathetic stimulation.

1.8.3 Animal experiments

A number of different species have been used to study the IAS. These include cats, rats, opossums, sheep and pigs. Both *in vivo* and *in vitro* studies have been performed.

1.8.3.1 In vivo studies

Kerremans *et al.* (1970) investigated the effects of α and β-adrenoceptor stimulation in anaesthetised cats. Their results indicate that stimulation of α-adrenoceptors via sympathetic stimulation causes a contraction in the IAS. The results for β-adrenoceptor stimulation are less clear but appear to have an inhibitory effect on the IAS [163]. These results were confirmed by Bouvier *et al.* (1981) [164] who performed stimulation of the hypogastric nerve and the presacral nerves in anaesthetised cats. Their results show a contraction in response to the hypogastric nerve (sympathetic) which was abolished by the addition of phentolamine. Stimulation of the presacral nerves (parasympathetic) caused a relaxation which was neither adrenergic nor cholinergic. They concluded that sympathetic stimulation produced contractions in the IAS mediated by noradrenaline acting on α-adrenoceptors and parasympathetic stimulation caused a relaxation mediated by acetylcholine acting on pre-ganglionic neurones stimulating post-ganglionic neurones to release a NANC transmitter.

Nurko *et al.* (1989) investigated the role of VIP in anaesthetised opossums and found a significant relaxation in the IAS in response to VIP [165].

1.8.3.2 In vitro studies

1.8.3.2.1 Opossum

Lynn *et al.* (1995) demonstrated the presence of VIP-containing and NO-containing neurones in the opossum IAS tissues using immunohistochemistry. They found presence of these neurones in both the myenteric and submucous plexuses in the opossum [166].

Chakder *et al.* (1996) demonstrated that the mechanism by which VIP caused relaxation of the IAS was by stimulating the production of NO via NO synthase. In their experiments on the opossum IAS, they found a significant increase in NO production in the myenteric plexus of the IAS in response to VIP stimulation [167].

Rattan *et al.* (1992) confirmed that NO was the primary mediator of relaxation in the IAS of opossums [168]. In their study, the relaxation of the IAS in response to VIP was also inhibited by inhibition of NO synthase. This would further support the findings of Chakder who concluded that VIP causes a relaxation by upregulating NO synthase activity [167]. The same authors later confirmed these findings in the opossum by demonstrating that NO was the neurotransmitter released during EFS of IAS strips [169].

1.8.3.2.2 Rat

De Luca *et al.* (1999) [170] investigated the IAS in rats and also found a significant nitrergic component to the relaxations induced by EFS. However, in their experiments, NO was not the only neurotransmitter identified. They found a reduction in EFS-induced relaxations to NO synthase inhibitor only in the presence of tubocurarine (an antagonist at the nicotinic *acetylcholine* receptor).

These results were confirmed by Opazo *et al.* (2011) who found a dual inhibitory stimulation in the rat IAS from both NO and purinergic pathways acting via P2Y receptors [171]. Evidence for purinergic relaxations acting via P2Y receptors were also provided by McDonnell *et al.* (2008) in their experiments on mice [172].

Keef *et al.* (2013) blocked both NO and purinergic pathways in their experiments on rat IAS. They found that in the wild type species, long trains of EFS stimulation produced relaxations independent of the nitergic and purinergic pathway. This response was sensitive to VIP antagonists. Further, this non-nitrergic-non-purinergic relaxation was absent in VIP receptor–ve mice. They concluded that VIP plays a role independent of NO and purinergic pathways in ultraslow relaxation of the rat IAS [173].

1.8.3.2.3 Sheep

The sheep IAS has been extensively investigated. Sheep IAS develops tone and displays transient relaxation in response to EFS which is sensitive to tetrodotoxin [174]. Mundey *et al.* (2000) also noted that the addition of L-NAME, an inhibitor of NO synthase, completely abolished the relaxatory response and unmasked a contractile response to EFS. This response was abolished by phentolamine and prazosin. These results indicate that sheep IAS relaxes to neuronal stimulation mediated by NO and there is a contractile element mediated by α_1-adrenoceptors. Acheson *et al.* (2009) confirmed the findings of Mundey *et al.* (2000) [174]. In their series, relaxation to EFS was increased in the presence of bretylium (an adrenergic neurone blocker) and prazosin. They also found that MRS2179 (P2Y1 receptor antagonist) had a modest inhibitory effect on the relaxation produced by NO. As with

the rat, there may be a component of purine induced relaxation in the neurogenic relaxation in sheep although the response is primarily mediated by NO [175].

Resting myogenic tone of the sheep IAS has been shown to be affected by nitric oxide donors and calcium blocking agents. Jonas-Obichere *et al.* (2005) found a significant reduction in resting IAS tone after the addition of sodium nitroprusside, diltiazem, glyceryl trinitrate (GTN), nifedipine and verapamil [176] indicating that NO caused relaxation in the sphincter and $[Ca^{2+}]_i$ caused a contraction.

1.8.3.2.4 Guinea-pig

The guinea-pig IAS has been shown to relax in response to EFS mediated by NANC neurotransmitters [177]. These NANC neurotransmitters have been identified as NO and a purine receptor agonist [178].

1.8.3.2.5 Summary of animal *in vitro* studies

Most of the studies on animals concur with those on humans – parasympathetic stimulation causes a relaxation mediated by NO, sympathetic stimulation causes a contraction via α-adrenoceptors. In the guinea pig, rat and sheep, there may a small contributory role of purine receptors in the relaxation to EFS.

1.9 Neuromyogenic properties of the rectum

The rectum has been investigated in both *in vivo* and *in vitro* studies in both human and animal experiments.

1.9.1 *In vivo* studies (human rectum)

Carlstedt *et al.* (1988) [153] stimulated the presacral HGN (sympathetic) and lumbar colonic nerves (parasympathetic) in anaesthetised patients – IAS contracted to HGN

stimulation preceded by relaxation. The rectum mostly contracted but weak responses were noted. Epidural anaesthesia in patients encompassing thoraco-lumbar region blocking sympathetic discharge relaxed the IAS and contracted the rectum. They concluded that sympathetic stimulation contracts IAS and both contracts and relaxes the rectum.

1.9.2 *In vitro* studies (human rectal tissue)

Numerous authors have studied the *in vitro* properties of the IAS and the rectum [146, 147, 159, 162]. Both O'Kelly [159] and Cook [146] demonstrated that the IAS developed spontaneous activity and tone in response to stretch whereas the rectal smooth muscle developed spontaneous activity only and no tone. In both studies, NA caused a contraction in the IAS while it abolished spontaneous activity in the rectum. The rectum contracted in response to carbachol, a stimulator of acetylcholine receptors, whereas the IAS relaxed. O'Kelly further noted that the addition of NA to pre-contracted rectal strips induced a relaxation [159] which was reproduced by the addition of both phenylephrine, an α-adrenoceptor agonist, and isoprenaline, a β-adrenoceptor agonist. Cook investigated the role of calcium and found that spontaneous activity and tone was abolished in both tissues in calcium-free solutions and by the addition of nifedipine, a calcium channel blocker [146]. However, after store loading with calcium, nifedipine inhibited contraction to agonists more in the rectum than in the IAS which indicated that contraction in the IAS was more dependent on release of calcium from intracellular stores whereas in the rectum it was primarily mediated by calcium ingress from extracellular sources. In contrast to the two studies mentioned, Glavind *et al.* [162] noted that rectal

circular smooth muscle developed intrinsic tone. Glavind used EFS to categorise the response to neural stimulation in IAS and rectal circular smooth muscle. Both tissues relaxed in response to EFS and the relaxation was inhibited by the addition of Nω-nitro-L-arginine, an inhibitor of NO synthase, in a dose-dependent manner. After antagonising NO, they found that most tissues contracted in response to EFS. This contraction was abolished by phentolamine in the IAS and by atropine in the rectum. They concluded that EFS induces relaxations in both rectum and IAS via NO and cholinergic excitatory stimulation via muscarinic receptors causes contractions in the rectum whereas adrenergic excitatory stimulation via α-adrenoceptors causes contraction in the IAS.

The effect of EFS has also been investigated by Stebbing *et al.* (1998) [179] and Brading *et al.* (1997) [180]. Stebbings found that, like O'Kelly and Cook, rectal smooth muscle did not develop intrinsic tone but demonstrated spontaneous activity. Increasing concentrations of carbachol caused increasing magnitude of contractions sensitive to atropine but not hexamethonium, a ganglion blocking agent. NA caused a reduction in the frequency of spontaneous activity in both longitudinal and circular smooth muscle. Stable increase in tone was achieved by the addition of histamine. EFS produced relaxations in precontracted strips which were reduced in a dose-dependent manner by the addition of Nω-nitro-L-arginine. This was reversed by the addition of L-arginine. Sodium nitroprusside caused dose-dependent relaxations in pre-contracted strips. Immunohistochemistry showed staining for NO-containing neurones in the myenteric plexus of nerves. In their experiments Brading *et al.* (1997) investigated the response of the longitudinal layer of rectal smooth muscle in

human tissue [180]. In their experiments, the tissue developed spontaneous activity and no baseline tone. Tone was increased by the addition of carbachol (sensitive to atropine) and spontaneous activity was reduced by the addition of both phenylephrine and NA. Histamine was used to produce stable baseline tension. In pre-contracted tissue, EFS produced relaxations which were abolished in a dose-dependent manner by L-NOArg.

1.9.2.1 Summary

Of the five studies on the human rectum *in vitro*, two compared IAS to rectum (O'Kelly [159], Cook [146]) while the remaining three studies investigated the rectum only. All studies barring one (Glavind [162]) mention that the rectum does not develop tone on stretch but develops spontaneous activity. Three studies investigated the effect of EFS on rectal smooth muscle, and all demonstrated a relaxation in response to EFS mediated by NO (Glavind [162], Brading [180], Stebbing [179]). Three studies demonstrate either abolition of spontaneous activity (Brading [180], Cook [146]) or relaxation of the rectum in response to NA (O'Kelly [161]). Two studies demonstrate a contraction in response to carbachol (O'Kelly [161], Stebbing [179]).

We conclude that on the basis of the available evidence, nerve stimulation of the rectum causes a relaxation mediated by NO. Stimulation of mAchR causes a contraction whereas stimulation of adrenoceptors causes a relaxation.

Both the studies comparing IAS to rectum note that the IAS contracts to α-adrenoceptor stimulation where as the rectum relaxes, and the rectum contracts in response to acetylcholine whereas the IAS relaxes.

1.9.3 Animal experiments

1.9.3.1 In vivo experiments

Helund and Carlsted performed experiments on anaesthetised cats [181, 182]. In both cases, the pelvic parasympathetic nerves were stimulated. Both experiments found a primarily excitatory response in the rectum to parasympathetic stimulation. In both cases, contraction was mediated through mAchR. Carlsted also investigated the response in the IAS in the same set of experiments and found a dual response – with high intensity stimulation, the IAS would contract whereas with low intensity stimulation the IAS would relax (whereas the rectum would contract to both). However, if the sympathetic nerves were cut, stimulation at all frequencies would elicit a contraction in the IAS. They concluded that at low intensity, parasympathetic stimulation caused a relaxation mediated via the sympathetic nerves in the IAS. The relaxation of the IAS was mediated by NANC transmitters and the contraction was via both adrenergic and cholinergic transmitters.

Anderson *et al.* (2006) investigated the IAS and the rectum in anaesthetised minipigs [183]. Sympathetic stimulation produced a contraction in the IAS mediated by adrenergic receptors and no response in the rectum. Parasympathetic stimulation caused the IAS to relax but had a different response in the rectum dependent on site – the proximal rectum contracted whereas the distal rectum relaxed. Møller also investigated IAS and rectal response to parasympathetic stimulation in pigs [184] and confirmed the findings of Andersen – contraction in the proximal rectum, relaxation in the distal rectum and IAS in response to parasympathetic stimulation. Further, they confirmed that the relaxation in distal rectum and IAS was mediated by NO.

1.9.3.2 In vitro studies

Cook *et al.* (1999) investigated the role of calcium in pig rectal and IAS tissue [147]. Their experiments show that IAS developed tone whereas rectal smooth muscle did not. IAS contracted to NA and rectal smooth muscle contracted in response to carbachol. Contractions were abolished by nifedipine and in calcium free solutions. However, after loading of calcium stores, nifedipine inhibited carbachol induced contractions in the rectum significantly more than NA induced contractions in the IAS. These results indicate that rectal smooth muscle in pigs relies on extracellular calcium for agonist-induced contractions whereas the IAS primarily relies on calcium stored in the sarcoplasmic reticulum.

Stebbing *et al.* (1995) also performed *in vitro* studies on porcine rectal tissue [185]. They found that porcine rectal tissue did not develop tone but displayed spontaneous activity. Histamine was used to produce stable contractile state. EFS in pre-contracted strips produced relaxations in the presence of atropine and guanethidine. The relaxation was abolished by L-NOArg, a competitive inhibitor of NO synthase, in a dose-dependent manner. Addition of sodium nitroprusside to pre-contracted strips caused a relaxation similar to that noticed with EFS. These results suggest that in response to EFS (a surrogate for neuronal stimulation), NO is released which causes a relaxation in the rectal tissue of pigs.

1.10 The role of α-adrenoceptors

Recent evidence suggests that in human colonic muscle, α-adrenoceptors are not involved in mediating either contraction or relaxation [186]. While previous authors have suggested a role for α-adrenoceptors [187] more recent authors suggest that

contractions in response to neuronal stimulation occurs by cholinergic stimulation while relaxation is mediated by nitregic nerves [186].

In the rectum and the IAS, α-adrenoceptors play a significant role. *In vitro* experiments on human IAS tissue investigating α-adrenoceptors have been reported by a number of authors [158, 159, 162]. Glavind (1997) demonstrated that relaxation to EFS was increased significantly in the IAS following the addition of phentolamine (an α-adrenoceptor antagonist) indicating that NA released during EFS stimulation was contractile in the IAS [162]. Furthermore, when the relaxation to EFS was abolished by antagonising NO, the further addition of phentolamine significantly reduced the contractions secondary to EFS. O'Kelly (1993) demonstrated that isolated IAS strips contracted when stimulated with NA but relaxed when NA was added in the presence of phentolamine indicating that α--adrenoceptors caused contraction while β -adrenoceptors mediated relaxation in the IAS [159]. Parks (1969) demonstrated a contraction in the IAS with addition of NA which was changed into a relaxation with addition of phenoxybenzamine (an α -adrenoceptor antagonist), the relaxation was abolished by adding pronethalol (a β -adrenoceptor antagonist) [158]. Therefore, all three authors demonstrated a contraction to α-adrenoceptor activation and relaxation following stimulation of β-adrenoceptors. Also, all authors note that there was no change in the intrinsic tone of the IAS following addition of either α or β-adrenoceptor antagonists indicating that baseline tone is independent of neuronal stimulation.

In vitro experiments on rectal tissue investigating α-adrenoceptors have been performed by two authors [159, 162]. Glavind *et al.* (1997) [162] investigated rectal

tissue with EFS and found a relaxation to EFS which was unaffected by the addition of phentolamine. After antagonising the effect of NO, a contraction to EFS was unveiled which was also unaffected by the addition of phentolamine but abolished by the addition of atropine (an mAchR antagonist). They therefore concluded that α-adrenoceptors do not play a significant role mediating the neuronal response in rectal tissue. O'Kelly *et al.* (1993) [159] found that rectal tissue did not generate tone on passive stretching. In the relaxed state, NA did not affect tone in rectal smooth muscle. Once pre-contracted with carbachol (an mAchR agonist), NA caused significant relaxation in rectal smooth muscle. This relaxation could be reproduced by isolated stimulation of both α and β-adrenoceptors using phenylephrine and isoprenaline respectively. Therefore, both authors agree that in the rectal tissues, α-adrenoceptor stimulation produced a relaxation. Other authors [146, 188] have shown a reduction in spontaneous activity of the rectal smooth muscle with the addition of noradrenaline and α-adrenoceptor agonists supporting the theory that α-adrenoceptor stimulation causes relaxation in the rectum.

One criticism of the above-mentioned studies is that none of them used inhibitors to confirm the action of α-adrenoceptors. Where NA was used to induce relaxation/reduce spontaneous activity [146, 159, 180, 188], the effects noted may well have been due to a predominant action of β-adrenoceptors. Where α agonists were used, O'Kelly [159] used phenylephrine at a concentration of 0.1 mM and Stebbing [188] used phenylephrine at a concentration of 10 μM – both concentrations are capable of producing dual α and β stimulation [189]. Of note,

Stebbing et at confirmed that the β agonist isoprenaline also produced the same effect at 10 μM phenylephrine.

In vivo experiments on human IAS tissues investigating α-adrenoceptors are described in the section on phenylephrine and methoxamine.

Traditionally, α adrenorecptors have been noted to cause relaxation in intestinal smooth muscle. In their seminal paper on the classification of adrenoceptors, Ahlquist [190] first sub-classified adrenoceptors in to α and β receptors. They found that α-adrenoceptors were excitatory in the majority of tissues studied but were inhibitory in the case of the intestine. Of note, they used strips of terminal ileum and not colon in their experiments. Two further papers from the same author [191, 192] confirmed the relaxatory effect of α-adrenoceptors in the terminal ileum in *in vitro* and *in vivo* experiments respectively. However, more recent evidence suggests that α-adrenoceptors may be associated with contraction in the colon. Lukensmeyer [193] investigated the colon in the rat *in vitro* and found that (sympathetic) nerve-mediated contractions in the circular muscle of the colon was completely inhibited by 3 μM phentolamine (an α antagonist) and was unaffected by 0.1 μM Propranolol (a β-adrenoceptor antagonist). Venkova *et al.* (1993) [194] studied the cat colon *in vitro* by stimulating the lumbar colonic nerves. They found a contraction to stimulation which was inhibited by phentolamine (a non-specific α antagonist) and prazosin (a selective α_1 antagonist) and potentiated by propranolol (a β antagonist) also confirming that α-adrenoceptor stimulation in the colon causes a contraction. Venkova *et al.* (1994) also investigated the effect of noradrenaline on cat colonic tissue in the absence of neuronal stimulation [195] and found a significant increase

in tone antagonised by prazosin. In this study, Venkova also noted that there was no response to α_2 stimulation of the colonic circular smooth muscle. In their two studies, Venkova *et al.* (1994) [195] also found evidence of purinergic receptors mediating the response to noradrenaline and the neuronal stimulation.

From the available evidence, there appears to an unresolved debate on the action of α-adrenoceptors in the rectum. We will attempt to investigate this in our experiments.

1.11 Evolving drug treatments for FI

Given the paucity of effective treatments for FI, new drug targets have focussed on modulating the tone of the IAS in an attempt to improve symptoms of incontinence by locally raising anal tone. The IAS contracts in response to NA in a response mediated by α-adrenoceptors. Two locally acting α-adrenoceptor agonists have been investigated – phenylephrine and methoxamine – and will be discussed in the following sections.

1.11.1 Phenylephrine

Intravenous phenylephrine has been described as increasing IAS resting pressures in anaesthetised opossums [196]. In their study, Yamato *et al.* (1990) [196] noted that the rise in IAS pressure was reversed by prazosin, a selective α_1-adrenoceptor antagonist. Further, clonidine, an α_2-adrenoceptor agonist, did not affect baseline IAS tone. They concluded that the IAS contracts in response to α_1-adrenoceptor agonists. Based on these findings, Carapeti *et al.* (1999) used topical phenylephrine gel at the anal verge of healthy volunteers and found a significant rise in anal resting pressure with the use of 10% topical phenylephrine gel [197]. Following this,

Cheetham *et al.* (2001) demonstrated a significant, sustained rise in anal resting pressure in a dose-dependent manner following administration of varying concentrations of phenylephrine gel to patients with FI [198].

In a later study, Carapeti *et al.* (2000) [199] investigated the use of topical phenylephrine gel in a randomised clinical trial (RCT) on patients with FI following ileo-anal pouch reconstruction. Their results demonstrated significant improvement in continence scores and increased mean anal resting pressure (MARP) following the use of phenylephrine gel compared to placebo. They did not report any significant adverse events. However, in a further RCT testing 10% topical phenylephrine gel in patients with normal sphincters and FI, Carapeti *et al.* (2000) found no significant differences between the treatment group and the placebo group with regards to MARP, incontinence scores or anodermal blood flow [200].

Badvie *et al.* (2005) reported a significant improvement in patients with radiation induced FI with the use of topical 10 or 20% phenylephrine gel [201]. However, this small retrospective study was contradicted by Park *et al.* (2007) who found no significant change in faecal incontinence severity index (FISI) or faecal incontinence quality of life (FIQoL) scores in their RCT investigating 30% phenylephrine gel in patients with FI following low anterior resection for rectal cancer. Further, Park *et al.* found a proportion of patients developed allergic dermatitis or headaches [202].

α_1-adrenoceptors are also present in the vascular tree. A theoretical concern with high concentrations of topical preparations is the potential for systemic absorption and consequent hypertension with reflex bradycardia. Perineal burning may be a

sign of systemic absorption and headaches may be a feature of underlying vasoconstriction.

1.11.2 L-erythro-methoxamine (LEM)

Jones *et al.* (2003) examined the relative potency of phenylephrine and methoxamine on porcine IAS strips [203]. They found a comparable concentration-dependent contraction in IAS strips to both phenylephrine and methoxamine which was inhibited by phentolamine, an α_1-adrenoceptor antagonist. Methoxamine is a racemic mixture of 4 stereoisomers. Isolation of L-erythro-methoxamine showed that it was 4 times more potent than either methoxamine racemate or phenylephrine in causing IAS contractions.

Nissar *et al.* (2005) investigated perianal, intra-anal and rectal application of L-erythro-methoxamine in healthy volunteers and measured MARP and cardiovascular parameters [204]. They found that perianal application did not increase MARP. Intra-anal application of 1-3% gel caused a significant rise in MARP but there was also a consequent increase in systolic blood pressure and a significant drop in heart rate. Six out of sixteen volunteers reported nausea and three reported headaches following gel application. Following this, Nissar *et al.* (2007) conducted a study on 10 patients with FI using intra-anal 0.3% and 1% LEM gel [205]. They found a significant and rapid increase in MARP to near normal resting pressures in all patients. The increase in MARP was accompanied by a significant decrease in heart rate but no other adverse events were reported.

Rayment *et al.* (2010) confirmed the application of LEM *in vitro* using a sheep model [206]. In their experiments, they confirmed the presence of α_1-adrenoceptors in the

sheep IAS using immunohistochemistry and confirmed the action of LEM in contracting the IAS in sheep.

Recently, a multiple-dosage RCT has been completed investigating the safety and efficacy of LEM in the treatment of FI (unpublished data). This is an industry run study using a modified-release preparation of LEM (NRL-001) used as a daily suppository in patients with FI. The patients recruited at our centre for this study will be presented as part of this thesis.

1.12 Thesis aims

The aims of this thesis are to evaluate new drug treatments for FI. As described, conservative and surgical measures for the treatment of FI have less than satisfactory outcomes. The established drugs for FI aim to increase colonic transit time in order to make the stool firmer, thereby aiding continence, but do not augment any of the mechanisms of continence.

Of the two new drugs being developed, LEM will be the main object of this project. As mentioned, LEM suppositories are being investigated in an international multicentre randomised control trial on patients with FI to establish efficacy and safety of the drug. The patients recruited to this trial from our centre will form the basis of the clinical element of this research project.

The neuromyogenic properties of the human IAS have been extensively investigated and do not require further revision. However, the properties of the rectum have not been investigated as thoroughly. There is also a disagreement in the published evidence on the properties of the rectum. As mentioned previously, LEM is an α_1 agonist and would cause vasoconstriction in the systemic circulation giving symptoms of headache and hypertension. Therefore, LEM use as suppositories has the best chance of achieving the contraction of the IAS which would ease the symptoms of FI while not causing systemic side-effects. However, the effect of α agonists in the rectum have not been adequately researched. Ideally, the best agent for the treatment of FI would cause a contraction in the IAS while causing a relaxation in the rectum. The laboratory-based section of this project aimed to identify the

neuromyogenic properties of the rectum and to study the effects of methoxamine on rectal tone to establish this.

Human rectal tissue is difficult to obtain. Therefore, two surrogate species were studied in the course of this project – sheep and pig. In both cases, tissue from freshly culled animals was obtained from a local abattoir. The initial aims were to validate our experimental model against that of published studies on the IAS and then use this validated model to investigate rectal tissue. Sheep tissue was used in the first set of experiments and pig tissue was used for the second set of experiments. These form chapters 2 and 3 of this thesis respectively. The final chapter presents the data on the 10 patients recruited in the clinical trial on LEM performed at our centre.

The aims of the thesis were:

- To identify a surrogate tissue for human rectal tissue – comparing sheep and pig rectal tissues with published data on human rectal tissue
- To investigate the neuromyogenic properties of the rectum in the animal model
- To identify the role of α_1-adrenoceptors in the animal model and postulate the role of α_1-adrenoceptors in the human tissue
- To test the safety and efficacy of LEM suppositories in the treatment of patients with FI

2 Chapter 2 – Studies on sheep rectum and IAS

2.1 Aim

The aim of the first set of experiments was to develop a reproducible protocol to investigate the neuronal responses in the sheep IAS and rectum. We then intended to use the protocol to investigate the neuronal mediators in the sheep IAS and rectum.

2.2 Introduction

Sheep IAS has been extensively investigated in the past. In summary, sheep IAS relaxes in response to electrical field stimulation (EFS), the relaxation being mediated by nitric oxide; contraction to EFS is due to α-adrenoceptor stimulation [174, 175]. This correlates well with human IAS tissue which also relaxes in response to EFS [159, 160, 162], the relaxation being mediated by NO [161, 162], and contracts in response to α-adrenoceptor stimulation [158, 159, 162].

In the first set of experiments, we sought to reproduce the results from previously published studies on the sheep IAS, thereby validating our experimental protocol. Investigating the sheep IAS was not the primary aim as this has been extensively investigated in the past. The aim was to use our validated experimental protocol and use the same protocol to investigate sheep rectal tissue. To the best of our knowledge, sheep rectal tissue has not been investigated in this fashion in the past. The aim was to investigate the effects of EFS on sheep rectal tissue and to identify the neuronal mediators involved in the response of rectal tissue to nerve stimulation

(simulated by EFS). This would allow us to investigate the effects of methoxamine on sheep rectal tissue.

2.3 Experimental material and methods

2.3.1 Aims

1. Develop a reproducible experimental protocol
2. Use sheep IAS to compare results with published data
3. Use this protocol to investigate the neuromyogenic properties of the sheep rectum
4. Compare the neuromyogenic properties of the sheep rectum to published data to the human rectal tissue
5. Investigate the effects of α_1-adrenoceptor agonists on the sheep rectum

2.3.2 Preparation of the tissue

Sheep rectal tissue was obtained from a local abattoir within one hour of culling. Tissue obtained consisted of distal colon and perineum of culled sheep.

This tissue was transported in Krebs-Henseleit solution in ice. The composition of Krebs–Henseleit solution was (mmol L^{-1}) NaCl 118.4, KCl 4.7, $CaCl_2$ 1.25, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1. Within 4 hours of culling, the tissue was further dissected to isolate strips of circular smooth muscle of the rectum or strips of the IAS as appropriate to the experiment planned.

Initially, the distal colon along with perineum was dissected from the abattoir specimen. The colonic 'tube' was cut to lay open the mucosa. The external pelvic floor musculature was identified and dissected free. The mucosa was dissected

exposing the underlying circular smooth muscle layer. The underlying longitudinal layer of muscle was dissected identifying the internal anal sphincter until only a layer of circular smooth muscle and sphincter remained. For experiments on the rectal smooth muscle, strips of the smooth muscle were cut along the length of the muscle fibres between 1 and 2 centimetres from the edge of the IAS. For IAS experiments, strips of muscle were cut 0.5cm from the anal verge along the length of the muscle fibres. The final tissue samples were 1cm (length) x 0.5cm (width) strips cut along the line of the circular smooth muscle/sphincter muscles. Tissues were allowed to equilibrate at room temperature for one hour following dissection.

The initial experimental results showed that often tissues did not respond to EFS at all, or that the EFS response was of low magnitude. In the first few months, the tissues had been preliminarily dissected in the afternoon following delivery from the abattoir. Final dissection of the smooth muscle layer and suspension in the organ bath was performed the following day, the tissues having been stored in the fridge suspended in Krebbs'-Henseleit solution. After the initial few months, a trial of full dissection and experimentation on fresh tissue demonstrated that the tissue response was more vigorous if the tissues had not been stored overnight, and therefore all following experiments were performed on fresh tissue – i.e. tissues would be dissected the same afternoon of culling and experiments performed straight after dissection. The results from the initial experiments were discarded and all the reported results follow this new protocol. The organ bath setup is described next.

2.3.3 Setting up the organ bath

The tissue was then tied at both ends using silk thread. This was then tied to a Perspex 'arm' in between 2 electrodes. This was then suspended in an organ bath containing Krebs-Henseleit solution kept at a constant temperature of 37^{o}C. Solution oxygen tension was maintained by constant infusion of 95% Oxygen/5% Carbon-Dioxide gas maintaining a constant pH of 7.4. The long end of the string was attached to a pressure transducer. After preliminary experiments, it was noted that the responses to tissues varied significantly on qualitative analysis. This was confirmed by adding 1 µM tetrodotoxin – it was noted that some tissues continued to respond to EFS while in other tissues there was no response following the addition of tetrodotoxin. We surmised that in tissues where the response continued despite tetrotodotoxin, the tissue was touching the electrodes and the response noted was direct electrical stimulation of the muscle fibres. Therefore, a method was required for standardisation of the tissue sizes although they were not weighed initially. A jig was designed using a wooden plank to ensure that all tissues were equally sized. The jig was fashioned so that the tissue could be laid out flat on its surface with markings corresponding to 0.5 cm width and 1 cm length and grooves cut into the wood to facilitate tying the tissue at appropriate lengths. After the tissue was sized (0.5cmx1cm), silk thread was placed in the grooves previously fashioned and the tissue was tied at each end. This resulted in identically-sized tissues (0.5cmx1cm) tied at either end. Experiments following this showed a consistent response to EFS and all tissues tested with tetrodotoxin following the use of the jig stopped responding to EFS stimulation.The final setup is shown in the figure (Figure 2-1).

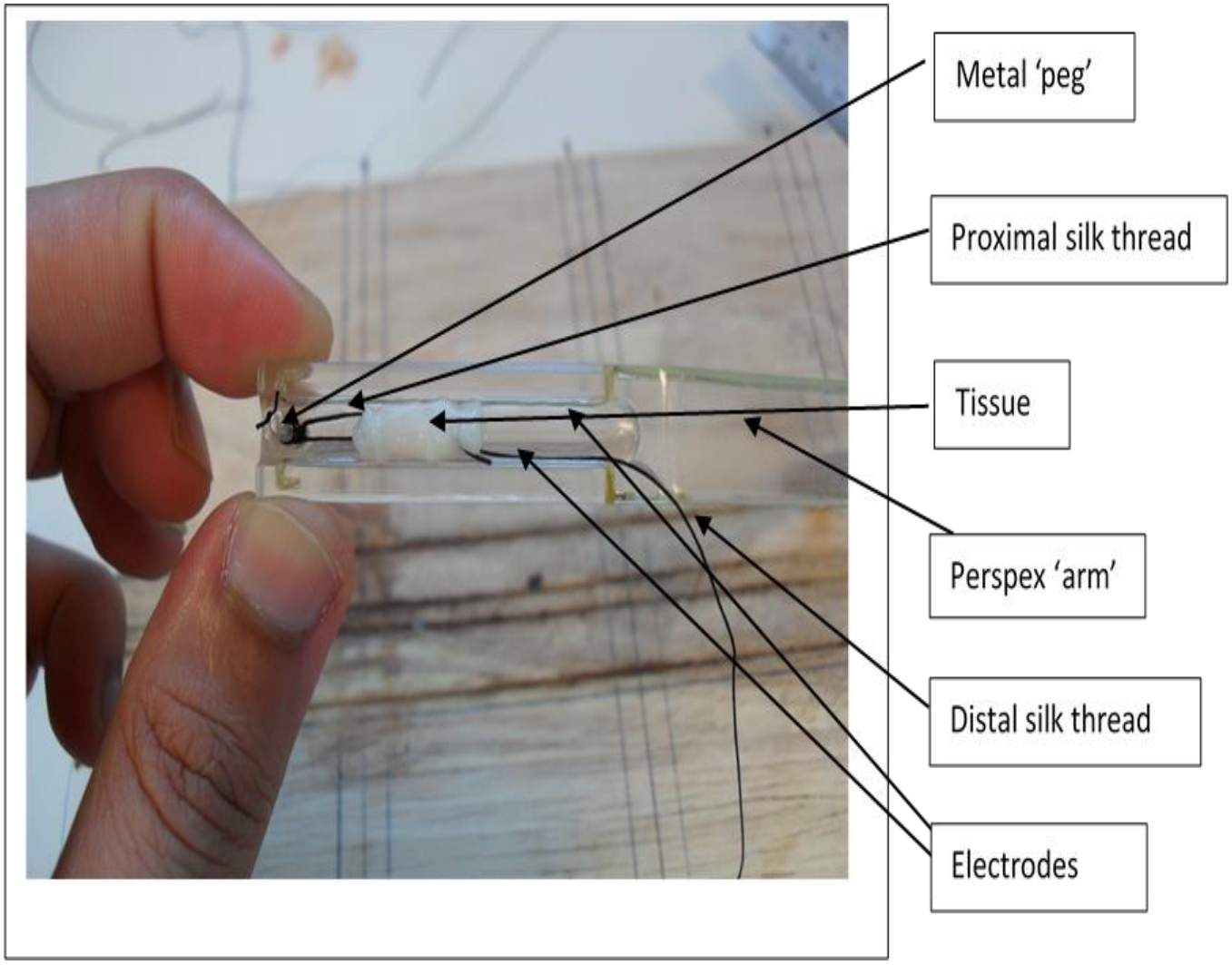


Figure 2-1– Organ bath setup

As the figure demonstrates, the tissue was tied at both ends using silk thread. The proximal end of the thread was tied to a metal 'peg' on the arm and the tissue was laid out in the groove between the two electrodes. The distal silk thread was left long and eventually attached to the transducer to measure tension in the tissue. The Perspex arm was then inserted into an organ bath so that it was vertical (horizontal in this picture); the transducer was calibrated; the distal silk thread was then attached to the transducer and the setup was complete.

2.3.4 Tissue setup

Transducers were calibrated prior to each experiment using a 20g weight. The transducers used were AD Instruments MLT050/D.

In the initial experiments two tissue samples from the same animal were used – one as the experimental sample and the other control. However, it was noted that tissue samples from the same animal did not always respond comparably – one tissue sample may respond while the other from the same animal failed to respond to EFS, or the magnitude of the responses was markedly different. The response to EFS qualitatively did not vary. The reason for this variance was probably due to injury to the tissue during dissection – the circular smooth muscle layer of the rectum is a very thin, translucent layer of muscle fibres and if these fibres are damaged or 'button-holed' during dissection, it would adversely affect the EFS response. Unfortunately, it was impossible to detect such an injury to the tissue prior to subjecting it to EFS. As a result, future experiments were conducted after establishing a baseline response for each tissue and then modifying the conditions (e.g. adding drugs to the organ bath) and comparing responses to the baseline previously recorded for the same tissue. Thus, each tissue functioned as its own control.

The tissue was tensioned to 3-4 g and allowed to relax until it achieved a stable tone (approximately 60 minutes). It was then challenged with 60mM potassium chloride (KCl) solution twice with wash-out following each KCl challenge.

An overview of smooth muscle contraction has been provided (section 1.7.1). The addition of KCl muscle tissues causes a contraction by depolarising the membrane and increasing $[Ca^{2+}]_i$ [207]. This allows the tissue to replenish stores of electrolytes

and glucose following a period of inactivity (from culling and transportation in cold solution to equilibration in the organ bath). The KCl challenges elicit a contractile response in viable muscle tissues and if the response is absent or poor, it indicates dead/dying tissues. In our set of experiments, two KCl challenges were performed to stimulate tissue that had been in non-physiological conditions and to replenish intracellular stores [208]. Initial experiments demonstrated that there was a significantly greater contraction to the second KCl compared to the first KCl – this indicated adequate store loading of the tissues following the first KCl challenge. Further KCl challenges did not produce any further significant increases in contraction and therefore for the remaining experiments, only two KCl challenges were performed. Following each KCl challenge, the tissue was allowed to develop a sustained contraction and once stable tone had been achieved, the organ bath was washed out with Krebbs solution three times to ensure that all KCl had been removed.

The tissue was then allowed to relax for 60 minutes (or longer until stable baseline tone was achieved) after the KCl challenges.

Baseline responses were established by stimulating the tissues with 30 Hz and 10 Hz EFS (300mA, 0.3ms pulse width, duration 30s), three times for each frequency. Drugs were added to the organ baths and concentrations were calculated to final bath concentration and allowed to equilibrate for approximately 30 minutes. Further stimulation with 30 Hz and 10 Hz EFS supplied the experimental results.

The EFS parameters were chosen based on the work of previous authors – Acheson *et al.* (2009) [175] used the same EFS stimulation parameters used in our experiments and numerous other authors have used similar EFS patterns [160-162].

All drugs were diluted in dissolved in distilled water.

Drug concentrations were chosen to provide complete inhibition of the neurotransmitter being examined and were based on previous authors' experiences. O'Kelly *et al.* (1993) [161] used 1 µM atropine and 3 µM guanethidine in their experiments and the same concentrations were used in our experiments. Acheson *et al.* (2009) [175] used 0.1 µM prazosin and 100 µM L-NAME in their experiments to achieve complete inhibition and therefore the same concentration was used in our experiments. Glavind *et al.* (1997) [162] used 1 µM phentolamine to achieve complete inhibition in their EFS experiments and this was the same concentration of phentolamine used in our experiments.

2.3.5 Data analysis

Changes to tissue tone with either EFS or following the addition of drugs are described as changes from the baseline tension in g wt. Where there was significant variability in baseline tone, a mean baseline tone over the one minute prior to the observation was taken. All values are reported as mean ± SEM. Relaxation and contraction were measured as change from baseline tone in grams.

The following measurements were taken for each response to EFS (Figure 2-1Figure 2-2):

- Baseline tension immediately prior to stimulation beginning

    - Where there was a variable baseline (spontaneous activity), an average baseline was calculated for 1 minute immediately preceding stimulation

- Relaxation measured as the change from baseline tension in grams
- Contraction measured as a change from baseline tension in grams
- After-contraction measured as a change from baseline in grams
    - This was the contraction (if any) immediately following the cessation of stimulation
- Time to 50% recovery of tone (RoT_{50}) measured in seconds
    - This was calculated only when there was a relaxation and was used as a measure of the duration of relaxation
    - Following maximal relaxation, the time at which the tissue reached 50% of the maximum relaxation was noted
    - Therefore, a longer RoT_{50} indicated a longer duration of relaxation

Figure 2-2 demonstrates all the measurements taken for each stimulation. Each tissue served as its own control – after the control responses were recorded, drugs were added to the organ baths and subsequent tissue responses served as the test responses. The following figure (Figure 2-3) demonstrates a trace of the tissue from the beginning of the experiment including the KCL challenges and one each of the 30Hz and 10 Hz responses. As demonstrated, tone stabilised following the KCL challenges and remained constant during the duration of the experiments.

As demonstrated in Figure 2-2, the contraction and relaxation would be easily described by the measurement of maximal tone and minimal tone following the EFS

stimulation. However, the duration of the relaxation could not be measured in this fashion. Relaxation of the rectum and IAS play a key role in the normal physiology of continence and defecation and if some neurotransmitter were to modulate the duration of relaxation without influencing the magnitude of relaxation and/or contraction, that would still be a potential target for pharmacological manipulation. The RoT_{50} was therefore used to measure the duration of the relaxation.

A minimum of three responses were recorded at every stage – for control response and after the addition of drugs. Where it was noted that there were evolving changes to the tissue response, no new changes were made to the stimulation until response of the tissue had stabilised to a reproducible and predictable response. A mean response was calculated and recorded for the three representative values.

All values were tested for normality. As each tissue functioned as its own control, either the paired T test (for parametric data) or the Wilcoxon signed rank test would have been appropriate. However, as there was variability between the data from within the same experiment (e.g. the relaxation in response to EFS may have been parametrically distributed but the contraction was not), we decided to use the Wilcoxon test for all variables.

All datasets were analysed for outliers and these were excluded from statistical analyses if present.

All statistical analyses were performed using SPSS statistical software.

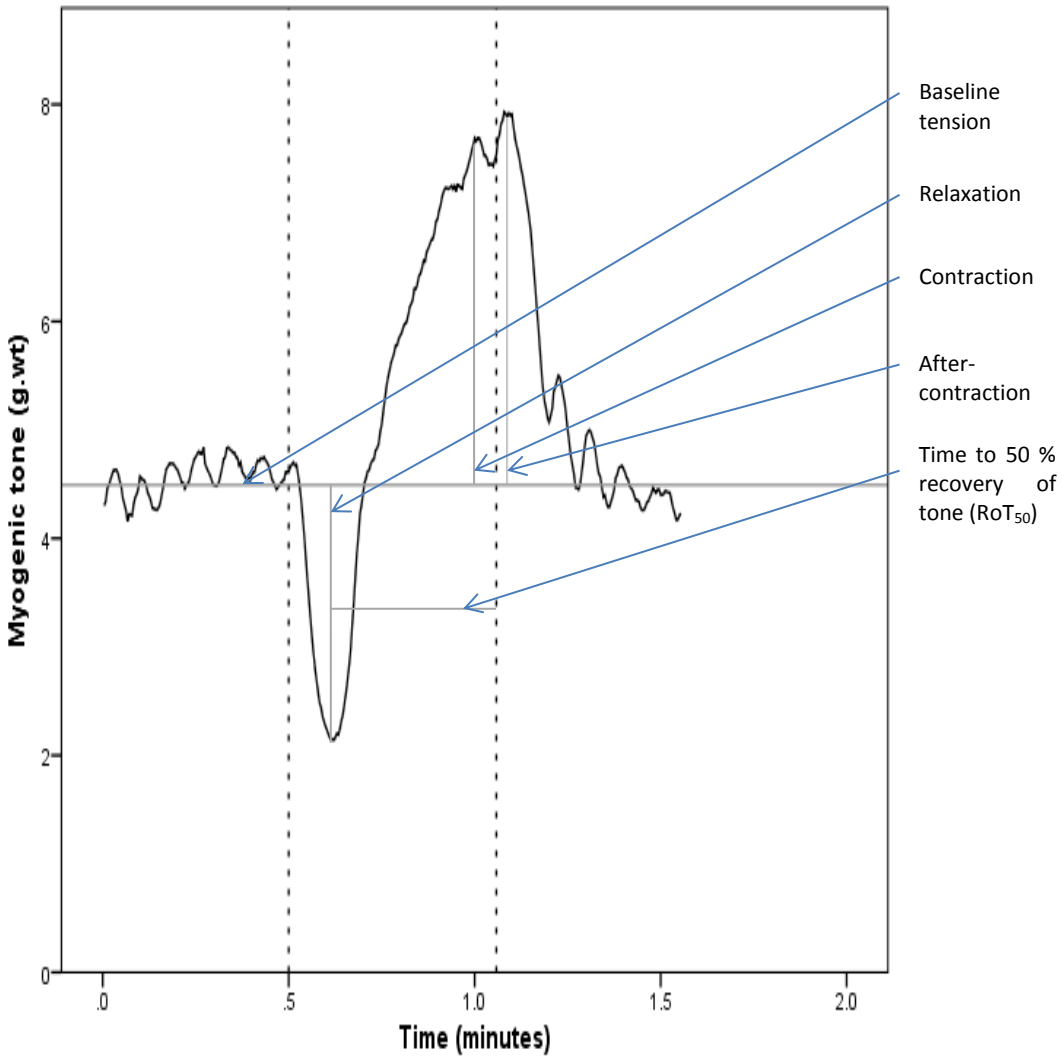


Figure 2-2 – Response to EFS 30 Hz (sheep IAS) demonstrating measurements taken for responses

The trace demonstrates the response of the tissue. The vertical dashed lines indicate the period of stimulation. The solid grey lines show the measurements taken. Relaxation is measured from the baseline at the point of maximal relaxation. Contraction is measured as the maximal contraction during the period of stimulation. After-contraction is the contraction immediately following cessation of stimulation. Time to 50% recovery of tone (RoT_{50}) is the time taken to recover 50% of baseline tone after maximal relaxation and is measured in seconds.

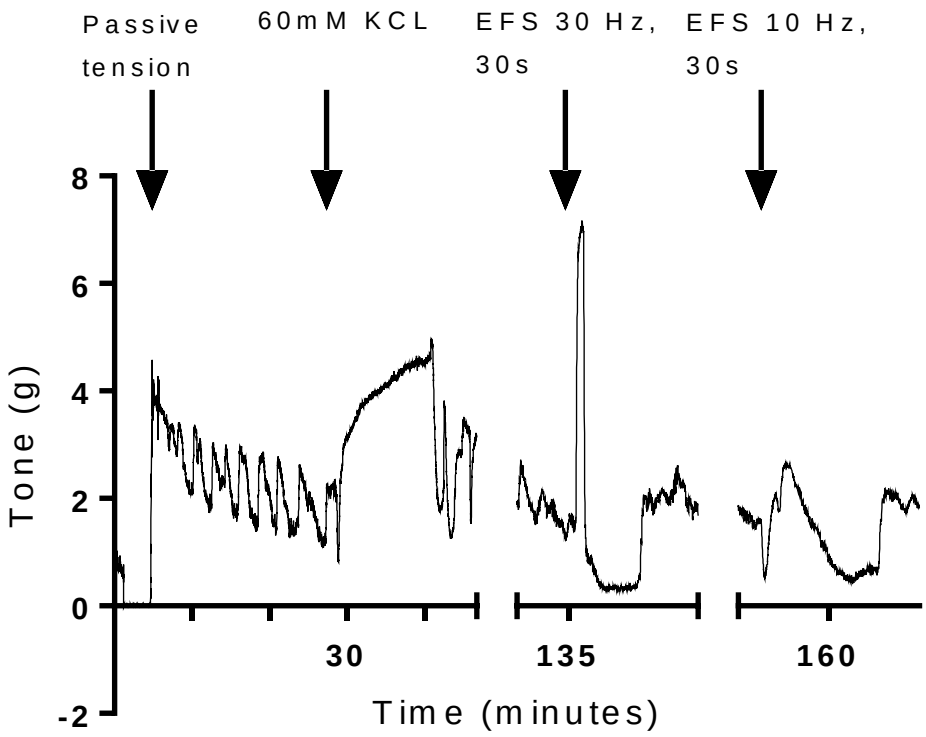


Figure 2-3 – Tissue setup and response to EFS, sheep IAS

The figure demonstrates a representative trace of sheep IAS starting with 0g tone, tension to 4 g, one KCL challenge and one each of EFS 30 Hz and 10 Hz stimulations. Following a period of relaxation after passive tension, the tissue was challenged with 60mM KCL and then washed out. One further challenge was given (not shown). After further equilibration, the tissue was stimulated with 30 Hz EFS for 3 responses (one shown) and then with EFS 10 Hz for 3 responses (one shown) – these served as the control responses. Drugs were then added as per the protocol and further EFS responses noted – these served as the experimental responses.

2.3.6 Experimental protocol

2.3.6.1 Control experiments

Control experiments were performed after tissue preparation as described. The initial aim was to identify if the tissues developed spontaneous tone and/or spontaneous active on passive stretch. Following this, tissues were stimulated with EFS. The tissue was stimulated initially at 30Hz (pulse strength 300mA, pulse width 0.3 ms, 30 seconds at 10 minute intervals) for three stimulations. The tissue was then stimulated at 10Hz (pulse strength 300mA, pulse width 0.3 ms, 30 seconds at 10 minute intervals) for 3 stimulations. This established the baseline (control) responses for each tissue and identified the response of tissues to baseline EFS stimulation. Tetrodotoxin was added to the tissues at the end of the first few control experiments to ensure that the responses to EFS were not due to direct stimulation of the muscle tissue.

2.3.6.2 The role of NO if mediating the response to EFS

After establishing the control responses, L-NAME (an antagonist to NO synthase) was added to the organ baths at a final concentration of 0.1 mM. Tissues were allowed to stabilise for 60 minutes following this and the EFS responses following this were taken to be the test responses.

2.3.6.3 The role of NA in the EFS response

Guanethidine (an inhibitor of NA release from nerve terminals) was used at a concentration of 3 µM. The test responses were compared to control responses prior to the addition of guanethidine and the change in relaxation, duration of relaxation and contraction were noted. In the sheep rectal experiments, guanethidine was also used in tissues pre-incubated with 0.1 mM L-NAME to isolate the effect of NA from

the background effect of NO release. In these cases, EFS responses after pre-incubation with L-NAME served as the control responses. 3 µM guanethidine was added to the tissues and the responses to EFS following this served as the test responses.

2.3.6.4 The role of acetylecholine in the response to EFS and in baseline tone

Atropine (an mAchR antagonist) was added at 1 µM concentration following control responses and the change in EFS responses was noted. Atropine was also investigated in tissues pre-incubated with guanethidine to isolate the effect of Ach from that of NA. EFS responses in tissues pre-incubated with 3 µM guanethidine served as the control responses and EFS responses following the further addition of 1 µM atropine served as the test responses.

In sheep rectal tissue, we investigated the effect of atropine in tissues pre-incubated with L-NAME to remove the effect of NO from the EFS response. Tissues pre-incubated with 0.1 mM L-NAME served as controls and EFS responses following the further addition of 1 µM atropine served as the test response.

In the sheep rectal tissue, carbachol (an acetylcholine receptor agonist) was used to investigate the change in baseline tone following addition of 3 µM carbachol. Tissues were allowed to stabilise for 120 minutes over which time the change in baseline tone was observed.

2.3.6.5 The effect of methoxamine on sheep rectal tissue

Methoxamine was added tissues cumulatively starting with a bath concentration of 0.01 µM to a bath concentration of 30 µM in the following increments – $1x10^{-8}$M, $3x10^{-8}$M, $1x10^{-7}$M, $3x10^{-7}$M, $1x10^{-6}$M, $3x10^{-6}$M, $1x10^{-5}$M, $3x10^{-5}$M. No EFS stimulation

was performed. The tissue was allowed to stabilise for a minimum of 15 minutes (or longer if necessary) to achieve stable tone prior to the next addition. The change in tone was plotted against a logarithmic scale to achieve a concentration-response curve.

2.4 Results

2.4.1 Sheep internal anal sphincter

After the application of 3 g wt, the sheep IAS developed sustained tone but did not display any spontaneous activity. Mean baseline tension was 2.66±0.21 g wt. (n = 23) and this stable tension was maintained throughout the experiment after an initial 60 minutes of equilibration.

2.4.1.1 Control experiments

Following passive stretch and stimulation with KCL, IAS tissue was allowed to equilibrate for approximately 60 minutes. Over 23 experiments, mean baseline tone was 2.66g ± 0.21.

Figure 2-4 demonstrates the response of the tissue to EFS. Electrical field stimulation (EFS) was used at two frequencies (n = 23) – 10 Hz and 30 Hz – for 30 seconds at 10 min intervals. The initial response to EFS was that a relaxation reached a peak within 10s and, depending on the frequency of stimulation, either slowly recovered towards baseline (10 Hz) or developed an overt contraction (30 Hz) before cessation of stimulation. This was followed by a contraction after the cessation of stimulation in 95% of cases with 10 Hz stimulation and only 17% of cases with EFS 30 Hz (after-contraction). The addition of 0.1 µM tetrodotoxin completely abolished the response to EFS confirming that the responses were neuronally mediated. The after-contractions are described here but are not referred to any subsequent analysis of responses to EFS as they were noted to be variable with no significant relationship with either baseline tone or stimulation response.

The magnitude of the relaxation was significantly greater for EFS 10 Hz compared to EFS 30 Hz (1.98g ± 0.18 vs 1.1g ± 0.14, p = 0.004) and the relaxation was sustained for a significantly longer duration at 10 Hz compared to 30 Hz (RoT_{50} 23.2s ± 1.6 at 10 Hz vs 6.3s ± 0.5 at 30 Hz, p < 0.001). The contraction to EFS was significantly lower at EFS 10 Hz compared to EFS 30 Hz (0.26g ± 0.08 at 10 Hz vs 5.15g ± 0.64 at 30 Hz, p < 0.001).

The response to EFS 30 Hz was easier to study in all cases than that at EFS 10 Hz because there was consistently a greater contraction during the period of stimulation at EFS 30 Hz compared to EFS 10 Hz. Therefore, the 30 Hz response is analysed only in the following sections. The 10 Hz responses are still demonstrated in the graphs to aid comparison.

2.4.1.2 The role of NA in the response to EFS

Figure 2-5 demonstrates the response to EFS before and after the addition of 3 µM guanethidine. Addition of guanethidine at 3µM did not affect the baseline tension (n = 16). The relaxation to EFS was increased by 64% (1.03g ± 0.17 to 1.69g ± 0.18, p = 0.002) and was sustained for longer by 158% (6.95s ± 1.18 to 17.91s ± 2.09, p = 0.001). Contraction was significantly reduced by 81% (5.08g ± 0.8 to 0.99g ± 0.32, p <0.001). At EFS 10 Hz, there was no difference in the response post 3 µM guanethidine.

2.4.1.3 The role of Ach in the response to EFS

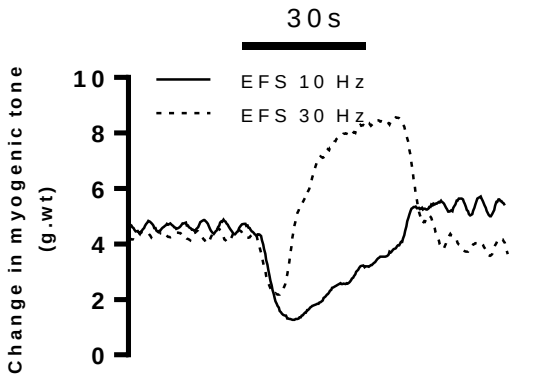
Figure 2-6 demonstrates the EFS response before and after the addition of atropine. Atropine was added to the organ baths at a final concentration of 1 µM (n = 8). Baseline tone remained unchanged following addition. Relaxation was marginally greater by 40% (1.29g ± 0.24 to 1.8g ± 0.3, p = 0.054) following Atropine but did not

reach statistical significance. Relaxation was also sustained for longer by 41% (7.11s ± 0.84 to 10.05s ± 1.25, p = 0.002) and this was statistically significant. Contraction was also significantly smaller by 34% (4.74g ± 1.05 to 3.11g ± 0.81, p = 0.034) after Atropine addition. However, these changes were small in magnitude compared to the changes following the addition of guanethidine. At EFS 10 Hz, there was no significant change in the EFS response post addition of 1 µM Atropine.

2.4.1.3.1 Effect of Atropine in tissues pre-incubated with Guanethidine

Eight experiments were performed where EFS responses in tissues pre-incubated with 3 µM guanethidine were treated as control and 1 µM atropine was added to the organ baths. The EFS responses following the addition of atropine were the test responses.

No significant changes were noted following the addition of atropine to tissues pre-incubated with guanethidine. Baseline tone, relaxation, duration of relaxation and contraction remained unchanged.



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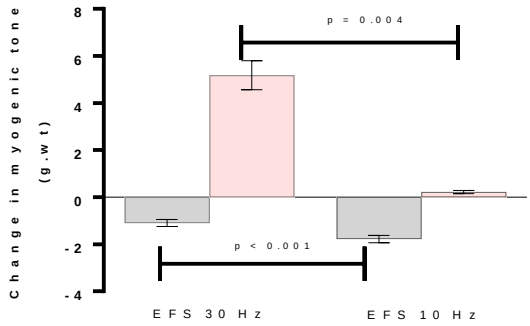


(b)

Figure 2-4 – Response to EFS 30 Hz and EFS 10 Hz, sheep IAS

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. Figure (b) illustrates the differences in contraction and relaxation for EFS 30 Hz and EFS 10 Hz (n = 23). SE bars are shown, statistically significant differences are highlighted. Compared to EFS 30 Hz, relaxation was significant greater at EFS 10 Hz (1.1g vs. 1.98g) and the contraction was significantly smaller (5.15g vs. 0.26g).

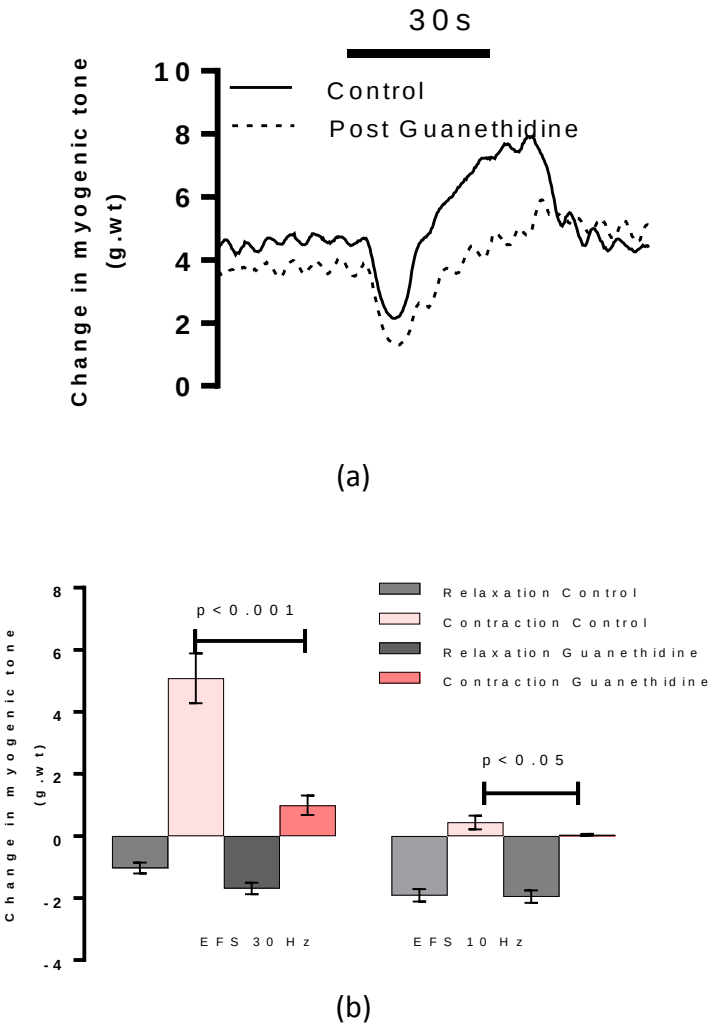
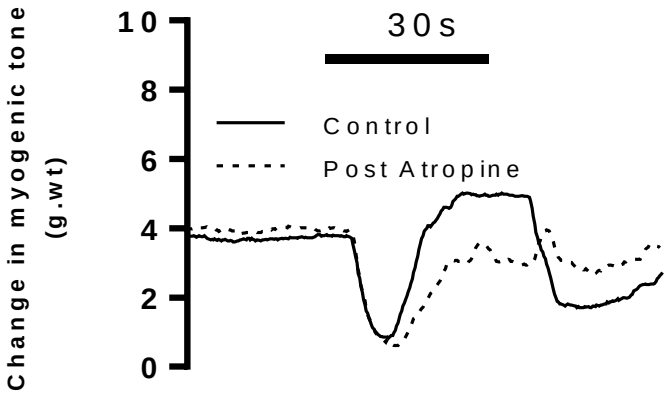
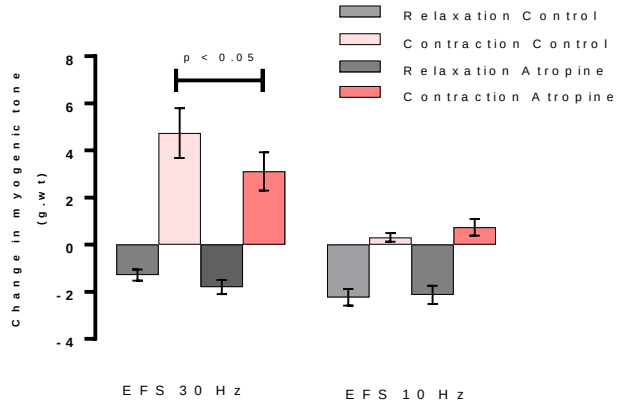


Figure 2-5 – Effect of guanethidine on the EFS response, sheep IAS

Figure (a) is a representative trace of the 30 Hz response in sheep IAS tissue before and after the addition of 3 µM guanethidine. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation. Figure (b) illustrates the difference in contraction and relaxation with EFS before and after the addition of guanethidine at EFS 30 Hz and EFS 10 Hz (n = 16). SE bars are shown, statistically significant differences are highlighted. At EFS 30 Hz, contraction was significantly reduced following the addition of guanethidine (5.08g vs. 0.99g, p <0.001) and a similar response was noted at EFS 10 Hz (p = 0.012). The duration of relaxation was also significantly increased at both stimulation frequencies following the addition of guanethidine (not shown).



(a)



(b)

Figure 2-6 – Effect of atropine on the EFS response, sheep IAS

Figure (a) is a representative trace of the 30 Hz response in sheep IAS tissue before and after the addition of 1 µM atropine. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation. Figure (b) illustrates the difference in contraction and relaxation with EFS before and after the addition of atropine at EFS 10 Hz and EFS 30 Hz (n = 8). SE bars are shown, statistically significant differences are highlighted. At EFS 30 Hz, the contraction was significantly reduced following the addition of atropine (4.74g vs. 3.11g, p = 0.034); relaxation was increased following atropine addition but did not reach statistical significance; the duration of relaxation was significantly increased following atropine addition (not shown). At EFS 10 Hz, no statistically significant differences were noted in contraction or relaxation following the addition of atropine

2.4.2 Summary

A table summarising the effects of drugs on the EFS 30 Hz response in Sheep IAS is provided.

	Baseline (g)	Relaxation (g)	Duration of relaxation (s)	Contraction (g)
Control	2.66 ± 0.21	1.1 ± 0.14	6.2 ± 0.5	5.15 ± 0.64
Guanethidine	No change	↑↑	↑↑	↓↓
Atropine	No change	No change	↑	↓
Atropine post guanethidine	No change	No change	No change	No change

Table 3 – Summary of results from Sheep IAS

We concluded that our experimental protocol was valid as it produced reproducible results comparable to previous authors. We therefore used the same experimental protocol to investigate the sheep rectum in our following experiments.

2.4.3 Sheep rectum

Following the experiments on the sheep IAS, sheep rectal tissue was prepared using the same technique described previously for the sheep IAS. Dissection and organ bath set-up were identical as for the IAS experiments. An overview of the tissue setup and response is shown in Figure 2-7.

2.4.3.1 Control experiments

On tensioning the tissues to 3g, sheep rectal tissue did not develop any baseline tension or spontaneous activity. Mean baseline tension was 0.5g.

Response to EFS was contractile only. No relaxations in response to EFS were noted (Figure 2-8). In approximately half cases, there was no after-contraction following the cessation of stimulation (32 out of 67 cases).

Response to 10 Hz and 30 Hz demonstrated no significant differences in terms of the magnitude of the contraction – mean contraction was 3.5g ± 0.9g at 10 Hz (n = 30), mean contraction 3.83g ± 0.69g at 30 Hz (n = 57), p = 0.226. Responses to EFS were abolished completely by the addition of 1 µM tetrodotoxin (n = 8) confirming that the responses were neurogenic in nature.

In the results following, only the 30 Hz response is analysed although the 10 Hz response is shown in the figures for comparison.

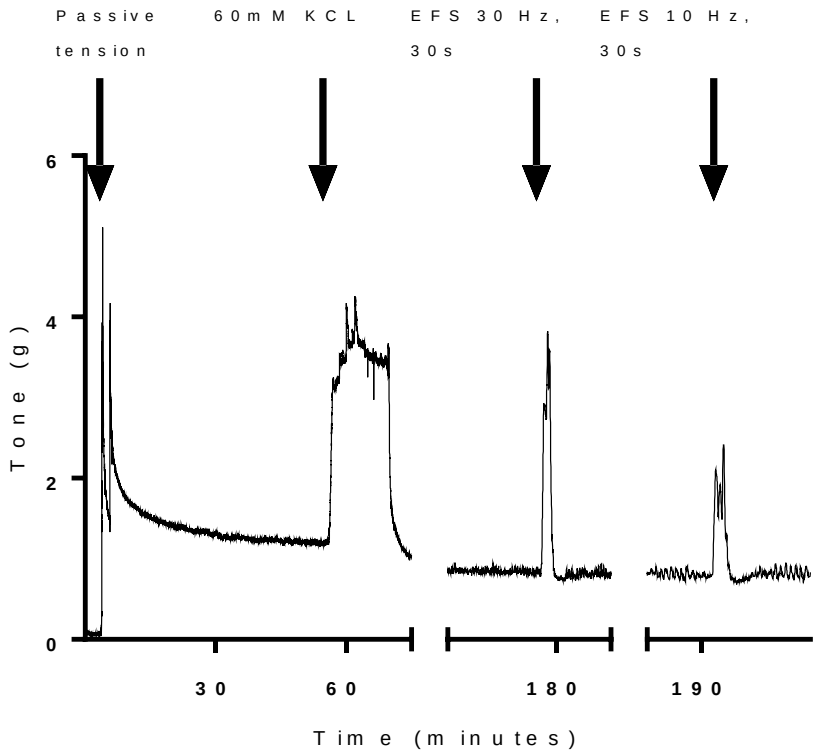
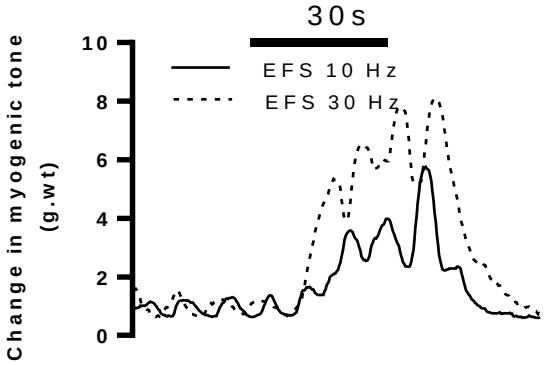
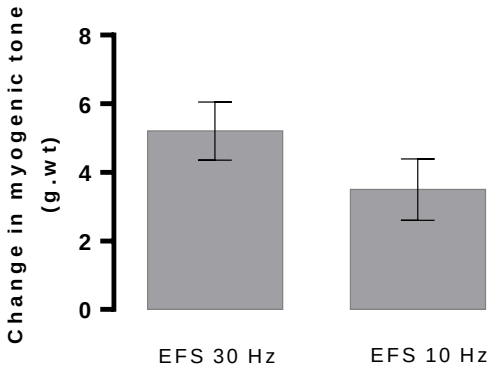


Figure 2-7 – Tissue setup and EFS response, sheep rectum

The figure demonstrates a representative trace of sheep rectum starting with 0g tone, tension to 4 g, one KCL challenge and one each of EFS 30 Hz and 10 Hz stimulations. Following a period of relaxation after passive tension, the tissue was challenged with 60mM KCL and then washed out. One further challenge was given (not shown). After further equilibration, the tissue was stimulated with 30 Hz EFS for 3 responses (one shown) and then with EFS 10 Hz for 3 responses (one shown) – these served as the control responses. Drugs were then added as per the protocol and further EFS responses noted – these served as the experimental responses



(a)



(b)

Figure 2-8 – Response to EFS 30 Hz and EFS 10 Hz, sheep rectum

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, there was no relaxation response demonstrated to EFS in the rectal tissues.

Figure (b) illustrates the differences in contraction and relaxation for EFS 30 Hz (n = 57) and EFS 10 Hz (n = 30). SE bars are shown. Compared to EFS 30 Hz, the contraction was smaller at EFS 10 Hz (3.8g vs. 3.5g) but did not reach statistical significance (p = 0.226).

2.4.3.2 The role of NO

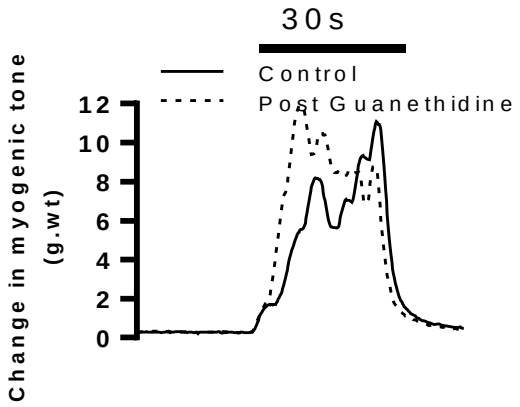
L-NAME was added at a bath concentration of 0.1 mM to tissues after the control EFS 30 Hz and 10 Hz responses (n = 10). Tissues were then allowed to equilibrate for 1 hour.

L-Name did not significantly change the baseline tension but did increase the contraction to EFS nearly fourfold at EFS 30 Hz (from 1g ± 0.55 to 3.98g ± 1.55, p = 0.009). A similar response was noted at EFS 10 Hz where the contraction was significantly greater following 1 mM L-NAME addition

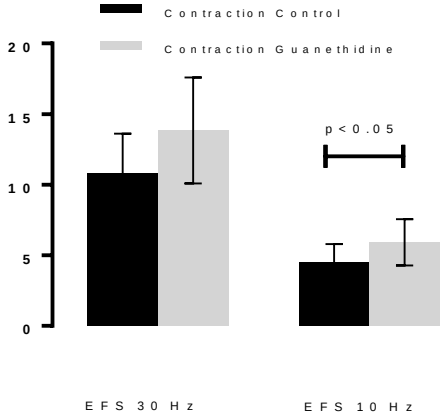
2.4.3.3 The role of NA in mediating the EFS response

Figure 2-9 demonstrates the change in the EFS response following the addition of guanethidine. After control responses, addition of 3 µM guanethidine at EFS 30 Hz (n = 11) caused a small (22%), non-significant increase in the contraction (10.79g ± 2.84 to 13.84g ± 3.76, p = 0.129). At EFS 10 Hz, the small increase in contraction following the addition of Guanethidine (31%) was statistically significant, but as with EFS 30 Hz, the change was small in magnitude.

Guanethidine was also investigated in tissues pre-incubated with 0.1 mM L-NAME (n = 8). Addition of Guanethidine did not cause a significant change in the contraction to either EFS 30 Hz or EFS 10 Hz.



(a)



(b)

Figure 2-9 – Effect of guanethidine on the EFS response, sheep rectum

Figure (a) is a representative trace of the 30 Hz response in sheep IAS tissue before and after the addition of 3 µM guanethidine. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation.

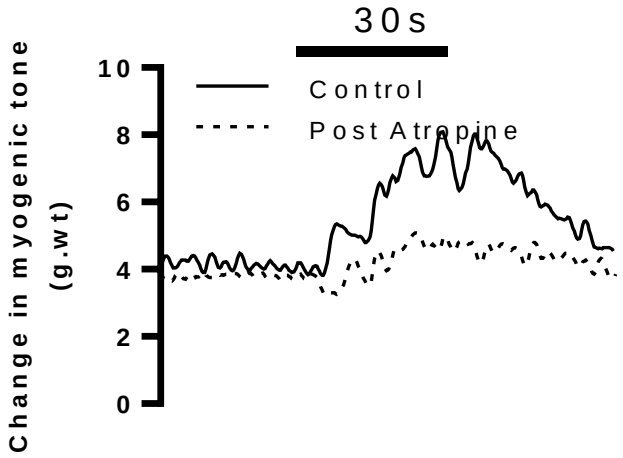
Figure (b) illustrates the difference in contraction with EFS before and after the addition of guanethidine at EFS 30 Hz and EFS 10 Hz (n = 11). SE bars are shown, statistically significant differences are highlighted. At EFS 30 Hz, contraction was increased following the addition of guanethidine (10.79g vs. 13.84g, p = 0.129) but did not reach statistical significance. At EFS 10 Hz contraction also increased (4.5g vs. 5.92g, p = 0.008) and this was statistically significant although the magnitude of change was small.

2.4.3.4 The role of Ach

Figure 2-10. Following control EFS responses, addition of 1 µM Atropine dramatically reduced the contraction to EFS 30 Hz (n = 7) by 94% (11.49g ± 3.06 to 0.72g ± 0.63, p = 0.018). A similar dramatic reduction in contraction was noted at EFS 10 Hz following the addition of 1 µM Atropine.

Atropine was also investigated in tissues pre-incubated with 0.1 mM L-NAME. 1 µM atropine caused a dramatic reduction in the contraction to EFS 30 Hz, reducing the contraction by 92% (4.15g ± 0.3 to 0.3g ± 0.34, p = 0.028).

Figure 2-11 demonstrates a representative trace of a tissue response to carbachol (no EFS stimulations). Carbachol (an acetylcholine receptor agonist) was added to tissues at a concentration of 3 µM with no EFS stimulation (n = 12). There was a sharp increase in tone initially which decreased gradually over the first 60 minutes but was stable following this at a significantly increased level (0.39g ± 0.09 to 5.89g ± 0.67). Carbachol also induced significant baseline variability in tone.



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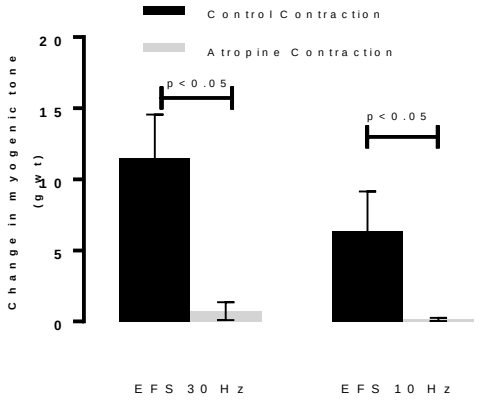


(b)

Figure 2-10 – Effect of atropine on the EFS response, sheep rectum

Figure (a) is a representative trace of the 30 Hz response in sheep IAS tissue before and after the addition of 1 µM atropine. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation. Figure (b) illustrates the difference in contraction and relaxation with EFS before and after the addition of atropine at EFS 10 Hz and EFS 30 Hz (n = 7 both). SE bars are shown, statistically significant differences are highlighted. At EFS 30 Hz, the contraction was significantly reduced following the addition of atropine (11.79g vs. 0.72g, p = 0.018) as well as at EFS 10 Hz (6.31g vs. 0.15g, p = 0.043)

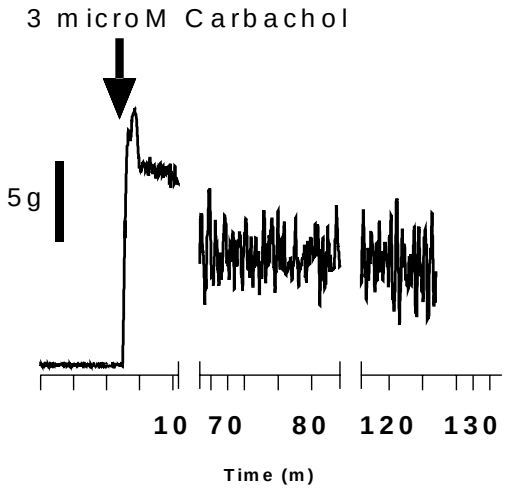


Figure 2-11 - Representative trace of the effect of carbachol, sheep rectum

Following addition of 3 µM carbachol the tissue was allowed to equilibrate over two hours (n = 12). Initially, there was a decrease in tone in the first hour but then stable tone was achieved with significant baseline variability in tone.

2.4.3.5 Effect of methoxamine

Methoxamine was the drug of greatest interest as it is one of the potential treatments for FI. We wished to investigate whether methoxamine would cause a change in baseline tone of the rectum. Tissues were prepared as before, but were not stimulated with EFS (n = 8).

Figure 2-12 demonstrates the concentration response curve to serial methoxamine additions. Methoxamine was added tissues cumulatively starting with a bath concentration of 0.01 µM to a bath concentration of 30 µM. Tissues were allowed to develop stable tone for 15 to 30 minutes following each successive addition of methoxamine. There was a steady increase in tone upto a concentration of 1 µM methoxamine followed by a sharp increase in tone upto 10 µM. Further addition of methoxamine caused a decline in baseline tone.

Log[EC_{50}] was -5.866 ± 0.999 and EC_{50} was 1.36 µM (n = 8) (Figure 2-12).

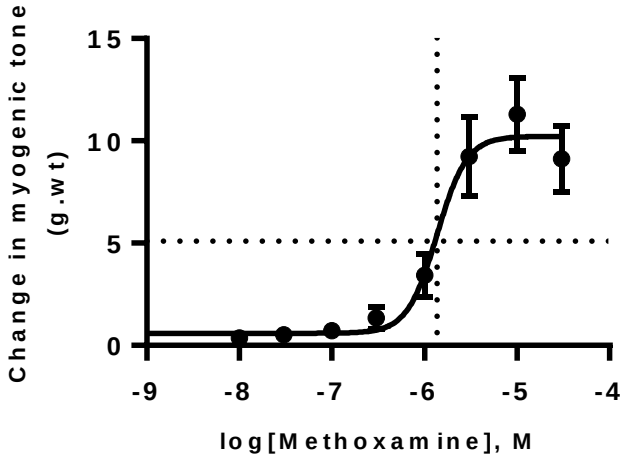


Figure 2-12 – Methoxamine concentration response curve, sheep rectum
The concentration response curve plotted against a log[Methoxamine] concentration is displayed along with SEM bars for each data point (n = 8). The dashed line indicates the EC_{50} (1.36 µM)

2.4.4 Summary

A table summarising the effects of drugs on the EFS 30 Hz response in sheep rectum is provided.

	Baseline (g)	Relaxation (g)	Duration of relaxation (s)	Contraction (g)
Control	0.5	N/A	N/A	3.83g ± 0.69g
L-NAME	No change	N/A	N/A	↑↑
Guanethidine	No Change	N/A	N/A	No change
Atropine	No change	N/A	N/A	↓↓
Carbachol	↑↑			
Methoxamine	↑↑			

Table 4 – Summary of results from sheep rectal experiments

Key – large reduction ↓↓
large increase ↑↑
N/A – no response, therefore not applicable

2.5 Discussion

2.5.1.1 Control experiments – evidence of homology between human and sheep tissue

Human IAS tissue has been shown to develop tone on passive stretch [158, 159, 174] and relaxes in response to EFS stimulation [155, 160-162, 174]. Our experiments with the sheep IAS tissues show similar characteristics with a tone developing on passive stretch and a relaxation (followed by a contraction) in response to EFS.

Results from human rectal tissue are more contradictory than from human IAS tissue. Some authors report that human rectal tissue developed no tone on passive stretch [146, 159, 180, 185] while Glavind *et al.* (1997) report that human rectal tissue developed myogenic tone on passive stretch [162]. In some experiments where tissue did not develop tone, histamine was used to induce stable tone in order to study the effects of EFS and other drugs [180, 185]. In experiments that investigated the effect of EFS (with or without histamine to pre-contract tissues), all authors report a relaxation to EFS [162, 179, 180]. In our experiments with the sheep rectal tissue, passive stretch did not generate myogenic tone. Previous experience from our centre showed that histamine did not induce stable tone in sheep tissues, therefore this was not used to generate tone in our experiments. The response to EFS was contractile only. While this may be because the tissue did not have any significant tone in order to manifest a relaxation, there was no delay between the start of EFS stimulation and the start of the contraction (Figure 2-8) – this is not conclusive evidence of the absence of

relaxation but indicates that relaxation was not a major component of the response to EFS.

For both IAS and rectal tissues, tetrodotoxin abolished the response to EFS, indicating that the responses were neuronally mediated and not due to direct stimulation of muscle, therefore our experimental protocol was valid.

2.5.1.2 The role of NO in the response to EFS

In our experiments, L-NAME was used to counter the action of NO.

In IAS tissue, the relaxation to EFS was significantly reduced and a contraction was unmasked to EFS. This agrees well with previous reports on human IAS tissue which also relaxes to EFS via the action of NO [161, 162].

In sheep rectal tissue, no relaxation was observed therefore the role of NO in producing relaxations to EFS cannot be reliably commented upon. However, addition of NO significantly increased the contraction to EFS indicating that NO was a co-secretory neurotransmitter which modulated the contraction observed. Human rectal tissue experiments investigating EFS have also shown that NO is released during stimulation and causes a relaxation *in vitro* [161, 162, 174].

2.5.1.3 The role of NA in the EFS response

We used Guanethidine to antagonise the release of NA in our experiments. In sheep IAS experiments, Guanthidine caused a very significant reduction in the contraction to EFS and increased the relaxation (both in magnitude and duration). This is similar to described findings in the human IAS where α-adrenoceptors have been shown to mediate the contraction to EFS [159, 162, 174, 175]. Some authors

have described a reduction in IAS tone when β-adrenoceptors are stimulated [158-160] but all these authors demonstrate a contraction to NA with relaxation only on isolated β-adrenoceptor stimulation. Burleigh *et al.* (1979) [160] also investigated EFS responses in the IAS but found no evidence of β-adrenoceptor involvement in the EFS response. We did not investigate β-adrenoceptors in isolation in our experiments.

NA did not appear to play a significant role in the EFS response in the sheep rectum in our experiments. Blocking NA caused a small increase in the contractile response to EFS but this was only significant in the case of EFS 10Hz and the change was small in magnitude (31% increase). This would agree with previous human rectal experiments where NA has been shown to cause a relaxation [146, 159, 179, 180].

2.5.1.4 The role of Ach in the EFS response

In the sheep IAS experiments we used atropine to inhibit the action of Ach. The results were contradictory – there was a small reduction noted in the contraction to EFS following the addition of atropine but this difference was not noted in tissues pre-incubated with Guanethidine. While we cannot account for this discrepancy, it appears that mAchR stimulation is not a major component of the EFS response. Two previous studies demonstrate a reduction in human IAS tone following stimulation of mAchR [159, 160] but in one of these studies there was an initial increase in tone followed by a reduction.

In sheep rectal tissue, the role of Ach was investigated by adding Atropine and noting the EFS response in control tissues and also in tissues pre-incubated with

L-Name. The effect of carbachol on baseline tone was also investigated without EFS stimulation. All the experiments indicate a significant contraction mediated by mAchR – the contraction to EFS was very significantly reduced following the addition of atropine (both in control tissues and in tissues pre-incubated with L-Name) and carbachol caused a significant sustained rise in baseline tone. A number of previous authors have described similar findings in human rectal tissue [146, 159, 162, 179, 180].

2.5.1.5 The effect of methoxamine on rectal tone

Methoxamine caused a concentration-dependant increase in rectal tone in our experiments. As mentioned, a number of authors have demonstrated a relaxation in human rectal tissue with NA stimulation and O'Kelly *et al.* (1993) [159] demonstrated a reduction in tone in pre-contracted rectal strips with the addition of phenylephrine. As previously discussed in the section on the role of α adrenoceptors in the colon and rectum, there is currently some evidence to suggest that α adrenoceptors may be involved in contraction in the colon and rectum. In the human rectal tissue experiments, authors report a reduction in tone/spontaneous activity after the addition of NA [146, 159, 180, 188] but this may be accounted for by stimulation of β adrenoceptors. The two authors who used α adrenoceptor agonists and observed a decrease in tone/spontaneous activity [159],[188] used phentolamine at concentrations of 100 µM and 10 µM respectively – both concentrations capable of producing dual α and β adrenoceptor stimulation. Therefore, the increase in tone that we observed with

methoxamine may well represent the action of α adrenoceptors in the human rectum as well as the sheep rectum.

2.6 Conclusion

Our results from sheep IAS experiments demonstrate that sheep IAS develops tone on stretch, relaxes in response to EFS. The relaxation is mediated by NO. Blocking NO unmasks/exaggerates the contractile response to EFS which is mediated by NA. These findings are in concordance with published data on human IAS tissue. Our experimental protocol is therefore taken to be valid.

Our results from sheep rectal tissue also show considerable homology with human rectal tissue. The tissue did not develop tone, which has been reported by a number of previous authors. The EFS response was contractile, the contraction being mediated primarily by mAChR which agrees well with human rectal experiments by other authors. The role of NA was less conclusive with only a small change in the EFS response noted. However, in our experiments, we were unable to demonstrated a relaxation to EFS in pre-contracted sheep rectal tissues and cannot comment on this. However, blocking NO did cause a significant rise in the contraction to EFS indicating that NO was a co-secretory compound in the EFS response which was involved in reducing the contraction to EFS. In conclusion, the sheep rectum has considerable homology with human rectal tissue. The response to methoxamine was contractile only and showed a dose-dependent contraction. This indicates that α_1 adrenoceptors are contractile in the rectum of the sheep. If this were translated to the human rectum, it would indicate that methoxamine may not be the ideal drug for the treatment of FI in humans. As

mentioned, the evidence for α adrenoceptors being relaxatory in the human rectum is not conclusive and our data contradicts 'received' wisdom that α adrenoceptors in the colon and rectum are relaxatory.

In our next set of experiments, we will investigate the pig IAS and rectum in order to elucidate if pig rectum is a better surrogate for human rectal tissue.

3 Chapter 3 – studies on pig rectum and IAS

3.1 Introduction

Following the experiments on sheep IAS and rectum, we had validated our experimental protocol. The aims with the pig experiments was to investigate the neuromyogenic properties of the pig rectum in an effort to find significant homology with human rectal tissue as described by previous authors. We also investigated the pig IAS to identify if there were any significant differences in the neuromygenic properties between the rectum and the IAS in pigs.

3.2 Comparison between human IAS and pig IAS tissues

3.2.1 Pig tissue

In vivo studies on pig IAS and rectum have been performed by Moller *et al.* (2008) [184] who report a relaxation in the distal rectum and anal canal in pigs when the pelvic parasympathetic nerves were stimulated. This response was removed after the addition of N^G-nitro-L-arginine, an inhibitor of NO synthase.

Cook *et al.* (1999) [147] performed *in vitro* studies on the pig IAS and rectum and report a contraction in the IAS following the addition of NA. Mills *et al.* (2008) [209] investigated the effects of α_1 adrenoceptor stimulation in the pig IAS and confirmed the dose-dependent increase in tone in pig IAS with α_1 stimulation. They further isolated the $\alpha_{1A/L}$ and α_{1L} receptors as mediating the contractile response. Jones *et al.* (2003) [203] have demonstrated contraction of the IAS in pigs using methoxamine, an α_1 adrenoceptor agonists.

Ramalingam *et al.* (2010) [210] performed *in vitro* experiments on pig IAS. They found that pig IAS developed tone on stretch and had a dual response to EFS – relaxation, followed by contraction. The contraction was abolished by adding guanethidine and the relaxation was sensitive to L-NOARG, an antagonist of NO. They concluded that in the pig IAS, neuronal stimulation causes a relaxation mediated by NO and a contraction mediated by NA. Opazo *et al.* (2009) [211] also investigated EFS in pig IAS tissue. They found a relaxation antagonised by NO and $P2Y_1$ antagonists but did not observe any contraction during the period of stimulation. In their experiments, pig IAS tissue also developed tone on passive stretch.

3.2.2 Human tissue

As previously demonstrated, there is good *in vivo* evidence to suggest that sympathetic stimulation causes a contraction *in vivo* in the human IAS (Table 1). *In vitro* studies on human IAS tissue have been summarised (Table 2) and confirm that human IAS tissue develops tone on passive stretch, contracts in response to NA stimulation and relaxes in response to mAchR stimulation. EFS stimulation causes a relaxation mediated by NO.

Therefore, there appears to be considerable homology between the human and pig IAS tissues.

3.3 Comparison between human rectum and pig rectal tissues

3.3.1 Pig rectum

Cook *et al.* (1999) [147] demonstrated a contraction in the pig rectum following the addition of carbachol. In their experiments, rectal tissue developed spontaneous activity but not tone on passive stretch. Stebbing *et al.* (1995) [185] investigated the effects of EFS on pig rectum and demonstrated a relaxation in precontracted strips in response to EFS mediated by NO. Of note, in their experiments, Stebbing found that pig rectal tissue did not develop spontaneous tone on stretching and they used histamine to induce stable tone.

3.3.2 Human rectum

As previously mentioned (*In vitro* studies (human rectal tissue)) there have been five previous studies investigating the human rectal tissue *in vitro* [146, 147, 159, 162, 179]. Only one study [162] reports that human rectal tissue developed tone in response to passive stretch. Our review of the literature suggests that in the human rectum, neuronal stimulation produces a relaxation mediated by NO; stimulation of mAchR causes a contraction; stimulation of adrenoceptors causes a relaxation. Most studies suggest a relaxation in response to α adrenoceptors – numerous authors have shown a reduction in the spontaneous activity in the human rectum with stimulation of α adrenoceptors [147, 159, 179, 180] while O'Kelly [159] also demonstrated a decrease in tone of pre-contracted human rectal strips. However, as mentioned in the section on α adrenoceptors, there remains a question as to the validity of these results.

3.4 Experimental materials and methods

3.4.1 Aims

In the first instance, we aimed to reproduce the same protocol for the pig tissues as in the sheep experiments. Following this, we aimed to investigate the neuronal mediators in pig tissues and to identify the role of α adrenoceptors in the pig IAS and rectum. Finally, we intended to investigate the effect of Methoxamine on the neuronal response as well as the effect of methoxamine on the baseline tone of tissues.

3.4.2 Tissue preparation and setup

This was identical to the sheep tissues. As with the sheep experiments, tissue was obtained from the abattoir and transported in Krebbs Hensleit solution at 4^{o}C within 1 hour of culling. Tissue consisted of distal colon and perineum of pigs.

Dissection was carried out exactly as described for the sheep experiments. The tissue was dissected to yield 0.5x1cm strips of either IAS or rectum (as appropriate) using the jig previously designed in order to give consistent tissue sizes. As before, rectal tissue was cut along the line of muscle fibres between 1 and 2 centimetres from the end of the IAS and IAS tissue was cut along the line of muscle fibres 0.5 cm from the anal verge.

Tissues were setup as previously described (see Figure 2-1).

3.4.3 Data analysis

The same measurements taken for the sheep experiments were also taken for pig experiments.

A minimum of three stimulations at each test point were performed – in the control setting and following the addition of drugs – and a mean of the response was taken for each measurement. The measurements taken have been previously described in Figure 2-2.

All results were tested for normality and outliers were excluded after performing an outlier analysis. As with the sheep experiments, the commonest statistical test used was the Wilcoxon signed rank test to investigate the differences in the EFS response before and after the addition of drugs. All statistical tests were performed using SPSS or Graph Pad Prism statistical software.

3.4.4 Experimental protocol

3.4.4.1 Control experiments

The tissues were suspended in organ baths and allowed equilibrate for approximately 60 minutes. They were then stretched with a 3g tension and following this, two KCL challenges were performed with a washout after each challenge. Tissues were then allowed to equilibrate for a further 60 to 90 minutes until stable baseline tone was observed. Tissues were then stimulated with EFS 10 Hz and EFS 30 Hz (pulse strength 300mA, pulse width 0.3 ms, 30 seconds at 10 minute intervals), three stimulations each, 10 minutes apart. These formed the control responses. Drugs were then added to the organ baths and the responses to EFS following addition of drugs was noted. Each tissue served as its own control.

3.4.4.2 The role NO in the EFS response

L-NAME was added at a concentration of 0.1 mM and the tissues were allowed to equilibrate for approximately 60 minutes, or longer, until stable baseline was achieved. Following this, the response to EFS 10 Hz and EFS 30 Hz was noted.

3.4.4.3 The role of mAchR in the EFS response

Atropine was used at a concentration of 1 µM after recording the control responses and further responses to EFS were noted.

3.4.4.4 The role of NA in the EFS response

Initially, Guanethidine was used at a concentration of 3 µM to block the release of NA from post-ganglionic nerves and the change to the EFS response was noted. Following this set of experiments, another set of experiments investigated the effect of adding 1 µM Phentolamine to block α adrenoceptors on the EFS response. A third set of experiments investigated the role of α_1 adrenoceptors by comparing the EFS response before and after the addition of 0.1 µM Prazosin to tissues.

3.4.4.5 The effect of methoxamine

Two sets of experiments were performed. In the first set of experiments, 1 µM Methoxamine was added following the control EFS responses and the effect following the addition of Methoxamine was noted. In the second set of experiments, Methoxamine was added in a cumulative fashion to tissues in the absence of EFS stimulation and the concentration-response of methoxamine on baseline tone was investigated.

3.4.4.6 The role of α_2 adrenoceptors

Clonidine (a specific α_2 agonist) was investigated in the same fashion as Methoxamine – one set of experiments investigated the effect of 0.1 µM Clonidine on the EFS response and a second set of experiments investigated the concentration-response to Clonidine in the absence of EFS stimulation.

Brimonidine – another specific α_2 agonist – was investigated only in a cumulative concentration-response fashion in the absence of EFS stimulation and the effect on baseline tone was noted.

3.5 Results

3.5.1 Pig internal anal sphincter

3.5.1.1 Control experiments

On passive stretch, pig IAS tissues developed tone (6.17g ± 0.41) (n = 15) and spontaneous activity. The response to EFS was relaxation, contraction, after-contraction.

Figure 3-2 illustrates the differences between the EFS 30 Hz and EFS 10 Hz responses. Compared to 10 Hz EFS, at 30 Hz, the relaxation was significantly smaller by 68% (1.76g ± 0.29 vs. 0.56g ± 0.16, p = 0.001), was sustained for significantly less time by 76% (RoT_{50} 16.59s ± 3.32 vs. 3.94s ± 0.91, p = 0.002) and the contraction was significantly greater by 384% (1.99g ± 0.7 vs. 9.64g ± 1.73, p < 0.001).

As the response to inhibitors was better appreciated at the 30 Hz responses, only these are described with the 10 Hz responses provided in the charts for comparison.

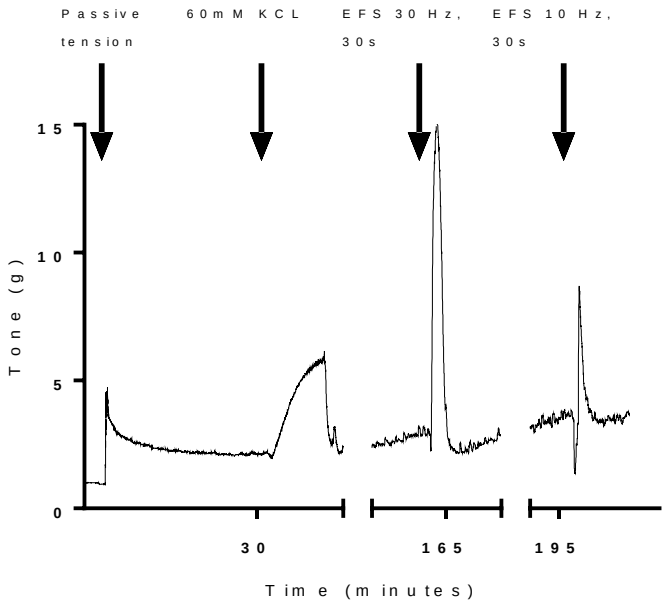
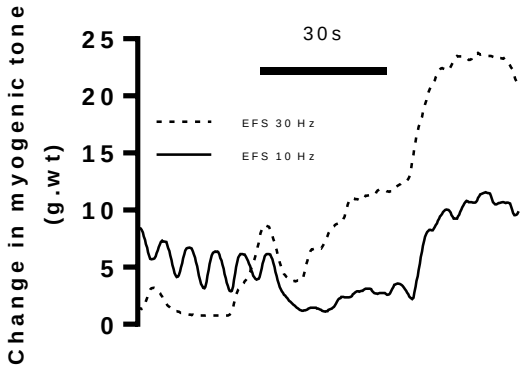


Figure 3-1 – Tissue setup and EFS response, pig IAS

The figure demonstrates a representative trace of pig IAS starting with 0g tone, tension to 4 g, one KCL challenge and one each of EFS 30 Hz and 10 Hz stimulations. Following a period of relaxation after passive tension, the tissue was challenged with 60mM KCL and then washed out. One further challenge was given (not shown). After further equilibration, the tissue was stimulated with 30 Hz EFS for 3 responses (one shown) and then with EFS 10 Hz for 3 responses (one shown) – these served as the control responses. Drugs were then added as per the protocol and further EFS responses noted – these served as the experimental responses



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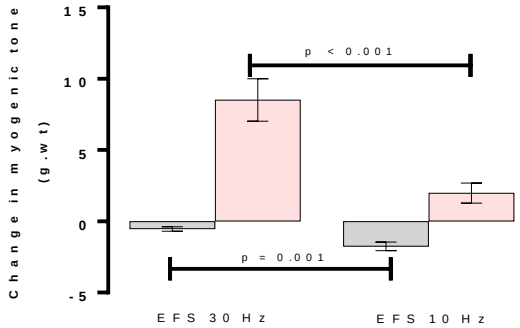


(b)

Figure 3-2 – Tissue response to EFS 30 Hz and EFS 10 Hz, pig IAS

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, there was a greater relaxation and smaller contraction to EFS 10 Hz compared to EFS 30 Hz.

Figure (b) illustrates the differences in contraction and relaxation for EFS 30 Hz (n = 15) and EFS 10 Hz (n = 15). SE bars are shown and significant differences are highlighted. Compared to EFS 30 Hz, the relaxation was significantly greater at EFS 10 Hz (0.56g vs. 1.76g, p = 0.001) and the contraction was significantly smaller (9.64g vs. 1.99g, p<0.001). The relaxation at EFS 10 Hz was also significantly longer in duration compared to EFS 30 Hz (not shown).

3.5.1.2 The role of NO in the EFS response?

Figure 3-3 demonstrates the change in the EFS response following the addition of L-NAME. 0.1 mM L-NAME caused a significant rise in baseline tone by 18% (5.93g ± 0.77 to 7.00g ± 0.96, p = 0.017) (n = 8). Relaxation was reduced by 88% but did not reach statistical significance. Contraction did not change significantly.

A similar response was noted at EFS 10 Hz where the relaxation was smaller but did not reach statistical significance.

3.5.1.3 The role of mAchR in the EFS response

Figure 3-4 illustrates the change in the EFS response following the addition of atropine. Tissues pre-incubated with 0.1 mM L-NAME served as the control responses, 1 µM Atropine was then added to tissues and further EFS responses served as the test responses. Following the addition of L-NAME (n = 8), relaxation was abolished in the majority of tissues and therefore was not analysed further. Atropine caused significant reduction in baseline tone by 12% (7.1g ± 0.73 to 6.35g ± 0.85, p = 0.006) but the magnitude of the change was small. There was a significant decrease in the contraction to EFS 30 Hz following the addition of Atropine by 17% (7.87g ± 1.88 to 6.55g ± 1.73, p = 0.05) but again, the magnitude of the change was small.

At EFS 10 Hz, although there was a decrease in the contraction to EFS following the addition of 1 µM Atropine by 95%, this did not reach statistical significance (p = 0.068).

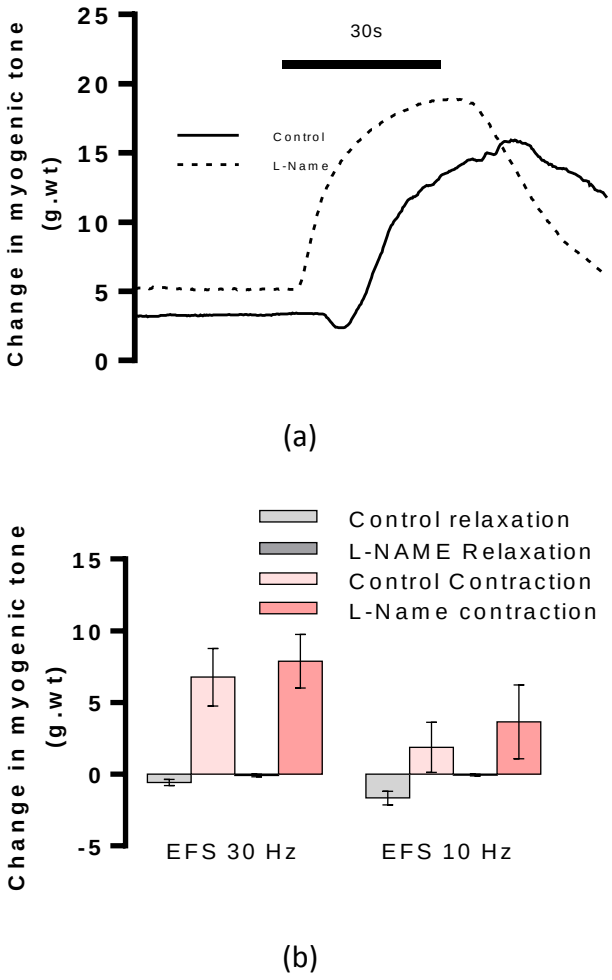
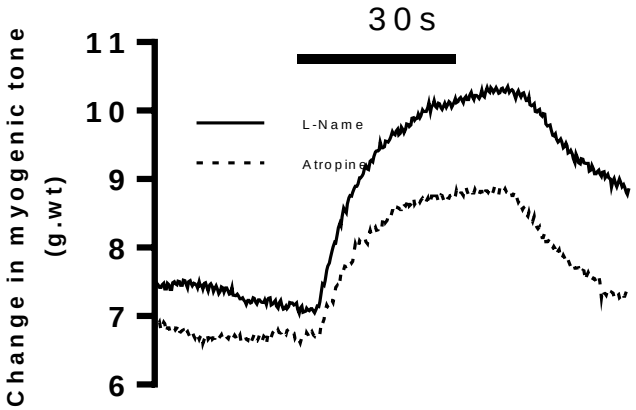


Figure 3-3 – Change in EFS response before and after the addition of 1 µM L-NAME, pig IAS

Figure (a) is a representative trace of the 30 Hz response in pig IAS tissue before and after the addition of 1 µM L-NAME. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation. Figure (b) illustrates the difference in contraction and relaxation to EFS before and after the addition of L-NAME at EFS 30 Hz (n = 8) and EFS 10 Hz (n = 4). SE bars are shown. At EFS 30 Hz, relaxation was reduced following L-NAME (0.66g to 0.08g) and contraction was increased (6.26g vs. 7.87g) but neither reached statistical significance. At EFS 10 Hz, a similar response was noted with a non-significant increase in contraction (1.87g vs. 3.65g) and a non-significant decrease in relaxation (1.65g vs. 0.06g) following the addition of L-NAME



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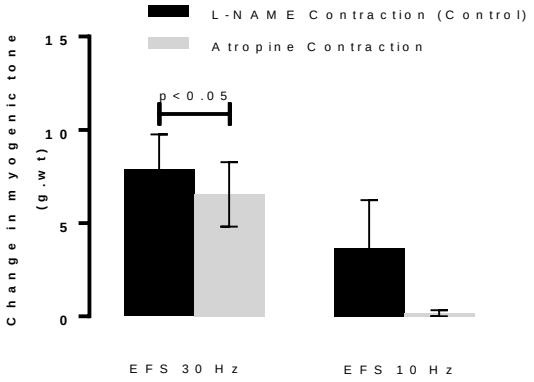


(b)

Figure 3-4 – Effect of EFS before and after the addition of 1 µM atropine in tissues pre-incubated with 0.1 mM L-NAME, pig IAS

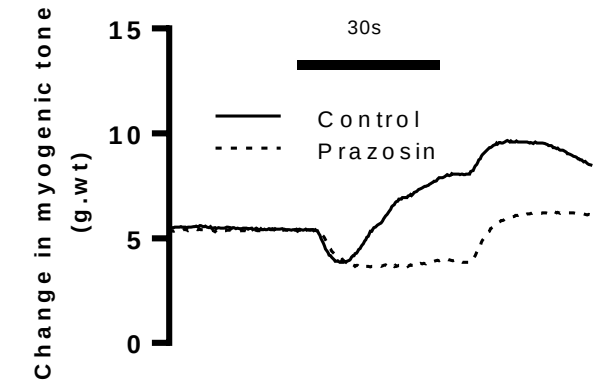
Figure (a) is a representative trace of the 30 Hz response in sheep IAS tissue (pre-incubated with 0.1 mM L-NAME) before and after the addition of 1 µM atropine. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation.

Figure (b) illustrates the difference in contraction and relaxation with EFS before and after the addition of atropine (tissues pre-treated with 0.1 mM L-NAME) at EFS 30 Hz (n = 8) and EFS 10 Hz (n = 4). SE bars are shown, statistically significant differences are highlighted. L-NAME abolished the relaxation to EFS in most tissues. At EFS 30 Hz, the contraction was significantly reduced following the addition of atropine (7.87g vs. 6.55g, p = 0.05) as well as at EFS 10 Hz (3.66g vs. 0.18g, p = 0.068) although the change was not statistically significant at EFS 10 Hz

3.5.1.4 The role of α_1 adrenoceptors in the EFS response

Figure 3-5 demonstrates the effect of prazosin on the EFS response. Prazosin was added at a concentration of 0.1 µM following the control EFS responses and the change in the EFS response was noted. Seven tissues were examined. Prazosin did not significantly alter baseline tone. Addition of Prazosin caused a dramatic change in the EFS response. At EFS 30 Hz, relaxation was increased by 413% (0.47g ± 0.24 to 2.41g ± 0.47, p = 0.018) and was sustained for significantly longer by 496% (RoT_{50} 3.32s ± 1.49 to 19.8s ± 3.99, p = 0.028). Contraction was significantly reduced by 96% (10.52g ± 2.12 to 0.42g ± 0.25, p = 0.018) and was completely abolished in 3 out of 8 tissues.

At EFS 10 Hz, a similar response was noted with a significant increase in relaxation and a significant decrease in contraction following the addition of 0.1 µM Prazosin.



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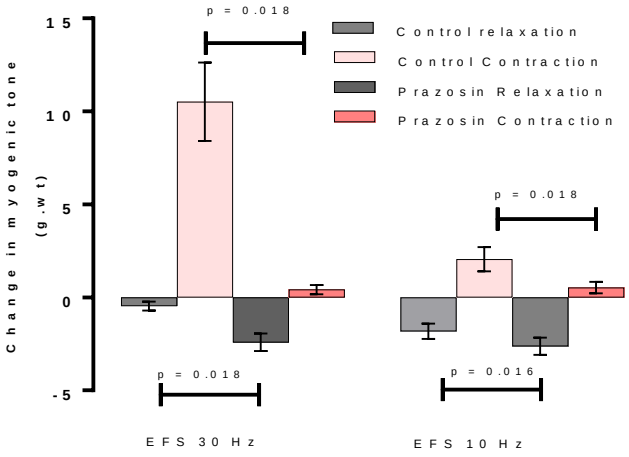


(b)

Figure 3-5 – Effect of 1 µM prazosin on the EFS response, pig IAS

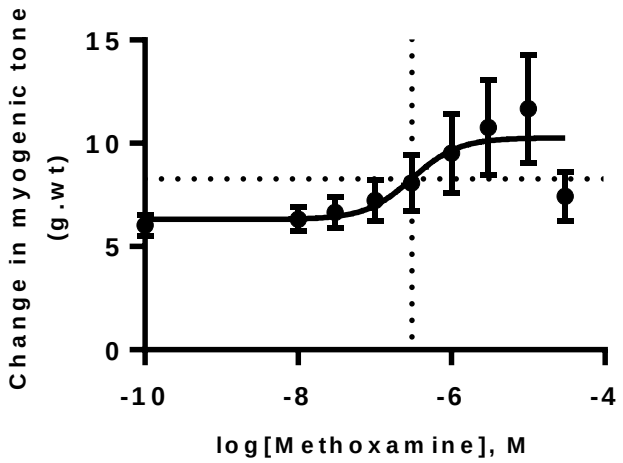
Figure (a) is a representative trace of the 30 Hz response in pig IAS tissue before and after the addition of 1 µM prazosin. The two traces are from the same tissue at different time points, the horizontal bar represents the period of stimulation. Figure (b) illustrates the difference in contraction with EFS before and after the addition of prazosin at EFS 30 Hz and EFS 10 Hz (n = 7 for both). SE bars are shown, statistically significant differences are highlighted. At EFS 30 Hz, contraction was significantly reduced following the addition of prazosin (10.52g vs. 0.42g) and relaxation was significantly increased (0.47g vs. 2.41g). At EFS 10 Hz contraction also decreased significantly (2.05g vs. 0.53g) and relaxation was significantly increased (1.82g vs. 2.62g)

3.5.1.5 The effect of methoxamine on baseline tone

A concentration-response experiment was performed (Figure 3-6) starting with a methoxamine concentration of 0.01µM and continuing serial additions of methoxamine to a maximum bath concentration of 30 µM (n = 6). Methoxamine caused a steady rise in baseline tone with increasing concentrations. EC_{50} was 303.1 nM (CI 32.9 nM to 2.79 µM); $logEC_{50}$ -6.51 ± 0.48.

3.5.1.6 The effect of α_2 agonists on the baseline tone

Clonidine was used to perform a concentration-response experiment (n = 6) in the absence of EFS stimulation (Figure 3-6) starting with a clonidine concentration of 10 nM to a concentration of 30 µM in incremental increases. As with methoxamine, a steady increase in baseline tone was noted with increasing concentrations of clonidine. EC_{50} was 91.6 nM (CI 8.8nM to 954 nM), $logEC_{50}$ -7.038 ± 0.506.



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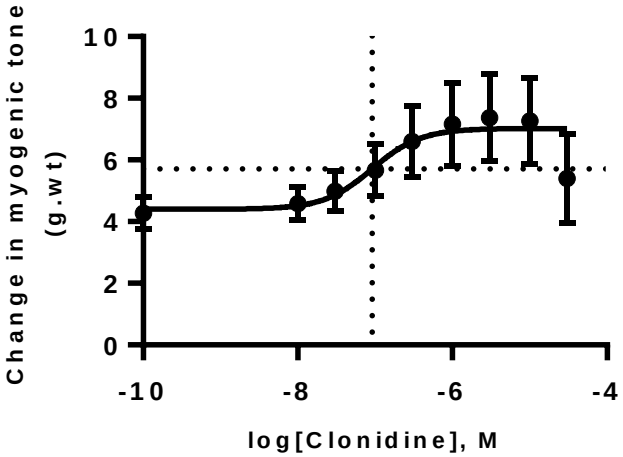


(b)

Figure 3-6 – Concentration response curves for methoxamine and clonidine, pig IAS

Figure (a) demonstrates the concentration response curve for methoxamine (n = 6) and figure (b) shows the concentration response curve for clonidine (n = 6). SE bars are shown for each concentration and the values are plotted against a log concentration axis. In both tissues, a steady rise in baseline tone was observed with serial additions of methoxamine or clonidine. The dashed lines indicate the EC_{50} values for each. For methoxamine, EC_{50} was 303.1 nM and for clonidine EC_{50} was 91.6 nM.

3.5.1.7 Summary

A table summarising the effects of drugs on the EFS 30 Hz response in pig IAS is provided.

	Baseline (g)	Relaxation (g)	Duration of relaxation (s)	Contraction (g)
Control	6.17	0.56	3.94	9.64
L-NAME	↑	↓	N/A	No change
L-NAME + Atropine	↓	NA	NA	↓
Prazosin	No change	↑↑	↑↑	↓↓
Methoxamine	↑↑			
Clonidine	↑↑			

Table 5 – Summary of 30 Hz responses in pig IAS

Key – large reduction ↓↓
large increase ↑↑
small but significant increase ↑
N/A – no response, therefore not applicable

Our experiments on pig IAS show that the tissue develops tone and spontaneous activity similar to that of the rectum on passive stretch. The response to EFS is initially a relaxation, mediated by NO. EFS also causes a contraction which is masked by NO in some cases, and is more prominent when NO is blocked. The contraction to EFS is primarily mediated by NA acting through α_1 adrenoceptors and only a small contributing component of Ach acting on mAchR was observed in our experiments.

3.5.2 Pig rectum

3.5.2.1 Control experiments

Figure 3-7 illustrates the setup of the tissue along with a representative EFS 30 Hz and EFS 10 Hz response. In response to stretch, pig rectum developed intrinsic tone and had significant baseline variability. Baseline tone was 4.63g ± 0.29 (n = 114). The EFS response during the period of stimulation was initial relaxation followed by contraction. At the end of stimulation, there was often a variable after-contraction. As the after-contraction did not appear to be related to the magnitude of the EFS response, no further analysis on this was performed.

There were significant differences in the responses to EFS 10 Hz (108) and EFS 30 Hz (n = 113) (Figure 3-8). At EFS 10 Hz, the relaxation was 32% greater (2.75g ± 0.27 vs. 2.08g ± 0.26, $p = 0.051$), was sustained for 47% longer (26.36s ± 0.92 vs. 17.85s ± 0.83, $p < 0.001$) and the contraction was significantly smaller by 70% (1.08g ± 0.31 vs. 3.64g ± 0.53, $p < 0.001$) when compared to EFS 30 Hz.

As the response to inhibitors was more easily appreciated in the EFS 30 Hz responses, the EFS 10 Hz responses are not further mentioned but they are still provided in the figures to aid comparison.

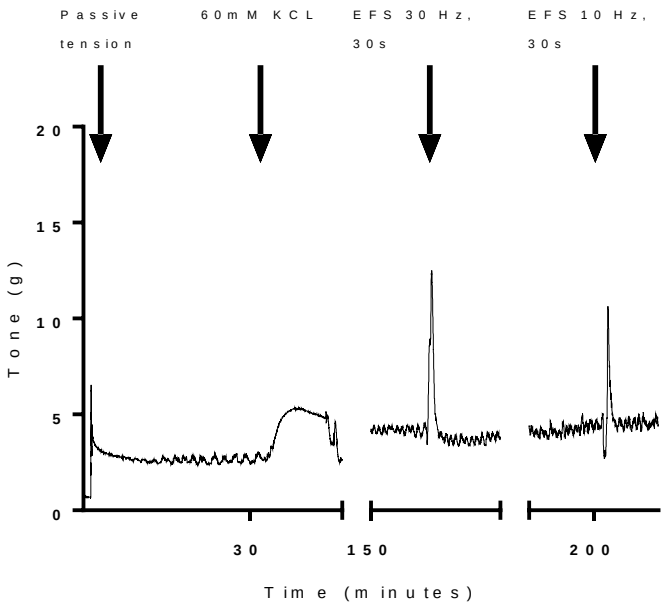
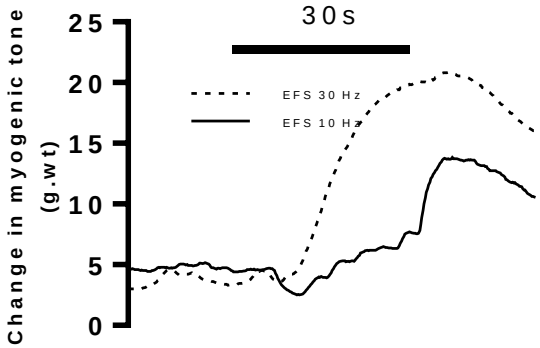
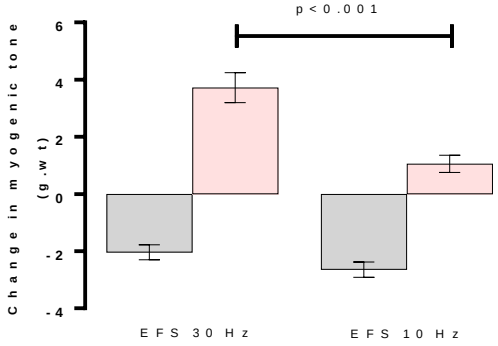


Figure 3-7 – Tissue setup and EFS response, pig rectum

The figure demonstrates a representative trace of pig rectum starting with 0g tone, tension to 4 g, one KCL challenge and one each of EFS 30 Hz and 10 Hz stimulations. Following a period of relaxation after passive tension, the tissue was challenged with 60mM KCL and then washed out. One further challenge was given (not shown). After further equilibration, the tissue was stimulated with 30 Hz EFS for 3 responses (one shown) and then with EFS 10 Hz for 3 responses (one shown) – these served as the control responses. Drugs were then added as per the protocol and further EFS responses noted – these served as the experimental responses



(a)



(b)

Figure 3-8 – Tissue response to EFS 30 Hz and EFS 10 Hz, pig rectum

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, there was a greater relaxation and smaller contraction to EFS 10 Hz compared to EFS 30 Hz.

*Figure (b) illustrates the differences in contraction and relaxation for EFS 30 Hz (n = 113) and EFS 10 Hz (n = 108). SE bars are shown and significant differences are highlighted with * (** p<0.001). Compared to EFS 30 Hz, the relaxation was greater at EFS 10 Hz (2.08g vs. 2.75g, p = 0.051) but did not reach statistical significance. Compared to EFS 30 Hz, the contraction was significantly smaller (3.64g vs. 1.08g, p<0.001) at EFS 10 Hz. The relaxation at EFS 10 Hz was also significantly longer in duration compared to EFS 30 Hz (not shown).*

3.5.2.2 The role of NO in the EFS response

Figure 3-9 demonstrates the change in response to EFS following the addition of L-NAME. L-NAME was added at a final bath concentration of 0.1mM following the EFS 10 Hz (n = 45) and EFS 30 Hz (n = 55) control responses. The tissues were then allowed to equilibrate for 60 to 90 minutes until stable baseline tone was observed. Further EFS 10 Hz and EFS 30 Hz responses were noted.

L-NAME significantly increased baseline tone by 43% (4.26g ± 0.32 to 6.1g ± 0.44, $p < 0.001$). Relaxation was significantly reduced by 75% (2.03g ± 0.44 to 0.49g ± 0.19, $p < 0.001$) and was completely abolished in 36 out of 55 cases. RoT_{50} was not calculable because of the reduction in relaxation. Contraction was significantly increased by 130% (3.43g ± 0.68 to 7.92g ± 1.01, $p < 0.001$).

At EFS 10 Hz, a similar response was noted with a significant reduction in relaxation and significant increase in the contraction.

3.5.2.3 The role of mAchR in the EFS response

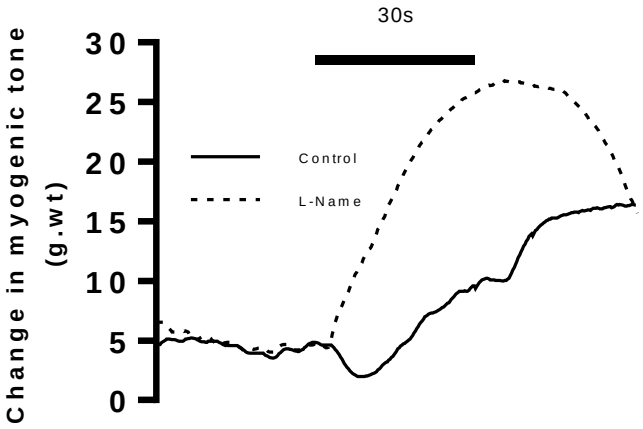
Atropine was added at a final bath concentration of 1 µM and the change in the EFS response was noted.

Baseline tone was not affected with the addition of atropine (n = 5). At EFS 30 Hz (n = 5), relaxation did not change significantly, but the duration of relaxation was significantly longer by 35% (RoT_{50} 17.37s ± 4.24 to 23.42s ± 3.3, $p = 0.046$). Contraction was smaller but not statistically significant.

At EFS 10 Hz, no significant changes were noted in the relaxation, duration of relaxation or contraction to EFS following the addition of 1 µM atropine.

Atropine was also investigated in tissues pre-incubated with L-NAME (n = 11). Figure 3-10 illustrates the effect of atropine on tissues pre-incubated with L-NAME. Tissues were suspended in an organ bath infused with 0.1 mM L-NAME and allowed to equilibrate as previously stated. EFS responses in the presence of L-NAME provided the control responses. 1 μM atropine was then added to the organ baths and the EFS responses following this provided the test responses.

In the presence of L-NAME, the majority of the tissues did not demonstrate any relaxation. At EFS 30 Hz, contraction following further addition of 1 μM atropine was significantly reduced by 32% (3.91g ± 1.45 to 2.64g ± 1.09, p = 0.005) – one value was excluded after outlier analysis. At EFS 10 Hz, the contraction following 1 μM atropine was also significantly reduced.



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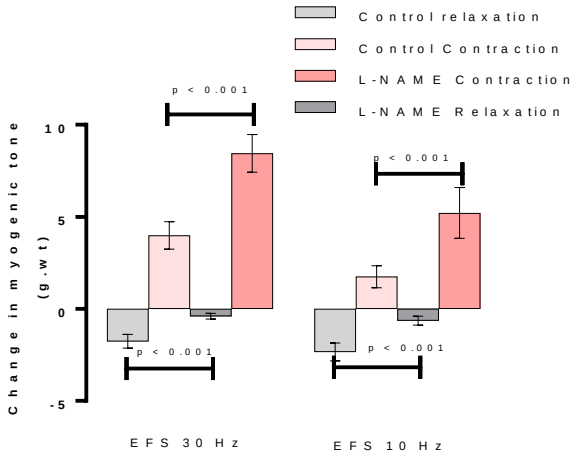
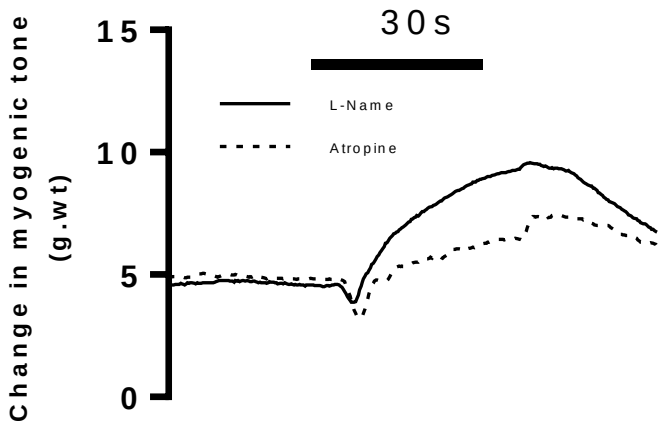


(b)

Figure 3-9 – Tissue response to 0.1mM L-NAME, pig rectum

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, following the addition of L-NAME, relaxation was abolished and contraction to EFS was increased.

Figure (b) illustrates the differences in contraction and relaxation for EFS 30 Hz (n = 55) and EFS 10 Hz (n = 45). SE bars are shown and significant differences are highlighted. Following the addition of 0.1mM L-NAME, relaxation at 30 Hz was significantly reduced (2.03g vs. 0.49g) and the contraction was significantly increased (3.43g vs. 7.92g). Similarly, at 10 Hz, relaxation was significantly reduced (2.9g vs. 0.88g) and contraction was significantly increased (0.95g vs. 3.36g) following the addition of L-NAME



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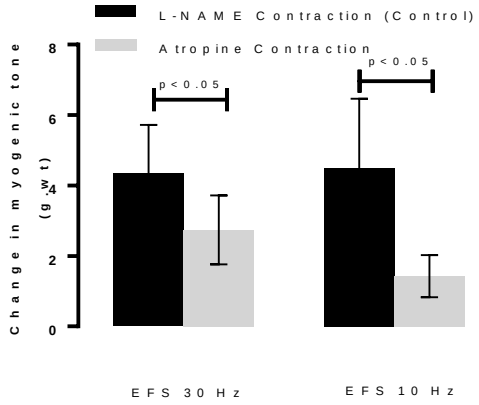


(b)

Figure 3-10 – Change in EFS response to 1µM atropine in tissues pre-treated with 0.1mM L-NAME, pig rectum

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, following the addition of atropine, contraction to EFS was reduced.

Figure (b) illustrates the differences in contraction for EFS 30 Hz (n = 11) and EFS 10 Hz (n = 8). SE bars are shown and significant differences are highlighted. Following L-NAME, relaxation was abolished in a majority of tissues, therefore not further analysed. Following the addition of 1µM atropine, contraction at 30 Hz was significantly reduced (3.91g vs. 2.64g, p=0.005) as well as at EFS 10 Hz (4.5g vs. 1.43g, p = 0.012)

3.5.2.4 The role of α adrenoceptors in the EFS response

3.5.2.4.1 Guanethidine

Guanethidine was added to tissues at 3 µM concentration (n = 9) and the change in the EFS response was noted.

Baseline tone was not affected by the addition of guanethidine. At EFS 30 Hz, relaxation did not change significantly following addition of guanethidine but the duration of relaxation was significantly increased by 104% (RoT_{50} 14.1s ± 1.97 to 28.77s ± 2.01, $p < 0.001$). The contraction to EFS 30 Hz was also dramatically reduced by 89% (1.99g ± 0.38 to 0.22g ± 0.04, $p = 0.02$) and was completely abolished in 6 out of 9 tissues . At EFS 10 Hz, a similar response was noted with a significant increase in the duration of relaxation but no significant change in the magnitude of the relaxation; contraction at baseline was very small (0.34g ± 0.24) therefore a change in contraction was not statistically significant.

Guanethidine was also examined in tissues pre-incubated with 0.1 mM L-NAME. EFS response post addition of L-NAME served as the control response. 3 µM guanethidine was then added to tissues and the EFS responses following this served as the test response.

L-NAME abolished the relaxation in 11 out of 13 tissues, therefore relaxation and RoT_{50} were not analysed further. At EFS 30 Hz (n = 13), addition of 3 µM guanethidine caused a significant reduction in the contraction to L-NAME by 44% (8.05g ± 2.09 to 4.52g ± 1.18, $p = 0.04$). A similar response was noted at EFS 10 Hz with a reduction in the contraction but this did not reach statistical significance.

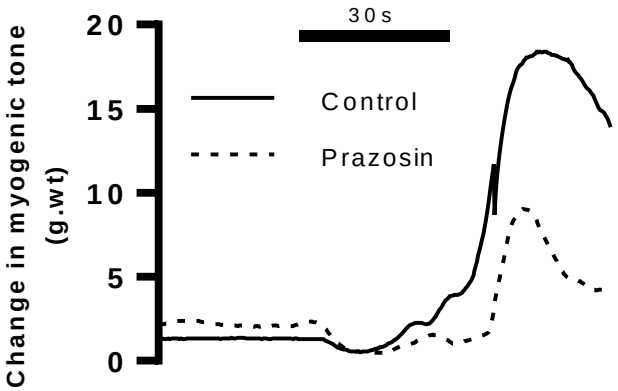
3.5.2.4.2 Phentolamine

Phentolamine was added to the organ bath at a concentration of 1 µM and the change in the EFS response was noted. Baseline tone was not affected. At EFS 30 Hz (n = 6), there was no significant change in the relaxation to EFS but the duration of relaxation was significantly increased by 87% (RoT_{50} 14.69s ± 2.81 to 27.44s ± 3.86, p = 0.016). Contraction was reduced by 62% but did not reach statistical significance. At EFS 10 Hz, no significant change was noted.

Phentolamine was also investigated in tissues pre-incubated with L-NAME. As before, L-NAME completely abolished the relaxation to EFS in 5 out of 7 tissues, therefore relaxation and RoT_{50} were not further analysed. At EFS 30 Hz (n = 7), 1 µM phentolamine caused a significant reduction in the contraction by 57% (9.15g ± 2.56 to 3.96g ± 1.33, p = 0.05). There were no significant differences noted at EFS 10 Hz.

3.5.2.4.3 Prazosin

Figure 3-11 illustrates the effect of prazosin on the EFS response. Prazosin was added to the organ bath at 0.1 µM concentration did not significantly affect baseline tone (n = 10). At EFS 30 Hz, 0.1 µM prazosin did not change the relaxation or the duration of relaxation significantly. The contraction to EFS was significantly reduced by 92% (4.9g ± 2.12 to 0.35g ± 0.12, p = 0.01). At EFS 10 Hz, no significant change was noted. A similar response was noted in tissues pre-incubated with 0.1 mM L-NAME (n = 11). At EFS 30 Hz, addition of 0.1 µM prazosin caused a significant reduction in the contraction by 36% (10.47g ± 2.29 to 6.65g ± 1.68, p = 0.003). At EFS 10 Hz, no significant changes were noted.



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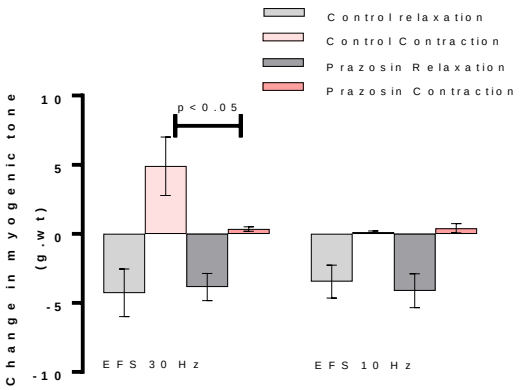


(b)

Figure 3-11 – Change in tissue response to 1µM prazosin, pig rectum

Figure (a) is a representative trace of the response to EFS from the same tissue at different time points. The horizontal bar denotes the period of EFS stimulation and EFS 10 Hz and EFS 30 Hz responses are denoted by separate lines. As shown, following the addition of prazosin, contraction to EFS was reduced and relaxation was increased.

Figure (b) illustrates the differences in contraction for EFS 30 Hz (n = 10) and EFS 10 Hz (n = 9). SE bars are shown and significant differences are highlighted. Following prazosin, relaxation was not significantly different at either EFS frequencies. At EFS 30 Hz, contraction was dramatically reduced (4.9g vs. 0.35g, p = 0.011) following prazosin. At EFS 10 Hz, the baseline contraction was too small to allow any significant differences to be appreciated

3.5.2.5 The effect of methoxamine on the EFS response and on tone

In the first set of experiments, 1 µM Methoxamine was added to the organ baths after the control responses and the effect on EFS responses was noted (n = 15). 1 µM methoxamine significantly increased baseline tone by 122% (4.1g ± 0.39 to 9.09 ± 1.17, $p < 0.001$). Relaxation was significantly increased by 195% (2.22g ± 0.38 to 6.56g ± 1.33, $p = 0.001$) and the duration of relaxation was significantly greater (RoT_{50} 14.08s ± 1.59 to 23.54s ± 1.39, $p = 0.004$). Contraction was also significantly reduced by 91% (4.98g ± 2.13 to 0.46g ± 0.15, $p = 0.026$). However, these results need to be interpreted with caution. As the relaxation and contraction were measured as differences from the baseline tone, a significantly increased baseline tone (as following the addition of methoxamine) could produce spurious results.

At EFS 10 Hz, a similar response was noted with a significant increase in the relaxation but no significant change in the contraction to EFS.

In the second set of experiments, methoxamine was added in a cumulative dose fashion to the organ bath in the absence of EFS stimulation to produce a concentration-response to methoxamine (n = 10). Figure 3-12 illustrates the concentration response curve for methoxamine. Methoxamine was added in a cumulative concentration starting with a concentration of 10nM and increased in increments – 30nM, 100nM, 300nM, 1µM etc upto a maximal concentration of 30µM. Maximal contraction was achieved at 0.3mM methoxamine concentration. EC_{50} was calculated at 371nM (CI 79.5nM to 1.74µM), $logEC_{50}$ -6.43 ± 0.336.

3.5.2.6 The effect of α_2 agonists on the EFS response and baseline tone

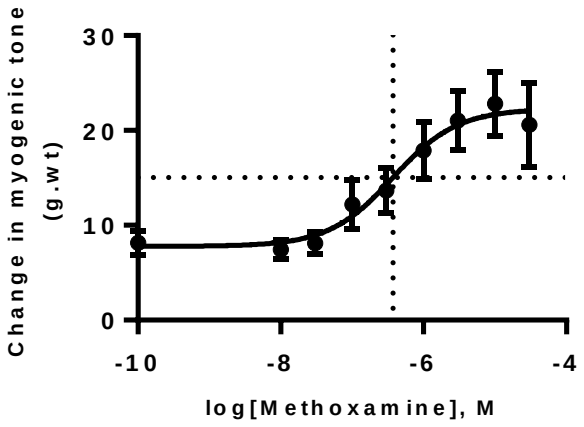
3.5.2.6.1 Clonidine

Two sets of experiments were performed with clonidine, as with methoxamine. In the first set of experiments, 0.1 µM clonidine was added to tissues following the control EFS responses and the change in the EFS response was noted (n = 12). Clonidine significantly increased baseline tone by 70% (4.17g ± 0.44 to 7.07g ± 0.85, $p < 0.001$). Relaxation was significantly greater by 94% (2.66g ± 0.62 to 5.18g ± 1.05, $p = 0.035$) and contraction was significantly reduced by 60% (1.6g ± 0.63 to 0.72g ± 0.29, $p = 0.017$). However, as with methoxamine, these results need to be interpreted with caution given the significant rise in the baseline tone.

In the second set of experiments, a dose response was calculated starting with a dose of 10nM and incrementally increasing the concentration to 30 µM (n = 6). A steady increase in tone was noted with EC_{50} calculated at 79 nM (CI 4.57nM to 1.37 µM), $logEC_{50}$ -7.102 ± 0.615.

3.5.2.6.2 Brimonidine

For Brimonidine, only a dose response experiment was performed. Brimonidine was added in a cumulative fashion starting with a concentration of 1 nM to a concentration of 30 µM. EC_{50} was 788 nM (CI 33.3 nM to 18.6 µM), $LogEC_{50}$ -6.014 ± 0.691 (n = 8).



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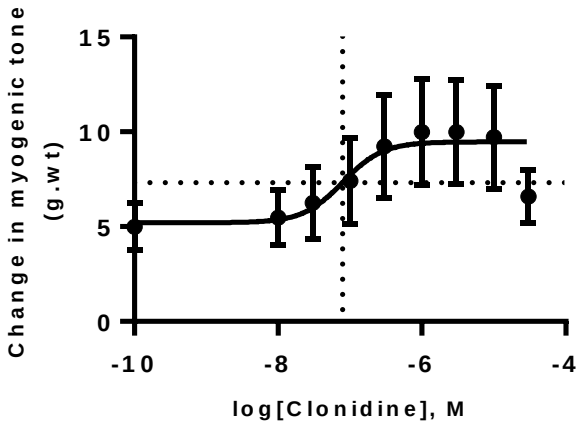

(b)

Figure 3-12 – Concentration response curves, pig rectum

Concentration response curves for methoxamine (Figure (a)) and clonidine (Figure (b)) are demonstrated. Standard error bars are demonstrated for each concentration point plotted against a log[concentration] axis. The dashed lines indicate EC_{50} values.

Figure (a) shows the concentration response curve for methoxamine (n = 10) EC_{50} was 371nM.

Figure (b) shows the concentration response curve for clonidine (n = 6). EC_{50} was 79 nM.

3.5.2.7 Summary

A table summarising the effects of drugs on the EFS 30 Hz response in pig rectum is provided.

	Baseline (g)	Relaxation (g)	Duration of relaxation (s)	Contraction (g)
Control	4.63	2.08	17.85	3.64
L-NAME	↑	↓↓	N/A	↑↑
Atropine	No change	No change	↑	No change
Guanethidine	No Change	No change	↑↑	↓↓
Prazosin	No change	No change	No change	↓↓
Methoxamine	↑↑			
Clonidine	↑↑			

Table 6 – Summary of changes to EFS stimulation, Pig Rectum

Key – large reduction ↓↓
large increase ↑↑
small but significant increase ↑
N/A – no response, therefore not applicable

3.5.3 Comparing pig rectum and pig IAS

A comparison of the results from the rectal and IAS tissues is provided.

		Rectum	IAS
Baseline		4.25 ± 0.20	6.18 ± 0.41
L-Name	Contraction	⬆ 130%	➡
	Relaxation	⬇ 75%	⬇ 87% (not significant)
L-Name + Atropine	Contraction	⬇ 32%	⬇ 17%
	Relaxation	⬆ 75% (not significant)	NA
Prazosin	Contraction	⬇ 93%	⬇ 96%
	Relaxation	➡	⬆ 412%
Methoxamine	Baseline Tone	⬆ EC_{50} 371 nM	⬆ EC_{50} 303 nM
Clonidine	Baseline Tone	⬆ EC_{50} 79 nM	⬆ EC_{50} 91.6 nM

Table 7 – Comparison of effects of drugs on EFS 30 Hz response, pig IAS and rectum

In order to compare the two tissues more objectively, we performed identical experiments on both IAS and rectal tissues from the same animal.

Dissection was identical to that previously described with one difference – the smooth muscle layer identified at the end of the dissection was dissected to yield

an identically-sized segment of IAS and rectum. These tissues were then suspended in separate organ baths and exactly the same experiment was performed on both.

3.5.3.1 Control experiments

Fifteen paired tissues were examined. Baseline tone did not vary significantly between rectal and IAS tissues (5.86g ± 0.58 vs. 6.24g ± 1.55, p = 0.799). At EFS 30 Hz, compared to rectal tissues, relaxation was significantly smaller in IAS tissues (0.66g ± 0.17 vs. 4.35g ± 1.83, p = 0.024) and was sustained for significantly shorter time (RoT_{50} 5.89s ± 2.11 vs. 14.14s ± 3.21, p = 0.015). Contraction did not vary significantly between the two tissues (8.84g ± 1.77 vs. 8.84g ± 3.18).

At EFS 10 Hz, no significant differences were noted.

3.5.3.2 The relative role of NO in IAS compared to rectal tissues

No paired experiments were performed with L-NAME. However, comparing the response from unpaired tissues between rectal and IAS tissues, we noted that addition of 0.1 mM L-NAME, there was a significant increase in baseline tone with both IAS and rectal tissues. However, in the case of rectal tissue, there was a 43% increase in baseline tone (4.26g to 6.1g) while for IAS tissue, there was only a 21% increase (5.92g to 7.19g).

In both tissues, there was a significant reduction in relaxation and an increase in contraction. With IAS tissues, the reduction in relaxation was 88% (0.66g to 0.08g) while in rectal tissue, the reduction was 76% (2.03g to 0.49g). Contraction

increased by 20% (6.26g to 7.87g) in IAS tissues (not statistically significant), and by 130% in rectal tissue (3.43g to 7.92g).

3.5.3.3 The relative effect of mAchR in IAS and rectal tissues

Eight tissue pairs were examined. Tissues were pre-incubated with 0.1 mM L-NAME and the EFS responses following this served as the control responses. Atropine was added at a concentration of 1 μM and the EFS responses following this were the test responses.

Atropine, in the presence of L-Name, caused a reduction in the contraction to EFS in both IAS and rectal tissue but the difference was smaller in IAS tissue (17%, 7.87g to 6.55g, $p = 0.05$) compared to rectal tissue where the difference was a lot greater (32%, 3.91g to 2.64g, $p = 0.005$).

3.5.3.4 Effect of prazosin

Seven pairs of tissues were examined. Prazosin had a greater effect on the relaxation and contraction in IAS tissues compared to rectal tissues. At EFS 30 Hz, in IAS tissues, relaxation was significantly increased by 413% (0.47g to 2.41g) whereas there was no statistically significant difference in rectal tissue. Contraction was significantly decreased by 96% (10.52g to 2.12g) in IAS tissues and by 92% (4.9g to 0.35g) in rectal tissues.

3.5.3.5 Effect of methoxamine

Six experiments were performed with paired rectal and IAS tissues investigating the concentration-response of each of these tissues to Methoxamine. Starting

with a concentration of 10 nM incremental increases in Methoxamine to a concentration of 30 µM was performed and the effect on baseline tone was noted.

Methoxamine caused a rise in the baseline tone in both IAS and rectal tissues. EC_{50} values for IAS and rectal tissues was similar (400nM for rectal and 303.1nM for IAS). The values were normalised to range from 0 to 100 for both sets and a best fit model was employed plotting log[Methoxamine] concentrations against the normalised increase in tone. Using a comparison of fits, there was no significant difference in the EC_{50} values between IAS and rectal tissues ($p = 0.828$).

3.5.3.6 The comparative effect of α_2 agonists in IAS and rectal tissues

Six paired tissues were examined. Clonidine was added cumulatively from a concentration of 10 nM to a concentration of 30 µM and the effect on tone was observed. The results were normalised to range from 0 to 100 for each of the tissues and a best fit model was employed comparing the concentration-response curves of both IAS and rectal tissues. EC_{50} was 79 nM in the rectal tissues and was 92 nM in the IAS tissues. Using the comparison of fits model, there was no significant difference between the response from either tissue to Clonidine ($p = 0.945$).

3.6 Discussion

3.6.1 Evidence of homology between human and pig tissues

In our experiments, the pig rectal tissue developed tone. The response to EFS was relaxation followed by a contraction during the period of stimulation. As

mentioned previously, there is disagreement among authors with regards to the human rectal tissue – some authors describe no tone to the human rectum on passive stretch [146, 159, 180, 185] while others describe the development of tone [162]. In some experiments, histamine was used to induce stable tone. In all experiments where EFS was investigated – with or without inducing tone – the authors describe a relaxation to EFS followed by a contraction during the period of stimulation [162, 179, 180].

Pig IAS tissue also developed tone on passive stretch in our experiments. The response to EFS was again relaxation followed by contraction during the period of stimulation. This agrees well with the findings of previous authors investigating the human IAS [155, 160-162, 174].

3.6.2 The role of NO in mediating the EFS response

In the pig rectum, 0.1 mM L-NAME significantly increased baseline tone indicating that NO was involved in modulating baseline tone in pig rectal tissue. Addition of L-NAME significantly reduced the relaxation to EFS and abolished it in the majority of tissues indicating that NO was the primary mediator of relaxation to EFS in this tissue. This correlates well with the results of previous authors on human rectal tissue [161, 162, 174] who also found a reduction in EFS induced relaxations following NO blockade and two of the three authors also noted a significant rise in baseline tension following NO blockade.

In the pig IAS tissues, blocking NO also caused a significant rise in baseline tone although this was small in magnitude. While there was a large reduction in the relaxation to EFS (88%), this did not reach statistical significance in our dataset. In

the human IAS, previous authors have demonstrated a significant reduction in relaxation to EFS following NO blockade [161, 162].

3.6.3 The role of mAchR in the EFS response

In the pig rectum, there was a significant reduction in the contraction to EFS following the addition of Atropine but the change in the response was relatively small in magnitude (30-40%). This was observed both in the presence and absence of L-NAME. This would indicate that while Ach is involved in mediating the contraction to neuronal stimulation, it is not the primary mediator. A number of previous authors have shown a contraction in the human rectum in response to AchR stimulation [146, 159, 188]. However, all these authors demonstrated a contraction of the human rectal tissue in response to Carbachol stimulation. Our results would agree with this as our dataset demonstrates that Ach stimulation is partly responsible for the contraction during EFS. In the sheep experiments, we performed Carbachol stimulations and demonstrated sustained contraction in rectal tissues, but this set of experiments were not performed in pig rectal tissue. Glavind *et al.* (1997) [162] performed a similar experiment to our experiments on human rectal tissues – they stimulated the human rectal tissue with EFS in the presence and absence of NO blockade and tested the effects of Phentolamine and Atropine on the EFS response. They found that in the control state, Phentolamine and Atropine did not significantly affect the EFS response. In the presence of NO blockade, Atropine completely abolished the contraction to EFS in human rectal tissue. It is worth noting that in their experiments, Glavind *et al.* (1997) [162]did not find did not find any contractions during the period of EFS stimulation in

human rectal tissue and only noticed contractions when NO had been blocked. Our results contradict these findings as the reduction in contraction in the presence of L-NAME in our tissues was modest and point to a minor role of mAchR in mediating the EFS contractions.

In the pig IAS tissue, in tissues pre-incubated with L-NAME, Atropine caused a small but statistical significant change in baseline tone (12%). We do not think this indicates a significant role of Ach in maintaining baseline tone in the pig IAS. In the presence of L-NAME, Atropine caused a small but statistically significant reduction in the contraction to EFS (17%). In the human IAS, two previous authors have noted a slight reduction in tone following mAchR blockade [159, 160] however, Burleigh *et al.* (1979) [160] noted that Atropine did not affect the EFS response. Glavind *et al.* (1997) [162] noted no change in the human IAS response to EFS stimulation in the presence or absence of mAchR blockade. These results compare well with our findings where the change in the EFS response was very small following the addition of Atropine.

3.6.4 The role of α adrenoceptors in the EFS response

In the rectum, the role of α adrenoceptors were investigated using Guanethidine, Phentolamine and Prazosin. The intention was to investigate the effect on the EFS response first by blocking NA release (Guanethidine), then by blocking α adrenoceptors in the rectal tissue (Phentolamine) and finally by specifically blocking α_1 adrenoceptors. Our results consistently demonstrate that NA is the primary mediator of contraction to EFS in the pig rectum, mediated via α_1 adrenoceptor action. Guanethidine, Phentolamine and Prazosin all significantly

reduced the contraction to EFS and either increased the relaxation significantly or significantly increased the duration of the relaxation. In tissues pre-incubated with L-NAME, a similar response was noted with a significant decrease in contraction following the addition of the three antagonists. Furthermore, the reduction in contraction following the addition of Guanethidine (89%) was similar in magnitude compared to the reduction in the contraction following the addition of Prazosin (92%). These results conclusively demonstrate that α_1 adrenoceptors are the primary mediator of contraction in the pig rectum. As noted previously, in investigating the human rectum, three authors have suggested that NA stimulation causes a relaxation by noting that spontaneous activity in the rectum was reduced by the addition of NA [146, 188]. O'Kelly *et al.* (1993) [159] added NA to pre-contracted human rectal tissue and found a relaxation. Discussion of these studies has been provided previously (The role of α-adrenoceptors). None of the mentioned studies used antagonists to confirm the action of α adrenoceptors and the response noted may well have been due to stimulation of β adrenoceptors. Glavind *et al.* (1997) [162] used α antagonists on the EFS response and noted no change in the response following α blockade. One observation is that in the control state, Glavind *et al.* (1997) did not observe any contractions during the period of EFS, and this may have been the cause of not observing any change. However, Glavind *et al.* (1997) also used α antagonists in tissues pre-incubated with L-NAME and found no change in the contraction to EFS. We are unable to account for this difference as our results demonstrate the predominant role of α_1 adrenoceptors in mediating the contraction to EFS in the rectum.

In the pig IAS, we noted a dramatic reduction in the contraction and increase in the relaxation to EFS stimulation following the addition of Prazosin which indicates that in the pig IAS α_1 adrenoceptors are the primary mediators of the contractile response to EFS. This agrees well with previous authors' findings in the human IAS [159, 162, 174, 175].

3.6.5 The effect of methoxamine

Methoxamine was investigated both in relation to its effects on the EFS response and in a concentration-response experiment. The significant rise in baseline tone with the addition of Methoxamine made the results from the EFS response spurious. The concentration-response experiments demonstrated a significant rise in baseline tone following serial Methoxamine concentrations with an EC_{50} of 371 nM. This confirms our findings previously that α_1 adrenoceptors cause contraction in the rectum. We do not know of any previous studies investigating Methoxamine in the human rectum *in vitro* but our results would suggest that Methoxamine would cause a decrease in rectal compliance (by increasing smooth muscle tone in the rectum) and would therefore be unsuitable for use as a treatment for FI.

In the pig IAS, we also confirmed a significant rise in tone following the addition of Methoxamine in a concentration-response experiment. This agrees with previous studies on the sheep IAS *in vitro* [206] and with *in vivo* human studies which showed a significant increase in mean anal resting pressure (MARP) following the application of perianal methoxamine [204].

3.6.6 The effect of α_2 agonists

In the pig rectal tissue, Clonidine and Brimonidine were investigated in a concentration-response experiment. Both caused a significant rise in baseline tone with EC_{50} of 79 nM and 788 nM respectively. These experiments were done to see if α_2 agonists could be used in conjunction with α_1 agonists and if they would be able to modulate the contractile effect of Methoxamine. Unfortunately, we found that α_2 agonists are contractile in the rectum as well.

In the IAS, Clonidine also produced significant rises in baseline tone with EC_{50} of 91.6 nM.

3.6.7 Comparing pig rectal and IAS tissues

These experiments were performed to investigate if there were any significant differences between the responses of the two tissues which might provide targets for pharmacological manipulation. We found that L-NAME caused a greater increase in the contraction to EFS in rectal tissue compared to IAS tissue (130% vs. 20%) and that Atropine caused a greater reduction in contraction in rectal tissue compared to IAS tissue (32% vs. 17%). However, the reduction in contraction with Prazosin was similar in rectal and IAS tissues (96% vs. 92%).

The normalised concentration-response curves for Methoxamine and Clonidine showed no significant differences between rectal and IAS tissues. This indicates that Methoxamine and Clonidine are both equally potent in causing contractions in both these tissues.

3.7 Conclusions

Our results from pig rectal tissue shows considerable homology with human rectal tissue but there were some differences noted with previous authors. We noted that pig rectal tissue did develop spontaneous tone and spontaneous activity – while this has been reported in human rectal tissue by one author, while three other authors have not reported human rectal tissue developing tone on stretch. The EFS response was relaxation (mediated by NO), followed by a contraction during the period of stimulation. This agrees well with numerous authors on the human rectal tissue. The contraction to EFS was mediated in small part by Ach but primarily by NA acting on α_1 adrenoceptors. This does not agree with previous authors' findings in human rectal tissue where one previous study has demonstrated that the contraction to EFS was primarily mediated by Ach and other authors have shown that NA causes a relaxation in the human rectum. We believe that the response to NA in the studies performed previously may well have been the result of β adrenoceptor stimulation rather than a true reflection of α adrenoceptor stimulation. The one study which reported an abolition of EFS induced contraction with Ach blockade does not agree with the results of our experiments and we do not have a satisfactory explanation for this difference.

We believe that there exists enough homology between pig and human rectal tissue for pig rectal tissue to be used as a reasonable substitute for human rectal tissue. We believe more work needs to be done on the human rectal tissue with regard to α adrenoceptors to confirm their role in mediating neuronal responses.

4 Conclusion from experiments on sheep and pig tissue

A table summarising the *in vitro* results of various authors on human IAS tissue has been previously cited (Table 2)

The following table summarises the *in vitro* results on human rectal tissue.

Study	Tone	NA	α adreno-receptors	β adreno-receptors	NO	mAchR	EFS
O'Kelly [159]	No	Relax	Relax	Relax		Contract	
Cook [146]	No	Relax				Contract	
Glavind [162]	Yes				Relax	Contract	Relax
Stebbing [179]	No	Relax			Relax	Contract	Relax
Brading [180]	No	Relax			Relax	Contract	Relax

Table 8 – Summary of in vitro experiments on human rectum

Most authors agree that mAchR stimulation causes a contraction in the rectum (Table 8). In the IAS, the role of mAchR is less clear with only one study demonstrating a relaxation to Carbachol antagonised by Atropine. With regards

to α adrenoceptors, the studies suggest a relaxation with α adrenoceptor stimulation in the rectum but we have already demonstrated that this requires further clarification. In the IAS, most authors agree that α adrenoceptor stimulation causes a contraction (Table 2).

The results from our experiments are summarised in Table 9.

	Tone	α adrenoceptors	NO	mAchR	EFS
Sheep IAS	Yes	Contraction	Not tested	Contraction	Relax
Pig IAS	Yes	Contraction	Relax	Contraction	Relax
Sheep rectum	No	Contraction	? Relax	Contraction	Contract
Pig rectum	Yes	Contraction	Relax	Contraction	Relax

Table 9 – Summary of findings from IAS and rectal tissue in sheep and pigs

In our experiments the IAS tissue from both pig and sheep showed good homology to human IAS tissue results from previous authors. As noted previously, the role of mAchR in the IAS tissue is not clear from the published studies – in our experiments, muscarinic acetylcholine receptors mediated *contraction* rather that *relaxation* in the responses to EFS. With rectal tissue, there was less homology with previous authors' experiments on human rectal tissues. Pig rectal tissue developed tone on passive stretching in our hands, whereas only one author describes similar findings in human rectal tissue. Furthermore, whereas previous authors have suggested that human rectal tissue *relaxes* in response to NA, our

experiments show that the contractile element of EFS is mediated primarily by NA in both the sheep and the pug. The effect of Methoxamine and α_2 agonists was exclusively contractile with a predictable concentration-response curve. In paired experiments between pig rectal and IAS tissues, we did not note any significant differences in the response to either Methoxamine or Clonidine.

For the treatment of FI, the ideal drug would relax the rectal smooth muscle and contract the IAS thereby increasing anal tone and increasing rectal compliance. In our experiments, we have not found any pharmacological targets that would potentiate both a relaxation in the rectum and contraction in the IAS. Our results with Methoxamine would suggest that this would not be the ideal drug to treat FI.

Our experiments have uncovered inconsistencies in the reports on the action of mAchR in the IAS and α adrenoceptors in the rectum. We believe further work is required on the human rectal and IAS tissues to clarify this.

5 Chapter 4 – Clinical trial of LEM in patiens with FI

As part of this project, we took part in an industry-sponsored trial investigating the efficacy and safety of LEM on patients with FI. The subjects recruited at our site and the data generated from our centre form the basis of this chapter.

5.1 Introduction

The presence of contractile α_1 receptors in the IAS has been known since the 1980s [212] and confirmed in an *in vitro* model by O'Kelly *et al.* in 1993 [159]. Phenylephrine, a selective α_1 adrenoceptor agonist, has been used on healthy volunteers at a dose of 5% and 10% perianally and has been shown to cause a significant rise in mean anal resting pressure (MARP) [213]. However, using a dose of 10% perianally, there was no significant improvement in continence scores in patients with FI in a randomised trial [200] with a number of subjects experiencing localised dermatitis at the areas of application. The suggestion of repeating the study with increasing doses of phenylephrine risks the potential side-effects of high doses of α_1 adrenoceptor agonists – hypertension and bradycardia. Methoxamine, another α_1 adrenoceptor agonist, has two chiral centres and the 1R,2S configuration (L-erythro methoxamine, LEM) was first discovered by Fujita *et al.* in 1988 [214] and was first synthesised by Barras *et al.* in 2001 [215]. The relative potency of LEM was compared to phenylephrine in an *in vitro* study on pig IAS by Jones *et al.* (2003) [203]. It was found that methoxamine racemate (1:1:1:1 concentration of all stereo-isomers) had equal potency with phenylephrine in contracting the pig IAS but LEM was four times as potent as either phenylephrine or methoxamine racemate in contracting the IAS. This provided a potential drug

for FI as it could be used in doses high enough to give a clinical effect but not have damaging cardiovascular side-effects.

LEM has been trialled in healthy volunteers in the form of perianal, intra-anal and rectal gel formulations [204]. It was noted that while perianal application did not increase MARP, intra-anal and rectal application of gel caused a rapid rise in MARP. However, a significant increase in blood pressure and a significant decrease in heart rate was noted in both groups of subjects. The same authors trialled intra-anal application of LEM gel in patients with FI [205] and found a significant rise in MARP in subjects receiving both 0.3% and 1% LEM gel. They also noted a significant decrease in heart rate following LEM gel and one patient developed hypertension in their study requiring nifedipine administration at 1 hour post dosing.

A slower, sustained release preparation of LEM was trialled by Bell *et al.* (2014) using LEM at doses between 5mg and 15mg in increments of 2.5mg in the form of 1g and 2g suppositories [216]. They noted a time to maximal concentration (T_{max}) at approximately 4.5hours for all doses of LEM and a significant decrease in heart rate following dosing with a trend towards a greater change with increasing doses of LEM. They also noted a prolongation of the QT interval in subjects receiving 15mg suppositories but no clinically significant serious adverse events were reported. Simpson *et al.* (2014) performed a similar study investigating varying doses of LEM in the form of 1g or 2g suppositories in healthy volunteers and found no significant difference in the maximal concentration (C_{Max}) between 10mg and 15mg doses of LEM, but a significantly shorter T_{Max} with the 1g suppository

compared to the 2g suppository. They also noted a significant decrease in heart rate following dosing but no clinically significant serious adverse events [217]. There was a dose-dependent increase in MARP between the 5mg and 10mg groups, but no significant change between the 10mg and the 15mg groups.

A tolerability study of using 2g suppositories of LEM in healthy volunteers was performed by Bell *et al.* (2014) over a period of 14 days with LEM doses of 7.5mg, 10mg, 12.5mg and 15mg. Over a period of 14 days, the drug was well tolerated with no accumulation of LEM over this period of time. T_{Max} was between 4 and 5 hours for all doses but weak dose proportionality was noted [218]. There was again a significant reduction in heart rate following dosing but no clinically significant adverse events.

A recently published meta-analysis investigated the cardiovascular side-effects of LEM [219]. This study pooled data from 4 studies and included 148 healthy volunteers. Overall, this study found a dose-dependant reduction in heart rate with LEM. A prolongation of the QT interval was noted post-dosing but this did not correlate well with the LEM concentration. However, no clinically significant effect on blood pressure or other parameters were observed.

Methoxamine is rapidly metabolised to physiologically inactive O-dealkylated metabolites and cleared from the body via the kidneys. There is no noted build-up in concentrations noted and no active metabolites [220].

Therefore, while LEM has been shown to be clinically safe in doses upto 15mg as suppositories and has been shown to increase MARP in both healthy volunteers

and in patients with FI, no clinical study so far has been performed to investigate the efficacy of LEM in reducing the number of episodes of FI in patients or its effect on the quality of life in patients with FI.

The current study was aimed at investigating the efficacy and safety of LEM in patients with FI over an 8 week period. The primary objective was to assess efficacy using the Wexner score and also to investigate the effect of treatment on the number of episodes of FI in each group.

This was an industry-sponsored study across the whole of Europe. The subjects recruited in this study at our centre are included in the analysis of results provided in the following sections.

5.2 Methods

This was an industry-sponsored multicentre study across Europe titled – A multicentre, Phase II, double–blind, randomised, placebo–controlled investigation to evaluate the efficacy, safety and tolerability of locally applied NRL001 in patients with faecal incontinence. The study was funded by Norgine Limited.

The rationale and study design were published in 2014 [221]. The study protocol synopsis is attached as an appendix (Appendix 1).

5.2.1 Inclusion criteria

- Adults over the age of 18 with passive faecal incontinence with greater than 2 episodes per week.
- Wexner score of 8-20

- An ultrasonographically intact internal anal sphincter with less than 60^{o} of scarring
- In women of post-menopausal age, a confirmation of menopause using serum FSH. In pre-menopausal women, strict contraception. In men, abstinence or use of condoms during intercourse

5.2.2 Exclusion criteria

- Disruption of external anal sphincter on endo-anal ultrasound (as this would best be treated with an overlapping sphicteroplasty and would also hamper the effect of the study drug)
- Complicating GI disorder which may pre-dispose to incontinence – e.g. any condition causing chronic diarrhoea, rectal prolapse etc.
- Uncontrolled cardiovascular risk factors including – uncontrolled hypertension, abnormal 24-hour holter ECG recordings, fixed cardiac output states, mitral regurgication and heart failure
- Severe asthma or chronic obstructive airway disease
- Chronic liver disease
- Vascular claudication with a claudication distance of less than 50 metres
- Chronic kidney disease with an estimated GFR < 30
- Benign prostatic hyperplasia (in men)
- Clinically significant electrolyte imbalances
- Clinically significant haemorrhoids and fissures
- Use of Loperamide greater than 16mg per day

5.2.3 Study drug

The study drug was packaged as a 2g suppository of either placebo, 5mg, 7.5mg or 10mg in identical packaging. The drug was dispensed following randomisation labelled for a specific subject. Subjects had to account for each suppository and the number of remaining suppositories were checked at each study visit. The remaining suppositories were returned at the end of the study.

5.2.4 Randomisation

Subjects deemed eligible for the study following screening were randomised using a computer model using block randomisation on the second visit. The study drug was dispensed to the subjects during the second visit and the first dose was taken in the presence of a study investigator and the subjects were observed for 4 hours following this.

5.2.5 Blinding

This was a double blind study. The investigator and the subjects were not made aware of the subjects' treatment with either placebo or any of the LEM doses. The medication was identically packaged and all subjects followed the same study protocol.

5.2.6 Study objectives

The primary objective was to test the efficacy of LEM in treating patients with FI. The primary objective was measured as an improvement in the Wexner score. Patient response was arbitrarily classified as either a 20% or 50% improvement in the Wexner scores.

Secondary objectives included:

- Evaluate the improvement in number of episodes of FI in patients over an 8 week treatment period
- Evaluate the improvement in the Vaizey and Faecal Incontinence Quality of Life (FIQoL) over an 8 week treatment with LEM
- Assess the tolerability of LEM in 5mg, 7.5 and 10mg doses – one dose taken every day at the same time, preferably in the morning.
- Identify the concentration-response relationship to LEM in patients with FI

5.2.7 Sample size and power calculation

The formal power calculation has been published in the previous paper explaining the rationale for the study [221]. As mentioned, there were no previous pilot studies to base a power calculation on. On the assumption that the placebo response would be small (25% at the outer limit), the study was powered to detect a 20% difference in number of incontinence episodes compared to placebo. The authors acknowledge that there is no previous indication if this would correlate with an improvement in the quality of life indexes measured in the study. Powered at 80% to detect a 20% difference in number of FI episodes, the study required 98 patients in each arm.

5.2.8 Study protocol

A figure demonstrating the study time-course is provided (Figure 5-1). The study required five visits over an 8 week period of treatment (including a 2 week pre-treatment screening period) and a follow-up telephone call at day 64 (± 3 days) of treatment. Subjects were given an electronic diary in which each episode of

incontinence was recorded (including date and time) and the application of the study drug was also recorded.

At each visit, a full physical examination was performed including all cardiovascular parameters and a 12-lead ECG. Compliance was checked at each visit and a review of all adverse events was performed.

Safety laboratory tests were performed at screening, at visit 3 (2 weeks post-treatment) and at visit 5 (8 weeks post-treatment). A urine analysis was performed at each visit.

Blood sampling for serum levels of LEM were performed at visit 2 – fist dosing visit when samples were taken at 0 hours, 1 hour, 2 hours and 4 hours post dosing – and at visit 4 (4 weeks post treatment).

Holter assessment over 24 hours was performed at visit 1 (screening) and at visit 3 (2 weeks post treatment).

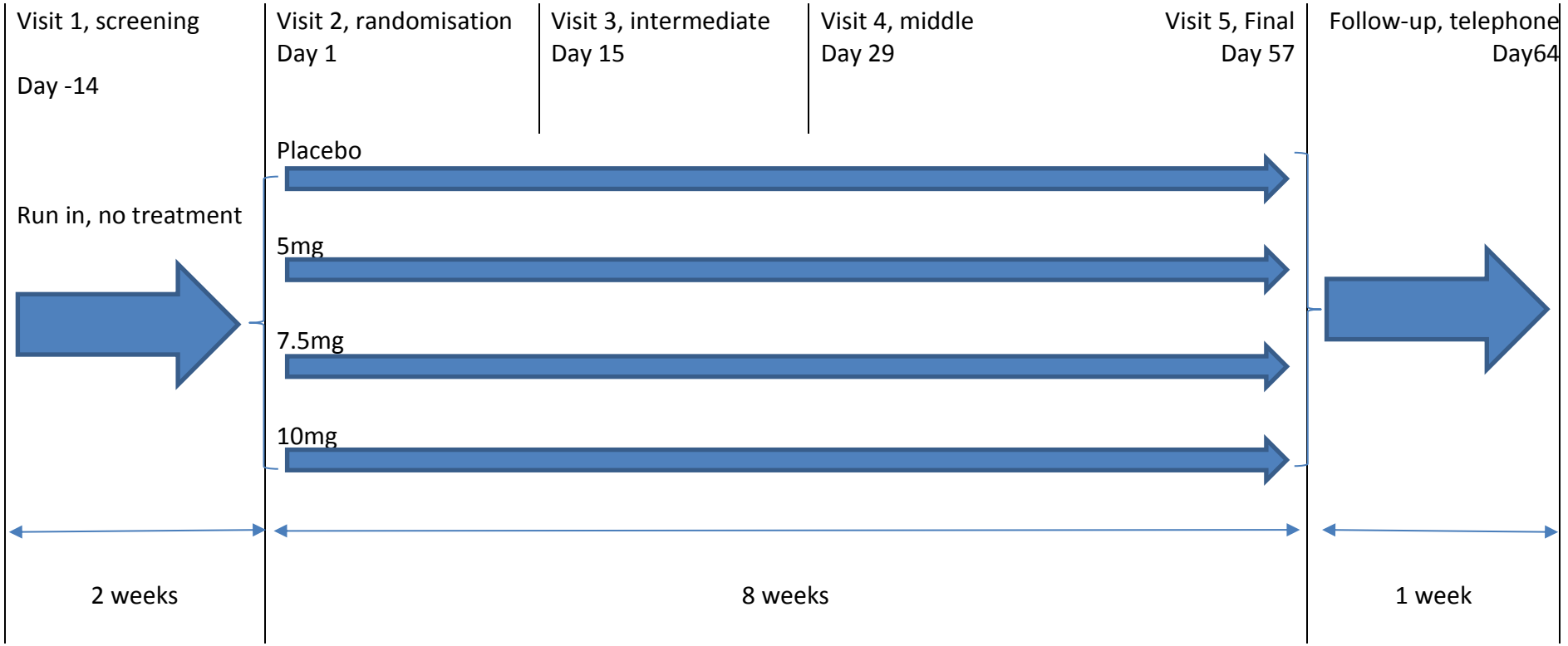

Figure 5-1 – Study design

There was a 2-week screening period prior to commencing treatment when subjects completed the eDiary as baseline. Randomisation was at Visit 2. The final dose of study drug was taken at Visit 5 and the study was completed following a final telephone call at day 64 ± 3 days

5.2.8.1 Visit 1

This was the screening visit during which eligibility to the study was assessed and if the inclusion and exclusion criteria were met then the subject was educated in the use of the eDiary and completed the Wexner, Vaizey and FIQoL questionnaires. Blood tests were performed including a full blood count, urea and electrolytes, liver function tests, lipid profile and bone profile. Urine analysis was carried out including a pregnancy test for pre-menopausal women. A full physical examination was performed. All cardiovascular parameters were assessed including heart rate, blood pressure (lying and standing) and ECG assessment. A holter monitoring over the next 24 hours was initiated at this visit. If the subject had not had an endoanal ultrasound assessment of their sphincter complex within the past year, then this was organised at this visit.

5.2.8.2 Visit 2

This was the randomisation visit. Investigations from the first visit were reviewed and inclusion/exclusion criteria were re-evaluated. Those subjects who completed the screening visit with no exclusion criteria were randomised and the first dose of drug/placebo was administered by the investigator. Prior to dosing, consent, compliance with eDiary, concomitant medications and urine analysis were performed. Post dosing, subjects were monitored over the next four hours with blood sampling for serum levels of LEM at 0 hours, 1 hour, 2 hours and 4 hours. At each time-point, an ECG was performed including a standard set of observations including pulse rate and oximetry analysis, lying and standing heart rate and blood pressure and ECG. The study drug for the next 4 weeks was

dispensed to the subject and a further appointment in 2 weeks' time was arranged.

5.2.8.3 Visit 3

This was performed at 2 weeks (± 3 days) following randomisation visit. Compliance with eDiary was checked, compliance with study medication was checked. A repeat set of bloods sampling was performed as at Visit 1. A 24 hour holter assessment was arranged over the next 24 hours including a 12-lead ECG during the visit. A complete physical examination and check of cardiovascular parameters was performed. A further appointment in 2 weeks was arranged.

5.2.8.4 Visit 4

This was performed at 4 weeks (± 3 days) from the randomisation visit. A full physical examination was performed and cardiovascular parameters were checked including an ECG. The Wexner, Vaizey and FIQoL questionnaires were completed. Urine analysis was performed. A blood sample was taken to measure serum concentration of LEM. Compliance was checked. The study drug was dispensed for another 4 weeks and an appointment was made for another 4 weeks' time.

5.2.8.5 Visit 5

This was performed at 8 weeks (± 3 days) post randomisation. A full physical examination was performed and cardiovascular parameters including a 12-lead ECG were checked. Compliance was checked and all study drugs were accounted for. The Wexner, Vaizey and FIQoL questionnaires were completed. A full set of

blood tests as at visit 1 was performed. A urine analysis was performed. The eDiaries were returned and a follow-up telephone call was organised.

5.2.8.6 Follow-up telephone call

This was performed at 64 days (± 3 days) post randomisation. Any emergent adverse events were checked and the subject was formally removed from the study.

5.2.9 Adverse events

An adverse event was defined as "the development of an undesirable medical condition or the deterioration of a pre-existing medical condition following or during exposure to a pharmaceutical product, whether or not considered causally related to the treatment". Adverse events were categorised as – definitely related to study drug, probably related to study drug, unrelated to study drug. Adverse events were reported by subjects at each study visit or over the telephone to the investigator at the site if in between visits.

5.2.10 Conduct of study at our centre

This was a reasonably complicated clinical study with multiple study visits, drug dosages and multiple assessments at each visit.

At our site (Queens Medical Centre, Nottingham), I was the Primary Investigator (PI) with oversight from the Chief Investigator (CI). No other staff were employed in the conduct of the study at our site. All study visits were conducted in a treatment room in the Queens Medical Centre, Nottingham, University department of Surgery.

As PI I was responsible for procuring approval for the study from the Trust Research and Development department. A full costing template for the study was created and I was responsible for negotiating between Norgine and the R&D department to procure all the necessary equipment for the study. The ECG machine, the holter monitors and the eDiaries were provided by Norgine directly but other equipment such as heart rate, blood pressure and oximetry machines were provided by the Trust. All blood sampling (including bottles, needles, syringes etc.) was conducted on site by the NHS except for the Pharmacokinetic samples (PK samples) which were stored on site in a monitored freezer and were collected by Norgine when requested by the local PI. These were then shipped by special courier to a Norgine approved laboratory for the measurement of serum LEM concentrations.

I contacted all the colorectal surgeons in the Trust to aid recruitment to the study. Any interested, potentially eligible patients were asked to get in touch with me directly in order to formally go through inclusion/exclusion criteria for the study. I contacted the local continence group in the community explaining the rationale for the study – again potential subjects were directed to me to undergo inclusion/exclusion criteria checks. Finally, a number of patients who had previously volunteered for clinical studies on FI in the past at our centre were contacted by me with a formal invitation to discuss potential inclusion in the study.

All study visits were conducted by me. Prior to the screening visits, I met with patients to explain the rationale for the study and check their consent and answer

any potential questions. Interested and potentially eligible patients were then given an appointment for Visit 1, screening visit. Those patients who had not had an endo-anal ultrasound in the preceding year were referred to a local nurse specialist who performed the endo-anal ultrasound for all the patients in the study. I reviewed the ultrasound images with the nurse specialist to confirm if there was any disruption to the EAS which would exclude patients from inclusion.

For each study visit, the physiological assessments (lying and standing HR and BP, ECG, urine analysis and venesection) were performed by me. The ECG was assessed by me during the study visit but following this was also faxed to a dedicated Cardiology laboratory (CardioAnalytics) who had been contracted by Norgine for the conduct of the study. Once the formal report of the ECG was issued, I checked this with my own assessment of the ECG to ensure that there were no discrepancies noted. For the 24-hour Holter measurements, I attached the holter to the patient during the visit and trained them on removing and safely packaging the holter device. The device was posted back to me by the patient following the 24 hours of monitoring. I was not able to review the holter data personally but uploaded it to CardioAnalytics electronically. A report was issued by CardioAnalytics and this was scrutinised by me to check if there were any clinically significant abnormalities noted. For the blood samples (not the PK samples), following venesection the blood bottles were sent to the hospital laboratory and the results were scrutinised by me the following day. For the PK samples, the blood tubes had to be centrifuged and the supernatant extracted and frozen. This was performed by me within one hour of venesection as per the

protocol. Once a significant number of PK samples had been collected, I would inform Norgine who would send a special courier to transport the samples to the accredited laboratory.

Once the study visit was completed, I was responsible for completing the Case Report Form (CRFs) online for each of the parameters tested during the visit. The blood parameters were entered the following day as they were not available on the day of the visit. Similarly, the ECG clinical interpretation was uploaded the same day and compared with the formal report from CardioAnalyitics. Holter data was reviewed and uploaded when available. Paper copies of all CRFs were kept securely during the conduct of the study. I was also responsible for performing safety/calibration checks on all the equipment used in the study and this was performed on a monthly basis. Freezer temperature was checked on a daily basis. Appropriate conduct of the study was checked by Norgine who would send a representative on a monthly basis to review the paper CRFs with the online entered data and would check the equipment calibration with agreed protocols.

5.2.11 Statistical tests

Our target number of subjects was 10. With such a small sample size, only descriptive analyses could be performed on most of the variables. Where pooled analysis of data from all groups was possible, statistical tests were performed with either using the T test (or Wilcoxon test), paired T test (or non-parametric equivalent). All datasets were tested for normality before performing the appropriate tests.

5.3 Results

5.3.1 Demographics

Patients were recruited from surgical (colorectal) clinics. We contacted the local incontinence group in the community to help with recruitment. A few patients who had taken part in previous studies at our centre were also contacted.

5.3.1.1 Difficulties with recruitment

FI is well recognised as being a silent problem with patients putting up with symptoms rather than seeking medical advice. Recruiting patients for this study proved particularly difficult as a result. The stringent inclusion/exclusion criteria also made recruitment difficult. The most commonly encountered barriers were the requirement for an intact external anal sphincter (EAS) on endo-anal ultrasound and the limit on the dosage of loperamide. A number of subjects from colorectal clinics had to be excluded even prior to screening as they were either noted to have too great a disruption of the EAS or were unwilling to reduce their dose of loperamide in order to enrol into the study.

5.3.1.2 Recruited subjects

Fifteen patients were initially assessed for suitability of inclusion into study. Six patients were deemed unsuitable and nine patients were screened. One patient failed screening as they were unable to use the diary to record the episodes of incontinence. This patient was therefore withdrawn and then re-enrolled. This provided ten patients who were screened (one patient screened twice) and nine patients who were randomised.

Of these nine patients, one patient was withdrawn at the 2 week visit due to persistent hypertension. Eight patients completed the study.

Of these nine patients, two were randomised to placebo, two were randomised to 5mg LEM, three to 7.5mg and two to 10mg.

Given this small number of subjects, the single subject in the 10mg group was excluded from analysis of the data at 4 weeks and 8 weeks but the data collected upto 2 weeks (Visit 3) was valid and included.

Seven subjects were female with two males. Mean age was 64 ± 4.1 years. Mean BMI was 27.5 ± 2.12.

Over the 2 weeks of screening prior to randomisation, the mean number of episodes of FI was 19.1 ± 2.77 across all patients. The screening Wexner score was 13.0 ± 0.52 and the Vaizey score was 17 ± 1.11 across all patients. The Faecal Incontinence Quality of Life (FIQoL) has four components – lifestyle, coping, depressing and embarrassment – and a composite score was taken at the screening visit for each subject. The mean FIQoL score was 8.49 ± 0.99 for all patients. Given the small number of patients in each group (two in each group with only one patient in the 10mg group), no comparison was possible between groups for baseline characteristics.

5.3.2 Pharmacokinetic values

Figure 5-2 demonstrates the different serum concentrations of LEM following first dosing of the drug at Visit 2. Serum levels of LEM were tested at the randomisation visit and at 2 weeks into treatment. During the randomisation visit,

four samples were taken – pre-dose, one hour post dose, 2 hours post dose and 4 hours post dose. During the 4 weeks post randomisation visit, another serum sample was taken and the time of dosing on that day was noted.

No subjects had any detectable levels of the drug prior to administration of drug. Excluding the two subjects who received placebo, the mean levels of drug in the subjects receiving 5mg, 7.5mg and 10mg were – 1.1 ± 0.15ng, 1.1 ± 0.50ng, 3.52 ± 2.16ng at 1 hour respectively; 3.41 ± 1.16ng, 4.84 ± 2.39ng, 15.28 ± 10.07ng at 2 hours respectively; 4.71 ± 1.65ng, 9.52 ± 1.22ng, 20.11 ± 0.74ng respectively at 4 hours. With only two patients per group, statistical analyses were not performed between groups.

5.3.3 Cardiovascular parameters

At the randomisation visit, cardiovascular parameters were measured before dosing and also at 1 hour, 2 hours and 4 hours post dosing. Excluding the subjects receiving placebo (n = 7 – two patients each for 5mg, three in the 7.5mg group, two in the 10mg group), mean lying heart rates were – 73 ± 3.18 pre-dose, 69.71 ± 2.91 at one hour, 70.29 ± 3.92 at 2 hours and 69.29 ± 4.46 at 4 hours – and there was no statistically significant difference in heart rate between any of the time-points ($p = 0.824$, n = 7, Anova). Mean arterial pressure (MAP) was – 92.2 ± 3.63 mmHg pre-dose, 95.8 ± 4.2 mmHg at 1 hour, 90.0 ± 3.38 mmHg at 2 hours and 92.0 ± 3.41 mmHg at 4 hours – and was not statistically different at the 4 time-points ($p = 0.733$, n = 7, Anova). Between group analysis was not performed due to the small sample size.

Figure 5-3 illustrates the difference in PR intervals between the groups at the randomisation visit. At the randomisation visit, ECG recordings were taken pre-dose, at 1 hour, 2 hours and 4 hours – patients receiving placebo were excluded from analysis(n = 7). The ECGs were analysed for any clinically significant changes and the PR interval, QRS duration, RR interval and the QT durations were noted. There was no significant differences in the PR intervals between the 3 groups pre-dosing with NRL-001 ($p = 0.065$, $n = 7$, Kruskal-Wallis); the patients receiving placebo were not analysed. At 1 hour post dose, while there was no significant difference between the groups overall ($p = 0.052$, $n = 7$, Anova), post hoc analysis unveiled a significant difference between the 7.5mg and 10mg groups (138 ± 11.37ms vs. 199 ± 3 ms, $p = 0.048$, Anova with Dunnett's test). At 2 hours, a similar difference was noted with a significant difference between the groups ($p = 0.017$, Anova) and a significant difference between 7.5mg and 10mg groups (141 ± 11.21 ms vs. 201 ± 3 ms, $p = 0.016$, Dunnett's test). At 4 hours similarly, there was a significant difference between the groups ($p = 0.016$, Anova) and a difference between the 7.5mg and 10mg groups (140 ± 8.33 ms vs 204 ± 2 ms, $p = 0.014$, Dunnett's).

There were no significant differences in the QRS intervals between the groups at any of the time points. Similarly, there were no significant differences in the QT intervals between the groups at any of the time points.

The blood concentrations of drug during the randomisation visit and the concentration of drug at the 2 week post treatment visit were used to identify any correlations between the cardiovascular parameters measured (n = 14). There

was a significant negative correlation between heart rate and drug concentration noted ($r = -0.37$, $p = 0.030$, Pearson's correlation) and a significant positive correlation between the drug concentration and the QT interval on the ECG ($r = 0.43$, $p = 0.010$, Pearson's correlation). No other significant correlations were noted.

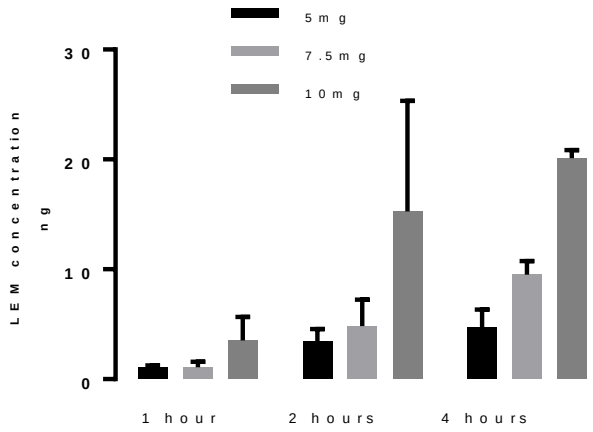

Figure 5-2 – Mean concentration of LEM during the randomisation visit

Mean serum concentration of LEM with SEM at 1 hour, 2 hours and 4 hours post dosing (n = 7, patients from our centre, 2 subjects in 5mg group, 3 subjects in 7.5mg group and 2 subjects in 10mg group). Baseline concentrations were 0. As there were only two subjects in each group, statistical analyses were not performed.

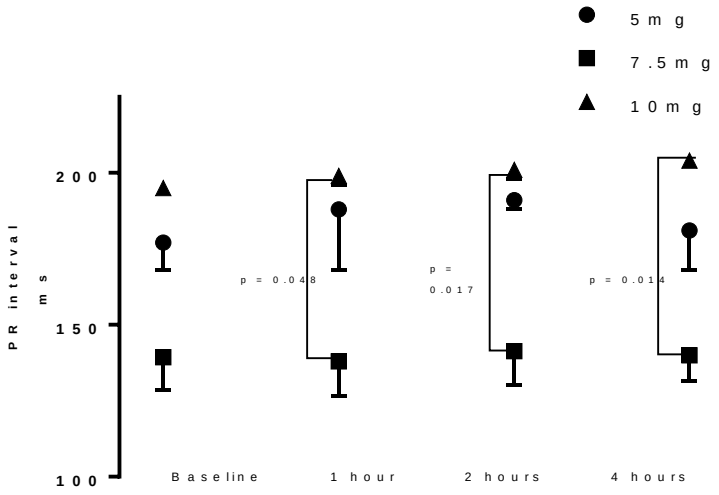

Figure 5-3 – Mean PR intervals during the randomisation visit

PR interval at baseline, 1 hour, 2 hours and 4 hours post dosing with LEM (n = 7 excluding 2 patients with placebo – 2 patients in each group, 3 patients in 7.5mg group). Lower error bars are shown. There was a difference between the groups at baseline, but this was not statistically significant. There was a progressive increase in the difference between the 10mg and the 7.5mg groups. At 1 hour, the difference between the 7.5mg and 10mg groups was significant only on post-hoc analysis. At 2 hours and 4 hours, there was a significant difference overall and on post hoc analysis (see text).

5.3.4 Episodes of incontinence

The total number of episodes of FI were recorded for each week in the trial. This included the two weeks of screening and the eight weeks of treatment. The total number of incontinence episodes in the two weeks of screening, week 3 and week 4, week 7 and week 8 were used to calculate the mean number of episodes at each time point to analyse any differences between the groups. In the screening period, the incontinence episodes for the placebo, 5mg, 7.5mg and 10mg groups were – 18.67 ± 1.67, 14 ± 3.54, 17.17 ± 5.62 and 39 ± 3 respectively. In treatment weeks 3/4, the incontinence episodes for the groups were 36.67 ± 11.46, 9.5 ± 5.23, 16.17 ± 3.59 and 31. In weeks 7/8, the incontinence episodes for the groups were 28.83 ± 7.33, 12 ± 6.64, 17.57 ± 4.23 and 39 ± 10. Due to the small sample size, it was not appropriate to perform within group or between group statistical analyses.

5.3.5 Questionnaire scores

As there was only one patient in the 10mg group who completed the study, the 10mg group was not analysed further. The Wexner scores at screening for placebo, 5mg and 7.5mg were – 11.5 ± 1.5, 12.5 ± 0.5 and 14 ± 1 respectively. At the 4 week post-treatment visit, the Wexner scores were – 11.5 ± 0.5, 8.5 ± 3.5 and 12.7 ± 1.9. At 8 weeks post treatment, the Wexner scores were – 11 ± 1, 8.5 ± 4.5 and 11.3 ± 3.2. Due to the small sample size, within group and between group statistical analyses were not performed.

5.3.6 Efficacy parameters

The primary outcome for the study was a greater than 50% improvement in the Wexner score after 4 weeks of treatment with NRL-001. Only one subject (subject 48-009), in the 5mg group, fulfilled this criteria and this improvement was sustained at 8 weeks; this particular subject also had a greater than 50% improvement in their episodes of incontinence at 4 weeks and 8 weeks. One further subject, from the 7.5mg group (subject 48-010), showed a greater than 50% improvement in the Wexner score at 8 weeks, but not at 4 weeks; this subject also had a greater than 30% improvement in their episodes of incontinence at 4 weeks but this was not sustained at 8 weeks.

With regards to the episodes of incontinence, other than the above two subjects, one subject from the 10mg group had a greater than 20% improvement in FI episodes at 4 weeks but not at 8 weeks. One subject from the placebo group had a greater than 20% improvement in FI episodes at both 4 weeks and 8 weeks post-treatment.

5.3.7 Adverse events

A total of 13 adverse events were noted over the 8 week period. Of these, 4 adverse events were considered being probably related to the study drug. One subject in the 5mg group and one subject in the 10mg group complained of piloerection, a recognised side-effect of the study drug. One subject in the 10mg group complained of perianal burning sensation, another recognised side effect. One subject in the 10mg group was noted to have asymptomatic hypertension on their 2-weeks post treatment visit and was therefore withdrawn from the study.

There were no clinically significant abnormalities noted in the safety blood parameters analysed. Two subjects were noted to have elevated lactate dehydrogenase levels (LDH) but we were not able to identify any relationship with the study drug. The rises in LDH were not clinically significant.

No serious adverse events were noted during the course of the study.

5.4 Discussion

This was the first study investigating LEM suppositories in patients with FI. Previous studies have investigated intra-anal or peri-anal gel preparations in patients with FI or investigated LEM suppositories in healthy volunteers. This was also the longest study so far investigating LEM suppositories – the longest previous study was for two weeks investigating suppositories in healthy volunteers. Therefore, one important aspect of the study was to assess feasibility of using suppositories as a treatment in patients with FI. In that regard, the study was successful as no subjects complained of any significant inconvenience with the treatment modality.

5.4.1 Study design

Given the very small dataset from our centre and the fact that we had only one subject in the 10mg group, no definitive statistical analyses could be performed. A critique of the pooled data from the entire study is provided in Section 5.5.

5.4.2 Pharmacokinetic data

Our results agree well with previous authors. We found significantly higher levels of LEM in serum sample from subjects in the 10mg group compared to the 5mg and 7.5mg groups at 4 hours. Previous authors have confirmed similar dose proportionality with a T_{Max} at between 4 and 5 hours. However, we did not note any significant differences between the 5mg and 7.5mg groups although there was a increased concentration of drug in the 7.5mg groups (4.71 ± 1.65ng vs. 9.52 ± 1.22ng). This may well have been a type 2 error due to our small sample size.

5.4.3 Safety data

There were no serious adverse events during the course of the study. However, one subject suffered from asymptomatic hypertension in the 10mg group and had to be withdrawn at their 2 week post-treatment visit.

Analysis of our cardiovascular parameters demonstrated similar findings to previous authors. There was a statistically significant negative correlation between heart rate and serum concentration of LEM. However, no subject experienced clinically significant bradycardia. Comparing of the 24-hour holter data between pre-dose and at 2 weeks post-treatment we found a significant difference in the 10mg group with a perceptible bradycardia post treatment compared to pre-treatment. However, no significant differences in the mean arterial pressure was noted.

As noted by previous authors, we also noted a statistically significant correlation between the QT interval and serum LEM concentration. However, no subjects suffered any clinically significant arrhythmias during their 2 week post-treatment holter recordings. There was a difference in the baseline PR values between the groups which was not statistically significant. It was noted that the significant difference in the PR interval at 2 hours and 4 hours was only noted between the 7.5mg and 10mg groups and not between the 5mg and 10mg groups. This may well have been because of the small number of subjects in each group.

No clinically relevant changes in the safety laboratory assessments were noted.

5.4.4 Tolerability assessment

Over the 8 week treatment period, LEM was well tolerated in all subjects. The two subjects who complained of piloerection and perianal burning did not find the side-effect very troublesome. One subject who was terminated early suffered asymptomatic hypertension.

5.4.5 Efficacy of LEM suppositories

Our data-set suggests that LEM suppositories are well tolerated at all doses. There was a statistically significant reduction in heart rated and increase in the PR interval and QT interval, but no clinically significant changes in cardiovascular parameters. The one subject who was withdrawn may indicate that some people are more sensitive to α agonists and need to be screened prior to regular dosing with the drug.

However, we failed to show any significant change in the efficacy parameters. As our sample size was too small to perform any meaningful statistical analyses, we cannot comment on whether this was a significant result or not.

5.4.6 Limitations

This was a small dataset of patients. Recruitment was limited by a number of factors including the difficulty in identifying patients with FI from surgical clinics, incontinence groups in the community and from previous studies on FI at our centre. The strict inclusion criteria disqualified a number of potential subjects from inclusion, primarily as the permissible degree of external sphincter disruption was quite conservative and disallowing the use of loperamide at 16mg per day was restrictive to a number of potential subjects. The sample size was not

large enough to confirm a negative study in terms of efficacy parameters. However, we have demonstrated adequate safety data with regards to laboratory assessments and cardiovascular parameters. Tolerability was also adequately assessed by this study at our centre.

5.5 Assessment of study

5.5.1 Study results

The results of the study with the pooled data from all centres was published in April 2016 [222]. A total of 466 patients were randomised during the study, exceeding the target of 400 patients. Of the 466, 463 patients received at least 1 dose of either placebo or drug. 23 subjects were excluded leaving a dataset of 440 subjects.

Over the 8 week study, the Wexner, Vaizey and FIQoL scores decreased for the subjects in all groups including the placebo and no statistically significant treatment effect was noted. The number of FI episodes also decreased across all groups with no significant differences between the treatment and placebo arms.

Overall, the drug was well tolerated with most of the adverse events mentioned being mild. A total of 494 adverse events were reported in 212 subjects (46%). In 20 subjects, adverse events led to the discontinuation of the study drug. One subject suffered severe heart failure following a urinary tract infection and required hospitalisation.

The high and sustained placebo response invalidated any treatment effect of the study drug and has been acknowledged as the principle finding in the study.

5.5.2 Critique of study design

This study was designed with two aims – to investigate the efficacy of LEM suppositories in patients with FI and to find the best dosage to achieve this effect. As previously mentioned, no previous study investigating the effects of LEM on patients with FI have been published. The only study to be used as an indicator was the RCT using phenylephrine gel [200]. In this study, Carpeti *et al* used 10% phenylephrine gel on 36 patients with FI in a cross-over study to investigate the effects on incontinence scores and found no significant difference between placebo and treatment arms. Their dose selection was directed by a previous study by the same authors [197] in 1999 where they tested topical phenylephrine gel on healthy volunteers and found 10% to be the optimal dose of drug beyond which further increase in drug concentration did not provide any meaningful increases in MARP. This present study was designed with the presumption that LEM would be more effective than phenylephrine given its greater potency in increasing IAS tone as demonstrated in porcine IAS tissue [203]. However, no previous study had been performed linking increased MARP with improvement in continence scores. In this context, a pilot study investigating LEM suppositories in patients with FI may have been more pertinent prior to the current phase II RCT.

In terms of finding the optimum dosage required for efficacy, this study used the previous data from Bell *et al* (2014) [216] and Simpson *et al* (2014) [217] who showed no significant increase in MARP between the 10mg and 15mg LEM suppository groups. However, both these authors reported their results only in healthy volunteers. The only study investigating LEM in patients with FI used

intra-anal LEM gel [205] and once again only noted changes in MARP and not in continence scores.

No studies exist investigating the effects of α_1 agonists on rectal compliance and few studies have adequately investigated α_1 adrenorecptors in the rectum in vitro. In retrospect, this appears to have been overlooked and has formed the basis of the laboratory sections of my thesis. Further, the conclusion that an increase in MARP will necessarily improve continence scores seems premature and one reason for the negative result both in this study and in the study investigating phenylephrine may well have been the reduction in rectal compliance due to α_1 adrenoceptor stimulation which is indicated by my laboratory experiments.

The rationale for the study published in 2014 [221] assumes a maximal placebo response at 25%. Previous studies have shown a sustained placebo response for FI treatments in testing injectable bulking agents[223], or PTNS [136, 224]. All these studies show a significant placebo response between 25% and 30% and the assumption for the power calculation of this study seemed to underestimate this.

The lower limit set for the Wexner score in the inclusion criteria of this trial was a score of 8. Previously Robath *et al* (2001) [86] found a good correlation for a Wexner score of 9 and greater with a poorer GIQLI score, indicating significant reduction in quality of life in these patients. While using a Wexner score of 8 as the inclusion criteria increased the number of recruitable subjects for this trial, it may also have included subjects who were less likely to benefit from the study intervention.

Barring these criticisms, the study was well designed overall and the methodology was rigorous. The number of recruited subjects was greater than the minimum number required by the power calculation. The blinding and assessment of subjects was good and the oversight from the Norgine Ltd was appropriately conducted at our site.

6 Afterword

As clinicians, we constantly prescribe drugs and are used to evaluating data from clinical studies regarding the efficacy of drugs for various conditions. However, we are rarely involved in the process by which a new drug is developed. In the course of this project, I believe I have had the opportunity to explore all aspects of drug development – from evaluating the basic scientific premise of drug development, reviewing the available evidence and identifying an area that has yet to be researched (the effect of α adrenoceptor agonists in the rectum in this instance). The experimental section of the project was entirely new to me and took me through the rigors of the scientific process anew. The results have highlighted areas which require clarification in the case of human rectum.

Running a study with multiple visits and assessments proved challenging to organise and implement. Running a study with an IMP was also a new experience and highlighted the degree of rectitude that the MHRA require for all such studies. The MHRA inspection was also a challenge but demonstrated reasonable compliance with their standards.

Clinicians are encouraged to get involved in research and I believe it provides valuable experience and has been very rewarding in my case. The NHS also relies on research to investigate and develop new drugs. Industry-sponsored studies are often a good source of revenue for participating trusts. For this particular study, our trust received approximately £4,500 per patient recruited and completed.

My research indicates that the drug that was being investigated may not be the ideal method for treating FI. The role of α_1 adrenoceptors in the GI tract have not been fully clarified in the literature and my results from sheep and pig rectal tissue suggest that α_1 adrenoceptors have the opposite effect on rectal smooth muscle compared to received wisdom. While more work needs to focus on clarifying the role of α adrenoceptors in human rectal tissue, combined with the results from the clinical trial I do not believe that α_1 adrenoceptors on their own would provide a viable solution to faecal incontinence treatment. Further clinical studies also need to focus on the correlation between measurable improvements in anorectal parameters (e.g. MARP, rectal compliance) and patient symptoms and faecal incontinence frequency. The clinical study has also highlighted the significant role of placebo in faecal incontinence treatment and this needs to be borne in mind when designing further treatments for this condition.

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8 Appendix 1 – FI questionnaires

8.1 Wexner score

Type of FI	Never	Rarely	Sometimes	Usually	Always
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Wear Pad	0	1	2	3	4
Lifestyle altered	0	1	2	3	4

8.2 Vaizey score

	Never	Rarely	Sometimes	Weekly	Daily
Solid	0	1	2	3	4
Liquid	0	1	2	3	4
Gas	0	1	2	3	4
Lifestyle change	0	1	2	3	4
				No	Yes
Wear pad/plug				0	2
Taking constipating medication				0	2
Unable to defer defaecation by 15 mins				0	4

8.3 FIQoL

1. In general, would you say your health is: (circle one number)
    1. Excellent
    2. Very Good
    3. Good
    4. Fair
    5. Poor
2. For each of the items, please check the appropriate box indicating how much of the time the issue is a concern for you, due to accidental bowel leakage.

Due to accidental bowel leakage:	Most of the Time	Some of the Time	A little of the time	None of the time
I am afraid to go out				
I avoid visiting friends				
I avoid staying overnight away from home				
It is difficult for me to get out and do things like going to a movie or to church				
I cut down on how much I eat before I go out				
Whenever I am away from home, I try to stay near a restroom as much as possible				
It is important to plan my schedule (daily activities) around my bowel pattern				
I avoid traveling				
I worry about not being able to get to the toilet in time				
I feel I have no control over my bowels				
I can't hold my bowel movement long enough to get to the bathroom				
I leak stool without even knowing it				
I try to prevent bowel accidents by staying very near a bathroom				

3. Due to accidental bowel leakage, indicate the extent to which you AGREE or DISAGREE with each of the following items

Due to accidental bowel leakage	Strongly Agree	Somewhat Agree	Somewhat Disagree	Strongly Disagree
I feel ashamed				
I cannot do many of things I want to do				
I worry about bowel accident				
I feel depressed				
I worry about others smelling stool on me				
I feel like I am not a healthy person				
I enjoy life less				
I have sex less often than I would like to				
I feel different from other people				
The possibility of bowel accidents is always on my mind				
I am afraid to have sex				
I avoid traveling by plane or train				
I avoid going out to eat				
When I go someplace new, I specifically locate where the bathrooms are				

4. During the past month, have you felt so sad, discouraged, hopeless, or had so many problems that you wondered if anything was worthwhile? (Circle a number)
   1 Extremely So - To the point that I have just about given up
   2 Very Much So
   3 Quite a Bit
   4 Some - Enough to bother me
   5 A Little Bit
   6 Not at all

9 Appendix 2 – Synopsis of protocol for clinical study

Study title	A multi-centre, phase II, double-blind, randomised, placebo-controlled, parallel group, dose-ranging study in patients with faecal incontinence; to evaluate the efficacy, safety and tolerability of locally applied NRL001 over an 8 week treatment period.
Phase of development	Phase II
Objectives	**Primary objective:** To evaluate the efficacy of NRL001 in faecal incontinence (FI) by assessing the improvement of the incontinence status after 4 weeks of treatment compared to baseline by means of the Wexner score. **Secondary objectives:** • To provide data on the efficacy of NRL001 in patients with faecal incontinence over an 8 week treatment period. • To provide preliminary data on the safety and tolerability of NRL001 (5 mg, 7.5 mg and 10 mg) over an 8 week treatment period compared to placebo. • To evaluate the population pharmacokinetics and to establish any pharmacokinetic/pharmacodynamic relationship with adverse events. • To evaluate the dose-response relationship in order to identify the appropriate dose(s) of NRL001 for future studies. • To evaluate the effect of treatment according to the patient's Faecal Incontinence Quality of Life questionnaire at 4 and 8 weeks.

	• To evaluate the effect of treatment according to the Vaizey score at 4 and 8 weeks.
Study design	**A multi-centre, phase II, double-blind, randomised, placebo-controlled, parallel group, dose-ranging study to evaluate efficacy, safety and tolerability of locally applied NRL001 during 8 weeks of treatment in patients with faecal incontinence.**
Study duration	An 11 week study period for each patient, comprising a 2 week run-in, an 8 week treatment period and 1 week post treatment follow up. The end of this study is defined as 7 days after Last Patient Last Visit (LPLV), i.e. after the last patient has been followed up by a scheduled phone call.
Study treatment	Treatment Arms (1:1:1:1 randomisation) • Placebo suppository • 5.0 mg NRL001 in a 2 g suppository • 7.5 mg NRL001 in a 2 g suppository • 10.0 mg NRL001 in a 2 g suppository Patients will receive a once daily (in the morning) suppository treatment for 8 weeks consisting of either 5 mg, 7.5 mg, 10 mg NRL001 or matching placebo. Treatment and Visit Schedule: Visit 1 – Screening Visit (Day -14 (+3days)) Visit 2 – Randomisation Visit (Day 1) Visit 3 – Intermediate Visit (Day 15 (+/-3 days)) Visit 4 – 4 Week Treatment Visit (Day 29 (+/-3 days)) Visit 5 – 8 Week Treatment /Early Termination (Day 57 (+/-3 days)) Day 78 (+/-3 days) – Follow-up Telephone Call

	First dosing on Visit 2 – Randomisation Visit will be in a controlled environment.
Number of patients	Approximately 580 patients will be screened to provide approximately 480 randomised patients to gain approximately 400 evaluable patients (100 per treatment arm).
Inclusion Criteria	1. An ultrasound assessment of the internal anal sphincter within the previous 12 months confirming an intact circular internal sphincter with minimal scars (maximum 60 degrees scarring circumferentially). 2. Diagnosis of faecal incontinence with a Wexner score of 8 – 20 inclusive at Visit 1 - Screening Visit. 3. Historical clinical evidence (past 6 months prior to Visit 1 – Screening Visit.) of faecal incontinence episodes (solids, liquid, gas or mucus). 4. Greater than or equal to two faecal incontinence episodes (solids, liquid, gas or mucus) per week during the 4 week historical period prior to Visit 1 – Screening Visit. 5. Able and willing to receive rectal examinations and treatments.

	6. Patients must be aged ≥18 without significant acute or uncontrolled chronic disease. 7. Patients must understand the purpose and risks of the study and be able to provide written informed consent and willing, able and competent to complete the entire study and comply with study instructions as defined in the protocol. 8. Female patients must be postmenopausal (for at least one year and confirmed by serum FSH at screening), or surgically sterile (status post bilateral tubal occlusion, bilateral oophorectomy, or hysterectomy). Female patients of childbearing potential must be practicing one of the following methods of birth control and agree to continue with this regimen throughout the study period: • Oral, implantable, or injectable contraceptives (for a minimum of 3 months or 1 full menstrual cycle before study entry) in combination with a condom; • Intrauterine device in combination with a condom; • Double barrier method (condoms, sponge, diaphragm, or vaginal ring with spermicidal jellies or cream); • True sexual abstinence; and have a negative pregnancy test at screening.

	9. Sexually active male patients must use condoms with their partners throughout the study and for 90 days after completion of the study in addition to their partner's normal mode of contraception. 10. Male patients must not donate sperm during the study and for 90 days after the completion of the study. 11. Patients taking any continuous medication need to have been on a stable regimen for at least 1 month prior to Visit 1 – Screening Visit.
Exclusion Criteria	1. External anal sphincter disruption related to faecal incontinence caused by trauma. 2. Patients with complicating gastrointestinal (GI) disease including those with active inflammatory bowel diseases, patients that have received radiotherapy or surgery for anal cancer, patients with rectal prolapse, transanal surgery. 3. Relevant history of or presence of any significant or uncontrolled cardiovascular risk including: a. Systolic > 160mmHg or Diastolic > 100mmHg. Patients on a stable regimen for $\geq$ 3 months with controlled hypertension prior to Visit 1 – Screening Visit (Systolic $\leq$ 140mmHg or Diastolic $\leq$ 90mmHg) can be included. b. Abnormal 24 hour Screening Holter: corrected QT interval (QTc_f) prolongation with cut-off values of >460 ms for females and >430 ms for males, acute arrhythmia, nocturnal bradycardia with heart rate (HR) $\leq$ 40bpm, atrial fibrillation, AV block Type II and III, Sick Sinus Syndrome, vasovagal syncope. c. Fixed cardiac output states (severe aortic stenosis (AS), hypertrophic obstructive cardiomyopathy (HOCM). d. Significant mitral regurgitation (MR). e. Cardiac failure (New York Heart Association (NYHA) stage II-IV). 4. Severe or uncontrolled asthma or chronic obstructive pulmonary disease determined by clinical history, physical examination, lung function tests or exercise tolerance.

	5. Chronic liver disease (e.g. liver cirrhosis, chronic hepatitis, severe hepatic insufficiency). 6. Vascular claudication after <50 metres walking distance. 7. Severe renal impairment defined as glomerular filtration rate (GFR) ≤ 30 ml/min, uncontrolled and reno-vascular end stage renal disease. 8. Patients with diabetic polyneuropathies. 9. Any type of chronic diarrhoea or frequent diarrhoea (defined as > 5 loose stools per day) 10. Faecal impaction and overflow diarrhoea. 11. Male patients with clinically diagnosed and symptomatic prostatic hyperplasia. 12. Clinically significant electrolyte abnormalities, e.g. clinically significant low/high potassium or low sodium. 13. Presence of clinical symptomatic haemorrhoids (grade III and IV), anal fissures or anorectal fistulas. 14. Less than 2 episodes of faecal incontinence episodes (solids, liquid, gas or mucus) per week during the 4 week historical period prior to Visit 1 – Screening Visit. 15. Participation in a clinical drug study during the 90 days preceding the initial dose in this study. 16. Known history of allergy to methoxamine or any other ingredients of the Investigational Medicinal Product. 17. Patients who, in the opinion of the Investigator, are unsuitable for participation in the study due to any dependencies, medical conditions or significant illness within two weeks prior to randomisation. 18. Use of any disallowed concomitant medication or other medication that the Investigator believes may affect the study including over-the-counter (OTC) products within 30 days prior to the Investigational Medicinal Product administration. 19. A personal or family history of QTc_f prolongation or sudden death.

	20. Patients taking Loperamide (2mg) ≥8 tablets per day for faecal incontinence either alone or in combination with codeine phosphate and/or paracetamol. 21. Patients using any device for the treatment of faecal incontinence. 22. Patients who suffer from closed-angle glaucoma or patients with other diseases with light sensitivity and/or mydriasis. 23. Patients with uncontrolled hyperthyroidism
Study endpoints	**Primary endpoint:** • The primary endpoint is defined as the change in Wexner score at Visit 4 – 4 Week Treatment Visit compared to baseline score obtained on Visit 1 – Screening Visit. **Secondary endpoints:** • **Change in the mean number of faecal incontinence episodes (defined as any leakage/seepage from the anus of gas, mucus, liquid stool or solid stool) in the week prior to the 4 Week Treatment Visit compared to baseline, where the baseline is the last week prior to randomisation.** • Responder analysis based on a ≥50% reduction in faecal incontinence episodes in the week prior to the 4 and 8 week visits compared to baseline, where baseline is the last week prior to randomisation. • Responder analysis based on a ≥30% reduction in faecal incontinence episodes in the week prior to the 4 and 8 week visits compared to baseline, where baseline is the last week prior to randomisation. • Responder analysis based on a ≥20% reduction in faecal incontinence episodes in the week prior to the 4 and 8 week visits compared to baseline, where baseline is the last week prior to randomisation. • Change in Quality of Life as measured via the FIQoL scale after 4 and 8 weeks of treatment from baseline (Screening Visit). • Change in the Wexner score after 8 weeks of treatment from baseline (Screening Visit).

	• Change in the Vaizey score after 4 and 8 weeks of treatment from baseline (Screening Visit).
Pharmacokinetic parameters	Pharmacokinetic samples will be taken from all patients and used to estimate PK parameters (eg C_{max}, $t_{1/2}$ and AUC). An exploratory analysis of population PK will be described in a separate analysis plan.
Safety parameters	• Adverse Events (AEs). Spontaneously reported AEs will be categorised into preferred terms and associated system organ classes according to Medical Dictionary for Regulatory Activities (MedDRA) version 14.0 or higher. • Laboratory safety tests: Blood samples will be taken at Visit 1 - Screening Visit to confirm patient eligibility for the study, Visits 3 and 5 (Final Visit). All blood samples for the safety laboratory tests will be evaluated at the local study site laboratory. Sample collection will be carried out according to each local laboratory practice. • Urine samples will be collected at Visit 1 - Screening Visit and Visits 2, 3, 4 and 5 and tested with dipsticks for the following as a minimum: glucose, protein, blood, bilirubin, urine specific gravity, leukocytes, nitrites and female pregnancy test if applicable. • Physical examination: A full physical examination will be performed at Visit 1- Screening Visit, with any changes from this assessment then being noted in the e-CRF at the subsequent study visits (Visits 2, 3, 4 and 5). The following body systems will be examined: cardiovascular, respiratory,

	abdominal, genitourinary, gastrointestinal, musculoskeletal, neurological and dermatological. • Blood pressure and pulse rate: specific cardiovascular safety assessment will include 12-lead Electrocardiogram (ECG), pulse rate and blood pressure measurements at Visits 1, 2, 3, 4 and 5. • 24-hour Holter measurements will be performed at Visit 1 – Screening Visit and Visit 3 – Intermediate Visit.
Statistical Methods	Populations Safety population The safety population will include all patients who receive any dose of study medication and will be used for the analyses of safety and baseline data. Intent to treat (ITT) population The ITT population will include all patients who are randomised. Modified Intent to treat (mITT) population The mITT population will include all patients who receive any dose of study medication and have data on the Wexner score at both baseline and Visit 4. Pharmacokinetic (PK) population The PK population will include all patients who receive any dose of study medication and have valid PK data from at least Visit 2.

	This will be the population used for the primary analysis of the PK data. Per Protocol (PP) population The per-protocol (PP) population will include patients who took at least 80% of the expected 28 doses in first 4 weeks of the study without any major protocol violations. Demographics and Other Baseline Characteristics Descriptive summaries of the demographic and other baseline characteristics will be completed for all populations by treatment group and overall. Analyses of Efficacy variables The efficacy analyses will be performed on the ITT, mITT and PP populations. Primary Efficacy Variable The primary endpoint is defined as the change in Wexner score at the four week Treatment Visit compared to baseline score (Screening Visit). The Wexner score will be derived and summarised for each treatment group at each visit. The change from Baseline will be calculated and summarised. Variation between the four treatment groups will be tested using an

	analysis of covariance (ANCOVA) with the baseline value as covariate and including treatment and country effects. Differences between placebo and each of the three active treatment groups will be presented along with a 95% confidence interval. Where significant variation is seen, pairwise differences between placebo and each of the 3 active treatment groups will be tested by calculating simultaneous confidence intervals for the difference in least square means between placebo and active using Dunnett's method within the ANCOVA. Dose proportionality will be tested using a linear contrast within the ANCOVA. Secondary Efficacy Variables A faecal incontinence episode is defined as any leakage/seepage from the anus of gas, mucus, liquid stool or solid stool. The number of faecal incontinence episodes per week and in the week prior to each visit will be calculated and summarised by treatment group. Differences between treatments will be tested using the same methods as for the primary efficacy parameter. A patient will be defined as a responder based on a ≥50% reduction in the number of faecal incontinence episodes in the week prior to the 4 and 8 week visits compared to baseline,

	where baseline is the last week prior to Randomisation. Similar responder rates will be defined using ≥30% and ≥20%. Variation between the four treatment groups will be tested at each visit using a logistic regression including treatment and country effects and the baseline score as covariable. Quality of life as measured via the FIQoL and the Vaizey Score will be summarised for each treatment group at each visit. The Wexner score will be summarised at Week 8 for each treatment group. The change from Baseline (Screening) will be calculated and summarised for each treatment group. Differences between treatments will be tested using the same methods as for the primary efficacy parameter. Pharmacokinetics The plasma concentrations will be listed and summarised for each treatment group at each time point. The PK variables will be estimated using noncompartmental analysis using WinNonlin or similar recognized software. The relationship between the estimated AUCs and reported AEs will be examined; full details will be given in the SAP. An exploratory analysis of population PK will be described in a separate analysis plan. Safety

	Adverse Events The primary safety parameter is the occurrence of SAEs and AEs. All data will be summarised within each treatment group. All SAEs and AEs will be listed using coding for System Organ Class and Preferred Term (using the MedDRA version 14 or higher). AEs will be summarised as follows: • Number and percentage of patients with AEs classified by System Organ Class and Preferred Term • Number and percentage of patients by strongest relationship to randomised study medication, System Organ Class and Preferred Term • Number and percentage of patients by maximum severity, System Organ Class and Preferred Term • Number and percentage of patients with serious adverse events (SAEs) classified by System Organ Class and Preferred Term • Number and percentage of patients with serious drug-related adverse events (SAEs) classified by System Organ Class and Preferred Term All safety parameters (physical examination, laboratory tests, ECGs, Holter data, blood pressure and pulse rate, concomitant medication) will be summarised appropriately. Treatment Exposure

	The total number of doses taken in weeks 1-4, weeks 4-8 and weeks 1-8 will be calculated from the data in the e-Diary and percentage compliance calculated. Descriptive statistics will be presented by treatment group.
Sample size determination	The sample size has been calculated using a responder rate. The placebo response is expected to be small (around 25%) and an active treatment is classed as a success if it shows a 20% improvement over placebo. With 80% power and a two-sided level of significance of 0.05 (5%), 98 patients are required per treatment group to show a difference between rates of 25% and 45%. The software nQuery Advisor v6.1 was used for the calculation.