A role for Glucagon-like peptide-1 in the Pathophysiology of Irritable Bowel Syndrome
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## Non-Standard Abbreviations

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<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cm</td>
<td>centimetres</td>
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<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
</tr>
<tr>
<td>DPP IV</td>
<td>dipeptidyl peptidase-4</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>ENS</td>
<td>enteric nervous system</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
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<tr>
<td>GIT</td>
<td>gastrointestinal tract</td>
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<tr>
<td>GLP-1R</td>
<td>glucagon like peptide-1 receptor</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic Pituitary Axis</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable Bowel Syndrome</td>
</tr>
<tr>
<td>IL-6,8,10,1β</td>
<td>interleukin-6,8,10,1β</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>monopotassium phosphate</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>mmol/L</td>
<td>millimoles per litre</td>
</tr>
<tr>
<td>MS</td>
<td>maternal separation</td>
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<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>sodium bicarbonate</td>
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<tr>
<td>NaH₂PO₄</td>
<td>monosodium phosphate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>disodium hydrogen phosphate</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>μm</td>
<td>micrometres</td>
</tr>
<tr>
<td>WKY</td>
<td>Wistar Kyoto</td>
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Abstract

Introduction: Irritable bowel syndrome (IBS) symptoms include visceral hypersensitivity and altered gut motility. Additionally absorptive-secretory function is altered resulting in constipation and/or diarrhoea. There is evidence which suggests altered immune cytokine profiles may contribute to symptom flares. Interleukin-6 (IL-6) has been proposed as a potential biomarker for IBS as it is elevated in IBS plasma. It also activates submucosal neurons and stimulates colonic secretion. However other molecules may be important in a specific characterisation of IBS, post-prandial exacerbated of symptoms. A possible candidate for this is Glucagon-like peptide-1 (GLP-1), an incretin hormone which is secreted following a meal and has antispasmodic effects on the gut. GLP-1 receptors are also expressed in submucosal neurons and modulate neurally evoked chloride secretions. Therefore the role of GLP-1 and the potential cross-talk between GLP-1 and IL-6 was investigated further.

Methods: Whole mount preparations of the submucosal plexus from male Sprague Dawley (SD) rats were fixed and stained with GLP-1R and dual labelled with IL-6, calbindin, calretinin, nNOS, S100, Synapsin I or PSD-95. Cross sections were also prepared from adult male SD and Wistar Kyoto rats and dual labelled with GLP1 and GLP-1R or IL-6. GLP-1 levels were determined using an ELISA and results were correlated with previously determined levels of circulating IL-6, TNF-α, IL-1β and IL-8.

Results: In immunohistochemistry studies GLP-1R were expressed in a punctate manner in the neuronal fibres, cell bodies and glial cells. 26% (n=9/35) of calbindin neurons, 21% (n=18/84) of calretinin neurons, 37.5% (n=48/128) of S100 cells and the majority, 63% (n=27/43) of nNOS were GLP-1R positive. Dual labelling with GLP-1R and Synapsin I or PSD-95 showed that GLP-1R is present in the synapses. GLP-1R co-localised with 30% (n=26/87) of IL-6 positive neurons revealing potential crosstalk between the molecules. In Elisa studies (n=9 samples per group) GLP-1 levels were significantly elevated in IBS-A (p<0.05) plasma and there was a trend towards significance in IBS-D plasma samples. Moreover plasma GLP-1 levels correlated with IL-6 levels.

Conclusion: These data demonstrate that GLP-1Rs are expressed on colonic submucosal neurons, where GLP-1 induced cellular signalling is likely to be mediated by nitrergic mechanisms. Moreover many of these cells co-express IL-6 revealing the potential cross-talk between IL-6 and GLP-1. Correlation between IL-6 and GLP-1 levels in human IBS plasma implicates this incretin hormone in the pathogenesis of IBS.
Introduction

The Gastrointestinal Tract

Fig 1: Schematic diagram of the gastrointestinal tract (Tharakan et al., 2010)

The gastrointestinal tract (GIT) provides the body with a continual supply of water, nutrients and electrolytes. Food is propelled through the tract, where it is digested by secreted digestive juices facilitating nutrient and water absorption. Regulation of this fundamental process is both by extrinsic and intrinsic neuronal control and through endocrine and paracrine processes. Figure 1 shows the various parts to the GIT and digestive system. Each part of the GIT is adapted for specific functions: the oesophagus is needed for the passage of food, the stomach functions as a temporary storage of food and also digests food, the small intestine is needed to digest and absorb food and the large intestine is needed for the absorption of water and electrolytes (Guyton and Hall, 2001).
The large intestine consists of the cecum, colon, rectum and anal canal. The colon can be divided into four parts; the ascending colon, the transverse colon, the descending colon and the sigmoid colon. The ascending colon and proximal transverse colon are known as the proximal colon and the distal transverse, descending and sigmoid colon are known as the distal colon. The distal colon is the anatomical part under investigation in this study. The wall of the colon as well as most of the GIT, typically includes, from outer to inner layer; the serosa, a longitudinal muscle layer, a circular muscle layer, the submucosa and the mucosal layer. As figure 2 shows, the mucosal lining of the large intestine consists of simple columnar epithelium that has numerous crypts, known as crypts of Lieberkuhn. These crypts contain three cell types; absorptive, goblet and granular, with goblet cells as predominating cell type (Seeley et al., 2003).

![Fig 2: Histology of the large intestine (Martini et al., 2012)](image)

Goblet cells of the colon are responsible for the mucosal excretions. The function of the mucous is to lubricate the wall of the lumen and to help the faecal matter stick together as well as protecting the intestinal wall from bacterial activity that occurs within the faeces. The mucous contains bicarbonate ions which is responsible for protecting the intestinal wall from acids formed (Guyton and Hall, 2001). The colonic absorption of water occurs by a molecular pump which exchanges HCO$_3^-$ for Cl$^-$ in epithelial cells in response to acid
produced by colic bacteria. Also another pump exchanges Na+ for H+, water then crosses the colon by osmosis due to the NaCl gradient.

Irritation to the wall of the colon causes an increase in mucous secretion by triggering local enteric reflexes. The submucosal plexus and myenteric plexus are both part of the enteric nervous system (ENS). As well as the enteric nervous system, the GIT is supplied with parasympathetic and sympathetic innervations (Seeley et al., 2003). The submucosal plexus which was under investigation in this study in relation to Irritable Bowel Syndrome (IBS) only has parasympathetic input and controls mainly the secretion and absorption functions. The myenteric plexus is responsible for contractility of the GIT and has both sympathetic and parasympathetic input (Guyton and Hall, 2001).

The Enteric Nervous System (ENS)

The ENS is one of the main divisions of the autonomic nervous system and governs the function of the gastrointestinal tract. The ENS is collected into two types of ganglia the myenteric (Auerbach’s) plexus and the submucosal (Meissner’s) plexus. The myenteric plexus is contained between the longitudinal and circular muscle layer. This plexus contains many interconnecting neurons that extend the entire GIT. When this plexus is stimulated there is increased tonic contraction of the gut wall, increased intensity of rhythmical contractions, an increase in the rhythm of the contraction and increased velocity of conduction of excitatory waves. The myenteric plexus has excitatory as well as inhibitory neurons. The inhibitory neurotransmitter of this plexus is possibly vasoactive intestinal polypeptide.

The submucosal plexus is contained within the submucosa. It controls the function within each minute segment of the intestine. The plexus controls local intestinal secretion, local absorption and local contraction of the submucosal muscle as the sensory signals that originate in the epithelium are integrated into the submucosal plexus. The local contraction causes various degrees of infoldings of the mucosa.

The different neurons within the submucosal and myenteric plexus release different types of neurotransmitters. The neurotransmitters include acetylcholine, norepinephrine,
adenosine triphosphate, serotonin, dopamine, cholecystokinin, substance P, vasoactive intestinal polypeptide, somatostatin, leu-enkephalin, met-enkephelin and bombesin (Guyton and Hall, 2001). A colonic mucosal biopsy would contain the above mentioned neurotransmitters as well as proteases and histamine. An increased activation of submucosal neurons is seen in the presence of colonic mucosal biopsy supernatants from Irritable Bowel Syndrome (IBS) patients (Buhner et al., 2012). Therefore it is important to study the submucosal plexus further in relation to IBS.

What is Irritable Bowel Syndrome?

IBS is a functional gastrointestinal disorder. Symptoms include abdominal pain, discomfort and altered bowel habits (Yale et al., 2008), affecting approximately 10% of the population (Corsetti et al., 2004). IBS is a heterogeneous, multisystem, multidomain disorder as opposed to a single disease entity (Crentsil, 2005) with several mechanisms being proposed to explain the altered sensory responses observed (Yale et al., 2008). It is also a polygenic disorder and the IBS phenotype can be determined by many common genetic variants (Saito et al., 2010). To evaluate the role of genes in IBS, candidate genes are studied that have an association with the intermediate phenotypes of IBS such as altered colonic transit, colonic motility and compliance and decreased sensation thresholds and ratings. Some of the candidate genes include genes involved in the alteration of serotonergic mechanisms (Camilleri and Katzka, 2012). Studying genes involved in serotonergic mechanisms show polymorphisms in the promoter region of serotonin transporters in IBS (Murphy et al., 2004).

Genetic abnormalities along with abnormalities in the enteric and the central nervous system which lead to altered pain and motor functions are all thought to account for the symptoms of IBS (Drossman, 2005). Also psychological stressors and ongoing enteric infections have been implicated in the onset and maintenance of IBS (FitzGerald et al., 2009). Indeed 7-31% of patients develop IBS after gastroenteritis (McKendrick, 1996) and this is termed post infectious IBS, thus revealing the importance of immune activation in this disorder.
As there is no biochemical marker or structural abnormality for IBS, diagnosis is based on the presence of clinical symptoms according to Rome III criteria (Dorn et al., 2009). However groups can be either distinguished as being IBS-D (diarrhoea predominant), IBS-C (constipation predominant) or IBS-A (alternating between diarrhoea and constipation). There is also a variability of symptoms between male and females and within each group (Yale et al., 2008).

**Irritable Bowel Syndrome Models**

**Wistar Kyoto Rat Irritable Bowel Syndrome Model**

The Wistar Kyoto rat is a pre-clinical model of IBS. IBS is associated with an enhanced visceral sensitivity in response to altered brain gut axis signalling such as that occurs during stress. The WKY rats are a viscerally hypersensitive rat model of IBS as they are genetically predisposed to pathological stress responses with alterations in colonic accommodation and morphology (Martínez et al., 2007) which leads to enhanced colonic motility and faecal output following a psychological stressor (O'Malley et al., 2010). Also following stressful stimuli WKY rats display exaggerated corticosterone levels (O'Mahony et al., 2010). A study has also shown that while the plasma Interleukin-6 (IL-6) levels are similar, mucosal scrapings from WKY rats contain higher levels of IL-6 and excised colons secrete more IL-6 than Sprague Dawley (SD) colons. The WKY secretions have also been shown to excite submucosal neurons more so than SD secretions (O'Malley et al., 2011a). O’Malley et al. also observed that under resting conditions the WKY rat in comparison to the SD rat displays a pro-absorptive phenotype as was elicited by the decrease in secretory currents. However this reversed in the presence of IL-6 where WKY colonic tissues evoked a larger secretory current (O'Malley et al., 2012).

**Maternal Separation Rat Irritable Bowel Syndrome Model**

The maternal separation (MS) rat model is another model of IBS. This model involves a postnatal stress that induces alterations in the hypothalamic-pituitary-adrenal (HPA) axis of the adult offspring (O'Malley et al., 2010). Like the WKY rat model the MS model exhibit symptoms of anxiety and depression (O'Mahony et al., 2008). The MS rat also exhibits
exaggerated GI response to inflammatory stimuli and colorectal distension, showing increased stress induced faecal output. This model shows an increased level of circulating IL-6 following an immunological challenge as well as altered 5-HT levels, and visceral hypersensitivity (O'Mahony et al., 2009).

The two rat models exhibit alterations in colonic motility (O' Mahony et al., 2011) which is a prominent phenotype of IBS (Camilleri and Katzka, 2012). As gut motility is a major function of glucagon-like peptide-1 studying GLP-1 in relation to IBS is of clinical interest (Hellström, 2009).

**Glucagon-like Peptide-1**

GLP-1 is formed in the L cells following cleavage from a pro-glucagon precursor molecule by post-translational processing and is a C-terminally amidated 7-37 amino acid peptide (Hellström, 2011). GLP-1 regulates numerous gastrointestinal functions, it decreases gastric acid secretions (Schjoldager et al., 1989), gastrointestinal transit, motility (Hellström et al., 2008) and gastric wall tone (Schirra et al., 2002).

The MMC are waves of activity that sweep through the intestine during the fasting state and they help trigger peristaltic waves which facilitate transport of the bolus through the GIT (Tolessa et al., 1998b). Helstrom et al. showed that GLP-1 inhibits the migrating myoelectric complex (MMC) during fasting in healthy and IBS patients (Hellström et al., 2008).

Along with regulating gastrointestinal motility functions GLP-1 has anti-hyperglycaemic actions. GLP-1 is released from L-cells in the ileum and colon following intake of carbohydrates and fat and is considered an important regulator of postprandial insulin secretion from the pancreas in response to a meal (Hellström et al., 2008). GLP-1 acts on β-cells of the pancreas to cause an increase in insulin secretion even before blood sugar levels become elevated (Hellström, 2009). GLP-1 has also been shown to suppress glucagon release from the pancreas and to enhance the sensitivity of pancreatic B-cells to glucose (Gutniak et al., 1994).
GLP-1 has a half-life of about 1-2 minute and is degraded by depiptidyl peptidase IV (DPP- IV) which is an enzyme present in the plasma (Hellström, 2011). The fact that it has such a short half-life together with the fact that GLP-1 has no effects on intestinal muscle strips in vitro concludes that direct endocrine or autocrine actions are unlikely. Therefore neurocrine actions of GLP-1 are most likely (Tolessa et al., 1998a).

GLP-1 has also been shown to be implicated in stress induced alteration in colonic transit via central CRF pathways and peripheral cholinergic pathways in rats (Nakade et al., 2007). IBS symptoms are exacerbated following a meal as lipids in the intraduodenum are shown to increase visceral sensitivity (Caldarella et al., 2005) and as GLP-1 is released following intake of carbohydrates and fat (Hellström et al., 2008) it is important to investigate GLP-1 in relation to IBS.

**Treating Irritable Bowel Syndrome**

Despite the prevalence and economic burden of IBS treatment options remain limited. Current treatment options include dietary adjustment, for example increasing the amount of fiber in the diet, the use of anti-diarrhoeals and the use of laxatives (Dalrymple and Bullock, 2008). IBS pain is due to visceral hypersensitivity and patients who have this pain as their most common complaint are most impacted by IBS and there are currently no treatments targeting this pain (Olden, 2003).

As previously described GLP-1 plays a role in the regulation of gut motility and therefore could be important clinically in treating pain that is associated with abnormal motor activity in the gut. A clinical trial was formulated where the GLP-1 hormone analogue ROSE-010 was administered to suitable IBS patients. The results found that the GLP-1 analogue ROSE-010 caused meaningful pain relief in a significant proportion of the patients and delayed gastric emptying of food without retarding small bowel or colonic transit (Hellström et al., 2009).

**The Brain Gut Axis**

The Brain gut axis includes the parasympathetic innervation and the sympathetic innervation, which supplies nerve fibres from the brain and spinal cord to the GIT. The brain gut axis also includes the previously described enteric nervous system (Saulnier et
A system which has a major influence on the brain gut axis is the HPA axis (Dinan, 1994).

**The Hypothalamic-Pituitary-Adrenal Axis**

![Diagram of the HPA axis and central and peripheral role of CRF pathways influencing immune processes](image)

**Fig 3:** The HPA axis and central and peripheral role of CRF pathways influencing immune processes (Kiank et al., 2010).

Figure 3 outlines the HPA axis. The HPA axis includes the hypothalamus, the pituitary gland and the adrenal gland. Corticotropin releasing factor (CRF) is released from the paraventricular nucleus of the hypothalamus at the median eminence into the primary capillary plexus of the hypothalamo-hypophyseal portal system. It is then carried to the
anterior lobe of the pituitary where it stimulates corticotropes to release adrenocorticotropic hormone (ACTH). ACTH stimulates the synthesis of cortisol, glucocorticoids and mineralocorticoids (Gillespie and Nemeroff, 2005). CRF containing neurons are widely distributed throughout extrahypothalamic brain areas and acts as a neuromodulatory agent in coordinating behaviour, endocrine, autocrine and immune responses to stress (Corsetti et al., 2004). CRF release is also controlled by neurotransmitters such as 5-HT and norepinephrine in response to stress (Dinan, 1996).

Patients with IBS show an exaggerated stress response when administering CRF and this could be seen by the enhanced levels of ACTH and cortisol (Dinan et al., 2006, FitzGerald et al., 2009). The increase in ACTH could be due to the exogenous CRF acting synergically with the pro-inflammatory cytokines and other secretogogues to bring about ACTH release. The increase could also be due to the CRF1 receptor on the anterior pituitary being up-regulated in IBS (Gillespie and Nemeroff, 2005). The enhanced level of ACTH and pro-inflammatory cytokines were shown not to be due to a decreased sensitivity of the glucocorticoid receptor (Dinan et al., 2006). There is also evidence of a similar upregulation of CRF1 receptor in the gut as a result of stress. This upregulation of CRF1 receptors warrants further investigation of CRF1 receptor antagonists as a treatment option for IBS (Fukudo et al., 1998, Gillespie and Nemeroff, 2005).

**Irritable Bowels Syndrome and the Immune System**

It is well established that acute gastrointestinal infections initiate the onset of IBS symptoms in at least a sub-group of patients (McKendrick and Read, 1994). While some IBS patients show persistent levels of low grade inflammation with increased levels of pro-inflammatory cytokines and activated T lymphocytes and mast cells (van der Veek et al., 2005). It is also shown that IBS patients have altered GI permeability (Camilleri et al., 2012) and most pro-inflammatory cytokines have the capacity to alter intestinal epithelial permeability (Al-Sadi et al., 2009).

Figure 3 outlines CRF pathways and the influence they have on immune processes. Immune cells such as macrophages, T lymphocytes, mucosal mast cells, and dendritic cells
respond to CRF receptor signalling mainly by promoting inflammation. However central CRF evoke an anti-inflammatory response, which may counter-regulate inflammatory responses in the gut, by increasing glucocorticoids and catecholamines and decreasing parasympathetic activity (Kiank et al., 2010). In response to infection CRF containing neurons also respond to pro-inflammatory cytokines (Chrousos, 1995).

Cytokines are small cell signalling protein molecules secreted by leukocytes and various other cells. Fundamentally cytokines influence innate and adaptive immunity (Ortiz-Lucas et al., 2010). Specifically pro-inflammatory cytokines such as IL-6 and IL-8 are shown to be increased in patients with IBS and are shown to activate the HPA axis (Dinan et al., 2006, FitzGerald et al., 2009). Analysis has also shown that HPA hyperresponsivity is related to enhanced IL-6 levels (Dinan et al., 2006). In turn activating the HPA axis causes an increased release of glucocorticoids which depresses inflammatory processes of the gut (Turnbull and Rivier, 1999). It is unclear whether the increase in IL-6 and IL-8 are biological markers for a trait of the syndrome or IBS itself (Dinan et al., 2006).

**Interleukin-6**

Interleukin-6 (IL-6) is a pro-inflammatory and anti-inflammatory cytokine (Scheller et al., 2011) and stimulates secretion into the colonic lumen by modulating submucosal neurons. IL-6 is elevated in the mucosa and plasma of the pre-clinical model of IBS, the WKY rat (O'Malley et al., 2012, O'Malley et al., 2011a). IL-6 levels are also increased in the plasma of IBS patients which may reflect an important mechanism which leads to symptom flares (Dinan et al., 2006).

Following administration of cholinesterase inhibitor there is an increase in IL-6 synthesis which correlates to an increase in abdominal bloating (Dinan et al., 2008). IL-6 along with IL-1 β was shown to act as excitatory neuromodulators of myenteric neurons via presynaptic inhibition by inhibiting acetylcholine release from pre-synaptic neurons (Keller et al., 2001). It has also been shown that IL-6 inhibits nicotinic and noradrenergic neurotransmitter release in guinea pig submucosal neurons (Xia et al., 1999). IL-6 receptors are expressed on rat submucosal neurons and following exposure to recombinant IL-6 there is an increase in extracellular calcium and colonic excretion that overrides the pro-
absorptive phenotype, which is regulated by epithelial cholinergic activity at rest (O'Malley et al., 2011b).

Adipose tissue is a major source of IL-6 concentration and therefore contributes to the induction of insulin resistance. Contracting skeletal muscles during exercise also increases circulating IL-6 concentrations. It is also proposed to promote nutrient availability and improves whole body insulin sensitivity. Pancreatic alpha cells are also a primary target of IL-6 action. IL-6 promotes alpha cell proliferation and inhibits apoptosis. In a high fat diet alpha cells mass expands in an IL 6 dependent manner. IL-6 was also shown to promote GLP-1 secretion and production from intestinal L cells and pancreatic alpha cells (Ellingsgaard et al., 2011)

Other cytokines such as interferon γ, tumour necrosis factor (TNF), transforming growth factor and interleukin-10 (IL-10) regulate inflammatory and immune responses (Gonsalkorale et al., 2003a). A UK study showed that frequency of the high IL-10 producer genotype for IL-10 (an anti-inflammatory cytokine) were significantly reduced in patients with IBS as compared with healthy controls (Gonsalkorale et al., 2003b).

**Aims of the Study**

The aims of the study were to indentify the location of GLP-1R and IL-6 within the submucosal ganglia of the distal colon. After getting location of GLP-1R and IL-6 within the submucosal ganglia we wanted to observe the quantity and location of GLP-1, IL-6 and GLP-1R across the entire cross section of the distal colon. To relate this to the human condition we investigated levels of GLP-1 in IBS patients and compared them to healthy controls and aimed to correlate these values with levels of other pro-inflammatory cytokines.
Materials and Methods

Animals
Male Sprague Dawley rats were purchased from Harlan UK weighing approximately 250-350 g. The rats were housed in groups of four to six per cage. They were maintained on a 12/12 h dark–light cycle and were given food and water ad libitum and kept at a room temperature of 21-23°C. Principles of laboratory animal care were followed, and all procedures were carried out in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation and Ethics Committee.

Tissue Preparation

Whole mount preparation of the Submucosal Plexus
A section of the colon was excised from each rat, cut 8cm proximal from the anus. The excised distal colon was stored in ice cold Krebs (pH7.4) solution at 4°C; containing in mmol/L: 117 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄ and 11 D-glucose. The pellets were then expunged using a 10 ml syringe filled with Krebs. Using a forceps the section of colon was pulled over a glass rod allowing for the mesenteric blood vessels to be removed. The colon was lightly scored along where the mesentery was removed, using a blade.

Figure 4: Layers of the colon. JD Wood et al

Figure 4 shows the various layers of the colon. The longitudinal and circular muscle containing the myenteric plexus were removed using a cotton swab. A scissors was used to
cut all the way through the mesentery and the remaining submucosal layer was pinned out mucosal side up on a sylgard lined plate filled with Krebs saline. Figure 4 also shows that the submucosal ganglion is contained within a separate layer from the mucosa. Using forceps the mucosal layer was removed and the remaining submucosal plexus was prepared for Immunofluorescent Imaging as detailed below.

**Cross Section Preparation of the Distal Colon**

Sections of colon from four separate Sprague Dawley rats (the control model) and four separate Wistar Kyoto rats (the pre-clinical IBS model) had been fixed and frozen from a previous study. Short sections of the colons were cut and brought up to -25°C in the cryostat (Leica, Nussloch, Germany; CM1900 UV). Using the cryostat, sections were prepared. Firstly Tissue-Tek® O.C.T. (optimum cutting temperature) compound embedding medium was applied to the stage and left to freeze for ten minutes. The colon was then mounted vertically onto the stage and more mounting medium was applied over the colon, again the medium was left to freeze for another 10 minutes. 10 μm sections were taken with 30 μm between sections. Eight sections were taken from each colon and applied to two separate Fisherbrand glass slides, four sections were applied to a slide to be stained for anti-GLP-1R and anti-GLP-1 and four sections were applied to a slide to be stained with anti-IL-6 and anti-GLP-1.

**Immunofluorescence**

**Immunofluorescence of whole mount SMP preparations**

Using 96-well plates seven separate sections of the submucosal plexus were stained from each of the four Sprague Dawley rats. The whole mount preparations were first fixed overnight in Zamboni’s fixative [paraformaldehyde (from 16% stock solution), picric acid (saturated aqueous), 10M NaOH, 0.1M phosphate buffer] at 4°C. The sections were washed three times for 15 minutes in phosphate buffered saline (PBS) (containing in mmol/L: 137 NaCl, 2.7 KCl, 10 Na₂HPO₄·2H₂O, 2.0 KH₂PO₄) and agitated. The preparations were then permeabilised for 1 hour in 0.1% Triton X-100 in PBS at room temperature (R.T.). Again the sections were washed three times (X3) for 15 minutes in PBS. For 1 hour the sections were incubated in blocking solution (1% donkey serum in PBS) at R.T. All sections were incubated overnight at 4°C with a 1:250 dilution of anti-
GLP-1 receptor (GLP-1R) antibody (affinity purified rabbit polyclonal antibody a synthetic peptide conjugated to KLH derived from within 250-350 of Human GLP1R; Abcam, Cambridge, UK). The sections were washed again in PBS for 15 minutes X3. The GLP-1R antibody labelled tissues, were incubated with a 1:250 dilution of Texas Red conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). The sections were washed for 10 minutes in PBS X3. For dual labelling the sections were incubated overnight with a separate primary antibody including neuronal markers for calbindin (1:300; mouse, Swant, Bellinzona, Switzerland) calretinin (1:300, goat; Swant) and neuronal nitric oxide synthase (nNOS, 1:300; goat, Abcam), the glial cell marker S100 (1:300, mouse; Sigma-Aldrich, St. Louis, MO) and the anti-rat IL-6 antibody (affinity purified goat antibody immunised E.coli-derived rIL-6, 1:300; R&D Systems, Abingdon, UK). Sections were also stained with the pre-synaptic marker Synapsin I (1:250, goat; Santa Cruz Biotechnology, Santa Cruz, CA) and the post-synaptic marker PSD-95 (1:200; mouse, Santa Cruz Biotechnology). Slides were again washed for 10 minutes in PBS X3. The sections were then incubated for 2 hours with Fluorescin isothiocyanate (FITC) conjugated antibody (1:250 goat anti mouse Jackson ImmunoResearch) and (1:250 donkey anti-goat; Milipore). Dako fluorescent mounting medium was used to mount the sections onto glass slides and a cover slip was placed over them.

Immunofluorescence of Cross Sections of Colon

The cross sections were prepared for immunofluorescent imaging. Two slides containing four sections were prepared for each rat. One of the two slides from each rat was incubated with a 1:200 dilution of GLP-1 antibody (mouse antibody; Antibody Shop). A 1:300 dilution goat anti-mouse rhodamine conjugated secondary antibody was used as a fluorophore (Jackson ImmunoResearch). This slide was then dual labelled with a 1:250 dilution of GLP-1R antibody (Abcam) with FITC conjugated donkey anti-rabbit secondary antibody as the fluorophore. The second slide from each rat was incubated with a 1:200 dilution of GLP-1 antibody and with a 1:300 dilution of the anti-mouse rhodamine conjugated secondary antibody. The second slide was also incubated with the anti-rat IL-6 antibody (1:300; R&D Systems) and with a FITC conjugated anti-goat secondary acting as the fluorophore.
**Image analyses**

Neurons in submucosal ganglia were imaged using Cell-F software (Soft Imaging Solutions) and an Olympus BX51 fluorescent microscope [Olympus America, Inc, Melville, NY, USA] with an Olympus DP71 digital camera [Optronics, Goleta, CA, USA] with filter sets for TRITC [excitation (557nm), emission (576nm)] and FITC [excitation (470nm), emission (525nm)]. Images were also obtained from the FLUOVIEW FV10i-Olympus-confocal. Three ganglia per tissue were imaged from four separate rats. With the Cell-F software co-localisation between the cell markers (calbindin, calretinin, nNOS and S100) and GLP-1R was determined by counting the number of cells positive for the cell markers and calculating the percentage of these cells that co-localised with GLP-1R. Co-localisation between the synaptic markers (PSD-95 and Synapsin I) and GLP-1R was determined using the confocal. Co-localisation between and location of GLP-1R and GLP-1 was also determined using confocal microscopy as was co-localisation between and location of GLP-1 and IL6 on the colon cross sections.

**Enzyme Linked Immunosorbent Assay (ELISA)**

A sandwich ELISA was carried out to determine the GLP-1 concentration in plasma samples from 9 healthy, 9 IBS-C, 9 IBS-D and 9 IBS-A patients following manufacturer’s guidelines (Milipore; Cat. EGLP-35K). The assay was run in duplicate. DPP-IV (dipeptidyl peptidase-IV) was added to the samples to inhibit GLP-1 degradation. A multi-pipette was used to ensure accurate measurements of solutions were made. The plates were read on a Synergy HT fluorescent microplate reader with an excitation/emission wavelength of 355 nm and 460 nm respectively and the lowest level of GLP-1 that could be detected was 2 pM. Having solutions of known GLP-1 concentrations, from 0 to 100 pM, a standard curve was created. From this curve the GLP-1 concentrations of the plasma samples were determined.

**Statistical Analyses**

Experiments were conducted in at least four different animals and in the case of the ELISA at least 9 patients per group. ELISA data was analysed using GraphPad prism for windows (version 7). Paired or unpaired two-tailed Student’s t-test were used where appropriate, P<0.05 was considered significant.
Results

Immunofluorescence labelling of GLP-1Rs in SMP preparations

Immunohistochemistry techniques were carried out on whole mount preparations taken from the distal colon of male, adult SD rats. To determine the neuronal cell type that expressed GLP-1R co-localisation, dual labelling studies were conducted with calbindin antibodies to label cholinergic sensory neurons, anti-calretinin for cholinergic motor neurons and anti-nNOS for nitrergic neurons. Anti-S100 labelled glial cells in the submucosal ganglia.

In Figure 5 the images show GLP-1R (red stain) dual labelled with calbindin (Fig 5(a) n=9/35), calretinin (Fig 5(b) n=18/84), nNOS (Fig 5(c) n=27/43) and S100 (Fig 5(c) n=48/128) (green stain), (Fig 5; n= 3 ganglia per tissue from 4 different rats). GLP-1R expression is found in the cell bodies but is also prominent in the neuron fibres and glial cells. The prominent staining in the neuronal cell fibres is also quite punctate indicating that GLP-1Rs are clustered together.

In Figure 7 it shows the percentage of total neurons that are positive for the different cell markers which co-localises with GLP1R. Dual labelling with GLP-1R and calbindin antibodies show a 26% (n=9/35) co-localisation in the cell bodies of neurons. Dual labelling with GLP-1R and calretinin shows a 21% (n=18/84) co-localisation in the cell body of neurons. Dual labelling with GLP-1R and nNOS shows a 63% (n=27/43) co-localisation in the cell body of neurons. There was also GLP-1R staining of glial cells as dual labelling with GLP-1R and S100 shows a 37.5% (n=48/128) co-localisation with the glial cells of neurons.
The punctate nature of the GLP1-R staining suggested that the receptor was potentially expressed at synapses. To investigate this further, tissues were dual labelled with GLP1-R antibody and the presynaptic marker Synapsin I and also dual labelled with GLP-1R antibody and the postsynaptic marker PSD-95. GLP-1R expression (red stain) and Synapsin I or PSD-95 (green stain) dual labelling can be seen in the confocal images in Figure 6. Some co-localisation was seen between GLP-1R and the presynaptic marker Synapsin I (Fig 6(a)) and the postsynaptic marker PSD-95 (fig 6(b)).
To determine cross-talk, co-localisation studies were conducted between IL-6 and GLP-1R. Dual labelling with GLP-1R and IL-6, shows that co-localisation is present in 30% (n=26/87) of neuronal cell bodies. In Figure 6(c) one neuronal body (indicated by arrow) shows co-localisation between GLP-1R and IL-6. GLP-1R is the red stain and IL-6 which is conjugated to the FITC secondary antibody is the green stain.

Fig 6(a): GLP1R (Texas Red) and Synapsin I (FITC). The area within the white box has been magnified. Regions of co-localisation (yellow staining) are indicated by the arrows. Scale bar = 20 μm

Fig 6(b): GLP-1R (Texas Red) and PSD-95 (FITC). The area within the white box has been magnified. Regions of co-localisation (yellow staining) are indicated by the arrows. Scale bar = 20 μm
**Fig 6(c):** GLP-1R (Texas Red) and IL-6 (FITC). The area within the white box has been magnified. A cell co-expressing and IL-6 and GLP-1Rs is indicated by the arrow (n=3 ganglia per tissue from 3 different rats). Scale bar=20 μm

The histogram below (Figure 7) shows the percentage of cells expressing GLP-1Rs. GLP-1R co-localised with a greater percentage of nNos positive neurons in comparison to other cell markers. From Figure 7 you can also see that GLP-1R co-localises with a lesser percentage of calretinin positive neurons in comparison to the other cell markers.

**Fig 7:** Graph showing percentage of cell marker positive cells that co-localise with GLP-1R
**Colonic Expression of L-cells and GLP-1R and IL-6 expressing cells**

To determine whether GLP-1 secreting L-cells were in close proximity to GLP-1R expressing submucosal neurons, transverse sections were immunolabelled with GLP-1 and GLP-1R, and sections were also labelled with GLP-1 and IL-6 antibody. The immunohistochemistry techniques were also used to compare the GLP-1, IL-6, and GLP-1R staining between the IBS pre-clinical model, the WKY rat, which has altered GI morphology, and the control model, the SD rat. Co-localisation can be seen in Figure 8(a). The co-localisation is between GLP-1 (red staining) and GLP-1 receptor (green staining) and shows a couple of L-cells (indicated by arrows) in the mucosal layer of the WKY rat.

The co-localisation between IL-6 (green staining) and GLP-1 (red staining) can be seen in Figure 8(b). A GLP-1 expressing L-cell that does not co-localise with IL-6 staining is indicated by the arrowhead and two L-cells co-expressing GLP-1 and IL-6 are indicated by the arrows.

**Fig 8(a):** Transverse section of GLP-1 and GLP-1R staining from distal colon of a WKY rat. Scale bar = 5μm
**Fig 8(b):** Transverse Section of GLP-1 and IL-6 staining from distal colon of a SD rat.  
*Scale bar= 5μm*

**Plasma Concentrations of Glucagon-like peptide-1**

![Graph showing standards curve of GLP-1 concentrations.](image)

*Fig 9: Standard curve of GLP-1 Concentrations*

An ELISA was used to calculate the GLP-1 concentrations in plasma samples from healthy controls and from each subtype of IBS. Figure 9 is a standard curve of GLP-1 concentrations and the GLP-1 concentrations of plasma samples were determined from this curve. A Student’s t-test showed that there was a significant difference between plasma GLP-1 content in IBS-A patients in comparison to controls (n=9 samples, p<0.09, figure 10). The Student’s t-test did not show a significant difference between IBS-C patients and
healthy controls (n=9 samples, p>0.05), however there was a trend toward a significant
difference between IBS-D patients and healthy patients (n=9 samples, p=0.09, figure 10).

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![Graph showing GLP-1 concentrations (pM) in healthy controls, IBS-C, IBS-D, and IBS-A.]

**Fig 10:** GLP-1 concentrations (pM) in healthy controls, and IBS patients. A significant
elevation in GLP-1 levels in IBS-A (n=9) in comparison to healthy controls (p<0.05, t-test).

Interestingly when compared to previously published data on the levels of cytokines in IBS plasma (McKernan et al., 2011) a significant correlation was found between the levels of IL-6 and GLP-1 in healthy controls, IBS-C, IBS-D and IBS-A ($r^2=0.12$, p<0.05, Figure 11). No correlation was detected between GLP-1 and IL-1β, TNF-α or IL-8.

**Figure 11:** Linear regression between levels of IL-6 and GLP-1 in healthy controls, IBS-C, IBS-D and IBS-A.
Discussion

The findings from this study show that GLP-1 concentrations are elevated in IBS-A plasma and this is correlated with raised IL-6 levels previously reported (Dinan et al., 2006). However it is likely that GLP-1 is more than just a biomarker of IBS as GLP-1 receptor staining is expansive and punctate among submucosal neuronal fibres and neurons where it may contribute to changes in colonic absorption and/or secretion. The data also show that IL-6 staining was in neuronal bodies among submucosal neurons and co-localised with GLP-1R in a number of cases, thereby revealing potential for cross-talk between the molecules.

GLP-1 has been shown to inhibit the MMC of the antrum of the stomach which leads to inhibition of gastric emptying and also inhibits MMC in the duodenum and jejunum of the small intestine in both healthy subjects and in IBS subjects. GLP-1 is also shown to inhibit gastrointestinal motility following a meal (Hellström et al., 2008). GLP-1 analogue ROSE-010 has also been shown in clinical trials to alleviate abdominal pain and inhibit gastric emptying without slowing colonic transit in IBS-C patients (Hellström et al., 2009). What is not clear however is what underlying mechanism GLP-1 is mediating these alleviating effects and to what extent does it differ among IBS subtypes. GLP-1 and GLP-1R have been shown to be present in the stomach and in the small intestine (Eissele et al., 1992), (Hellström et al., 2008) and distal colon (Eissele et al., 1992, Amato et al., 2010). While submucosal expression of GLP-1Rs has been reported in colonic submucosal neurons (Baldassano et al., 2012), our findings have shown the type of ganglionic cells expressing the receptor and co-localisation with the pro-inflammatory cytokine, IL-6.

The GLP-1 receptor co-localises with 63% of nNOS positive neurons. Neuronal nitric oxide synthase synthesises nitric oxide which is a free radical signalling molecule and acts as a smooth muscle cell relaxant in the GIT (Shah et al., 2004). GLP-1 causes gastric accommodation (Delgado-Aros et al., 2002) and this accommodation depends on a nitricergic link (Barragán et al., 1994, Tolessa et al., 1998a). Indeed applying a NO synthase inhibitor leads to a blockage in postprandial augmentation of gastric volume by GLP-1(Andrews et al., 2007). nNOS has been found to co-localise with GLP-1R in the myenteric neurons of the duodenum and proximal colon indicating that nitricergic pathways play a role in the
inhibitory effects of GLP-1 on muscle activity (Amato et al., 2010). nNOS has also been implicated in the pathogenesis of IBS (Reinders et al., 2005, Tjong et al., 2011), with elevated nNOS expression and NO production from the distal colon of MS rodents (Tjong et al., 2011). Clinical studies have also shown elevated rectal mucosal and plasma NO levels in IBS patients (Reinders et al., 2005, Yazar et al., 2005). Our data suggests the effects of GLP-1 on submucosal neurons in the distal colon may also be mediated by nitrergic pathways.

GLP-1R co-localises with 21% of calretinin neurons and 26% of calbindin neurons. Calretinin is a calcium binding protein and is predominantly in motor neurons. Calbindin is also a calcium binding protein and is predominantly in sensory neurons (Abalo et al., 2009). Our data showed the co-localisation between calbindin and GLP-1R was lower than the co-localisation between nNOS and GLP-1R. Like-wise co-localisation between calretinin and GLP-1R was lower than the co-localisation between nNOS and GLP-1R. Our data therefore suggests that the effects of GLP-1R on submucosal neurons of the distal colon is mediated to a greater extent by nitrergic pathways than motor or sensory pathways.

A previous study has shown that IL-6 and IL-6R staining was primarily cytosolic and co-localised with the neuronal cell markers calretinin, calbindin and the glial cell maker S100 (O'Malley et al., 2011b). We have demonstrated that GLP-1R is primarily expressed in nNOS positive neurons. Interestingly IL-6 receptor has been shown to co-localise with a similar percentage of nNOS labelled cells as does GLP-1R (O'Malley et al., 2011b, O'Malley et al., 2011a). In this study IL-6 is shown to be in the neuronal bodies showing cytosolic expression. Dual labelling with IL-6 and GLP-1R showed that co-localisation is present in 30% of neuronal bodies revealing a potential area of cross talk. Further studies to investigate if IL-6 receptors and GLP-1 receptors are in close proximity would also provide valuable information.

The GLP-1R staining was punctate along the neuronal bodies and fibres. To determine if these receptor clusters were at synapses neurons were dual labelled with the pre-synaptic marker, Synapsin I and the post-synaptic marker, PSD-95. GLP-1R clusters appeared to co-localise with both Synapsin I and PSD-95 indicating that GLP-1 may be important in
the synaptic excitation of submucosal neurons and may directly influence the secretion of a neurotransmitter or act as a neurotransmitter itself.

Cross Sections were obtained in his study to demonstrate the location and quantity of GLP-1, GLP-1R and IL-6 across the entire cross section of the distal colon. GLP-1 and IL-6 were shown to co-localise within L-cells in the colon. L-cells within the gut secrete GLP-1 and a study has shown that IL-6 receptors are also expressed on L-cells. IL-6 has also been shown to cause an increase in GLP-1 secretion from L-cells (Ellingsgaard et al., 2011), hence with elevations in IL-6 in IBS patients this may result in increased secretion of GLP-1. Indeed the correlation seen in our study between IL-6 and GLP-1 levels in the IBS samples would substantiate this theory.

In summary GLP-1R co-localised with calbindin, calretinin, nNOS and S100 and to the greatest extent with nNOS, therefore concluding that GLP-1 could be exerting its effects through a nitrergic pathway. Co-localisation studies also allowed us to conclude that a certain level of cross talk may exist between GLP1-R and IL-6 and this may result in functional changes in the gut such as altered secretion and absorption. In this way, GLP-1 may contribute to the pathogenesis of IBS.

Further Studies
As the ELISA data of IBS-C and IBS-D plasma samples were trending towards significance further experiments could be carried out with more plasma samples.

The functional role of GLP-1 in IBS may be further characterised by carrying out calcium imaging studies. Exposure of submucosal neurons to GLP-1 would determine whether GLP-1 excites or inhibits submucosal neurons. Using an animal model of IBS, the sensitivity of submucosal neurons in a model of altered GI function could be assessed, as has been carried out for IL-6 (O’Malley et al., 2011b). Moreover supernatants pooled from WKY rats could be added to the submucosal preparation from both SD and WKY rats. Following neuronal excitation by the added supernatant, an anti-GLP-1 neutralising antibody could then be added to the supernatant. If there is a reduced calcium response it would show whether GLP-1 in secretions are also involved in exciting submucosal neurons.
To analyse effects of GLP-1 on absorption and secretion across the colonic epithelial membrane Ussing Chamber electrophysiology experiments could be conducted using SD and WKY rats. The effects of GLP-1 levels on secretion would be compared between both rat models. The Ussing chambers design could be exploited to investigate the capacity that IL-6 has on GLP-1 secretion and also to investigate the capacity that GLP-1 has on IL-6 secretion.

Also as CRF expression is seen to be prominent in the submucosal plexus of the proximal and distal colon (Yuan et al., 2010) and GLP-1 has been shown to be mediated in stress induced alteration of colonic transit via CRF mediated pathways (Nakade et al., 2007), further studies could be carried out to determine potential cross-talk between GLP-1 and CRF within the submucosal ganglia. Immunofluorescent techniques could be carried out to determine co-localisation between CRF and GLP-1. Also Ussing chamber experiments could be carried out to investigate the capacity that CRF has on GLP-1 secretion.

The Immunofluorescent results in this study could be further investigated by carrying out Western Blot Analysis. Western Blot Analysis would be carried out on colonic human biopsies to determine the concentration of IL-6 and GLP-1 in the submucosal plexus and other layers of the colon. It would allow for a comparison of concentrations of IL-6 and GLP-1 between the different layers of the colon.
References


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