MODULATION OF MECHANOSENSITIVE GASTRO-OESOPHAGEAL VAGAL AFFERENTS BY NOVEL TARGETS

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ABBREVIATIONS

α,β-meATP; *α,β*-methylene adenosine 5'-triphosphate

AMPA; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

AP-5, D-(-)-2-amino-5-phosphonopentanoic acid

ASIC; Acid Sensing Ion Channels

BK; Bradykinin

C; carboxyl terminus

CCK; Cholecystokinin

CNS; central nervous system

CRD; colorectal distension

CT; Cycle threshold

DEG/ENaC; Degenerin/Epithelial Na^+ Channel

ΔCT; (Cycle threshold (CT) of GalR/iGluR/ghrelin receptor transcript - Cycle threshold (CT) of β-actin)

DRG; dorsal root ganglia

GalR; galanin receptor

GABA; γ-Amino butyric acid

Glu-IR; Glutamate immunoreactivity

IMG; inferior mesenteric ganglion

IGLEs; intraganglionic laminar endings

IMAs; intramuscular arrays

iGluR; ionotropic glutamate receptors

LSN; lumbar splanchnic nerve

mGlur; metabotropic glutamate receptor

MTEP, 3-[(2-Methyl-1, 3-thiazol-4-yl)ethynyl]-pyridine

N; amino terminus

NBQX, 2,3-dioxo-6-nitro-1, 2, 3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide

NO; Nitric oxide

-/- ; null mutant

NMDA; *N*-methyl-D-aspartate

PN; sacral pelvic nerve

PCR; polymerase chain reaction

QRT-PCR; Quantitative reverse transcription polymerase chain reaction

RA; rapidly adapting mechanoreceptor

rIGLEs; rectal intraganglionic laminar endings

RNA; ribonucleic acid

RT; reverse transcription

5-HT; serotonin

spikes / sec; spikes per second

SD; standard deviation

TM; transmembrane domain

TLOSRs; transient lower oesophageal relaxations

TRP; transient receptor potential

TRPV1; transient receptor potential vanilloid receptor 1

VIP; Vasoactive intestinal peptide

ANOVA; analysis of variance

+/+; wild-type

SUMMARY

Modulation of signals from peripheral vagal afferent mechanoreceptors to the central nervous system has been identified as the most accessible target for control of neuronal pathways and reflexes central to gastrointestinal disorders such as GORD, disordered food intake and functional dyspepsia.

There are numerous candidates for modulation of vagal afferent signals from the gastrointestinal tract to the CNS, all of which may represent novel targets for therapeutic treatment of gastrointestinal disorders. These candidates include excitatory ionotropic receptors as well as inhibitory and excitatory (metabotropic) G-protein coupled receptors. Four were chosen for study in this thesis. These are:

1) **Galanin receptors,** which may be excitatory or inhibitory GPCRs depending on their subtype
2) **Excitatory ionotropic glutamate receptors**, and their relative contribution compared with excitatory metabotropic glutamate receptors.
3) **Ghrelin receptors**, which may have excitatory or inhibitory actions on nerves elsewhere.

Aims

Determine the roles of four groups of identified receptors in modulation of mechanosensitivity of peripheral gastro-oesophageal mechanoreceptors and to identify endogenous ligands and receptors in vagal cell bodies to complement their known location in stomach.

Methods:

Novel *in vitro* mouse and ferret vagal gastro-oesophageal preparations have been previously reported. Accurate quantification of mechanical responses was performed according to the primary stimulus for the type of afferent. Mechanical sensitivity of primary afferents was established by mechanical stimulation of the preparation via circumferential tension (0.5-7g) or mucosal stroking with von Frey hairs (10-1000mg). Afferent responses to mechanical stimulus were tested in the presence of selective agonists and antagonists of galanin, ionotropic and metabotropic glutamate as well as ghrelin receptors. In additional studies, the effects of galanin and selective receptor agonists and antagonist on GalR1 wild type (+/+) and null mutant (-/-) mice were determined.

Results:

Two types of vagal afferent mechanoreceptors were identified in the mouse model, decribed as tension and mucosal sensitive afferents. An additional sub-type, tension-mucosal was identified in the ferret oesophagus.

1) Galanin induced potent inhibition of mechanosensitivity of both types of mouse afferent, an effect mimicked by a GalR1/2 agonist but was absent in null mutant GalR1 (-/-) mice. A GalR1/2 agonist demonstrated minor potentiation of mechanosensitivity in null mutant GalR1 (-/-) mice. There was no significant effect of GalR3 selective ligands observed however.
2) Selective iGluR receptor agonists AMPA and NMDA dose dependently potentiated responses of vagal afferents to mechanical sensitivity, an effect reversed by both selective and non-selective antagonists, whilst the mGluR5 antagonist MTEP concentration dependently inhibited mechanosensitivity.

Efficacy of agonists and antagonists for the various receptor sub-types differed between mucosal and tension receptors. No role for Kainate receptors was observed in this study.

3) In a mouse model ghrelin significantly reduced the response of tension sensitive afferents to circumferential tension, an effect reversed by a selective receptor antagonist. This effect was not observed in mouse mucosal receptors. In the ferret model, ghrelin significantly reduced the response of mucosal and tension mucosal receptors to mucosal stroking however did not affect responses to circumferential tension.

Conclusions:

The current study highlights the complex interaction between excitatory and inhibitory receptors, located on peripheral vagal afferent terminals, that serve to modulate afferent signalling to the CNS and thus allows precise control over gut reflex and secretory function. This study further adds to an expanding list of modulators of peripheral vagal afferent mechanoreceptors, providing additional possible novel therapeutic candidates for treatment of upper gastro-intestinal dysfunction.

INTRODUCTION

1. Anatomy of Innervation of the Gastro-intestinal tract

The gastrointestinal tract (GIT) is innervated by several neuronal systems that ensure precise control over digestive function. The rich afferent innervation of the GIT convey sensory information regarding intra-, and extra-luminal environment as well as initiating gut reflex functions, and includes both extrinsic and intrinsic primary afferents [1]. Most basic motor and secretory functions of the GIT are controlled by the intrinsic nervous system, which is composed of three neuronal structures contained entirely within the gut wall namely, a) submucosal plexus, which is predominantly involved in nutrient signalling through the gut epithelium, b) myenteric plexus, located between longitudinal and circular smooth muscle of the GIT and primarily involved in co-ordination of motor pattern, and c) intestinofugal fibres, that convey sensory signals as far as sympathetic pre-vertebral ganglia, have their cell bodies contained within myenteric ganglia and form synapses in sympathetic ganglia, as well as forming part of the afferent limb of intestine-enteric reflexes [2]. Enteric (intrinsic) primary afferent neurones form connections with interneurones and motor neurones of enteric nerve pathways as well as with other intrinsic afferent neurones [1-3]. Given their synaptic connections, none of these afferent pathways can make a direct contribution to mechanisms of intestinal sensation. These intrinsic neuronal plexuses allow the intestine to have a considerable degree of independent neural control, however the stomach and oesophagus are almost completely dependent on extrinsic nervous inputs arising from the central nervous system (CNS) [4].

CNS control of gut function is mediated via parasympathetic or sympathetic pathways that either originate in, or are controlled by neural circuits in the caudal

brainstem [4]. Extrinsic innervation of the GIT provides the connection between the viscera and the CNS and includes afferent and efferent neurones of two major systems, namely vagal and spinal nerves, the latter comprised of thoracolumbar splanchnic nerves (splanchnic pathway), and via paired pelvic nerves (sacral spinal afferent pathway) [5].

1.1 Vagal Afferents

Vagal afferents carry a considerable volume of information regarding the physiological status of the gut directly to brainstem circuits regulating GI function. Previous studies in a number of animal models have shown the vagus to contain between 18500 and 45000 afferent and efferent fibres, with approximately 90% being afferent. The afferent limb of the vagus nerve is particularly important in the sensory innervation of the upper GIT with afferent endings largely concentrated in the upper GIT, whilst innervation caudal of the splenic flexure becomes sparse [4, 6]. Visceral sensory vagal axons are almost exclusively thin myelinated Aδ or unmyelinated C fibres and vary in diameter from 1-28 μM [6].

Two separate vagus nerves contribute to the innervation of the gastro-intestinal tract. The anterior and posterior vagi exit the thoracic cavity via the diaphragmatic hiatus and contribute to the oesophageal plexus, thereafter sending multiple branches to the rest of the gastrointestinal tract. The two vagus nerves ascend with the internal jugular vein, then the common and internal carotid arteries. The nerve then tracks between the olive and inferior cerebral peduncle before reaching the anterolateral surface of the upper part of the medulla oblongata [7]. The nerve contains its cell bodies within the nodose and jugular ganglia close to its entry into the cranial fossa. Neurones in the nodose ganglion are bipolar and

connect the gut directly with nucleus tractus solitarius (NTS) neurones in the brainstem with no intervening synapse [4].

Stimulation of sensory vagal afferent pathways activates second order neurones particularly in subnuclei of the lower part of the NTS as well as the area sub-postrema [8]. Projections to the area sub-postrema have not been found in rat studies possibly explaining the lack of emetic reflex in the rat [8]. Visceral sensory afferents are organised in an organised topographic manner within the NTS subnuclei. Terminal fields from the intestine are represented in the subnuclei commisuralis and medialis, the stomach terminating in the subnuclei medialis and gelatinosus, and the subnucleus centralis receives afferents almost exclusively from the oesophagus [4, 9-11]. Efferent fibres then ascend to the thalamus and a number of hypothalamic nuclei, with remaining fibres eventually reaching the postcentral gyrus [7]. Studies have also shown extensive information exchange from the abdominal vagus to the hypothalamus, which is responsible for coordinating autonomic reflexes. Studies showed the hypothalamus receives information from the gut via vagal afferents concerning the chemical nature of its contents, the degree of distension and absorbed glucose levels [12-14]. Importantly, neurones ascending from the NTS control output of dorsal motor nucleus of the vagus (DMV) cells, which, in turn, control gastric function and completes the vago-vagal loop (Figure 1.) [4].

The DMV contains the cell bodies for the majority of parasympathetic efferent motor fibres that project to the upper GIT. The DMV is a paired structure in the dorsal caudal medulla adjacent to the central canal, the majority of whose cells project to neurones in the myenteric plexus or onto interstitial cells of cajal (ICC) of

the proximal GIT, with the highest density of efferent fibres terminating in the stomach [4, 15-19].

Using an *in vitro* vagus-gastric myenteric plexus preparation, Schemann and Grundy, (1992) [9], demonstrated nicotinic acetylcholine receptors located on myenteric ganglia are the likely termination point for vagal efferent neurones.

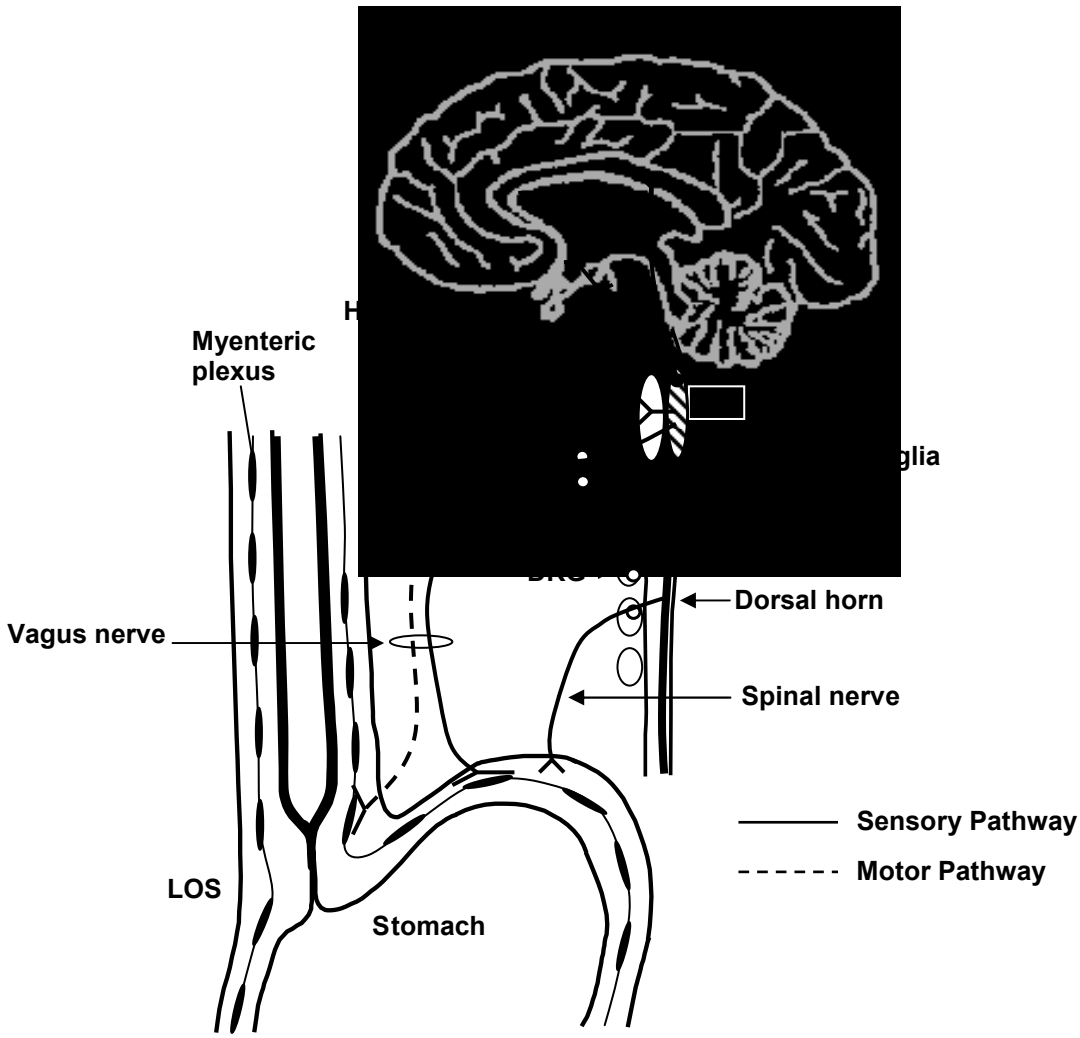


Figure 1. Vagal afferent and efferent pathway.

Representative demonstration of vagal afferent fibres that densely innervate the proximal gastro-intestinal tract and have their cell bodies contained within

nodose ganglia. Ascending afferents terminate in the medulla oblongata of the midbrain. Projections from the medulla oblongatta make connections with the hypothalamus, thalamus and the NTS in the brainstem. Output of the nucleus tractus solitarius (NTS) to synapse with dorsal motor nucleus of the vagus (DMV) cells is critical in this investigation as a program generator within the DMV controls efferent neurones that project to the gut, in particular to the lower oesophageal sphincter (LOS) and synapse with myenteric neurones or interstitial cells of cajal. Vagal efferents may activate nitrergic motor neurones and can cause smooth muscle relaxation. This figure also demonstrates spinal afferents that have their cell bodies contained within dorsal root ganglia. These neurones then synapse in the dorsal horn of the spinal cord. Central projections of spinal afferents synapse in the dorsal horn and ascending second order neurones make connections throughout the CNS.

Activation of vagal efferents can produce both excitatory and inhibitory effects on GI smooth muscle [4], therefore it is believed that both excitatory and inhibitory postganglionic neuroeffectors are released from enteric neurones in response to excitatory vagal input. Acetylcholine is the principal excitatory postganglionic neurotransmitter and acts on muscarinic receptors in gastric smooth muscle, augmenting smooth muscle activity driving peristalsis and tone [10], as well as parietal cells [4]. Inhibitory postganglionic neurones comprise the non-adrenergic non-cholinergic (NANC) link between the nicotinic preganglionic vagal efferent fibres and gastric smooth muscles and ICC's. The two most likely candidates for mediating this connection are nitric oxide (NO) or vasoactive intestinal polypeptide (VIP), other mediators have been implicated however [4].

Of key importance to the current investigation is the afferent limb of the vago-vagal pathway. The vagus has afferent endings located in all layers of the gut wall. There are several types of visceral receptors and afferent fibres. Vagal afferent terminals have been classified according to their morphology and anatomical location into mucosal endings [11, 12], intraganglionic laminar endings (IGLE), and intramuscular arrays (IMA) [13, 14]. These afferents have in common low thresholds of activation and reach maximal responses within physiological levels of mechanical stimulation [15, 16].

1.1 a) Intra Ganglionic Laminar Endings (IGLE)

Neuronal labelling techniques have been used to isolate origins of both vagal and spinal afferents in the distinct layers of the gut wall. A number of studies have utilised chemical coding with calretinin, a calcium binding protein, anterograde tracing with DiI, a red fluorescent carbocyanine dye, and wheat germ agglutinin-conjugated horseradish peroxidases to identify vagal afferents [11, 17], whilst calcitonin gene-related peptide (CGRP) largely labels spinal afferents, although a pitfall is that some vagal afferents also contain this peptide [11, 18].

IGLEs were first identified in the striated oesophageal muscle of the dog [19], and were further described in oesophageal and gastric cardia smooth muscle in cat and rat [20]. Rodrigo, et al (1982)[21], described IGLEs as nerve endings that surround myenteric ganglia and lie between the longitudinal and circular smooth muscle layers of the oesophagus. Anterograde tracing from nodose ganglia and nodose ganglionectomy techniques comprehensively described IGLEs to be of vagal origin [14, 21, 22].

IGLEs have dense concentration in the proximal GIT including in the stomach, where about one half to one third of myenteric ganglia receive at least one IGLE. However, they have been described throughout the gastrointestinal tract including small and large intestine of the rat [13, 14, 23-25]. It was found that whilst the jejunum contained the highest overall number of IGLE, the relative proportion of myenteric ganglia supplied by IGLE decreased throughout the small intestine, and the fewest identified in the caecum and colon [13, 14, 25, 26].

Later studies have found a close correlation in the morphology and regional distribution patterns previously mapped in the rat model in the mouse and guinea pig [26, 27].

They are localised to longitudinal, and to a lesser extent, circular smooth muscle layers, with fibres penetrating the muscle and running parallel to the respective fibres for several millimetres [23]. Individual axons appear to form multiple terminal branches forming varicosities which interdigitate with myenteric ganglia as well as having intimate association with surrounding connective tissue components, NADPH positive enteric neurones and interstitial cells of cajal [11, 26, 28].

Individual parent axons supply multiple IGLE fields to adjacent myenteric ganglia with proposed overlapping of receptive fields providing a network of afferent innervation to myenteric plexuses [26, 27, 29].

It was proposed that IGLE act as mechanoreceptors detecting intra-mural tension/shearing forces between orthogonal smooth muscle layers. Given their topographic distribution and morphology, IGLE were implicated in mediation of a number of reflex actions involving myenteric neurones, including rhythmical motor

programs such as coordination of peristalsis, swallowing peristalsis and emptying [11, 26].

In vitro preparations using guinea pig stomach have since shown, using a combination of electrophysiology and anterograde tracing techniques from vagus nerve branches to oesophagus and stomach, that IGLE correlate closely with mechanosensitive "hot spots". IGLEs are now considered the transduction sites of tension sensitive mechanoreceptors [27, 29, 30]. It is assumed that IGLEs found in other rodent species correspond also to tension receptive sites responsible for signalling active contraction and passive distension. Zagorodnyuk, et al (2003) [40], further demonstrated modulation of sensitivity of these afferents, with the transduction process itself involving mechanosensitive ion channels on the afferent ending and to be relatively independent of chemical transmission.

IGLEs are not unique to vagal afferents, rather similar structures have now been described on afferents of the pelvic nerve in the rectum [31]. Recordings from pelvic afferent bundles innervating rectum revealed low threshold, slowly adapting mechanoreceptors. Functionally similar to their vagal counterparts in the oesophagus and stomach, morphologically they typically appear as flattened leaf-like endings located in myenteric ganglia, however are smaller and simpler in function, having fewer leaflets and less extensive branching patterns than in the upper GIT [31].

1.1 b) Intramuscular Arrays

The other type of vagal afferent ending to the muscularis propria is the intramuscular array (IMA). IMA anterogradely labelled in vagus nerve stomach preparations were observed to be specialised endings consisting of several long and

straight varicose nerve fibres, ranging from hundreds of microns up to several millimetres in length. They too, are located between longitudinal and circular smooth muscle layers, branching and running parallel to smooth muscle fibres. Individual fibres bear close proximity, are connected by short oblique or perpendicular bridging collaterals and are commonly associated with interstitial cells of cajal [23, 26, 29, 32].

In contrast to IGLEs, IMAs appear to have a distinct distribution, and are densely concentrated innervating sphincter regions including the lower oesophageal and pyloric sphincters, as well as a dense concentration in the proximal stomach becoming more sparse toward the corpus and antrum [11, 13, 14, 23, 25, 26]. It was postulated that IMA act as 'in series' length or stretch receptors, responding to passive stretch and active contraction, due to their location where intraluminal matter collects (forestomach), or passes (sphincters) [26], however *in vitro* studies of the guinea pig stomach failed to correlate anterogradely labelled IMA and mechanosensory transduction sites, and thus direct morphological evidence for IMAs as length sensitive afferents is lacking [27, 29, 32].

1.1 c) Mucosal Afferents

The third morphologically distinct vagal afferent has its endings in the mucosal layer of the gut. These fibres penetrate the muscle layer and the submucosa, forming networks of branching axons in the lamina propria of crypts and villi [32, 33]. Vagal afferent mucosal innervation has biphasic distribution, being densely distributed to uppermost cervical oesophagus immediately distal to pharyngeo-oesophageal junction, and a second dense concentration of afferents in abdominal regions, with innervation sparse in the lower cervical and thoracic

oesophagus [11, 12, 22, 28]. This pattern is reflected in animal and human studies where variable sensory properties of afferents occur in different regions of the gut, with the upper oesophagus more sensitive to mucosal mechanical and chemical stimuli [11, 12].

Anterograde labelling techniques from the nodose ganglia have identified up to four types of fibre supplying the mucosa. Wank, et al (2001) [12], described small varicose intra-epithelial processes invading the superficial epithelial layers. Dutsch, et al (1998) [21], commented on similar structures densely innervating the upper oesophagus. These thick calibre fibres, running parallel to the oesophagus were highly branched laminar structures, with their terminal ramifications directed toward the basal epithelial layer and appearing to penetrate it, and are thus ideally positioned to sense luminal contact and chemical mediators released from local structures [32, 33]. Thinner axon fibres were also observed in the upper cervical oesophagus having dense networks in the submucosal layer, with finger like projections penetrating toward the basal epithelium. Other types of mucosal fibres supply submucosal and perivascular tissue and appear to have functional importance [12].

No attempt has been made to identify histologically vagal and spinal afferent endings in the mucosa of the large intestine, however functional evidence shows vagal, pelvic, and splanchnic afferents between them, to supply all regions of the GIT mucosa [32].

1.2 Spinal Afferents

Like vagal sensory afferents, spinal afferents innervating the GI tract are almost exclusively thin myelinated Aδ or unmyelinated C fibres. The spinal

pathway can be further divided into splanchnic and pelvic nerves despite the fact both pathways contains cell bodies in dorsal root ganglia (DRG) of the spinal cord, forming part of the sympathetic and parasympathetic nervous systems respectively. Spinal innervation of the abdominal viscera originates in the anterolateral horn of the spinal cord. Preganglionic axons from levels T5 through T12 exit the spinal cord with ventral spinal routes to join the white communicating rami *en route* to the sympathetic chain. In contradistinction from other sympathetic nerves, these axons do not synapse in the sympathetic chain, instead they pass through the chain and synapse at distal sites such as prevertebral (celiac, inferior mesenteric, and superior mesenteric ganglion) ganglia. Post-ganglionic nerves then accompany blood vessels to their respective visceral structures [34]. Splanchnic nerves have their cell bodies contained in the thoracolumbar DRG. Pelvic afferents, however, are limited to the distal colon and rectum and have cell bodies in lumbosacral DRG [32].

Central projections of spinal afferents synapse in the dorsal horn of the cord, and have ascending second order neurones that make connections throughout the CNS involved in sensory and reflex pathways, importantly making connections with thalamic nuclei, from which sensory input reaches conscious level [35] (Figure 1.).

As mentioned above, calcitonin gene-related peptide (CGRP) is a relatively specific marker for spinal afferents, being present in up to 85% of spinal gastric afferents in the rat [46, 47], and a similar percentage of oesophageal afferents in the rat [47] and mouse [21], however a drawback of CGRP is that it may also co-localise with calretinin, a marker for vagal afferents, in certain types of thin calibre neurones [21]. Immunocytochemical studies using CGRP have identified the anatomy of spinal afferents to the viscera in several species.

Spinal afferent innervation of the gastro-oesophageal region arises from a segment of the cord spanning upper cervical (C1) to upper lumbar (L2) (Table 1.). These afferents are conveyed in the greater splanchnic and the thoracic spinal nerves [36, 37], 51-53], and are generally considered to give rise to non-specialised free endings in the mucosa, muscle and serosa, arising from thin varicose fibres in apposition with myenteric ganglia, possibly connected to muscle tension receptors as well as distributed between or passing to other structures and also releasing neurotransmitter [49, 50]. These fibres are fairly evenly distributed along the gastrointestinal tract in contrast to vagal innervation as described previously [21, 37, 48].

Retrograde labelling studies have enabled mapping of distribution of these afferents in the oesophagus revealing bimodal distribution of afferents to the oesophagus with dense innervation at cervical (C2-C6) and thoracic (T2-T4) parts of the oesophagus [37, 38]. Fibres innervating the LOS are distributed in the cord from T1-L2, having a peak distribution in the T8-T12 region [36].

The sensory innervation of the gastro-duodenal segment extends from C2-L5 with a peak innervation field of the stomach spanning the cranial, middle, and the immediately adjoining caudal thoracic ganglia (T2-T10) in the dog, and T4-L2 in cat and rat [39, 40]. The duodenum has peak innervation originating in the middle and caudal thoracic ganglia and cranial lumbar (T6-L1) ganglia. There is a recognizable viscerotopic organization in the sensory innervation of the post-pharyngeal foregut; successively more caudal sectors of this region of the alimentary canal are supplied with sensory fibres from successively more caudal spinal dorsal root ganglia [37].

	Innervation	Peak Innervation	Reference
Oesophagus	C1-T4	C2-6 and T2-4	Clerc, et al (1994)
Lower Oesophageal Sphincter	T1-L2	T8-12	Clerc, et al (1983)
Gastro-Duodenal segment	C2-L5	T2-10 (Dog) T4-L2 (Cat and Rat)	Brtva, et al (1989) Ozaki, et al (2001)
Duodenum	T6-L1	T6-L1	Khurana, et al (1991)

Table 1. Spinal origins of afferent innervation of the proximal gastrointestinal tract

Spinal pathways innervate all viscera and are associated with sensations of pain, discomfort, bloating and urge to void, and respond over a wide dynamic range of distension pressures [41]. Neurones of the paired pelvic nerves are primarily concerned with regulation and control of local environment, and also conscious sensations such as sensation of stool, urge to defecate, as well as pain and nausea [57]. Retrograde labelling in mice revealed bimodal distribution of small to medium sized afferents in the spinal cord, one wide covering spinal levels T8-L1 representing predominantly the lumbar splanchnic nerve, and one narrow restricted to L6 and S1 likely to be due to innervation of the pelvic nerve. A distribution that is consistent across species of rodent [57].

Afferents from the guinea pig rectum, most likely to be pelvic nerve origin, have been shown to have specialized IGLE terminals in contact with myenteric

ganglia in the guinea pig rectum. These endings bear similar function to their vagal counterparts in the upper gut however are morphologically slightly different as mentioned previously. Rectal IGLEs are concentrated in rectal nerves and are absent in colonic nerves indicating their function in rectal reflexes such as defecation [31].

2. Functional properties of visceral afferent endings

Much of our knowledge about the electrophysiology and function of GI sensory afferents has arisen from *in vitro and in vivo* whole animal experiments using 'single fibre' recording techniques first established by Paintal, (1953)[42] and Iggo, (1955)[43]. Most visceral afferents are unmyelinated C fibres with few being Aδ fibres [5]. Visceral afferents were traditionally thought to be best described based on the layer of gut containing their afferent terminals, and their general response properties including response to mechanical stimulation, however Brierley, et al (2005)[60], demonstrated lumbar splanchnic, and pelvic nerve pathways differ in their chemosensitivity to known noxious stimuli. Also pertinent to this project however, is the general response profiles of visceral afferents to mechanical stimuli.

Visceral afferent responses to mechanical stimuli, acting within the GI wall, is consistent with the location in mucosal layer, muscle, and in serosal mesenteric attachments. With this in mind, functional studies using *in vitro* and *in vivo* electrophysiological techniques have enabled vagal and spinal afferents to be classified into different classes according to their response to mechanical stimulation [35].

Vagal and spinal extrinsic afferent nerves exhibit contrasting stimulus response properties, highlighting their differing roles in sensory signalling. Clinical

evidence demonstrates spinal nerves to be involved in the signalling of visceral pain, as any pain originating from visceral structures can be alleviated by splanchnic nerve blockade [34]. *In vitro* and *in vivo* animal studies have demonstrated spinal afferents in the upper gut to as far as the transverse colon, give rise to sensations of pain, discomfort, bloating and fullness. Afferents to the distal colon and rectum, give rise to urge to defecate, discomfort as well as pain evoked by more intense stimuli [42, 56].

In contrast vagal mechanoreceptors are thought to be involved in regulatory physiological processes such as reflexes involving intestinal secretion and motility including involvement in the emetic reflex [6], as well as contributing to nausea and malaise [44].

Afferent fibre labelling studies have enabled the localisation of the precise terminations of afferent endings and have enabled greater understanding of functional correlates of their endings. Tracing techniques have demonstrated the localisation of vagal and spinal afferents to numerous layers of the gastrointestinal tract. Functional roles correlate with layer of gut and exposure to mechanical and chemical stimuli. The focused distribution of vagal and pelvic afferents may correspond to regions of the gut where graded, innocuous sensations can be evoked by distension whereas regions that predominantly receive splanchnic innervation appear to generate less graded sensation, rather discomfort and frank pain are the first responses to increasing levels of distension [32].

2.1 Vagal Afferents

2.1 a) Mucosal Receptors

The general consensus is that mucosal receptors are implicated in the regulation of gastric motility and secretion. Mucosal afferents have endings in the mucosal lamina propria, where they are ideally positioned to detect material absorbed across the mucosal epithelium or released from epithelial and sub-epithelial cells [32].

The existence of these terminal endings has been verified by *in vivo* and *in vitro* electrophysiological studies in a number of animal models demonstrating them to be exquisitely sensitive to mechanical deformation of the mucosa by mucosal stroking with calibrated von Frey hairs (10-1000mg) or probing, as might occur with particulate material within the lumen. In general mucosal receptors do not show resting activity, and show rapidly adapting responses in a force dependent manner to mucosal stroking with calibrated von Frey hairs, whilst being insensitive to muscular stimuli such as contraction or distension [32, 45-51]. Mucosal receptors are polymodal with numerous studies demonstrating, in addition to light stroking, they are also responsive to luminally applied drugs and chemical stimuli including inorganic and fatty acids, bile, hypo- and hyperosmolality, as well as 5-hydroxytryptamine (5-HT), cholecystokinin (CCK), α,β-meATP, prostaglandins (PG) and bradykinin (BK) [44, 46-53].

These afferents have been extensively characterised in the vagus nerve where in addition to those functions mentioned above, are also thought to play a role in generation of sensations of satiety, nausea and vomiting [45, 47-49, 51, 53, 54].

2.1 b) Tension Receptors

Nerve terminals in the muscle and serosa convey mechanosensitive information corresponding to distension or contraction of the gut wall [35], however electrophysiological studies have demonstrated information originating from vagal and spinal mechanoreceptive afferents differs.

Vagal mechanoreceptors located in the muscle layer often have resting discharge and are specifically sensitive to muscular contraction and distension, but are unresponsive to mucosal stroking with calibrated von Frey hairs (10-50mg), have low thresholds of activation, and reach maximal responses within physiological levels of distension [6, 35, 43, 46, 47, 51, 55]. These basic characteristics of vagal distension sensitive afferents are conserved in dog [56], opossum [57], mouse [46] and ferret [45]. In contrast, sub-populations of spinal afferents have thresholds of activation within the physiological range, however are generally are considered to be critical in detecting stimuli in the supra-physiological range [58]. Vagal muscular afferents, like pelvic muscular afferents, show slowly adapting maintained responses to distension, in contrast to splanchnic muscular afferents which are more rapidly adapting [32].

Of particular importance in the current investigation are tension sensitive afferents, which have been comprehensively described in the muscle layer of the gastro-oesophageal region.

Vagal afferent terminals form specialized endings called IGLEs found throughout the length of the GI tract. Studies in the guinea pig have demonstrated IGLEs to be the transduction sites of vagal tension sensitive mechanoreceptors in the oesophagus and stomach.

Tension sensitive afferents are thought to serve as pure mechanoreceptors involved in physiological regulation such as mediating the non painful sensations of fullness, bloating and nausea, as well as gut reflex function such as the triggering of transient lower oesophageal sphincter relaxations (TLOSRs) which are of importance to the current investigation [56-59].

2.1 c) Tension Mucosal (TM) Receptors

The ferret gastro-oesophageal *in vitro* preparation has also identified a further vagal afferent receptor subtype with endings in the oesophagus, that responds not only to mucosal stimulation with calibrated von Frey hairs 10-1000mg, but also to circumferential tension, thus has both mucosal and tension sensitive afferent properties. There are two possible anatomical explanations for the site of the TM receptive field. There are either two receptive fields, topographically superimposed with one being in the mucosal and the other in the muscular layer, or like a vagal afferent, Davison et al, (1972)[53], had previous described in the duodenum, there is one receptive field interposed in the muscularis mucosae. These afferents are thought to be important in the detection of rapidly moving boli of food and/or liquid along the oesophagus [60].

2.2 Spinal Afferents

Gastro-Oesophageal:

Afferent innervation of the gastro-oesophageal region conveying sensory information to the central nervous system occurs via the splanchnic nerve, or thoracic sympathetic chain [58]. Characterisation of mechanosensitive splanchnic afferents have been studied in the rat stomach [40], and the opossum oesophagus

[58]. There exist separate populations of low- and high-threshold afferent fibres that innervate many viscera. The low threshold afferent fibres are assumed to mediate sympatho-sympathetic or sympatho-vagal regulatory reflexes which, under normal physiological conditions, are generally not sensed. Low threshold fibres also likely play a role in non-painful sensations such as bloating, fullness, nausea. High threshold afferent fibres are believed to mediate nociception [77]. Mechanosensitive afferents have been classified into low threshold (wide dynamic range) mechanoreceptors or high threshold (phasic) nociceptors depending on their ability to encode either non-noxious mechanical stimuli, noxious mechanical stimuli or both [58].

Low threshold (phasic) mechano-nociceptors were observed in both preparations and respond to a wide range of stimuli from innocuous to noxious intensity. They have a low threshold of stimulation, responding to peristaltic contractions and a linear stimulus response to graded distension of the viscera within a narrow physiological pressure range [58]. Discharge is saturated in opossum oesophagus at innocuous levels and therefore does not distinguish noxious from innocuous stimuli [58], in contrast to the low threshold mechanoreceptors population in the rat stomach that encoded distending stimuli throughout range of distending pressures and are thus considered to contribute to sensations such as discomfort and pain [40]. This subtype of mechanoreceptive afferent differs from gastro-oesophageal vagal mechanoreceptive afferents in magnitude of response and saturation of receptor activity at physiological pressures [40]. Given they are activated at low thresholds of mechanical stimulus they are implicated in non painful mechanical signalling

giving rise to sensations such as bloating, fullness and nausea as well as physiological regulatory functions such as storage, propulsion and emptying [40].

High Threshold (phasic) mechanoreceptors are insensitive to peristaltic contraction and are activated only by mechanical stimuli considered within noxious range [40, 58]. The majority of these afferents have little spontaneous resting activity; however resting activity could be altered by chemical or mechanical stimuli. These afferents also show ability to be sensitised by insult or even non injurious stimuli, and are thus considered to contribute to altered sensation arising from the gastro-oesophageal region such as central hyperexcitability and visceral hyperalgesia [40]. Given the response profiles of these afferents, their presence is taken as evidence for the presence of nociceptors that give rise to sensations of discomfort and pain [40]. Responses of either subtype of afferent to gastric distension was conserved across species, and generally exhibited a monotonic increase in firing with increasing distension pressure of the viscus, typically exhibiting slowly adapting responses during maintained distension [40, 58].

Distal Gastrointestinal tract:

Mechanosensitive afferents have been well described in the distal gastrointestinal tract where they have been identified using *in vivo* and *in vitro* electrophysiological techniques in lumbar splanchnic and pelvic nerves of the cat [61, 62], rat [63, 64] and mouse [65], including afferents that respond to mechanical stimulation of the colonic mucosa, muscle layer, serosa and/or mesentery. Major differences between anatomical location of receptive fields and response profiles of lumbar splanchnic and pelvic nerves to mechanical stimulus have been identified

[65]. Brierley, et al (2004)[65], identified five different classes of afferent fibre based on mechanical response profiles in the mouse colon. Three types were conserved across pelvic and splanchnic pathways (muscular, serosal and mucosal), and each nerve supplied a unique type of afferent. A general discussion of afferent types is presented here.

2.2 a) Mucosal Receptors

Mucosal receptive afferents have been previously described in the distal colon and perianal mucosa [52, 63, 65]. Duthie and Gairns, (1960)[66], provided the only morphological evidence of free nerve endings in the anal mucosa epithelium, exhibiting multiple branching and presence of varicose structures. An *in vitro* electrophysiological study of the rat identified pelvic nerve afferents in the distal colon mucosa which, like vagal afferents innervating the upper gastro-oesophageal tract, were not spontaneously active but all responded with rapidly adapting burst of discharge in an incremental manner to full range of von Frey hair stroking, and the majority were unresponsive to circular stretch [52]. These results are also consistent with those of mucosal afferents in the cat [67].

In vitro electrophysiological studies have also identified mucosal afferents in LSN innervating the colon. These colonic mucosal afferent fibres have a comparable response to von Frey hair stroking to that of the upper gastrointestinal mucosal fibres recorded *in vitro*, and similarly show no response to circumferential tension [45]. These colonic fibres exhibited limited or no resting discharge, consistent with previous findings in the ferret [45], mouse [65] as well as vagal mucosal afferents in a number of species [45, 47, 68]. Mucosal colonic afferents were also found to be polymodal with most responding to one of hypertonic saline,

HCL, bile and capsaicin, which is in contrast to what was found in ferret oesophageal vagal afferents where only a small proportion were responsive to chemical stimulus [52].

2.2 b) Muscular Receptors

The response profiles of LSN and PN afferents to colonic distension has been characterised extensively using a variety of *in vitro* and *in vivo* electrophysiological techniques [52, 61, 62, 69]. Spinal afferents with terminals in the muscle layers are likely to encode both physiological and noxious levels of stimulation.

Whilst both pathways have similar response properties to mechanical stimuli, and have been shown to have similar response profiles to noxious colorectal distension, discrepancies do exist [61, 69-72].

Lumbar Splanchnic Nerve:

Muscular afferents of the LSN do not respond to low intensity mucosal stroking but do respond to probing of the receptive field and most consistently to distension of the colon [63]. Blumberg, (1983)[61], categorised distension sensitive afferents in the cat LSN to exhibit different characteristic responses to phasic distension, classifying fibres into 4 types based on their adaptation to phasic colonic distension, ranging between tonic and phasic discharge in response to colonic distension. These afferents display responses to normal physiological stimuli (non-noxious) however they also are thought to encode stimuli into the noxious range, as well as being chemosensitive [62].

Pelvic Nerve:

Two populations of slowly adapting afferent fibre have been described arising from the pelvic nerve based on their responses to graded colorectal distension (CRD) in the rat [63]. Low threshold responding to ≤10mmHg, and high threshold responding to ≥28mmHg, indicating functionally different afferent fibres in the pelvic nerve [63]. Pelvic fibres innervating the colon of the rat responsive to noxious CRD were mainly C fibres, were active at rest and exhibited either a dynamic response followed by slow adaptation or only a tonic response to phasic CRD.

Pelvic high threshold afferents are thought to be important for visceral nociception, whilst low threshold afferents may mediate sensation such as fullness, as well as mediating sympatho-sympathetic or sympatho-vagal regulatory reflexes, but do not appear to elicit painful sensation.

Pelvic nerve afferents in the cat were subdivided into phasic and tonic afferents. Phasic being rapidly adapting and tonic being slowly adapting throughout distension of the colon. These fibres responded to low thresholds of intraluminal pressure [70].

Brierley, et al (2004)[65], identified a further receptor subtype unique to the pelvic nerve that possessed properties of both muscular and mucosal receptors, responding to both mucosal stroking with von Frey hairs (10-1000mg) and also to circumferential stretch and was termed muscular/mucosal receptors.

In contrast to the lower colon, the guinea pig rectum is richly innervated by distension sensitive afferents arising from the pelvic nerve which display low thresholds and are slowly adapting. The receptive fields of these stretch sensitive mechanoreceptive afferents have been morphologically identified as IGLEs. These

IGLEs termed rectal IGLEs (rIGLE) bear many similarities with IGLEs in the upper GIT however are less complex and have less branching [52].

2.2 c) Serosal and Mesenteric

Afferents to the serosa and mesenteric attachments of the colon are the most common afferent identified from the lumbar splanchnic nerve in animal *in vitro* preparations accounting for over 50% of recorded afferents [65]. Afferents have their endings located on or close to blood vessels or branching points of capillaries supplying the serosa and mesentery [65], and have the ability to respond beyond physiological levels and encode both physiological and noxious levels of intestinal distension [73].

Serosal afferents often have multiple punctuate receptive fields (2-4mm^2) that extend from the point of division of the left colic artery to the branch points beneath the serosa of the colon [68, 82, 90]. Previous studies have found afferents to respond to micromanipulator rod stimulation over receptive fields as well as responding to tension, exhibiting a phasic and tonic component of discharge [74]. Responses to circumferential tension are either absent or only seen at onset of stretch, bearing no relationship with tension or length of tissue. In addition they do not respond to mucosal stroking with calibrated von Frey hairs less than 50mmHg [50, 51, 63].

These afferents have sporadic resting discharge, and are characterised by their ability to respond to firm blunt probing on the mucosal surface, responding at lower mechanical threshold to stimulation of the reflected serosal surface than of the mucosal surface [68, 78, 79, 82, 91]. They demonstrate a burst of firing rather than a prolonged response [52], and have a high threshold for response to mechanical

stimulation [52, 75]. Mesenteric afferents are specifically sensitive to distortion of mesenteric attachments, therefore a force strong enough to distort the mesentery or serosa is required for activation of both types. Contraction and distension of the bowel wall are sufficient stimuli to activate these afferents post a sensitising event such as colonic inflammation [76]. It has been proposed these afferents may detect twisting and torsion of the colon and pulsatile changes in blood pressure in mesenteric blood vessels, possibly critical during plasma extravasation resulting from colonic inflammation [65].

In addition to their mechanoreceptive properties, these afferents are generally chemosensitive. Responsiveness to bradykinin, capsaicin, hypertonic saline, normal saline and hydrochloric acid (HCl) applied extra-luminally have been previously observed [62, 77], indicating possible roles for transmission of signals related to noxious and inflammatory events and sensing composition of luminal content [52].

3. Pharmacology of Visceral Afferents

In addition to mechanical stimuli, vagal and spinal afferents are also responsive to chemical stimuli, a characteristic vital for their role in participating in central and local reflex control of gastrointestinal motor and secretory function. The location of visceral afferent terminals supplying the gut near myenteric ganglia, blood vessels and luminal material, combined with the fact that these afferents express a variety of membrane receptors to a multitude of chemical mediators generated from both within and outside the gut wall [73], mean they are well suited to sense and be modulated by chemical stimuli [61].

Sensory signal transduction is highly dependent on the excitability of the sensory neurone, which in turn, is governed by the relative contribution of a variety of voltage-dependent ion channels controlling ion movement, thus charge, across the cell membrane. These and other channels present in nerve terminals transmit and amplify this code into generation of action potential, which conveys the message onwards toward the central nervous system [73].

Electrophysiological, immunocytochemical and molecular biological techniques have identified a variety of chemical mediators acting on functionally active receptors expressed on sensory nerve terminals. Several mediators have been shown to be active at nerve terminals of extrinsic afferents supplying the gut wall, altering the excitability of the sensory neurone; a general discussion is presented below.

These substances are thought to produce their effects via three distinct mechanisms. 1) Direct activation, which generally involves the opening of ion channels present on nerve terminals, 2) Sensitization, which may only occur in the absence of direct stimulation but which usually results in hyperexcitability to both chemical and mechanical modalities, and 3) Alteration of the phenotype of the afferent nerve, for example through alterations in the expressions of mediators, channels and receptors or modulating activity of these by changing the ligand-binding characteristics or coupling efficiency of other receptors [78].

3.1 Excitatory Receptors

3.1 a) Adenosine triphosphate (ATP)

Within the gut, there is an abundance of evidence to suggest that adenosine triphosphate (ATP) acts as a neurotransmitter, released from either extrinsic

sympathetic nerves (as a co-transmitter with nor-adrenaline and neuropeptide Y) [79, 80], intrinsic neurones [81], or from tissue injury, where by cell damage causes the release of cytoplasmic contents rich in ATP. ATP may further induce the synthesis of prostaglandins which are, in turn, mediators of inflammation [80, 82, 83].

ATP exerts its extracellular actions as a neurotransmitter via cell surface receptors, P2 purinoceptors. Purinoceptors are further divided into two major classes based on structural and pharmacological profiles. P2X are ligand-gated ion channels causing a voltage dependent calcium influx upon activation, thus mediating fast synaptic transmission. The second subclass, P2Y receptors are metabotropic G-Protein coupled receptors that mediate signalling via inositol trisphosphate, leading to intracellular calcium release. P2Y receptors are therefore considered to play a modulatory role rather than directly mediating purinergic transmission [82, 84].

P2X and P2Y receptors are both present in dorsal root and nodose ganglia [80, 84, 85]. Both P2X and P2Y receptors have been shown to be located on afferent sensory neurones including vagal [86], and splanchnic afferent fibres [80, 87]. A subclass of P2X receptors, the P2X3 receptor, is selectively expressed in sensory neurones and is implicated in the mediation of nociception [85]. Immunohistochemistry demonstrated binding of ATP throughout the human and rat nodose ganglion and brainstem, including the ventrolateral medulla and NTS, with ATP binding specifically P2Y receptors. Bilateral ligature of the vagus nerves and accumulation of both P2Y immunoreactivity and ATP binding sites present adjacent to ligatures of the nerve suggest bidirectional transport of the $P2Y_1$ receptor along the vagus nerve [84, 88].

Page, et al (2000, 2002) [46, 83], showed two distinct responses of gastro-oesophageal vagal afferents to the purinergic agonist α, β-meATP. A proportion of mouse afferents were directly excited by the purinergic agonist, in contrast to the ferret, where no gastro-oesophageal mucosal receptors were excited by direct application. However, mechanosensitivity was reduced in inflamed tissue in the ferret and was enhanced in the presence of a purinergic agonist back to uninflamed levels [83]. This phenomenon is also demonstrated by Dang, et al (2005)[87], who showed that inflammation increases excitability in both rat DRG and nodose ganglion neurones that innervate the stomach, increasing the fraction of neurones that are responsive, as well as their peak response to α,β-meATP.

This combination of morphological and functional electrophysiological evidence demonstrating the expression of purinergic receptors on peripheral sensory neurones implicate a role for purinergic receptors in not only mediation of nociceptive input into the spinal cord, but also in mechanosensation of gastro-oesophageal afferents, particularly post inflammation.

3.1 b) Bradykinin

Bradykinin (BK), a metabolite of the Kallikrein-Kinin system, is released from ischaemic and inflamed tissues and has been shown to be an important algesic stimulus on sensory nerves and mediates pain responses to tissue injury [89]. It is a well recognized nociceptive stimulus on skeletal muscle receptors and cutaneous nociceptors. Importantly, bradykinin stimulates visceral nociceptors including cardiac as well as splanchnic afferents innervating much of the gastrointestinal tract [59, 62, 77, 90-95]. Bradykinin exerts its effect via two different subtypes of G-protein coupled receptors termed B_1 and B_2. The B_2 receptor is extensively

expressed in a constitutive fashion whereas the B_1 receptor is expressed at low levels in healthy tissue, but becomes rapidly up-regulated following tissue injury by various pro-inflammatory kinin metabolites [89, 96]. Sengupta, et al (1992)[90], describes an additional three receptors termed $B_{3\text{-}5}$, B_3 and B_4 being excitatory receptors, involved in smooth muscle contraction and B_5, an inhibitory receptor involved in relaxation of the lower oesophageal sphincter.

Both vagal and spinal afferents innervating the viscera are responsive to bradykinin. Bradykinin directly increases firing of serosal afferents in the rat jejunum, with results consistent with an action directly on the B_2 receptor located on serosal afferent terminals [113]. Colonic distension sensitive splanchnic afferents of the cat were also sensitive to BK [62]. This result was reinforced in the opossum where all distension sensitive wide dynamic range and high threshold afferents were responsive to bradykinin in a dose dependent and pharmacologically reversible manner, an action believed to be directly mediated by the B_2 receptor [90]. In the abdominal viscera of the mouse, BK potently stimulates splanchnic afferents innervating much of the gastrointestinal tract [90], but few pelvic afferents [60]. In addition, mechanically insensitive afferents recruited by BK are exclusive to LSN [60].

Bradykinin also activates vagal afferent fibres supplying the guinea pig airway where all afferents originating in the jugular ganglion responded to BK, however in the nodose ganglion, whilst 100% of C fibres responded to BK, there were no Aδ responsive fibres [97]. This stimulation of vagal afferent fibres innervating the guinea pig airway was not dependent on smooth muscle tone nor on BK induced production of prostaglandins, and was mediated via B_2 type receptor. More importantly to the current investigation is that bradykinin has been shown to

increase signalling of vagal afferents innervating the gastrointestinal tract. Bradykinin was shown to cause excitation of vagal distension sensitive afferents innervating the duodenum of the sheep, and the oesophagus of the opossum [90, 98]. However in each case, there was close coupling of vagal afferent activation and longitudinal smooth muscle contraction, suggesting distortion of the tissue caused direct activation of mechanosensitive receptors. Sengupta et al, (1992)[90], demonstrated this effect in opossum to be reproducible by activation of a post junctional B_4 receptor, suggesting a role for B_4 receptor in this effect of contraction of longitudinal smooth muscle.

These results led to the proposition of two possible explanations for the actions of BK on sensory afferents innervating the GIT. Either BK acts directly on chemosensitive nerve endings or can stimulate pure mechanoreceptors secondary to muscle contraction. However, Page and Blackshaw (1998) [45], have demonstrated, using an *in vitro* ferret preparation, a tension sensitive afferent to be directly responsive to bradykinin, unrelated to smooth muscle contraction, as was found for vagal afferents innervating the guinea pig oesophagus [99].

3.1 c) Cholecystokinin (CCK)

Cholecystokinin (CCK) is a peptide hormone originally isolated from mammalian GIT, where the predominant form, CCK-octapeptide (CCK-8), exists in sulfated-, and non-sulfated states [100, 101]. CCK is a hormonal regulator of digestive processes produced by two separate cell types, enteroendocrine I-cells in the upper GIT and various neurones in the GIT and CNS [102, 103]. Receptors for CCK are classified into two distinct subtypes, CCK_1, and CCK_2. CCK_1 receptors occur in a few localised areas of central and peripheral nervous system, but

predominate in the peripheral tissue including the gastrointestinal tract where they influence feeding behaviour. The CCK_2 subtype predominates in the central nervous system where they are distributed throughout the CNS and spinal cord, and modulate anxiety, analgesia, arousal and neuroleptic activity [100, 101].

CCK is an important satiety factor, having a marked effect on reflex and behavioural responses to food, and this effect is dependent on an intact vagal afferent pathway [104].

CCK binding sites are present both within the vagus nerve proper, and also within the cell bodies in the nodose ganglia [103, 105, 106]. This result confirmed by the presence of CCK receptor mRNA isolated within nodose ganglia [103, 107-109], and by autoradiography demonstrating CCK binding sites at ligation points in rat vagal afferents, suggesting peripheral transport [110]. CCK has also been shown to activate gastric mechanoreceptors in both muscle and mucosal layers. Distension sensitive vagal afferents in both stomach and duodenum have been shown to be activated upon application of CCK, demonstrating integration of signals elicited by CCK and gastric loads at the level of the vagal afferent fibre [47, 104, 111, 112]. However, whilst Davison, et al (1988)[104] found this effect to be independent of intraluminal pressure, and thus gastric wall tension, Blackshaw & Grundy, (1990)[47], found mechanoreceptor discharge to closely follow intraluminal wall pressure, indicating sensitivity of mechanoreceptors could be secondary to a direct action on the muscle itself, or a reflex pathway involving muscle tone, rather than directly attributed to CCK [47, 105].

In contrast, Blackshaw and Grundy, (1990)[47], found CCK to similarly cause an increase in firing of mechanosensitive vagal afferents innervating the mucosa of the ferret gastroduodenal region, and found this to be an effect mediated

directly by CCK. Also, Richards, et al (1996) [105], recording from mesenteric nerve bundles found that 70% were responsive to CCK, demonstrating an increase rate of firing. This effect was abolished by vagal degeneration indicating the effect exclusively mediated by vagal, and not splanchnic and enteric nerves. These responsive nerves were believed also to have endings in the mucosal layer as these afferents were unresponsive to distension. Whilst there is no evidence for effects of CCK on spinal gastro-oesophageal afferents, evidence does exist for CCK receptors in dorsal root ganglia [113, 114].

3.1 d) Ionotropic Glutamate Receptors (iGluRs)

Glutamate is the major excitatory neurotransmitter in the central nervous system. In addition to mediating synaptic transmission, it is also involved as a modulator at both pre- and post-synaptic sites. Glutamate exerts its effects via metabotropic (mGluR) or ionotropic receptors (iGluR). Ionotropic glutamate receptors are ligand gated ion channels. There are three major types of iGluR, named after the selective agonists that were originally identified to activate them, and are thus called N-Methyl-D-Aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazoleproprionate (AMPA) and Kainate receptors. Each of the major functional subtypes of iGluR are made up of a family of genes forming an agonist binding domain, interrupted by insertion of three transmembrane pore loop ion channels [115]. Native receptors of all of these families are likely tetrameric assemblies comprising more than one type of subunit [116]. Binding of agonist to an iGluR triggers opening of an ion pore, allowing the flow of selected cations across the membrane. In the continuing presence of agonist, the receptor typically desensitises, closing the channel [117].

3.1 d i) NMDA receptors:

NMDA receptors are likely of tetrameric structure and are likely composed of pairs, or dimers, of dimers [116]. The NMDA receptor family is composed of seven subunits, NR1, NR2A-D and NR3A and B, which are all products of separate genes. Eight functional splice variants and one non-functional truncated splice variant of NR1 have been described [118].

All NMDA receptors contain an obligate NR1 subunit that serves two roles. NR1 contain a glycine binding site, with NMDA receptor activation requiring glycine occupation of this site. In addition, it is required for trafficking of heteromeric receptor assemblies to the plasma membrane [119]. Functional NMDA receptors appear to be comprised of at least one NR2 subunit, or NR1 and both NR2 and NR3 subunits [116].

Of the different iGluR subtypes, NMDA receptor channels are highly permeable to Ca^{++} ions giving them additional physiological importance as triggers in secondary messenger cascades [115]. An additional unique feature of NMDA receptors that demonstrates their important in synaptic physiology is that Mg^{++} is an extremely fast NMDA receptor channel blocker [115]. NMDA is the most commonly used agonist of NMDA receptors, however drawbacks such as poor selectivity between receptor subtypes, and being less potent than glutamate, mean NMDA is much more effective in intact preparations [116].

3.1 d ii) AMPA receptors:

AMPA receptors are composed of a four-subunit family (GluR1-4) that are products of separate genes and are believed to assemble as functional tetramers. Like NMDA receptors, AMPA receptors are likely heteromeric in composition. The

GluR2 subunit plays a critical role in determination of the permeability of heteromeric receptors to Ca^{++}. Thus, AMPA receptors that do not contain GluR2 are Ca^{++}-impermeable and show marked inward rectification, which is the result of a voltage dependent block of the ion channel by intracellular polyamines. Glutamate and AMPA are full agonists and induce rapidly desensitising responses. Kainate acts as a partial agonist and induces AMPA receptor responses that show little desensitisation [116].

3.1 d iii) Kainate Receptors:

The composition of native kainate receptor remains unclear. The study of kainate receptor physiology is restricted by the lack of selective ligands. In addition to glutamate, both recombinant and native kainate receptors are activated by agonists including kainate and domoate. However, both compounds also elicit non-desensitising responses at AMPA receptors and exhibit only moderate sensitivity for kainate receptors [116]. Kainate receptors are composed of two related subunit families GluR 5-7 and KA-1 and 2. Native receptors are likely tetrameric combinations, possibly of both homomeric and heteromeric combinations. KA-1 and KA-2 subunits don't form functional homomeric receptors but do form high-affinity kainate binding sites and combine in heteromeric assemblies with members of the GluR5-7 subfamily to form functional receptors resembling native kainate receptors. Unlike KA subunits, GluR5-7 subunits can form functional homomeric receptors as well as combine with KA-1 and 2 to form heteromeric receptors with distinct pharmacological properties. Similar to AMPA receptor subunits, GluR5, 6, 7 all undergo alternative splicing and RNA editing, introducing additional heterogeneity [116].

Expression of iGluRs in nodose ganglia has been demonstrated in several species with transport of receptors towards peripheral endings [135, 136], although there is no evidence to support the existence of functional glutamate receptors on guinea pig vagal afferents [40].

In vitro studies in the rat indicated that iGluR may be involved in excitatory modulation of vagal afferents [120], consistent with findings of Page, et al (2005)[121], who found an inhibitory effect of glutamate on metabotropic glutamate receptors in the mouse, an effect that was only apparent upon blocking iGluR effects, suggesting an excitatory (iGluR) and inhibitory (mGluR) balance of vagal afferent mechanosensitivity.

NMDA receptors have been implicated in signalling painful distension of the colon, as peripheral administration of an ionotropic receptor antagonist, memantine, caused a reduction in afferent and behavioural responses to colorectal distension [139]. However as yet, there are no studies that identify a role for iGluR activity on gastro-oesophageal spinal afferents.

3.1 e) Metabotropic glutamate receptors (mGluR)

In addition to fast synaptic transmission at ionotropic glutamate receptors (iGluR), glutamatergic neurotransmission is also mediated by metabotropic glutamate receptors (mGluR). These G-protein coupled receptors can be divided into 8 molecular subtypes, which fall into three pharmacological and functional groups. In general Group I (mGlu 1 & 5) activate phospholipase C and increase neuronal excitability, whereas group II (mGlu 2 & 3) and group III (mGlu 4, 6, 7, 8)

are negatively coupled to adenylyl cyclase and inhibit glutamatergic transmission, decreasing neuronal excitability and will be discussed separately [56, 140, 141]. All eight mGluR transcripts are present in nodose ganglia of a number of species including humans, with the exception of mGluR 3 and 6 in human nodose ganglion. Ligation studies further demonstrated the distal transport of mGluR 1, 4, 5, 7 & 8 along vagus neurones [121]. In addition, using both *in vivo* and *in vitro* techniques, a specific mGlu5 receptor antagonist has been shown to significantly inhibit mechanosensitivity of gastro-oesophageal tension sensitive vagal afferents, acting at peripheral terminals [142].

This effect has been shown to translate to potent inhibition of transient lower oesophageal sphincter relaxations in the ferret and dog [122, 123], and has implicated the mGlu5 receptor, like the $GABA_B$ receptor, as a potential novel target for the treatment of GORD.

Group I, II, and III receptors are expressed on DRG cells or their peripheral or central processes [124]. Particular focus has been placed on the mGlu5 receptors, as in addition to its effects on vagal afferents, the mGluR 5 receptor has also been implicated in nociceptive and mechanosensory signalling from the colon. An mGlu5 receptor antagonist was shown to potently inhibit responses to colorectal distension in conscious rats [125]. This was reflected in an *in vitro* preparation whereby an mGluR5 antagonist was shown to potently inhibit pelvic mechanoreceptive afferents in a similar manner to the *in vivo* preparation, indicating the effect observed was at least partly due to antagonism of mGluR5 at peripheral colorectal endings [143].

3.1 f) Prostaglandin Receptors:

Prostaglandins are phospholipid by-products, arising from the arachadonic acid pathway, being mediators in the response to immune challenges [126]. They are inflammatory mediators that excite and sensitise cell bodies [127, 128], and peripheral nerve terminals of primary vagal sensory neurones [129], as well as acting on central projections of vagal afferents to modulate glutamatergic transmission in the NTS [126].

Subtypes of prostaglandins, PGE_1, E_2, and $F_{2\alpha}$ have all been shown to sensitise vagal cardiac baroreceptors as well as enhance pulmonary vagal afferent signalling in response to chemical and electrical stimuli [130]. More specifically prostaglandin E_2 (PGE_2) has been shown to be involved in physiological reflexes initiated by primary vagal afferents by acting directly on receptors located at vagal afferent terminals [127, 130]. PGE_2 which binds four subtypes of G-protein coupled receptors $EP_{1\text{-}4}$, specifically located in both nodose and dorsal root ganglia [130, 131], has previously been shown to directly excite a proportion of gastro-oesophageal vagal afferents *in vitro* [45].

A number of studies have shown prostaglandins to have a sensitisation effect on sensory afferents innervating the viscera [127, 130, 131], including augmenting both vagal and spinal afferent responses to inflammation by increasing the number of action potentials, and decreasing the threshold of nociceptors. However, whether this effect of PGE_2 is directly on sensory endings or by augmenting actions of other inflammatory mediators is still unclear [130].

3.1 g) 5-Hydroxytryptamine (5-HT):

The primary source of the neurotransmitter 5-hydroxytryptamine (5-HT) in the human body is within the gastrointestinal tract. It is released from a population of enterochromaffin cells in the intestinal mucosa, as well as enteric neurones and mast cells in response to meals, toxins and chemotherapeutic agents [60, 132, 133]. 5-HT receptors represent a superfamily of receptors. To date fifteen receptor subtypes from seven families (5-HT_{1-7}) have been identified, all being G-protein coupled with the exception of the 5-HT_3 family which are ion channels [134]. To date, only 5-HT_{1-4} receptors have been shown to mediate actions within the gut [133].

5-HT has been previously shown to modulate extrinsic afferents innervating the gastrointestinal tract, an effect that was shown to be mediated by various 5-HT receptors. Importantly, the 5-HT_3 receptor subtype has been shown to be involved in direct activation of vagal afferent endings in the upper gastrointestinal tract mediating luminal stimuli [135], as well as mesenteric afferents containing both vagal and spinal afferents innervating the rat jejunum [133]. Functional evidence also exists to show 5-HT_3 receptors to be expressed on colonic distension sensitive afferents [132].

Blackshaw and Grundy, (1993)[49], showed 5-HT to activate vagal mechanosensitive afferents in the ferret, however this direct effect on mucosal afferents was thought to be mediated via changes in motor activity evoked by 5-HT rather than a direct effect of 5-HT on afferent endings. Since then, studies using mouse and ferret *in vitro* preparations have showed 5-HT to directly activate vagal tension, mucosal and tension/mucosal receptors consistent with expression of 5-HT

receptors on vagal afferent endings as opposed to a secondary effect via muscle contraction [45, 46].

3.1 h) Vanilloid Receptors: transient receptor potential channels:

Full transient receptor potential (TRP) channels are proposed to be primary transducers of thermal, mechanical and chemical stimuli. The TRP family is made up of 5 subfamilies (TRPV, TRPA, TRPC, TRPM and TRPP) of selective cation channels, generally composed of 6 transmembrane domains and exist as tetramers [136].

The TRPV family, or vanilloid family are best known for TRPV1 that is present only on sensory neurones, and is activated by heat (>43°C), protons and the exogenous ligand, capsaicin [137]. Capsaicin is a pungent vanilloid that is used as a tool for chronic ablation or functional impairment of sensory C-fibres, or as an acute stimulus for nociceptive afferents [138]. Thus the TRPV1 receptor can be viewed as an integrator of chemical and physical stimuli that elicit pain [139].

It has been demonstrated vagal afferent pathways can be activated by capsaicin infused intraluminally into the distal oesophagus of the ferret [138]. Functional studies demonstrated significant reduction in afferent responses to intraluminally applied HCl in TRPV1 -/- mice compared to wild type [140]. It has also been shown that TRPV1 -/- mice develop significantly less acid induced oesophagitis compared with wild type mice [137]. These results are reflected in the clinical setting whereby TRPV1 positive endings are upreguated in patients with oesophagitis [141].

An action of capsaicin on afferents has two phases; initial excitation which leads to transmitter release, followed by desensitisation and damage after prolonged

or repeated exposure [138]. In the ferret vagal afferent preparation, capsaicin caused desensitisation of responses to further capsaicin application in a proportion of afferents. In addition it caused cross desensitisation to mechanical stimuli, an effect also seen in afferents that did not directly respond to capsaicin [138].
Studies demonstrated acute excitation by capsaicin of all subclasses of vagal afferents innervating the gut in both *in vivo* and *in vitro* ferret preparations [138], with responses restricted to less than one third of fibres recorded in each subclass. These results are comparable with studies of vagal mucosal afferents supplying the rat stomach which showed 32% of afferents were directly excited by capsaicin [64]. In contrast to the vagus, all splanchnic afferents arising from the serosa and mesentery of the cat were activated by capsaicin [77].

TRPV1 receptor mRNA in the nodose ganglion was isolated in 80% of neuronal cells, however, it was considered only slightly more than half of these neurones functionally expressed TRPV1 receptors by eliciting a response to capsaicin [142]

Neurones retrogradely labelled from the gastrointestinal tract to both nodose and dorsal root ganglia contain TRPV1 receptors. Vagal afferents to the gastrointestinal tract express TRPV1 receptors, but at low level. Immunoreactivity was mainly limited to thoracic and cervical extensions of the vagus, while few fibres of the subdiaphragmatic portion of the vagus showed TRPV1 immunoreactivity.TRPV1 was only co-localised with vagal afferent endings of the mucosal layer, and there was no colocalisation with IGLEs, rather there was dense innervation of the myenteric and submucosal plexi with neurones that showed dense TRPV1 immunoreactivity, however these neurones showed colocalisation with CGRP indicating these afferents were of spinal origin [159]. These results are

consistent with the finding of a much greater TRPV1immunoreactivity in dorsal root compared with nodose ganglia [155].

3.2 Inhibitory Receptors

3.2 a) γ-Amino butyric acid (GABA):

γ-Amino butyric acid **(**GABA) is the most prevalent inhibitory neurotransmitter in the CNS. GABA exerts its effects through 3 main classes of receptor, GABA $_{A,B,\&C}$. Type A and C are ligand gated ion channels that allow entry of chloride into the cell leading to rapid hyperpolarisation, whereas $GABA_B$ receptors are coupled via G-proteins, giving rise to slower mechanisms of signalling [143]. $GABA_B$ receptors are functionally expressed on vagal afferents innervating the gastro-oeosophageal region as demonstrated in both *in vivo* and *in vitro* electrophysiological studies, whereby inhibition of mechansensory signalling from gastro-oesophageal vagal afferents in a dose dependent manner by a selective $GABA_B$ agonist was observed [144, 145]. Studies have also isolated $GABA_B$ receptors on 93% of vagal afferents retrogradely labelled from the ferret stomach [161-163]. An *in vitro* study in the ferret demonstrated the inhibitory effect mentioned in 100% of tension and mucosal but only 60% of oesophageal tension/mucosal afferents in the ferret [146]. Subsequent *in vivo* studies demonstrated a similar result for tension sensitive afferents but not for mucosal afferents, however the *in vivo* study evaluated only sensitivity of mucosal receptors to chemical stimulus, whereas the *in vitro* study evaluated only mechanical sensitivity, therefore it is not known whether $GABA_B$ receptors have differing effects *in vivo* compared to *in vitro* or chemo- versus mechanosensitivity [144].

The ability to modulate mechanosensitivity of vagal afferents innervating the gastro-oesophageal region has implicated the $GABA_B$ receptor as a possible target for the treatment of gastro-oesophageal reflux disease (GORD), via reduction in transient lower oesophageal sphincter relaxations in response to gastric distension [163-165], the major underlying pathophysiological mechanism of GORD.

3.2 b) Group II and III mGluR:

In addition to the observed excitatory effects on mechanosensitivity of mGluR5 receptors, group II and III metabotropic glutamate receptors (mGluR 2, 3, 4, 6, 7, 8), which are linked to inhibition of neuronal excitability have also been implicated in modulation of vagal afferent mechanosensation. After a conditioning mechanical stimulation of an *in vitro* preparation, a known trigger of endogenous glutamate release, a group III antagonist increased the response to the subsequent stimuli, however this antagonist had no effect on stimulus response without conditioning stimulation, indicating that endogenous glutamate release modulates mechanosensitivity, but resting levels of glutamate are insufficient [138]. Glutamate was shown to inhibit mechanosensitivity of gastro-oesophageal vagal afferents *in vitro* in both mouse and ferret via group II and III receptors, however this effect was only observed after the addition of a non-selective ionotropic glutamate receptor antagonist Kynurenate, indicating the inhibitory effect of mGluR is opposed by an excitatory effect of ionotropic or group I metabotropic glutamate receptors [138].

3.2 c) Galanin:

Galanin is a peptide found throughout both central and peripheral nervous systems. It may have either excitatory or inhibitory effects on motor function and neuronal excitability, depending on which of the three galanin receptors it binds to. The galanin receptors GalR1-3 are G-protein coupled with GalR1 and GalR3 being inhibitory, acting via adenylyl cyclase, whereas GalR2 is excitatory acting via phospholipase C [147].

Galanin has both inhibitory and excitatory effects in gastric vagal pathways [167, 168], and has been shown to potently influence responses to mechanical stimuli in several types of gastro-oesophageal vagal afferent in the ferret. Galanin was demonstrated to mostly inhibit gastro-oesophageal vagal afferent mechanosensitivity, although potentiation was observed in a minority of experiments [148]. Several endogenous sources of galanin have been identified, including cell bodies in the nodose ganglion as well as on vagal neurones that project to the stomach [148]. A role for endogenous galanin to modulate peripheral vagal afferent mechanosensitivity was observed when a non specific galanin receptor antagonist was shown to potentiate mechanosensitivity directly in specific types of ferret afferent [148].

Galanin has also been shown to have comparable effects on somatic afferents and skin nociceptors in the rat [170, 171]. Both the peptide and its receptors are localised in DRG and in the dorsal horn. In addition, 50% of primary sensory DRG afferents contain galanin [172, 173], however as yet there is no information regarding the effect of galanin on modulation of mechanical sensitivity of spinal afferents supplying the gastrointestinal tract.

3.2 d) Ghrelin:

Ghrelin is a 28-amino acid peptide, acylated by octanoic acid that was first characterised as the natural ligand of the growth hormone secretagogue receptor (GHS-R), and as an endogenous mediator of growth hormone (GH) release [174, 175]. Ghrelin is mainly produced by discrete types of endocrine cells in the oxyntic mucosa of the stomach implicating the peptide as a regulator of food intake [149]. The ghrelin receptor belongs to a rhodopsin-like, seven trans-membrane domain receptor family. Alternative splicing leads to the formation of two splice variants of GHS-R. The full length receptor, GHS-R1a, contains 366 amino-acids and has 7 trans-membrane spanning alpha helices, connected by alternating intra- and extra-cellular loops. The type 1b receptor is a carboxyl truncated form and contains 5 trans-membrane domains. Unlike GHS-R1a, the 1b receptor is not activated by the GHSs or ghrelin, and it is unclear whether it is actually a functional receptor [149]. Despite the unknown physiological significance of the GHS-R1b variant of the ghrelin receptor, mRNA of this subtype has more widespread expression than the type 1a variant [149]. The ghrelin receptor is highly constitutively active. Exposure of GHS-R to ghrelin results in rapid attenuation of the receptor responsiveness, resulting from uncoupling of the receptor from heteromeric G-proteins and of the internalisation of the cell surface receptors to intracellular compartments [174, 176].

Ghrelin is well known to be orexigenic, intricately involved in mechanisms influencing satiety and body weight, including via modulation of vagal afferent signalling and GH secretion [175]. Ghrelin and GHS-R immunoreactivity has been previously demonstrated within sensory neuronal cell bodies and fibres innervating the human stomach and colon. Date, et al (2002)[175], demonstrated both ghrelin and GHS-R to be present in cell bodies of mouse nodose ganglion, as well as

peripheral vagal afferents, transported to peripheral afferent terminals in the rat. Ghrelin has been previously shown to suppress vagal afferent discharge, which in turn led to a reduction in ghrelin induced feeding and GH secretion [175]. Studies have since demonstrated ghrelin to modulate vagal afferent mechanosensitivity of jejunal afferents, whereby ghrelin augmented jejunal afferent responses to distension, an effect reversed by vagotomy and a selective ghrelin antagonist, D-Lys-3-GHRP-6, confirming an action at ghrelin receptors on vagal afferent endings [150].

3.2 e) Opioids:

Opioid receptors located in central and peripheral neural pathways, are best known for their analgesic properties as targets for morphine and its derivatives. Three major subtypes of opioid receptor, μ, δ, and κ, have been identified, all are GPCRs. Both vagal and spinal neurones express all three subtypes, but at differing levels. Both μ- and δ- opioid receptors have been isolated in nodose ganglia cells and on vagal neurones, with relative expression studies demonstrating greater expression of μ- than of δ-opioid receptors. [177-179]. All three receptors have been isolated in DRG and along sensory neurones transported to peripheral endings from DRG [180-182].

Despite the inhibitory function of opioids in the CNS, opioids cause excitation of afferents from the small intestine, unrelated to muscle contraction [151]. Subsequent electrophysiological studies of rat vagal afferent response to gastric distension confirmed a dose dependent attenuation of response by κ- but not μ- or δ-opioid agonists [184]. Further studies elucidated the effect of opioid receptor analogues on all subtypes of vagal afferent, including mucosal and TM receptors in

the oesophagus of the ferret. Page, et al (2008)[185] demonstrated, like Ozaki, et al (2000)[184], inhibition of tension sensitive afferents by a κ-opioid agonist, whilst TM afferents were unaffected. In contrast to Ozaki, et al (2000)[184], tension sensitive afferents as well as mucosal afferents, were significantly inhibited also by a μ-opioid receptor agonist, but had no significant effect on TM afferents [152]. Like Ozaki, et al (2000) [184], there was no effect of δ-opioid receptor agonist on mechanical sensitivity of vagal afferents [185].

Modulation of spinal afferent sensitivity by opioid receptors has been observed in the rat colon, where pelvic afferent response to colorectal distension confirmed a dose dependent attenuation of response by κ- but not μ- or δ-opioid agonists, a similar effect to that seen in the proximal gastrointestinal tract on vagal afferents [186, 187].

4. Molecular Mechanisms of Mechanotransduction

4.1 Mechanosensory Ion Channels

Although a variety of mechanoreceptors have been described, little is known about the molecules utilized by any of these cells to transduce mechanical signals. Mechanically sensitive cells transmit valuable information about external and internal environment to the brain and are essential for the senses of hearing, touch, proprioception and interoception, therefore, to the life of an organism [153].
The identity of mechanotransduction mechanisms in the viscera remain unclear, however we gain insight from knowledge of mechanoreception in other systems. The major candidates as transducers of mechanical stimuli are two families of ion

channel, the degenerin/ epithelial sodium (DEG/ENaC) ion channels and the transient receptor potential (TRP) ion channels families [189].

Observations in vertebrate hair cells has led to models in which mechanosensory transduction is mediated by physical manipulation of a sensory channel or channel complex [154]. The identification of components needed to sense mechanical signals using biochemical or molecular biological approaches is not currently feasible [154], however genetic studies suppressing and enhancing mutations have hypothesised a general model of a mechanosensory apparatus, in which a mechanosensory complex is formed by a rigid cytoskeleton, an intracellular linking protein, a mechanosensitive channel, and an extracellular linking protein [190-192].

Electrophysiological studies have demonstrated that any molecule, for it to be the basis of mechanotransduction must satisfy several criteria. Firstly, rapid response times of vertebrate hair cells and drosophila mechanosensitive bristles led to the assumption that the mechanosensory transduction mechanism must be an ion channel rather than intracellular messaging cascades, although this is yet to be proven [188, 190]. Secondly, the molecule must direct the maximum amount of stimulus energy to the transducing channel, and thus be highly sensitive. Thirdly for mechanical forces to be directed toward specific ion channels, which can open rapidly and amplify the signal by permitting large number of ions to pass [153]. The channel should also be located in the correct cells and correct position within the cells. The channel must be necessary for the electrical response of the cell rather than for subsequent activity of the cell and mechanical forces must gate the channel expressed in both heterologous cells and in its native environment [154].

Candidate transduction mechanisms have arisen from electrophysiological and genetic screening studies of the two most commonly studied mechanoreceptor models, the nematode (Caenorhabditis elegans) and the fruitfly (Drosophila melanogaster). The identities and interaction of cloned mutant genes of C. elegans defective in mechanosensation, revealed a mechanoreceptive complex, with a mechanically sensitive ion channel connected to both intracellular cytoskeletal components and extracellular matrix proteins to form a transduction apparatus, consistent with the general model for mechanotransduction proposed [153]. Screening for a set of mutant genes responsible identified a set of genes encoding family of ion channels, the degenerins (DEG). The DEG family encode ion channels related to vertebrate epithelial sodium channels (ENaCs), responsible for sodium ion absorption, and are sensitive to amiloride. These molecules form heteromultimeric channels with combination of α, β, γ and δ subunits likely in either a tetrameric or octomeric configuration [188, 192, 193]. Vertebrates also have a large array of DEG/ENaC channels. Members of this family traverse the membrane twice, with a large extracellular loop thought to function as the channel pore [153]. Novel members of this ion channel family have since been identified, however the C. elegans sequence database still includes at least 14 uncharacterised degenerin homologues [155].

Amiloride and its analogue benzamil inhibit DEG/ENaC channels [156]. Consistent with a role for DEG/ENaC ion channels in mechanosensory transduction, both these drugs inhibit mechanosensory function of several types of visceral afferent in the vasculature, pulmonary and gastrointestinal systems [40, 195, 196].

Acid Sensing Ion Channels (ASIC):

The DEG/ENaC family in mammals is comprised mainly of acid sensing ion channels (ASICs) [188, 193]. ASICs have been localised to peripheral mechanosensory structures and sensory ganglia [189, 197-200]. They were implicated as candidates to serve as components of molecular transduction mechanisms in visceral and somatic mechanoreceptors by their close relationship to invertebrate channels, without which there is major deficits in body touch perception [188, 193].

These channels were originally shown to be involved in perception of pain that accompanies tissue acidosis as a result of inflammation, and ischemic tissue conditions. A total of four H^+-gated cation channel subunits have been cloned. ASIC1 has two variants (ASIC1a and 1b), and is activated by relatively high pH and inactivates rapidly. ASIC2, also known as BNC1 (brain Na^+ channel-1) or MDEG (mammalian degenerin) and ASIC3, originally named DRASIC and is isolated specifically in sensory neurones [188, 194].

All four subunits form heteromultimeric channels and have been shown to have a widespread effect on the sensitivity of mechanoreceptors and are therefore considered to be required for normal mechanotransduction. Evidence for this stems from targeted disruption of the acid sensing ion channel gene, which leads to major alterations in responses of afferents innervating the skin and viscera [189, 194, 198, 199, 201, 202].

ASIC mutations have both positive and negative effects on mechanosensitivity, suggesting a complexity in their contribution to mechanotransduction. The picture that emerges from these positive and negative

effects of ASIC mutations is of a complex interplay between individual members of the heteromultimer.

In the mouse, ASIC2 and 3 have been found in cutaneous sensory structures [197-199]. A specific role for ASIC2 and 3 in cutaneous mechanosensation was discovered when disruption of these channels impaired cutaneous sensation [198, 199]. In contrast however, ASIC1 had no effect on any of the 5 types of cutaneous mechanoreceptors

Electrophysiological studies recording from visceral afferents has shown contrasting effects of ASIC suggesting different mechanisms underly mechanosensory function in the gut and skin [189].

ASIC1 has been shown to be expressed in sensory ganglia [157]. A role for ASIC1 was shown by consistently increased mechanical sensitivity of gastric, oesophageal and colonic afferents from ASIC1-/- mice by single fibre recordings [189]. Therefore ASIC1 is required for normal visceral mechanosensory function of vagal neurones innervating the stomach and oesophagus, and DRG neurones innervating the colon [189], but not cutaneous mechanoreceptor function [198, 199]. The actual contribution to mechanotransduction of ASIC1 however, is debatable given, without it, mechanosensitivity is universally increased in visceral afferents, suggesting an inhibitory role of the ASIC1 channel, and that the heteromultimeric mechanotransducer becomes more efficient in its absence [158].

The role of ASIC2 differs widely across subclasses of afferent. Disruption of ASIC2 had variable effects on visceral mechanosensitivity. It increased mechanosensitivity in low threshold gastro-oesophageal tension receptors, but attenuated high threshold colonic serosal endings and had no effect on colonic mesenteric endings [201, 203].

ASIC3 makes a critical positive contribution to mechanosensitivity in three out of four classes of visceral afferent [203]. In ASIC3-/- mice, all colonic afferent classes arising from DRG had marked reduction in afferent mechanosensitivity. This effect was also seen in vagal tension sensitive afferents innervating the gastro-oesophageal region but not in mucosal receptors [158].

Identification of the contribution of specific ACIC channel subtypes to mechanosensory mechanisms in the colon was further elucidated by studies demonstrating ASIC3-/- mice to be significantly less responsive to colorectal distension than control [202]. As well, the ability of benzamil (a non selective DEG/ENaC antagonist) to inhibit colonic afferent mechanosensitivity was markedly reduced in ASIC2-/- and ASIC3-/- mice, but unchanged in ASIC1a-/- mice. Gastro-oesophageal afferents were shown however, to require none of the ASIC channels individually for inhibition by benzamil suggesting alternate mechanisms in upper gastrointestinal afferents with different pharmacology than afferents innervating the colon [203].

Transient Receptor Potential (TRP) Channels:

TRP channels were first described in Drosophila. As previously mentioned, the TRP family comprises 5 main members (TRPA, C, M, P and V) in mammals, and all are putative 6 transmembrane spanning polypeptide subunits that assemble as tetramers to form cation permeable pores. They are ubiquitously expressed and most have splice variants [204, 205]. TRP family members have been implicated in osmosensation, a similar sense to mechanotransduction. A C. elegans TRP channel (OSM-9) is expressed in dendrites of some ciliated sensory neurones and is required

for osmosensation and nose touch sensation [153]. Candidate TRP channels in mechanotransduction are TRPV1, V4 and A1 [156, 192, 206, 207].

TRPV1:

TRPV1, which has capsaicin as a ligand, is a Ca^{++} permeable cation channel that is potentiated by heat (>43°C) and decreased pH, and is inhibited by PIP_2 as described in 3.1h.

TRPV1-/- mice are deficient in nociception, inflammatory and hyperthermic responses to vanilloid compounds, supporting the impression that TRPV1 contribute to thermal nociception and hyper-algesia after tissue injury [159]. TRPV1 has also been shown to be essential for normal mechanically evoked purinergic signalling by the urothelium [206].

The TRPV1 receptor has been identified in both intrinsic and extrinsic neurones of the GIT [208, 209]. *In vitro* studies have shown only one quarter of gastric vagal mechanosensitive neurones showed TRPV1 immunoreactivity [210], suggesting TRPV1 does not play a major role in sensing mechanical stimulus under physiological condition. Supporting this, responses of jejunal afferents to tension were significantly lower in TRPV1-/- than wild type (or TRPV+/+) mice, with a marked downward shift in the pressure response curve for wide dynamic range (WDR) fibres in TRPV1-/- mice compared to control. In addition, the TRPV1 antagonist capsazepine significantly attenuated afferent nerve responses to distension, as well as spontaneous discharge in TRPV +/+ mice, and acute desensitisation of afferents by capsaicin resulted in profound reduction in afferent sensitivity in TRPV+/+ but not TRPV1-/- mice [140]. This effect, which is only seen in wide dynamic range afferents, points to a role for TRPV1 receptors in

mechanical transduction from splanchnic nerves innervating the small intestine. This effect is not observed in low threshold afferents, which are considered to be vagal afferents [211], suggesting TRPV1 receptors to be less important in mechanosensory transduction in vagal afferents [140]. However, TRPV1 has not yet been shown to be mechanosensitive, but alternatively may sensitise small intestinal afferent neurones [140].

TRPV4:

TRPV4, the mammalian homologue of the C. elegans gene OSM-9, was originally found to be involved in osmosensation. TRPV4 is critical in mechanical avoidance suggesting that it functions to detect noxious mechanical stimuli [205]. It has since been shown that temperature may sensitise the channel to other stimuli including mechanical stress, clearly pointing to a role of this channel in mechanosensory phenomena. The recent demonstration that mammalian TRPV4 is sensitive to activation by mechanical stress in a heterologous expressed system provides strong evidence that TRPV4 channels and perhaps TRPV4 related proteins such as OSM-9 are transducers of mechanical stimuli [212].

Recent studies have demonstrated mechanosensory responses of colonic serosal and mesenteric afferents to be enhanced by a TRPV4 agonist and dramatically reduced by a targeted deletion of TRPV4, or by a TRP antagonist. Other types of vagal and pelvic afferents in contrast, were unaffected, indicating a specific role for mechanotransduction in colonic serosal and mesenteric afferents [205].

TRPA1:

In addition to the TRPV family, the TRPA subfamily has also been implicated in mechanosensation. The TRPA family has one member, TRPA1, which is the mammalian orthologue of the *Drosophila* gene, Painless, where it is expressed in polymodal nociceptors endings and contributes to detection of mechanical stimuli [136]. TPRA1 is known to be localised to extrinsic afferents supplying the gut, being expressed in 55% of gastro-oesophageal, 54% of splanchnic colonic and 58% of pelvic colonic afferents [205]. Deletion of the TRPA1 gene has been correlated with reduced mechanosensitivity of mucosal afferents in vagal and spinal pathways as well as decreasing the response-function of high threshold colonic splanchnic, and pelvic serosal-mesenteric afferents. However, TRPA1 had no effect on vagal tension sensitive, as well as pelvic muscular, and muscular-mucosal afferents, suggesting TRPA1 may play a selective role in both tactile and nociceptive afferents [205, 207].

As yet there is no evidence demonstrating a role for the further TRP subfamilies in signalling from the gastrointestinal tract, however a role for the calcium channel effector TRPC is currently under investigation [136].

5. Clinical Aspects of Gastro-oesophageal Reflux Disease (GORD)

Abdominal vagal afferents that sense distension are a well known trigger of belching and transient relaxations of the lower oesophageal sphincter (TLOSR), allowing refluxate of gastric contents, which is the most common mechanism underlying gastro-oesophageal reflux disease (GORD) [213-215]. GORD as

generally defined, is a common condition that results from reflux of gastric material through the lower oesophageal sphincter (LOS), into the oesophagus or oropharynx, causing symptoms and/or injury to oesophageal tissue.

The term covers both symptoms and pathophysiological changes to the oesophageal mucosa, which occur as a result of exposure of the distal oesophagus to acidic gastric contents after episodes of gastro-oesophageal reflux.
Some degree of gastro-oesophageal reflux into the oesophagus is a normal occurrence in most individuals, however protective mechanisms such as oesophageal clearance and mucosal resistance are normally able to maintain a physiologically balanced state and such episodes are not usually associated with pathological signs or symptoms. When these mechanisms are overcome by the injurious properties of the refluxate which contains gastric acid, bile, pepsin, and duodenal contents, reflux becomes pathological.

Pathological reflux episodes are of longer duration and are more frequent. Pathological acid reflux can result in a spectrum of symptoms and oesophageal changes that characterise the disease, including most commonly heartburn and acid regurgitation, but also dysphagia/odynophagia and non cardiac chest pain. In addition to oesophageal symptoms, other symptoms include laryngitis/pharyngitis, chronic sinusitis, dental erosions, asthma and chronic cough.

Long standing cases of GORD can possibly lead to complications which may include oesophagitis, oesophageal erosions and ulcerations with resultant scar tissue contributing to stricture formation. Importantly, replacement of oesophageal squamous epithelium by a metaplastic columnar epithelium characterises development of Barrett's oesophagus, a known complication of long standing GORD, which significantly increases the risk of oesophageal adenocarcinoma

[160]. In fact, symptomatic GORD is a strong risk factor for oesophageal adenocarcinoma [161].

The most common presentation of reflux is heartburn, classically described as a retrosternal burning sensation radiating to the pharynx. Patients may also complain of unexplained substernal chest pain resembling myocardial infarction without evidence of coronary artery disease, termed non-cardiac chest pain (NCCP). GORD is the most common gastrointestinal cause of NCCP. This obviously presents a diagnostic dilemma to the physician.

5.1 Epidemiology of GORD

Longitudinal studies reporting the incidence of GORD in a population of patients aged 2-79 years, as diagnosed by physicians in the UK, reported an overall incidence of GORD to be 4.5 per 1000 person years (95% CI 4.4-4.7).
Using a strict symptom frequency criteria to define GORD, whereby symptoms must be present at least once per week, thus considered sufficient to impair quality of life, its prevalence in the western world may be as high as 20% [162]. GORD, therefore, is currently one of the most common clinical problems of our society [162].

Recent work in twin studies has also identified a familial vulnerability to GORD, demonstrating aggregation of GORD symptoms in monozygotic twins, compared with dizygotic twins [163]. Other studies have demonstrated there to be a correlation between patients with Barrett's oesophagus or adenocarcinoma and family members with GORD symptoms [164].

5.2 Pathophysiology: The lower oesophageal sphincter (LOS) and reflux

Constriction of the lower oesophageal sphincter (LOS) prevents regurgitation of stomach contents into the oesophagus. The LOS is the main anti-reflux barrier allowing the passage of boluses during food ingestion to the stomach, whilst preventing reflux of gastric content back into the oesophagus. The normal LOS is a 3-4cm segment of tonically contracted smooth muscle located at the gastro-oesophageal junction. Anatomically, the gastro-oesophageal junction is made up of two components: the true LOS in the distal oesophagus and the crural portion of the diaphragm, which both contribute to the gastro-oesophageal sphincter competence [165].

The primary aetiology of GORD has long been considered to involve disordered control of the gastro-oesophageal barrier, arising from motor dysfunction of the LOS, allowing gastric content passage to the oesophagus. Mechanisms thought to contribute to the development of gastro-oesophageal reflux (GOR), involve decreased LOS resting tone, which can be provoked by a variety of hormonal and neuronal agents, commonly used medications, food, impaired oesophageal acid clearance, delayed gastric emptying, decreased salivation, and impaired tissue resistance.

A minority of patients with GORD (20%) have, as their primary underlying motility disorder, LOS incompetence due to either decreased LOS pressure, increased abdominal pressure (obesity or pregnancy), or a shorter than normal LOS [221, 222]. Many patients with GORD however have normal LOS pressure. In this group of patients, frequent transient LOS relaxations (TLOSRs), are often found as the underlying cause of pathological reflux [166]. Although TLOSRs are not more frequent in GORD, a higher proportion of them are accompanied by reflux [165].

McNally, et al (1964)[167], first described non-swallow related LOS relaxations as a mechanism of belching, the freeing of gastric gas however, TLOSRs were not described until 1980 [168]. TLOSRs are brief episodes of LOS relaxation that are unrelated to swallowing or peristalsis [224, 225]. Lasting approximately 5-35 seconds, TLOSRs decrease LOS pressure to gastric levels and are associated with inhibition of the crural diaphragm, gastric fundus and oesophagus [213, 222]. Among patients with GOR, TLOSRs are considered the primary underlying cause of pathological reflux in the presence of a normal resting LOS tone.

They are strongly associated with both physiological and pathological GOR. TLOSRs are common to both healthy and GORD patients in order to vent gas trapped above gastric contents, during which a little gastric acid may reflux simultaneously, but not usually enough to cause oesophageal mucosal damage [226, 227].

The dominant pattern of LOS dysfunction in patients with GORD is an increased proportion of TLOSRs accompanied by reflux [169], as well as an increased number of inappropriate TLOSRs [170], thus the time that acid is in contact with oesophageal mucosa is increased in patients with GORD, increasing their risk of symptoms and oesophageal injury [165].

In normal subjects, gastro-oesophageal reflux occurs only during TLOSRs and swallow induced LOS relaxations, accounting for 94% of GOR episodes. Excessive acid reflux due to TLOSRs is the most common causative mechanism in patients with GORD, accounting for 48-73% of reflux episodes [225, 230-232].

Manifestations of GORD are thus dependent on rate of TLOSRs and composition of refluxate (acid vs non acid).

5.3 Neural pathway of transient LOS relaxations (TLOSR)

Whilst the precise mechanism responsible for TLOSRs remains to be elucidated, the most potent trigger among both normal individuals, and those with GORD, is known to be gastric distension caused by anything that increases intragastric volume, such as postprandial fullness or air [221, 233-236].

A number of lines of evidence exist to suggest that TLOSRs are neurally mediated. TLOSRs have an abrupt onset and brief time course, lasting from 5 to 35 seconds [170]. Moreover they are abolished by cooling of the cervical vago-sympathetic trunks [171], general anaesthesia [172], and during sleep [173], indicating they are mediated via a vagal pathway, and are under central control.

The fact that TLOSRs are absent in achalasia patients, a condition characterised by partial oesophageal denervation, reinforces the notion that TLOSRs are neurally mediated [174].

Neurophysiology studies indicate that TLOSRs are visceral motor programmes with vagal afferent and efferent pathways that transmit information to and from the dorsal motor nucleus of the vagus [240, 241]. TLOSRs are mediated by a vago-vagal reflex initiated by mechanoreceptor response to distension in the gastric wall [174]. The trigger zone was identified using partitioning studies of the dog stomach to be localised within the proximal (cardia) portion of the stomach [175]. Animal species possess mechanoreceptors located in this part of the stomach musculature that are responsive to local tension in smooth muscle, as opposed to its length [55]. These vagal afferents have central terminals in the nucleus tractus solitarius of the brain stem [243], whereupon they synapse with other neurones which constitute a central program generator [168]. There are several outputs from the program: first is a brief and powerful activation of vagal preganglionic motor

neurones (in adjacent dorsal vagal motor nucleus) projecting to the LOS, which activate nitrergic motor neurones of the enteric system, causing smooth muscle relaxation (Figure 1.). Second is a suppression of oesophageal body peristalsis [243], presumably due to interruption of excitatory vagal output [243]. Third is a suppression of motor output to the crural diaphragm leading to opening of the external striated muscle sphincter [213, 244-246].

5.4 Current treatments for GORD

GORD is typically a chronic condition, requiring continued management using medications and lifestyle modifications. In early stages of the disease, uncomplicated GORD can be treated by lifestyle modification and changing eating habits. Despite the fact that gastric acid secretion is actually normal in most GORD patients [176], recognition that symptoms and oesophagitis are mainly due to the damaging effects of acid and pepsin means focussed attention on gastric acid suppression is currently the mainstay of treatment, which has been effective in relieving symptoms and oesophageal lesions [177].

Pharmacotherapy, particularly the use of anti-secretory agents to minimise acid content of refluxate present in the oesophagus, has probably modified the natural history of GORD. The various agents currently used for treating GORD include mucoprotective substances, antacids, H_2 blockers, prokinetics, and the mainstay of treatment, proton pump inhibitors, which reduce gastric acid production. Proton pump inhibitor (PPI) use, in particular, has had an enormous impact on treatment, significantly improving erosive oesophagitis healing rates and bettering symptom control [165]. Although these drugs are effective, they do not necessarily influence the underlying causes of the disease, in particular reducing

frequency of TLOSRs [178]. In addition, GORD refractory to PPI therapy is very common, and may affect up to 40% of patients prescribed daily PPI therapy [179, 180]. Refractory GORD can present as incomplete or lack of response to PPI therapy, possibly due to weakly acidic reflux, duodenogastro-oesophageal/bile reflux, visceral hypersensitivity, delayed gastric emptying, and concomitant functional bowel disorders. Reduced PPI bioavailability, rapid PPI metabolism, PPI resistance, nocturnal reflux and Helicobacter Pylori infection status are not however thought to be responsible [248, 249].

The underlying aetiology of GORD is characterised by an increased number of reflux episodes, rather than acid hypersecretion [250]. TLOSRs are the mechanism underlying most episodes of gastro-oesophageal reflux in healthy subjects and in most patients with reflux disease [229-232]. It is thus an attractive target for drug therapy that could effectively decrease the occurrence of reflux episodes [251-253].

Gastric distension, activating tension sensitive mechanoreceptors (sensing gastric volume) appear to be most relevant factor in triggering transient LOS relaxations [213, 250]. Animal studies have demonstrated that tension mechanoreceptors are present in the stomach [71, 254-257]. Whilst it is still unknown which of these receptors is more relevant to triggering TLOSRs, the proximal stomach is an area of particular interest with regard to modulation of these events [168]. A treatment that addresses the fundamental cause of reflux would be expected to provide a better success rate, and of course be more appropriate to the disease aetiology.

5.5 Pharmacology of TLOSR pathways

Current pharmacological targets for management of GORD are aimed at acid suppression, prokinetic agents, or both. These approaches do not address the major mechanism underlying reflux of gastric contents into the oesophagus, transient LOS relaxation, which is clearly the most prevalent mechanism in most patients with reflux disease. Therefore control of reflux through pharmacological inhibition of TLOSRs would provide a novel pharmacological approach for reflux disease based on the pathophysiology outlined above [181]. Intense interest in the area has led to numerous studies that have demonstrated pharmacological reduction of the rate of TLOSRs [241, 259-263].

The gamma amino butyric acid (GABA) receptor type B agonist, baclofen, has been shown to be a potent inhibitor of TLOSRs in both normal subjects and patients with GORD. Baclofen, was first implicated to be a potent inhibitor of TLOSRs in the dog [264], these findings were subsequently confirmed in the ferret [165].

Baclofen has since been shown to significantly reduce the rate of TLOSRs in both healthy subjects and patients with reflux disease by up to 60%, with a similar rate of reduction of GOR [258, 265].

GABA is a major inhibitory neurotransmitter within the central nervous system. Evidence exists for modulation by $GABA_B$ receptors at several points along the vagal pathway controlling TLOSRs.

$GABA_B$ receptors are present at many sites in both the central, peripheral and enteric nervous systems, including expression at central and peripheral terminals of primary vagal afferents. There is dense distribution of inhibitory $GABA_B$ receptors along the central vagal pathways in the nucleus tractus solitarii

(NTS) and dorsal vagal nucleus [182], as well as in vagal LOS-projecting pre-ganglionic motor neurones [183].

This distribution implicates $GABA_B$ receptors in control of the oesophageal phase of swallowing at the level of the central program generator [268]. $GABA_B$ receptors have been implicated in attenuation of mechanosensory input into brain stem neurones [144, 184], inhibition of centrally evoked LOS relaxation via a site of action associated with vagal motor outflow to the LOS [268], as well as a discreet pre-synaptic inhibitory role for $GABA_B$ receptors on vagal preganglionic fibres serving inhibitory motorneurones in the ferret LOS [183].

Retrograde labelling and immunohistochemistry were used to identify $GABA_B$ receptors located on vagal afferent neurones in the nodose ganglion. Over 93% of retrogradely labelled neurones in the nodose ganglion expressed immunoreactivity for $GABA_B$ receptors [145]. Page, et al (1999)[146], provided the first direct evidence for the inhibitory modulation of peripheral mechanosensory endings via $GABA_B$ receptors expressed on vagal afferent fibres. Inhibition was dose dependent, pharmacologically reversible, and selective to certain aspects of mechanosensitivity [146]. Baclofen, acting at $GABA_B$ receptors expressed on vagal afferents also significantly inhibited the vagal afferent distension response curves in the ferret proximal stomach [145]. This is likely a contributing mechanism to the efficacy of $GABA_B$ receptor agonist in reducing TLOSRs and reflux episodes *in vivo* [145].

These findings suggest that $GABA_B$ agonists may be useful as therapeutic agents for the management of reflux disease and sparked intense clinical and basic interest [60].

GABA however, is an important inhibitory neurotransmitter within the central nervous system and is responsible for mediating at least 40% of all inhibitory synaptic events within higher centres of the mammalian brain [185], and is associated with numerous centrally mediated side effects. Recently, a peripherally acting $GABA_B$ receptor agonist AZD3355 potently stimulated recombinant human GABA receptors and inhibited TLOSRs in dogs [186]. Its peripheral effects were further confirmed by *in vitro* studies demonstrating AR-HO61719 (the racemate of AZD3355), to inhibit responses of ferret mechanoreceptors to gastric distension [186]. Several additional pharmacological agents have been shown to inhibit triggering of TLOSRs mediating gastro-oesophageal reflux.

Cholecystokinin (CCK) appears to play a role in triggering of TLOSRs in dogs and healthy subjects [187-189] . A CCK_1 receptor antagonist, Loxiglumide, was shown to antagonise mechanical and chemical induction of TLOSRs. This effect was possibly mediated by an effect on vagal afferents [188]. However, whilst loxiglumide has been shown to reduce meal induced TLOSRs in healthy subjects and GORD patients, its effects on GOR has been minimal [190]. This, as well as a side effect profile that includes inhibition of gall bladder emptying, leading to stone formation, means CCK antagonists have a limited role in the clinical setting.

Metabotropic Glutamate receptors (mGluR) are present in cell bodies of the vagus nerve in the nodose ganglia, as well as in vagal nerves themselves, where they are demonstrated to be transported peripherally [146]. A total of 8 mGLuRs belong to a family of G-protein coupled receptors and can be divided into three groups (I, II, III). Group I(mGluR 1 and 5) are excitatory, Group II (mGluR 2 and 3) and Group III (mGluR 4, 6, 7 and 8) are inhibitory. Electrophysiological *in vitro*

studies demonstrated modulation of certain subtypes of vagal afferent mechanosensitivity by these receptors [146, 191].

In vivo studies in ferret [122], and dog [123], have demonstrated that antagonism of the group I receptor mGlu5, produces a dose dependent inhibition of gastric distension induced TLOSRs, an effect which correlates with a reduction of reflux episodes. Frisby, et al (2005) [122], further identified an mGluR8 agonist to also reduce the number of TLOSRs, however its effect was not as potent as the mGluR5 antagonist which reduced TLOSRs by up to 90%.

Recent evidence comparing *in vitro* peripheral responses of ferret gastro-oesophageal vagal afferents to graded mechanical stimuli, and central responses of NTS neurones with gastric input *in vivo*, in the presence or absence of mGluR5 antagonist, demonstrated the principal site of action of the mGluR 5 antagonist previously seen on vagal afferent mechanosensitivity was on afferent terminals in the periphery. In contrast, minor central effects of the mGluR5 antagonist, as well as sparse receptor immuno-labelling in NTS subnuclei, suggest mGluR5 plays only a minor role in the central vagal pathway linked to gastric distension [191].

The *Muscarinic Receptor* antagonist atropine has been shown to reduce basal LOS tone in dogs and humans, presumably by a reduction in cholinergic neural input [192, 193]. However, atropine has been shown to reduce the frequency of reflux in normal subjects, and reflux disease patients, via inhibition of TLOSRs, but also having the unwanted side effect of inhibition of swallow induced LOS relaxations [193]. Atropine induced inhibition of TLOSRs is unlikely to be via an effect on the cholinergic supply of the proximal stomach causing relaxation, rather by a central action on the integrating mechanisms in the brainstem [194].

Nitric oxide (NO) is the major inhibitory neurotransmitter of post-ganglionic neurones innervating the LOS that cause it to relax. Boulant, et al (1994)[187], demonstrated a specific NO-synthase inhibitor to evoke a significant reduction of gastric distension induced TLOSRs in dogs. This result was reflected in human studies of normal subjects, whereby a NO-synthase inhibitor evoked a reduction in TLOSRs by greater than 75%, suggesting that NO is involved in the triggering of TLOSRs. In addition, there was no effect on the degree of swallow-induced LOS relaxations [195].

Opioid receptor agonist morphine, has been shown to increase residual LOS pressure during swallow induced TLOSRs [196]. In addition to this effect, morphine was the first drug shown to decrease the rate of TLOSRs in GORD patients, leading to a substantial decrease in the number of reflux episodes [197]. However, morphine also prolonged acid exposure to the oesophagus, presumably by the unwanted effect of inhibition of swallow induced LOS relaxations [197].

There are many theoretical points of intervention to inhibit TLOSRs. However the effects of morphine outlined above, as well as those of Mittal, et al (1995) [193], with atropine, on the motor limb of the TLOSR pathway, highlight the difficulty of devising a pharmacological approach that selectively decreases the rate of transient LOS relaxations without affecting the central or efferent limb of the vagal pathway responsible for TLOSRs, which are not desirable [197].

Signalling from gastric vagal tension receptors to the CNS is crucial in the initiation of this motor pattern [144]. The most accessible target in modulation of the neuronal pathway mediating TLOSRs is via the vagal afferent terminal mechanoreceptors. Findings such as demonstrated by inhibitory G-protein coupled $GABA_B$ and metabotropic glutamate receptors have important relevance to

strategies for selective reduction of sensory input to the CNS at a peripheral site. Such peripheral targets offer advantages by minimising interference with motor pathways that may produce unwanted side effects elsewhere in the GI tract as well as in other target organs of the vagus [198].

AIMS

General aim:

Three major classes of receptors are candidates for modulation of vagal afferent signals from the gastrointestinal tract: excitatory ionotropic receptors, inhibitory and excitatory (metabotropic) G-Protein coupled receptors (GPCR). Four were chosen for study in this thesis, since their endogenous ligands are present in the stomach. These are galanin receptors (which may be excitatory or inhibitory GPCR according to subtype), excitatory ionotropic glutamate receptors, and their relative contribution compared with excitatory metabotropic glutamate receptors, and finally G-protein coupled ghrelin receptors (which may have excitatory or inhibitory actions on nerves elsewhere). These receptors have defined roles elsewhere in the nervous system, but the critical focus of this investigation is their roles at a peripheral site, where they may represent novel targets for therapeutic modulation of gastro-oesophageal vagal signals to the central nervous system, and therefore potential treatments for diseases such as GORD, disordered food intake, and functional dyspepsia.

Specific aims of this study:

1) To identify and compare relative expression galanin receptors in vagal sensory ganglia and to evaluate the contribution of individual receptor subtypes involved in modulation of vagal afferent mechanosensitivity by galanin, using quantitative RT-PCR as well as an *in vitro* electrophysiological approach utilising transgenic knockout mice and receptor specific ligands.

2) To further understand and elucidate mechanisms of glutamatergic modulation of vagal mechanosensitive afferents by systematically identifying possible roles for individual ionotropic glutamate receptors in the excitatory modulation of vagal afferents using quantitative RT-PCR and an *in vitro* electrophysiological preparation, and to investigate a potential peripheral mechanism of action of group I mGluR, mGluR5, to inhibit signalling of mechanosensitivity.

3) To investigate the effect of ghrelin on the sensitivity of differing populations of mechanosensitive gastro-oesophageal vagal afferents to mechanical stimulation using RT-PCR and an *in vitro* electrophysiological preparation, and to determine if any effects observed are conserved across species.

CHAPTER 1 : Identification of Receptors Responsible for Neuromodulation of Mouse Gastro-oesophageal Vagal Afferents by Galanin

SUMMARY

Galanin, a 29-amino acid brain-gut neuropeptide, is known to have inhibitory and excitatory effects on neuronal excitability, acting via three galanin receptors, GalR1, 2 and 3. We have previously demonstrated a predominantly inhibitory effect of exogenously applied galanin in gastro-oesophageal vagal afferents, whilst potentiating a minority. These actions may have implications for therapeutic inhibition of gut afferent signalling. We performed quantitative RT-PCR on RNA from vagal (nodose) sensory ganglia, which indicated that all three GalR subtypes were expressed at similar levels. The responses of mouse gastro-oesophageal vagal afferents to graded mechanical stimuli were investigated before and during the application of galanin receptor ligands to their peripheral endings. Two types of vagal afferents were tested: tension receptors, which responded to circumferential tension, and mucosal receptors, which responded only to mucosal stroking. Galanin induced potent inhibition of mechanosensitivity of both types of afferent. This effect was absent in GalR1- null mutant mice. A GalR1/2 agonist was shown to mimic the inhibitory effect of galanin in GalR1+/+ mice, but this effect was reversed to cause modest, but significant potentiation in GalR1-/- mice, indicating a minor role for GalR2 in potentiation of vagal afferents. We observed no functional evidence of GalR3 involvement, despite its relative expression in nodose ganglia. The current study highlights the complex interaction of galanin with differing receptor subtypes, consistent with the function of galanin in other systems.
*I would like to acknowledge the work of Dr Stuart Brierley who assisted to develop PCR protocols used in this and other chapters.

INTRODUCTION

Much basic and clinical interest has been recently directed toward novel therapies for management of gastro-oesophageal reflux disease (GORD). The major mechanism underlying pathological reflux is transient lower oesophageal sphincter relaxations (TLOSRs), which are mediated by a vagal pathway and comprise a motor pattern initiated by the activation of gastric mechanoreceptors by distension of the proximal stomach [171, 172, 199, 200]. Therefore, modulation of peripheral vagal afferent signalling from the upper gastro-intestinal tract has important implications in the treatment of GORD.

Modulation of peripheral vagal afferent mechanosensation has been previously demonstrated by numerous amino-acid neurotransmitters, ion channels and peptides [146, 148, 157, 158, 201]. Importantly, potent inhibition of vagal afferent mechanosensitivity by metabotropic receptors to the amino-acids γ-amino butyric acid (GABA) and glutamate [121, 146], has been mirrored by *in vivo* animal and human trials to correlate with inhibition of TLOSRs and subsequently gastro-oesophageal reflux with therapeutic benefit [143, 181, 198, 202, 203].

We have recently identified an additional peptide target shown to modulate vagal afferent mechanosensitivity from the upper GIT in both ferret and mouse models [148]. Galanin is a 29-30 amino acid peptide found throughout the central and enteric nervous system, known to have either inhibitory or excitatory effects on motor function and neuronal excitability [147]. Galanin has previously demonstrated these divergent effects on nociceptive transmission in the spinal cord [204], gastric smooth muscle activity [205], and signalling in central gastric vagal pathways [206, 207].

This property of galanin was replicated in our *in vitro* gastro-oesophageal vagal afferent preparation whereby inhibition and potentiation of vagal afferent mechanosensitivity was observed. We observed galanin to be predominantly coupled to inhibition of vagal afferent mechanosensitivity [148]; prompting the question whether variable expression of different galanin receptor subtypes on gastro-oesophageal vagal afferents accounts for this phenomenon.

Vagal afferents that project to the stomach, including their cell bodies in the nodose ganglion, in addition to enteric neurones, contain galanin, implicating both extrinsic and intrinsic neurones as candidates for providing ongoing modulation of mechanosensitivity by an endogenous source of galanin. In keeping, an endogenous role of galanin on vagal afferent mechanoreceptors has been previously demonstrated [148].

Galanin exerts its effects via three different receptor subtypes. GalR1 is negatively coupled to adenylate cyclase via an inhibitory G_i G-protein, and can influence neuronal Ca^{++} and inwardly rectifying K^+ currents. GalR2 is coupled via G_o and $G_{q/11}$ G-proteins leading to activation of cellular inositol tri-phosphate production and protein kinase C activity. The physiological role of GalR3 remains unclear, however the receptor is known to utilize a signalling pathway linked through G-proteins to inhibition of adenylate cyclase or modulation of other effectors such as ATP dependent K^+ or Ca^{++} channels [208].

The divergent effects of galanin are likely to be due to a result of activation of multiple receptor subtypes with differing signal transduction pathways. It is highly likely that the inhibitory effects we observed are mediated via GalR1 or GalR3 and potentiating effects via GalR2. The resultant change in signalling from peripheral afferents could be accounted for by widely different expression of

different GalR subtypes on different afferent fibres; however this remains to be examined. The relatively higher frequency at which we encountered inhibitory effects compared to the excitatory effects suggests agonists aimed at GalR1 or GalR3 may have greater therapeutic potential by modulating mechanosensory function than antagonists for GalR2.

There are several theoretical points of intervention to inhibit TLOSRs, however, targeting specific receptors in order to reduce vagal afferent signalling is important as to minimise side effects of therapeutic intervention, and to gain a better understanding of mechanisms of modulation of vagal afferent endings. To date, the specific role of each receptor on vagal afferents innervating the upper gastrointestinal tract is yet to be determined. In order to better understand the contribution of individual receptor subtypes involved in modulation of mechanosensitivity we used specific galanin receptor ligands in conjunction with GalR1 -/- transgenic knock-out mice to identify receptors mediating modulation of mechanosensitivity caused by galanin.

MATERIALS AND METHODS

All studies were performed in accordance with the guidelines of the Animal Ethics Committees of the Royal Adelaide Hospital and Institute for Medical and Veterinary Science, Adelaide, Australia. These committees followed the Australian code of practice for the care and use of animals for scientific purposes, 6th edition 1997. Every attempt was made to minimise the number of animals required and to minimise their suffering.

Generation of GalR1-/- Mutant Mice

For exhaustive methods used to generate GalR1-/- null mutants, refer to Jacoby et al, (2002)[209], and Blakeman et al, (2003)[210]. Briefly, W9.5 embryonic stem cells were transfected with a GalR1 gene targeting construct and correctly targeted clones were isolated and injected into C57BL/6 blastocysts. The resultant chimeric mice were mated with C57BL/6 mice to produce heterozygous (Galr1+/-) mice that carried the GalR1-null allele in their germ line. These heterozygotes were backcrossed to C57BL/6 mice to generate GalR1 +/-for mating to produce GalR1-/- and GalR1 +/+ littermates for analysis. GalR1-/- mice on a C57BL/6 background exhibit normal growth rates and lifespan and display no over phenotype.

Nodose Ganglia Dissection and RNA extraction for RT-PCR and Quantitative RT-PCR

Nodose ganglia were removed from five male or female C57 mice (20-30g). Nodose ganglia were pooled in RNAlater® (QIAGEN, NSW, Australia) at -20°C for subsequent RNA extraction. RNA isolation involved homogenisation with a sterile glass pestle, of the nodose ganglia, after being snap frozen using liquid nitrogen. A total of 1ml of TRIzol reagent (Invitrogen, Vic, Australia) was added before the homogenized material was added to Qiashredders and centrifuged (12,000 rpm) at room temperature. Tubes containing homogenate were capped after Qiashredders were removed, vortexed and allowed to stand at room temperature for 15 minutes in order to promote dissociation of nucleoprotein complexes. After which 0.2 ml of chloroform was added, tubes were vortexed and left to stand at room temperature for a further 15 minutes. The samples were then cold centrifuged

(12,000 rpm, 4°C) for 15 minutes with the upper aqueous layer (containing RNA substrate) being removed and transferred to a sterile eppendorf tube followed by isopropanol precipitation of RNA, by which 0.5 ml isopropanol was added and cold centrifuged for 10min (38, 47). The upper aqueous phase was discarded leaving an RNA pellet to which 1 ml of 75% ethanol was added. A 1 min vortex and 4 min cold centrifuge and then supernatant was removed with the pellet being re-suspended in 100µl RNAase free sterile water and heated for 2 min at 60°C. RNA was separated into 5µl aliquots and stored at -80°C.

RNA quantification was determined by measuring the absorbance at 260nm (A260) using a spectrophotometer (Biorad, Sydney, Australia). RNA quality was estimated by the A260: A280 nm ratio.

Determination of galanin receptor transcript expression and relative galanin receptor transcript expression using Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reactions (QRT-PCR) were performed using a Qiagen QuantiTect® SYBR® green RT-PCR one step RT-PCR kit, using QuantiTect® primer assays (Qiagen) optimized for the detection of the known sequence of mouse GalR1, GalR2, GalR3 and β–actin transcripts contained in the NCBI reference sequence database (www.ncbi.nlm.nih.gov/RefSeq). All product lengths were restricted below 150bp to maximize efficiency of the SYBR® Green reaction.

Real-time PCR mater mix was prepared according to product manufactures specifications as follows: for each reaction 24.5µL of 2x QuantiTect SYBR Green RT-PCR Master Mix, (each master mix containing HotStarTaq® DNA polymerase, QuantiTect® SYBR green RT-PCR buffer, dNTP Mix including dUTP, SYBR®

Green I (Fluoresces in presence of double stranded DNA), ROX (passive reference dye), 5mM $MgCl_2$, and 0.5µL of QuantiTect® RT mix (Omniscript® Reverse Transcriptase and Sensiscript® Reverse transcriptase).

For each reaction 25µL of the master mix, 10µL (3µM) forward primer, 10µL (3µM) backward primer and 5µL of 50ng of template RNA were added in MJ White PCR tube (MJ Research). RNA template was replaced by RNAase free, sterile water in control samples.

QRT-PCR reactions were performed using a Chromo4 (MJ research, Biorad) real-time instrument (designed to detect SYBR green fluorescence, therefore amplified cDNA products) attached to PTC-200 Peltier thermal cycler (MJ Research) and analysed using Opticon Monitor Software (MJ Research).

The following amplification program followed: Reverse Transcription: 50°C for 30mins; Initial PCR activation: 95°C for 15 mins; PCR cycles; denaturing: 94°C for 15 sec, annealing: 47°C for 30 sec and extension: 72°C for 30 sec repeated for 44 cycles with final extension step of 72°C for 10 min. A melting curve program was performed to verify the specificity and identity of the RT-PCR products, whilst all other reactions were verified using gel electrophoresis. Assays were run in triplicate in separate experiments. The size of the amplified products (5µL PCR product + 2µL loading buffer) was confirmed by 1.5-3% agarose gel electrophoresis and visualised by ethidium bromide staining.

Results for QRT-PCR were viewed via Opticon software and were represented as cycle threshold (CT) required to achieve a certain level of fluorescence (amount of double stranded PCR product). In all experiments CT was obtained from the exponential/linear join of the PCR fluorescence curve. As CT is

proportional to the logarithm of total DNA in sample, comparing CT enabled determination of the relative expression of transcripts.

Titration curves were obtained to confirm amplification efficiencies and therefore the validity of the comparative cycle threshold. Template RNA of known concentration from nodose ganglia was used to create a five log dilution series of RNA. Equal amplification was indicated by the linearity of the plots assay over a range of input RNA concentrations for β-actin, galanin and galanin receptor subtypes. These curves demonstrated the efficiencies of targets and references were equal.

The relative concentration of a sample can be determined by the number of cycles required to reach a certain fluorescence level when compared with other samples. The β-actin sample was the first sample to reach CT and thus galanin receptor transcripts expression values were referenced relative to β-actin. The CT for each was compared with that of β-actin using the equation of CT of target transcript – CT of β-actin. To determine relative expression of these transcripts in whole nodose ganglia, the ΔΔCT was calculated using the formula: $\Delta\Delta CT = \Delta CT$ [β-actin] - ΔCT [galanin receptor] and the relative fold differences calculated using the formula $2^{-\Delta\Delta CT}$. CT values for β-actin were highly reproducible between samples and between methods of RNA preparation. Quantitative data are expressed as mean ± SD, and significant differences in transcript expression determined by a Mann-Whitney test, significance considered where $p < 0.05$.

In vitro mouse gastro-oesophageal afferent preparation

Male and female C57/BL6 and GalR1-/- mice (20-30g, 7-10 weeks) were killed via CO_2 inhalation. The thoracic and abdominal cavity was opened. At this

stage no overt phenotype was noted. The stomach, oesophagus and attached vagus nerves were then removed and placed in a modified Krebs' solution of the following composition (mM): 118.1 NaCl, 4.7 KCl, 25.1 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$, 7 H2O, 1.5 $CaCl_2$, 1.0 Citric acid and 11.1 glucose, bubbled with 95% O_2-5% CO_2. The preparation temperature was maintained at 4°C during dissection to preserve tissue integrity and prevent metabolic degradation. The peripheral organs, major blood vessels and diaphragm were removed by further dissection, leaving the stomach oesophagus and attached vagus nerves intact. The preparation was opened longitudinally and pinned out flat, mucosa side up in a Perspex organ bath (dimension 6 x 2.5 x 1.2 cm) lined with Sylgard® (Dow Corning) and perfused at a rate of 12ml/min with carbogenated Krebs' bicarbonate buffer solution maintained at 34°C. The vagus nerves were pulled into a separate isolate recording chamber which was filled with paraffin oil. The dimensions of the second chamber are 3.7 cm diameter and 1.2 cm deep. Under a dissecting microscope, the vagus sheath was removed exposing the underlying nerve fibres, which were teased using fine forceps into 16-24 bundles and one by one placed on platinum recording electrodes. A reference electrode rested on the mirror surface in the bath in a small pool of Krebs'. Nifedipine (1μM) was also added to the Krebs superfusate in order to prevent smooth muscle contraction. Previous preliminary studies have demonstrated nifedipine to have no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents [211].

Characterisation of gastro-oesophageal vagal afferent properties

Corresponding receptive fields of all types of afferent fibre were located by mechanical stimulation throughout the preparation with a brush and then more

accurately with a von Frey hair. Accurate quantification of mechanical responses was performed differently according to the primary adequate stimulus for the type of fibre as has been described previously [46, 148, 157, 158]. The mechanical sensitivity of afferents was characterised by mucosal stroking with calibrated von Frey hairs (10mg-1000mg) and applied circular tension (0.5-5g).

Two types of mechanosensitive afferent were studied, those responding to mucosal stroking but not to circular tension (mucosal receptors) and those responding to mucosal stroking and circular tension (tension receptors) as reported previously [46].

Mechanical thresholds of all types of fibre were determined using calibrated von Frey hairs. The most reproducible, stimulus dependent response of these afferents to mucosal stimuli was evoked when the probe was moved at a rate of $5mms^{-1}$ across the receptive field rather than being static. The mechanical sensitivity of the receptor response to mucosal stroking was recorded as impulses evoked per stroke of the von Frey hair for the middle 8 of 10 strokes given at 1s intervals as to minimise experimental error. A bent von Frey hair (10-1000mg) stroking over the preparation meant that an even force was distributed over the receptive field during stroking stimulus.

Tension-response curves were also obtained for fibres responsive to circular tension. Tension receptors also responded to von Frey hair stroking, however were distinguished from mucosal receptors by their clear responsiveness to circular tension which was slowly adapting.

Tension stimuli were applied via a thread attached to an unpinned point adjacent to the receptive field. The thread was attached to a cantilever and pulley system close to the preparation, with reference standard weights applied to the

opposite side of the cantilever. Each weight was applied in a step wise manner and maintained for duration of 1 minute. Sensory receptors response to circular tension was recorded as impulses evoked per second over a 1 min period. Because all responses to tension were similarly slowly adapting, this method of assessment was considered representative of physiological responsiveness to distension. The tension response curves were produced by applying weights to the cantilever system in a range of (1-5g) which was sufficient to evoke a maximum response without tissue damage. A recovery period of at least 1 min was allowed between each tension stimulus.

Effects of Galanin on mechanosensitivity of vagal afferents

After mechanical sensitivity of gastro-oesophageal vagal afferents has been established, the effects of galanin on mechanical sensitivity were determined. Galanin (1nM) was added to the superfusing Krebs' solution, and for each dose was allowed to equilibrate for 20 min, after which the tension-response curves and stroke response curves were re-determined. This equilibration period was to ensure complete penetration of the drug into all layers of tissue. This procedure was repeated for galanin at increasingly higher doses (3-10nM). Concentrations used were based on previous studies of ferret and mouse (C57BL/6) gastro-oesophageal afferents in which galanin had a dose dependent effect between 1 and 10nM [148]. Time controlled experiments were performed in which there was no significant change in the mechanical responses over a comparable duration (see Fig 6). Concentration response curves were obtained in tissue from matched GalR1+/+ and GalR1-/- mice for tension receptors. Because of the effects of galanin on tension receptors were similar in the GalR1+/+ littermate mice and unmatched C57BL/6

mice, the latter were used as controls for studies of mucosal afferents. This was necessary because of the limited availability of GalR1+/+ littermates.

Effect of a GalR3 antagonist on the inhibitory effects of Galanin

The effects of the selective GalR3 agonist SNAP 37889 (100nM) was also determined. After mechanical sensitivity of the gastro-oesophageal vagal afferent was established, the effect of galanin on mechanical sensitivity was observed. The maximal concentrations of galanin (1nM for tension receptors and 10nM for mucosal receptors) were added to the superfusing Krebs' and allowed to equilibrate for 20 mins. After which time, tension response and stroke response curves were then re-determined. SNAP 37889 (10nM for tension receptors and 100nM for mucosal receptors) was then added to the Krebs' superfusate in addition to galanin and allowed to equilibrate for 20 mins. Before tension and stroke response curves were once again re-determined.

These doses of SNAP 37889 were calculated based on published potency of SNAP 37889 [212].

Effects of AR-M961 on mechanical sensitivity of GalR1 -/- vagal afferents

In a separate experiment, the effects of selective GalR1 and GalR2 agonist AR-M961 (10-100n M) on tension receptor responses to circular tension was determined. AR-M961 was tested for afferents where galanin (100nM) had showed significant inhibition of tension receptor sensitivity to mechanical stimuli. The effects of galanin were then reversed with a 20 minute washout of the preparation with Krebs' superfusate before the addition of AR-M961 at increasingly higher doses (10-100 nM), and for each dose an equilibration period of 20 mins before

stimulus response curves were re-determined. AR-M961 has been shown to have potent agonist activity at both GalR1 and GalR2, with an IC_{50} of 0.403 nM and 1.74 nM, respectively [213].

In a separate series of experiments using GalR1-/- mice, the effect of AR-M961 alone was determined. After mechanical sensitivity had been established, AR-M961 (100nM) was added to the superfusing solution and the preparation was allowed to equilibrate for 20min. The tension-response curve was then re-determined.

Time control experiments (n=9) were preformed and showed there was no significant change in mechanical responses over a comparable time period (Fig 6). All experiments were performed in the presence of Nifedipine 1μM in order to limit the effects of these drugs secondary to smooth muscle responses or extra-cellular Ca^{++} - dependent mediator release.

Data Recording and Analysis

Afferent impulses were amplified with a biological amplifier (World Precision Instruments, Sarasota, FL, DAM 50) and scaling amplifier (JRAK, Melbourne, Australia), filtered (CWE Inc., Ardmore, PA band-pass filter-932) and monitored using an oscilloscope (DL 1200A; Yakogawa, Tokyo, Japan). Single unit action potentials were discriminated on the basis of size and shape using spike II software (Cambridge Electronic Design, Cambridge, UK).

Statistical Analysis: All data were recorded and analysed off-line using a personal computer. All data were expressed as mean ± SE with n = number of individual afferents in all instances. Statistical difference between stimulus-response curves were evaluated using a two-way ANOVA performed using graphpad Prism

(Version 3.00 for Windows, Graphpad Software, San Diego, California, USA). Differences were considered significant where $p < 0.05$.

Drugs

Stock solutions of all drugs were kept frozen (-80°C) and diluted to their final concentration in Krebs' solution on the day of the experiment. Galanin was obtained from SIGMA (Sydney, Australia) and SNAP 37889 was a kind donation from AstraZeneca (Montreal, Canada).

RESULTS

Expression of Galanin receptors in mouse nodose ganglion

Using RT-PCR and gel electrophoresis we were able to confirm the presence and size of amplified products generated by QuantiTect Primer Assays specific for galanin receptors GalR1, GalR2 GalR3, and β-actin (Figure 1A.). Isolation of single bands of correct predicted transcript size was achieved by UV analysis of the gel. Bands of correct predicted product size for each galanin receptor was observed in mouse nodose ganglia. Where template RNA was substituted with sterile water, no evidence of amplified products was observed. Expression of the reference β-actin gene was observed in all cases using template RNA.

Quantitative RT-PCR

Using gel electrophoresis we confirmed the size of amplified products generated by the QuantiTect® Primer Assays specific for galanin receptors and β-actin, showing intense single bands for all three galanin receptors and β-actin transcripts (Figure 1A.).

Separate quantitative RT-PCR experiments were performed to compare relative expression galanin receptors in whole nodose ganglion. Using β-actin as a reference CT value, QRT-PCR analysis of whole nodose ganglia revealed there to be no significant difference in relative transcript expression between galanin receptors (Figure 1B.).

We also measured quantitative expression of GalR2 and GalR3 in whole nodose ganglia of GalR1-/- mice, which showed no difference in their levels relative to β-actin (data not shown), compared with wild-types; these data indicate no adaptive regulation of other galanin receptors in GalR1-/- mice.

Electrophysiology

Effect of galanin on mechanosensitivity of gastro-oesophageal vagal afferents

The effect of galanin on mouse GalR1 +/+ and GalR1 -/- tension receptor sensitivity to mechanical stimulation is illustrated in Figure 2. Galanin (1-10 nM) significantly reduced the response of GalR1 +/+ tension receptors to circular tension (n = 7; Figure 2A. and C). The inhibitory effect of galanin was reversible by removing it from the Krebs' superfusate in all experiments (Fig 2Cc). Galanin (1-10 nM) however had no significant effect on GalR1 -/- tension receptors response to circular tension (n = 13; Figure 2B and D).

The effect of galanin on mouse C57BL/6 (GalR1 +/+ substitute) and GalR1 -/- mucosal receptor sensitivity to mechanical stimulation is illustrated in figure 3. Galanin (1-10 nM) significantly reduced the response of C57BL/6 mouse mucosal receptors to mucosal stroking with calibrated von Frey hairs (10-1000mg) (n = 7; Figure 3A and C). Galanin (1-10nM), however did not have any significant effect

on GalR1 -/- mice mucosal receptor response to mucosal stroking (n = 8; Figure 3B and D.).

Mechanosensitivity of mucosal and tension receptors in the absence of galanin showed no significant differences (P>0.05, two-way ANOVA) in control stimulus-response curves between GalR1-/- and wild type mice (Data from Figs 2A and B, and 3a and B)

Effect of a GalR3 antagonist on the inhibitory effect of galanin

The effect of a GalR3 selective antagonist SNAP 37889 on wild-type mouse gastro-oesophageal vagal afferents is illustrated in fig 4. Galanin (1nM) alone significantly reduced the response of tension receptors to circular tension (n=5; Fig 4A). When the GalR3 antagonist SNAP 37889 (10nM) was added to the superfusate along with galanin (1nM), there was no significant change in the response to circular tension compared with galanin (1nM) alone (Fig4A). Galanin (10nM) also significantly reduced response of mucosal receptors (Fig 4B), which were unchanged by SNAP 37889 (100nM; Fig 4B).

Effect of a GalR1/2 agonist on the mechanosensitivity of gastro-oesophageal vagal afferents

The effects of the selective GalR1 and GalR2 agonist AR-M961 on tension sensitive gastro-oesophageal afferents of GalR1 +/+ and GalR1 -/- mice is illustrated in Figure 5.

In preparations of wild-type mice, galanin (100nM) significantly reduced mechanical sensitivity of tension sensitive afferents to circular tension (n = 5; Fig 5A). After galanin had been washed out of the preparation by Krebs superfusate for

20 minutes and sensitivity had been shown to return to normal, the GalR1 and GalR2 agonist AR-M961 (10-100nM) was then added, which dose dependently and significantly inhibited mechanical sensitivity to circular tension mimicking the inhibitory effects of galanin (n = 5; Figure 5A.). In contrast, in preparations obtained from GalR1-/- mice, AR-M961 (100nM; n=6) caused a minor but significant potentiation of mechanical sensitivity of tension sensitive afferents to circular tension (Fig. 5B).

DISCUSSION

Modulation of vagal afferent signalling from the upper gastrointestinal tract has been implicated as a novel therapeutic approach for the treatment of a number of disorders of dysfunctional neuronal signalling from the gastro-intestinal tract to the central nervous system (CNS), particularly gastro-oesophageal reflux disease (GORD). The current data demonstrate potent, reversible modulation of vagal afferent sensitivity of mouse gastro-oesophageal vagal afferents by exogenous galanin. As we have observed previously [148], both inhibitory and facilitatory effects of galanin were seen, however again, we observed a predominantly inhibitory effect of galanin in our preparations. The inhibitory effect of galanin on both types of vagal afferent mechanoreceptor observed in wild-type and C57BL/6 mice was not observed in GalR1-/- mice, indicating the inhibitory effect observed was mediated through the GalR1 receptor. A dominant role for GalR1 to mediate the observed inhibitory effect of galanin was further established when a GalR3 selective antagonist SNAP 37889 [212], failed to reverse the inhibitory effects of galanin observed on both tension and mucosal sensitive afferents, suggestive of no

apparent functional role for GalR3 on gastro-oesophageal vagal afferent mechanoreceptors.

Confirmation of the role for GalR1 mediating the inhibitory effects came from studies using the selective GalR1/2 agonist AR-M961 [213], which mimicked the inhibitory effect of galanin on tension sensitive afferents after galanin had been washed out of the preparation.

Exogenously applied AR-M961 was also shown to significantly potentiate tension sensitive afferents in mice lacking GalR1, implicating a functional role for GalR2 to mediate the excitatory effects on vagal afferent mechanosensitivity observed in several preparations.

The dihydropyridine calcium channel blocker Nifedipine (1μM), was added to superfusate bathing the tissue in our electrophysiological preparations to prevent smooth muscle contraction. In previous preliminary studies we have shown that nifedipine has no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents [211]. Therefore the effects of galanin and its analogues we observed, was due to an effect on mechanically sensitive afferents directly, rather than distortion of gastrointestinal smooth muscle.

We identified the presence of all three receptor transcripts in whole mouse nodose ganglia, which represented the cell bodies of vagal afferents. These receptors have previously been shown to be present on vagal afferents and transported to peripheral afferent terminals [148]. Quantitative RT-PCR was unable to distinguish any difference in level of expression between the three receptors. These findings suggest that whilst all three receptors are found to have equal expression in nodose ganglia, their functional expression on vagal afferent mechanoreceptors varies.

Sources of endogenous galanin

Several sources of endogenous galanin are proposed. Circulating galanin levels found in previous studies are equal to or greater than doses used in this investigation [214]. In addition, detailed distribution of galanin immuno-reactivity in gut of the rat, mouse, guinea pig and pig as well as human oesophagus, reveal galanin exists in neuronal structures throughout the gastrointestinal tract. Galanin immunoreactive fibres were isolated in myenteric neurones of the submucosal and myenteric plexi, coursing through the lamina propria, submucosa and circular smooth muscle layers as well as innervating intramural ganglia [215-218].

Galanin immunoreactivity occurs in several functionally distinct classes of enteric neurones, amongst which are neurones controlling motility, intestinal blood flow as well as mucosal water and electrolyte transport [219].

Galanin has also been identified in the cell bodies of the nodose ganglion in a number of species and importantly, vagal afferent fibres projecting to the stomach have also been shown to contain galanin [148, 220].

These findings implicate a number of sources of endogenous galanin that may be released locally in an autocrine or paracrine fashion from vagal afferents themselves, or systemically in an endocrine manner by neurones or endocrine cells of the gut to modulate activity of galanin receptors located on vagal mechanoreceptive afferents.

Previous data identified a role for endogenous galanin acting on ferret tension sensitive gastro-oesophageal vagal afferents [148], where by a number of tension sensitive afferents were potentiated by a non-selective galanin receptor antagonist, galantide. Results from the current study contradict this finding,

demonstrating baseline mechanosensitivity of tension and mucosal sensitive afferents was unchanged in mice lacking GalR1 which argues against an ongoing role for endogenous galanin in modulation of vagal afferent mechanosensitivity, however a possible explanation for limited access of endogenous galanin to the endings may include several factors including, an intact vascular supply, dimension of tissue and species.

Galanin receptors on vagal afferents

Galanin exerts its effects on neuronal excitability by acting on specific G-protein coupled receptors which in turn affect intra-cellular second messenger signalling cascades. Three distinct receptor subtypes for galanin have been cloned; Gal-R1, -R2 and -R3, and pharmacological studies strongly suggest the existence of additional unidentified galanin receptors [221]. Activation of GalR1 and GalR3 receptors expressed in cell lines leads to modulation of inwardly rectifying calcium and potassium channels resulting in inhibition of cyclic AMP levels. In contrast, GalR2 has been shown to be excitatory, activating phospholipase C and increasing phosphoinositol and intracellular calcium levels leading to cellular excitability [147, 222].

The expression of GalR 1 and 2 in sensory neurones is well established [223-227], however, whilst it is widespread in central and peripheral sensory neurones, little evidence exists regarding the existence of functional GalR3 in sensory neurones [227, 228].

Previous reports have demonstrated galanin specific binding sites in the nodose ganglion [229], however ours are the first data demonstrating expression of galanin receptors in the nodose ganglion.

Role of GalR1

Both inhibitory and excitatory effects of galanin are well documented, however several lines of anatomical and functional evidence exist to demonstrate a predominant inhibitory role of galanin in neuronal processing including inhibition of local gut reflexes affecting feeding behaviour, as well as neurotransmitter and hormone release [230, 231], and modulation of nociceptive processing at the spinal level where galanin has been shown to exert an analgesic effect and inhibit spinal reflexes [205, 230-232]. In addition, studies of the effects of exogenous galanin on sensory endings also demonstrate a predominant inhibitory effect, causing modulation of peripheral nociceptive neurones [222], and importantly causing predominant inhibition of mechanosensitivity of somatic and visceral primary afferents [148, 233].

The predominant inhibitory effect of galanin in previous studies is reflected in our current studies, whereby galanin exerted a predominantly inhibitory effect on gastro-oesophageal vagal afferent mechanosensitivity. Based on the widespread distribution of GalR1 mRNA in central and peripheral nervous systems, the inhibitory actions of galanin in feeding [234], nociception [210, 235], gut motility and secretion [205, 208], have largely been attributed to the Gal1 receptor.
In the present study we set out to determine the relative contribution of the three galanin receptor subtypes in modulation of vagal afferent mechanosensitivity and had the opportunity to utilise a combination of GalR1-/- mutant mice and receptor specific ligands to test the hypothesis that GalR1 predominates in galanin's effect on mechanosensitivity. Previous studies in GalR1 -/- mice have focussed on roles for galanin in modulation of neuropathic pain at the level of the spinal cord [210], as well as its role for GalR1in the central nervous system where it plays an inhibitory

role in seizure activity, anxiety, learning and memory [209, 236-239]. The current data demonstrate actions of galanin in the peripheral nervous system where it mediates inhibition of vagal afferent mechanosensitivity via GalR1. As described in other studies of GalR1-null mutant mice raised on the same background, no other overt phenotypic differences could be observed, suggesting that the changes in mechanical responsiveness and effects of galanin were directly as a result of loss of the receptor

Role of GalR2

GalR2 was the only receptor of the three to be coupled to excitatory mechanisms, activating phospholipase C and increasing intracellular calcium levels [147, 222], and we therefore expected it to be responsible for the excitatory effects observed. Our findings with a GalR1/2 agonist AR-M961 [213], reinforced our initial findings with galanin, showing predominantly inhibition mediated through GalR1. In the GalR1-/- mice however, AR-M961 was shown to modestly but significantly potentiate mechanosensitivity, implicating a functional role for GalR2 in modulating mechanosensitivity of vagal afferents, with GalR1 having a greater functional activity than GalR2. This observation supported prior observations made of two wild type mice in which galanin augmented mechanosensitivity, where this effect was mimicked by selective GalR2 agonist AR-M1896 [213] (Data not shown). Both excitatory and inhibitory effects of galanin observed may perhaps reflect differing receptor activation or proportion of receptors on afferents.

Role for GalR3

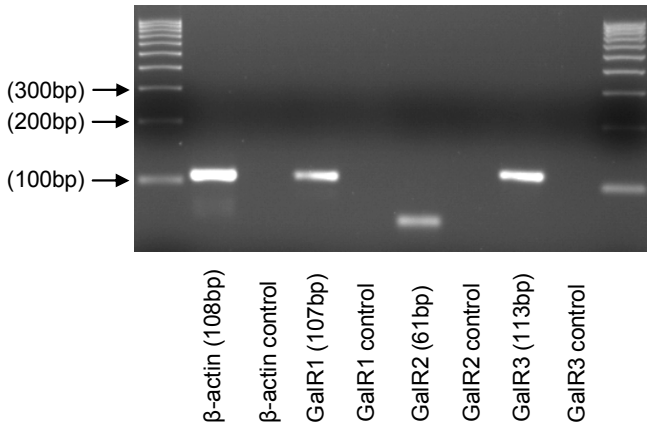
The other possible candidate for mediating the inhibitory effects of galanin was via GalR3, however we were unable to identify a functional role for this receptor in our preparation. A selective GalR3 antagonist SNAP 37889 [212], failed to reverse any of the inhibitory effects of galanin on both tension and mucosal afferents. This coupled with the fact that the inhibitory effect of galanin in GalR1-/- mice was absent, argues against any role for GalR3 in modulation of gastro-oesophageal vagal afferents. Surprisingly, the relative expression of all three receptors was not significantly different in cell bodies of the nodose ganglia. This contradicts our functional results, however our recent estimates of gastric-projecting neurones suggest that GalR3 containing neurones represent only 10-15% of the total number of neurones [148]. Consistent with this finding, measurement of GalR3 in gut tissue has shown considerably lower expression compared with GalR1 and GalR2 [240]. Therefore the possibility exists that GalR3 plays a predominant role in other organs supplied by the vagus nerve, however histochemical detection of GalR3 is equivocal and as yet no functional role for GalR3 has yet been identified in the gastrointestinal tract.

CONCLUSION

In conclusion, the divergent effects of galanin have again been observed in our preparation, facilitating both inhibitory and excitatory modulation of extrinsic vagal afferents supplying the upper gastrointestinal tract, and importantly that this effect is mediated by GalR1 and GalR2 respectively. However, we were unable to identify any evidence of functional involvement of GalR3. The current study highlights the complex interaction of not only galanin with multiple receptors, but

also the differential receptor activation which may reflect the proportion of receptors expressed on vagal afferent terminals. The complex actions of galanin observed are consistent throughout functional studies of galanin on sensory innervation of somatic and visceral structures, as well as roles of galanin in gastric motility and secretion. This study implicates a GalR1 agonist as the most likely candidate of a novel therapeutic target for modulation of extrinsic afferent signalling from the gastrointestinal tract, an effect which previous modulators of vagal afferent sensitivity have shown to translate to therapeutic use in diseases of altered extrinsic afferent signalling from the gastrointestinal tract.

A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(200bp)
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β-actin (108bp)
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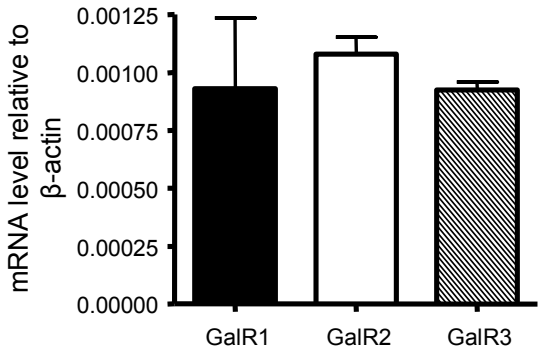
GalR1 (107bp)
GalR1 control
GalR2 (61bp)
GalR2 control
GalR3 (113bp)
GalR3 control

B


mRNA level relative to β-actin
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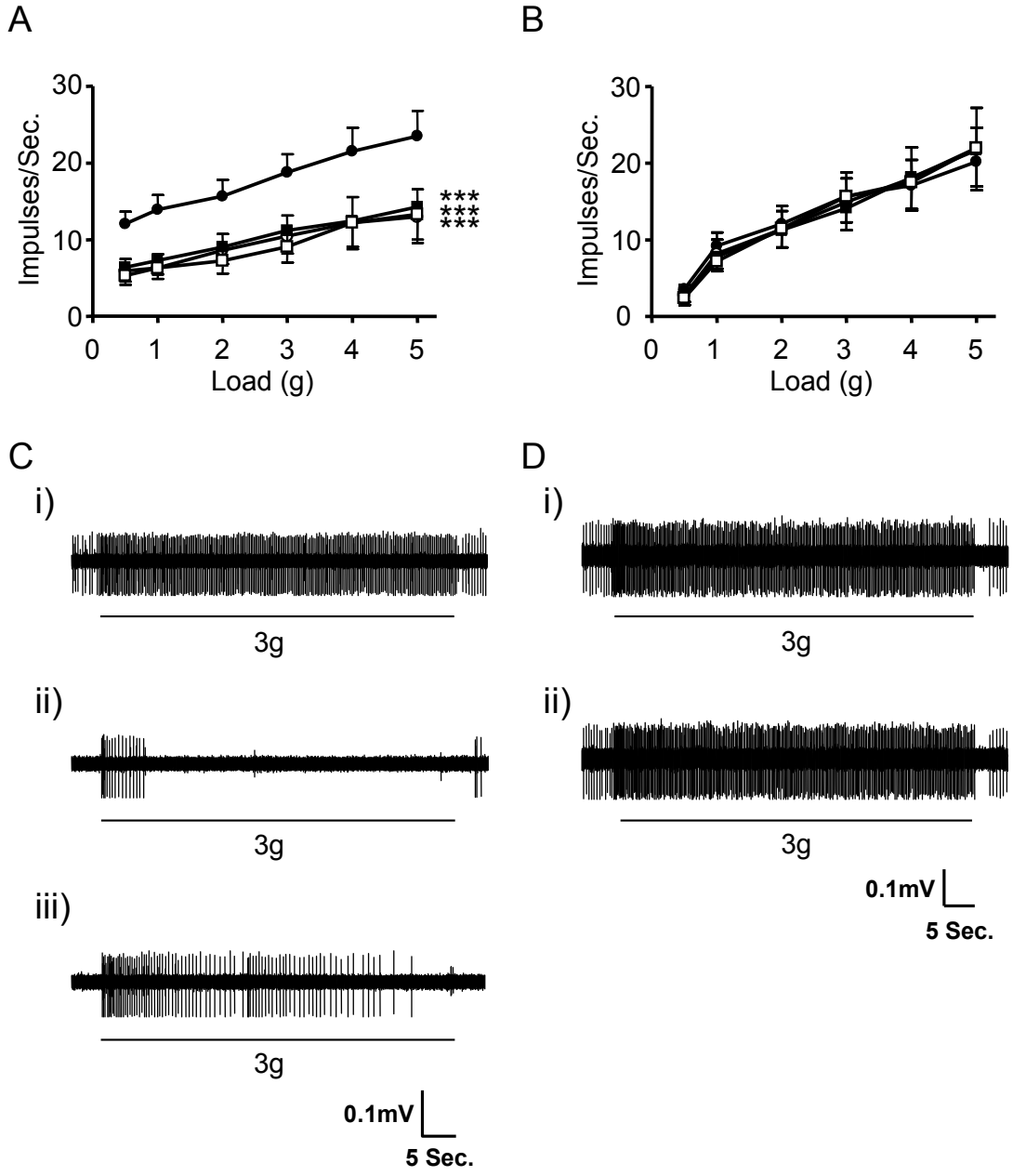
0.00100
0.00075
0.00050
0.00025
0.00000
GalR1
GalR2
GalR3

Figure 1

Transcript expression and relative expression of galanin receptors in whole mouse nodose ganglia

A. PCR products of quantitative RT-PCR experiments separated on a 3% agarose gel to confirm the transcript expression of galanin receptors GalR1, GalR2 and GalR3. The sizes of the amplified products were confirmed showing intense single bands corresponding to the predicted sizes of each of the receptors transcript as well as β-actin transcripts. B. Quantitative RT-PCR revealed there to be no significant difference in transcript expression between galanin receptor subtypes ($P>0.05$, Mann-Whitney U test). Experiments were performed in at least triplicate. Fold differences of GalR1, 2 and 3 receptor expressions are calculated relative to β-actin mRNA levels.


A
B
C
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Impulses/Sec.
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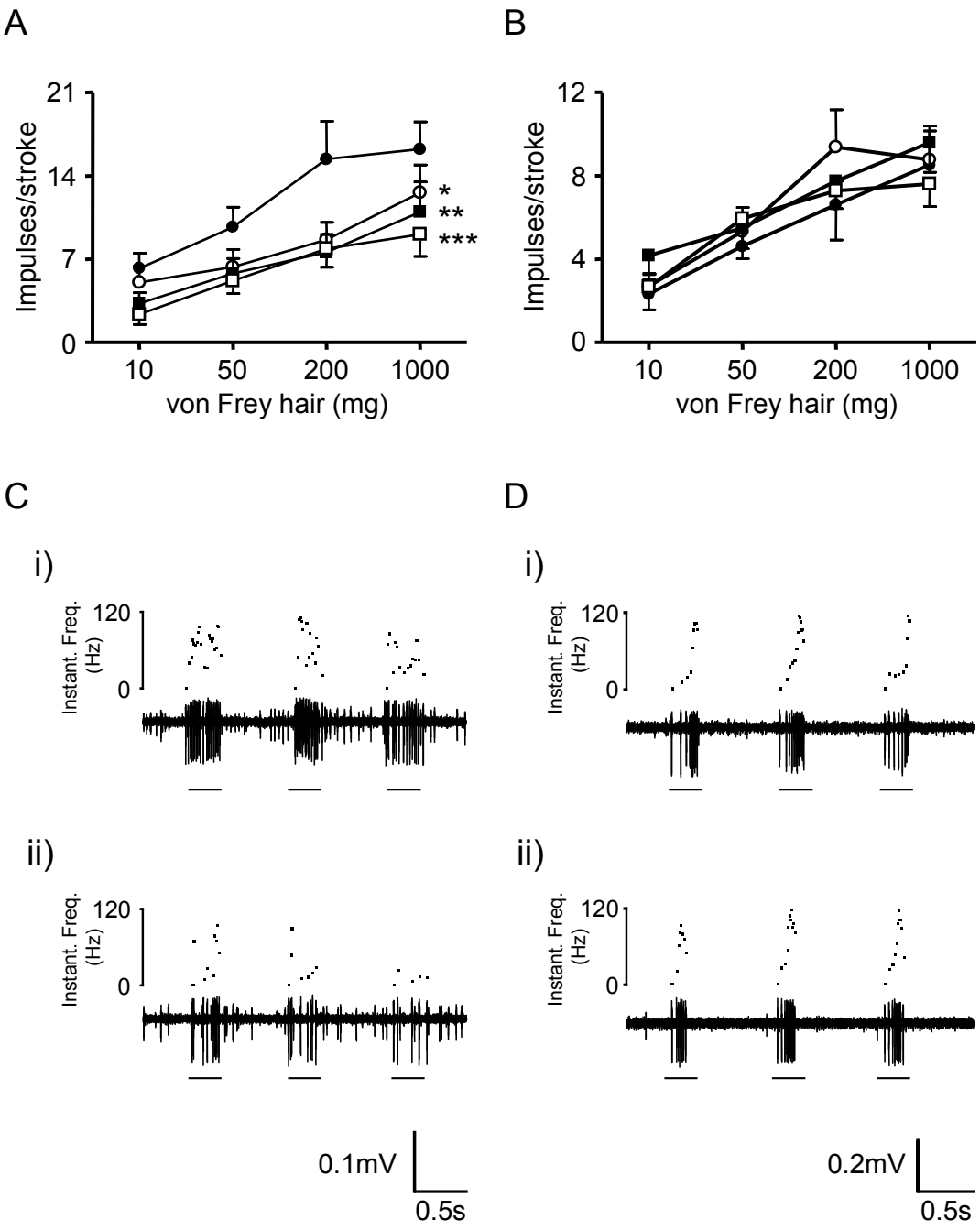
***
***
i)
ii)
iii)
3g
0.1mV
5 Sec.

Figure 2.

The effects of galanin on mouse gastro-oesophageal vagal afferent tension receptors Group data for responses of wild-type (A; n=7) and GalR1 -/- (B; n=13) stimulus-response functions of tension receptors to circumferential tension. The responses are before (●) and after exposure to galanin (1(○), 3(■) and 10nM (□).Galanin (1-10nM) significantly reduced mechanical sensitivity of wild type afferents to circular stretch however galanin (1-100nM) did not significantly effect the mechanical sensitivity of GalR1-/- tension sensitive afferents to circular stretch. The graphs show the mean ± SEM.

Significant differences between treated and control (assessed using two-way ANOVA) are shown nearest each treated response curve. (*$p<0.05$, **$p<0.001$, ***$p<0.0001$).

C, original recordings of a tension receptor response in GalR1+/+ animal to circular tension with a 3g weight before (Ci), during (Cii) and after washout (Ciii) of galanin (10nM). D, original recording in GalR1-/- animal of a tension receptor response to circular tension with a 3g weight before (Di) and during (Dii) exposure to galanin (10nM).


A
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Impulses/stroke
21
14
7
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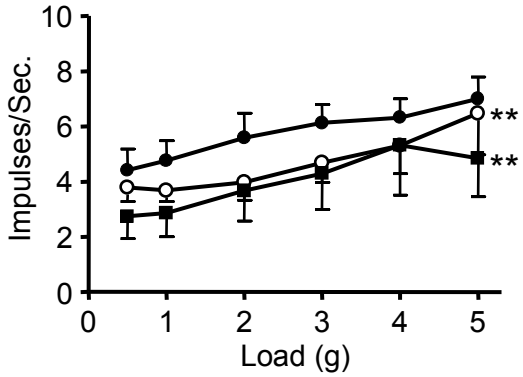
12
8
4
0
10
50
200
1000
von Frey hair (mg)
*
**
***
C
D
i)
ii)
Instant. Freq. (Hz)
120
0
0.1mV
0.2mV
0.5s

Figure 3.

The effect of galanin on mouse gastro-oesophageal vagal mucosal receptor

Group data for stimulus-response functions from GalR1 +/+ (A; n=7) and GalR1 -/- (B; n=8) mice of mucosal receptors to mucosal stroking with calibrated von Frey hairs. The responses are before (●) and after exposure to galanin (1(○), 3 (■) and 10 nM (□)). Galanin (1-100nM). Galanin significantly inhibited wild-type mouse mucosal receptor response to mucosal stroking however galanin had no statistically significant effect on GalR1-/- mouse mucosal receptors. Significant differences between treated and control (assessed using two-way ANOVA) are shown nearest each treated response curve. (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). C, original recording of a mucosal receptor response from wild-type mouse to mucosal stroking with a 200mg von Frey hair before (Ci) and during (Cii) exposure to galanin (10nM). D, original recording from a GalR1-/- mouse of a mucosal receptor response to mucosal stroking with a 200mg von Frey hair before (Di) and during (Dii) exposure to galanin (10nM)

A


Impulses/Sec.
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B

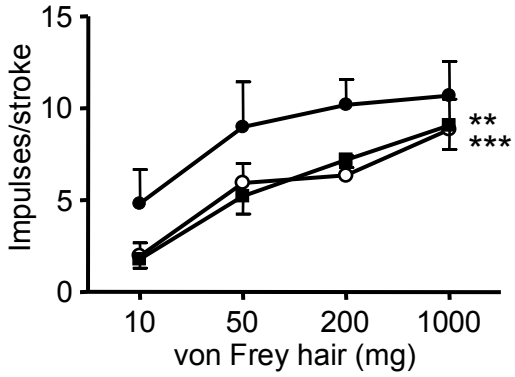
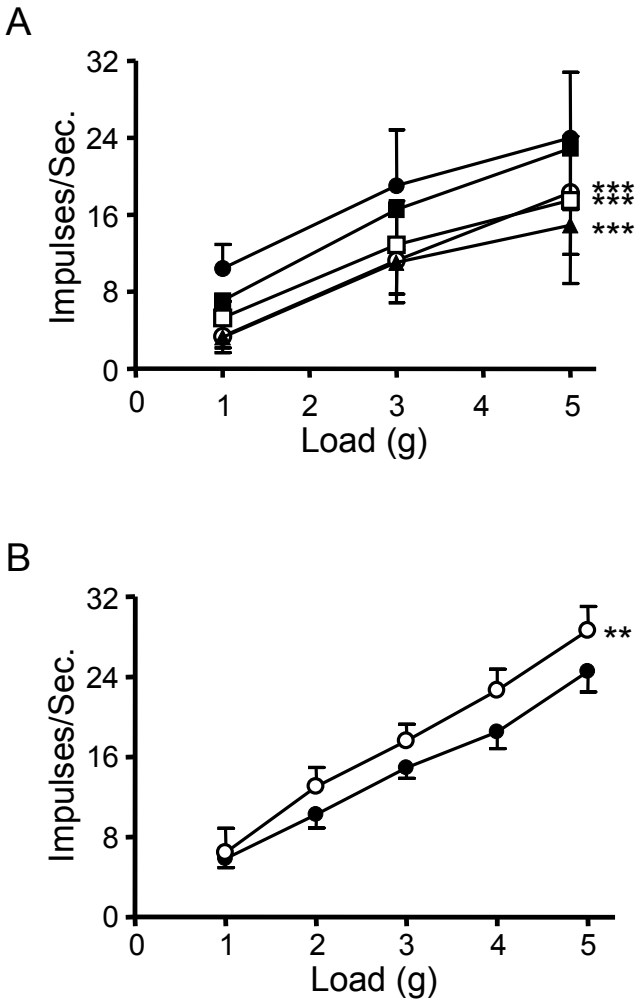

Impulses/stroke
15
10
5
0
10
50
200
1000
von Frey hair (mg)
**
***

Figure 4.

The effect of galanin and SNAP 37889 on the mechanosensitivity of gastro-oesophageal vagal afferents

Stimulus-response functions of tension (A; n=5) and mucosal (B, n=5) receptors to circumferential tension and mucosal stroking. The responses before galanin (●), after exposure to galanin (○; A, 1 nM; B, 10 nM) and after exposure to galanin and SNAP 37889 (■; A, 10 nM; B, 100nM). Significant differences between treated and control (assessed using two-way ANOVA) are shown nearest each treated response curve. (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). Significant inhibition of mechanical sensitivity of tension sensitive afferents by galanin was observed, however SNAP-37889 did not have any influence on reversing the inhibitory effect originally observed by galanin.


A
32
24
16
8
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Impulses/Sec.
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***
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32
24
16
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Impulses/Sec.
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3
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Load (g)
**

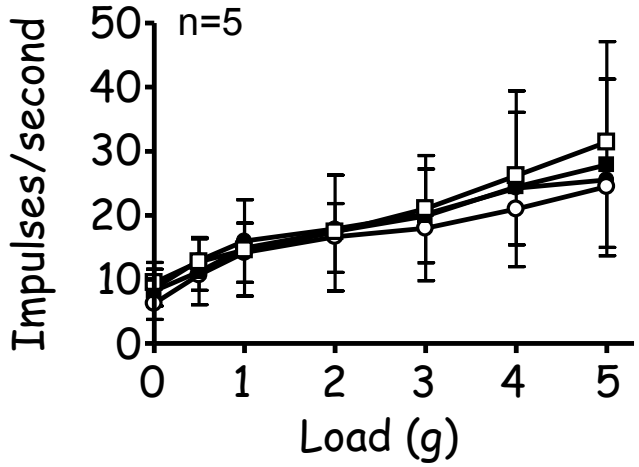
Figure 5.

The effect of galanin and AR-M961 on the mechanosensitivity of gastro-oesophageal vagal tension receptors

The effects of the selective GalR1 and GalR2 agonist AR-M961 are shown on the responses to circular tension in tension sensitive afferents of GalR1 +/+ (A; n=5) and GalR1-/- (B; n=6) mice. A, the responses of wild-type tension receptors before (●), after exposure to galanin (○; 100nM) after washout of galanin (■), after exposure to AR-M961 (▲; 100nM). Significant inhibition of mechanical sensitivity of tension sensitive afferents by galanin was observed, galanin was then completely washed out and the effect of AR-M961 was observed. AR-M961 (10-100nM) dose dependently and significantly inhibited mechanical sensitivity to circular stretch (average impulses s^{-1} over stimulus response of 1 min) of tension receptors when compared to both control and washout.

B, the responses of GalR1-/- tension receptors before (●) and after exposure to AR-M961 (○; 100nM). Significant differences between treated and control (assessed using two-way ANOVA) are shown nearest each treated response curve. (*$p<0.05$, **$p<0.001$, ***$p<0.0001$). AR-M961 (100nM) significantly potentiated the mechanical sensitivity of GalR1-/- tension sensitive afferents to circular stretch. Whilst the effect was not dramatic it was very reproducible.

A) Tension receptor



B) Mucosal receptor

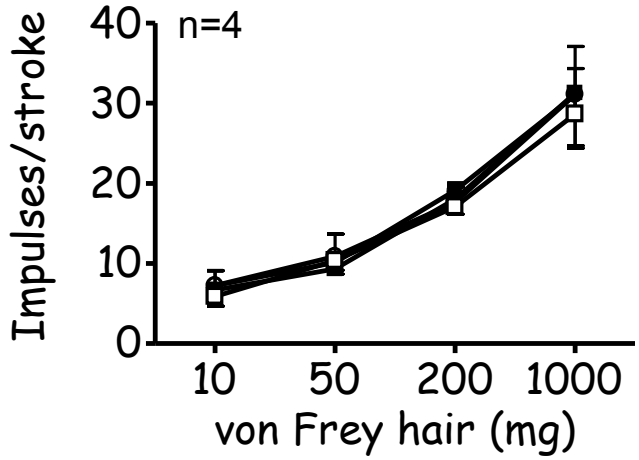


Figure 6.

Time control experiments were preformed and showed there was no significant change in mechanical responses over a comparable time period. A, the response of C57BL/6 mice tension receptor response to circular stretch (0.5-5g; n=5) at time 0; control (●), and at 20 min (○), 40 min (■) and 60 min (□) after control. B, the response of C57BL/6 mice mucosal receptors to mucosal stroking with calibrated von Frey hairs (10-1000mg; n=4) at time 0; control (●), and at 20 min (○), 40 min (■) and 60 min (□) after control. Two-way ANOVA found there was no statistical difference between time control groups and control.

CHAPTER 2: Potentiation of Vagal Afferent Mechanosensitivity by Ionotropic and Metabotropic Glutamate Receptors

SUMMARY

Glutamate exerts its effects at central synapses via ionotropic (iGluR-NMDA, AMPA & Kainate) and metabotropic (mGluR) receptors. Group I mGluR are coupled to excitatory, whilst group II and III mGluR are coupled to inhibitory mechanisms, but more importantly to the current investigation, inhibitory modulation of gastro-oesophageal vagal afferent sensitivity. Here we identified an opposing excitatory role for GluR in the modulation of gastro-oesophageal vagal afferent mechanosensitivity, and described the specific subunit expression of receptor subtypes in the nodose ganglion. The response of mouse gastro-oesophageal vagal afferent endings to mechanical stimuli was examined and the effect of agonists and antagonists to GluR was determined. Two types of vagal afferents were tested: tension receptors responding to circumferential stretch and mucosal receptors which responded only to mucosal stroking. The selective iGluR agonists AMPA and NMDA concentration dependently potentiated afferent responses. Their corresponding antagonists NBQX and AP-5 respectively, alone attenuated mechanosensitivity responses as did the non- selective antagonist Kynurenate. However the Kainate selective agonist had little effect on mechanosensitivity, and its antagonist UBP 302 was ineffective also. The mGluR5 antagonist MTEP concentration dependently inhibited mechanosensitivity. Efficacy of agonists and antagonist for the various receptor sub-types differed on mucosal and tension receptors. We conclude that NMDA, AMPA, and mGluR5 are coupled to excitatory modulation of afferent mechanosensitivity, and the role of each differs according to receptor subtypes. This data was reinforced by the finding of complete

complement of subtypes of all receptors located in the nodose ganglion, therefore implicating each receptor as a possible candidate for neuromodulation.

***I would like to acknowledge Dr Amanda Page who contributed the MTEP electrophysiological data used in this chapter.**

INTRODUCTION

Glutamate is the major excitatory neurotransmitter mediating synaptic transmission and modulation at pre- and post-central nervous system (CNS) synapses. Vagal afferent transmission from peripheral sites to the CNS is largely glutamatergic, and is tightly regulated pre-synaptically via a number of glutamate receptors [241-246]. The range of glutamate receptors in the CNS includes ionotropic (NMDA, AMPA, Kainate) receptors (iGluR), which are ligand gated ion channels [247], and metabotropic (group I, II, III) receptors (mGluR), which are G-protein coupled receptors [248]. Metabotropic GluR exist as eight different subtypes: group I mGluR (mGluR 1 and 5) exerting their effect through activation of phospholipase C, whilst Group II (mGluR 2 & 3), and group III (mGluR 4,6 ,7, 8) are coupled to inhibition of adenylate cyclase causing altered calcium and potassium current and slow hyperpolarisation [248]. The three mGluR groups were initially designated based on similarities in their sequence and pharmacology. In addition to effects in the CNS, a role for modulation of peripheral vagal gastro-oesophageal afferents by group II and III mGluR has been demonstrated in ferret and rodent studies [121]. Inhibition of peripheral gastro-oesophageal vagal afferents was observed by glutamate and group II and III mGluR agonists and a selective group III antagonist caused potentiation of mechanosensitivity. This data indicates both exogenous and endogenous sources of glutamate are able to negatively modulate vagal afferents.

Inhibition of gastro-oesophageal vagal afferents has been previously demonstrated by $GABA_B$ [144, 146], importantly corresponding to associated effects on gastro-oesophageal reflux *in vivo* [202]. Triggering of transient lower oesophageal sphincter relaxations (TLOSR) by gastric distension was markedly attenuated by $GABA_B$ receptor agonists, which correlated with a reduction in the incidence of gastro-oesophageal acid reflux in ferrets, dogs, humans and gastro-oesophageal reflux patients [181, 184, 198, 202, 249, 250]. This has focused attention on peripheral vagal afferent endings as important potential targets for the treatment of reflux disease. Importantly, the inhibitory effect of group III mGluR agonists on vagal afferents *in vitro* was conserved across species [121], and is translated into inhibition of TLOSR's *in vivo* [122].

A number of important observations were made in the course of these studies on mGluR. Firstly the effect of glutamate on vagal afferents was not seen unless the effects of iGluR was blocked, suggesting an excitatory (iGluR and mGluR) and inhibitory (mGluR) balance. Secondly, a group I (mGluR 5) antagonist potently inhibited transient lower oesophageal sphincter relaxations, however a central or peripheral mechanism for this effect remains unclear. Therefore, whilst gaining an understanding of inhibitory mechanisms of regulation of vagal afferents by mGluR, there remained major un-investigated possibilities regarding an excitatory balance by mGluRs and/or iGluRs.

Several lines of evidence exist that implicate a role for iGluRs in the mediation of the excitatory glutamatergic modulation of vagal afferent mechanosensitivity. Morphological evidence demonstrates iGluR subtype expression in nodose ganglia as well as peripheral sensory afferent neurones innervating the viscera of several species [120, 137, 138]. In addition, functional *in*

vitro studies have demonstrated an NMDA receptor antagonist to cause a reduction in afferent response to colorectal distension [141], and corresponding effects observed *in vivo* in behavioural studies. Importantly, *in vitro* rat studies have identified iGluR to play a modulatory role in vagal afferent mechanosensitivity, whereby non-specific iGluR receptor antagonists dose dependently attenuated mechanosensory properties of vagal afferents to mechanical stimulation [120], however precise contributions of select iGluR and the potential for involvement of excitatory group I mGluR remained undetermined.

Our aim in this study was to investigate systematically the iGluR that may be involved in the excitatory modulation of vagal afferents, and to determine the ability of mGluR 5 to have peripheral affects by influencing vagal afferent mechanosensitivity *in vitro*. We also sought to identify expression of iGluR and mGluR5 transcripts in the mouse nodose ganglion which contains the cell bodies of vagal afferents, and thus gain insight as to the subtypes that may be implicated in modulating afferent function.

MATERIALS AND METHODS

All studies were performed in accordance with the guidelines of the Animal Ethics Committees of the Royal Adelaide Hospital and Institute for Medical and Veterinary Science, Adelaide, Australia. These committees followed the Australian code of practice for the care and use of animals for scientific purposes, 6th edition 1997. Every attempt was made to minimise the number of animals required and to minimise their suffering.

Nodose Ganglia Dissection and RNA extraction for RT-PCR and Quantitative RT-PCR

Nodose ganglia were removed from five male or female C57 mice (20-30g). Nodose ganglia were pooled in RNAlater® (QIAGEN, NSW, Australia) at -20°C for subsequent RNA extraction. RNA isolation involved homogenisation of the nodose ganglia with a sterile glass pestle, after being snap frozen using liquid nitrogen. A total of 1mL of TRIzol reagent (Invitrogen, Vic, Australia) was added before the homogenized material was added to Qiashredders and centrifuged (12,000 rpm) at room temperature. Qiashredders were removed and tubes containing homogenate were capped, vortexed, and allowed to stand at room temperature for 15 minutes in order to promote dissociation of nucleoprotein complexes. After which 0.2 mL of chloroform was added, tubes were vortexed and left to stand at room temperature for a further 15 minutes. The samples were then cold centrifuged (12,00 rpm, 4°C) for 15 minutes and the upper aqueous layer (containing RNA substrate) was removed and transferred to a sterile eppendorf tube followed by isopropanol precipitation of RNA, by which 0.5 ml isopropanol was added cold centrifuged for 10min (38, 47). The upper aqueous phase was discarded leaving an RNA pellet to which 1 mL of 75% ethanol was added. A 1 min vortex and 4 min cold centrifuge and then supernatant was removed with the pellet being re-suspended in 100µL RNAase free sterile water and heated for 2 min. at 60°C. RNA was separated into 5µL aliquots and stored at -80°C

RNA quantification was determined by measuring the absorbance at 260nm (A260) using a spectrophotometer (Biorad, Sydney, Australia). RNA quality was estimated by the A260: A280 nm ratio.

RT-PCR analysis of iGluR and mGluR subunit expression in mouse nodose ganglia

Reverse transcription (RT) and polymerase chain reaction (PCR) was performed using a Qiagen® one-step RT-PCR kit, with primers used as indicated in Table 1. Previously published primers were used but were tested for specificity using NCBI's BLAST software. RT-PCR master-mix was prepared as follows: for each reaction 25μL RT-PCR Master Mix, 10μL RNAse free water, 10μL of 5x QIAGEN One-Step RT-PCR Buffer, 2μL dNTP Mix (containing 10 mM of each dNTP), 2μL QIAGEN One-Step RT-PCR Enzyme Mix and), 1μL of RNAse inhibitor (Ambion).

RT–PCR reactions were performed using an alpha unit block for PTC DNA engine systems (MJ Research) attached to a PTC-200 Peltier thermal cycler (MJ Research). The following amplification program was applied: Reverse Transcription: 50°C for 30mins, Initial PCR activation: 95°C for 15 mins, PCR cycles: denaturing: 94°C for 1 min, annealing: 47°C for 1 min and extension: 72°C for 1 min repeated for 40 cycles followed by a final extension step of 72°C for 10 min.

For each reaction 25μL of Master Mix was added to 0.2mL clear thin walled PCR tubes (Axygen) with 10μL (3μM) forward primer (for a final concentration of 0.6 μM), 10μL (3μM) backward primer (for a final concentration of 0.6μM) and 5μL of 50ng template RNA. For each reaction, RNA template was substituted with 5μL distilled RNA-free water as controls. Amplified products (5μL PCR product/2μL loading buffer) were resolved by 1.5-3% agarose gel electrophoresis and visualised by ethidium bromide staining.

In Vitro mouse gastro-oesophageal afferent preparation

Male and female C57/BL6 mice (20-30g, 7-10 weeks) were killed by CO_2 inhalation. The stomach, oesophagus and attached vagus nerves were removed and placed in a modified Krebs' solution of the following composition (mM): 118.1 NaCl, 4.7 KCl, 25.1 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$.7H2O, 1.5 $CaCl_2$, 1.0 Citric acid and 11.1 glucose, bubbled with 95% O_2-5% CO_2.

The preparation temperature was maintained at 4°C during dissection to preserve tissue integrity and prevent metabolic degradation. The peripheral organs, major blood vessels and diaphragm were removed by further dissection, leaving the stomach oesophagus and attached vagus nerves intact. The preparation was opened longitudinally and pinned out flat mucosa side up in a Perspex organ chamber (dimension 6 x 2.5 x 1.2 cm) lined with Sylgard® (Dow Corning) and perfused at a rate of 12mL/min with carbogenated Krebs' bicarbonate buffer solution maintained at 34°C. The vagus nerves were pulled into a separate isolate recording chamber which was filled with paraffin oil. The dimensions of the second chamber are 3.7 cm diameter and 1.2 cm deep. Under a dissecting microscope, the vagus sheath was removed exposing the underlying nerve fibres, which were teased using fine forceps into 16-24 bundles and one by one placed on platinum recording electrodes. A reference electrode rested on the mirror surface in the bath in a small pool of Krebs'. Nifedipine (1μM) was also added to the Krebs superfusate in order to prevent smooth muscle contraction. Previous preliminary studies have demonstrated nifedipine to have no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents[148].

Characterisation of gastro-oesophageal vagal afferent properties

Corresponding receptive fields of all types of vagal afferent were located by mechanical stimulation throughout the preparation with a brush and then more accurately with a von Frey hair. Accurate quantification of mechanical responses was performed differently according to the primary adequate stimulus for the type of fibre as has been described previously [46, 148, 157, 158]. The mechanical sensitivity of afferents was characterised by mucosal stroking with calibrated von Frey hairs (10mg-1000mg) and applied circular tension (0.5-7g).

Two types of mechanosensitive afferent were studied, those responding to mucosal stroking but not to circular tension (mucosal receptors) and those responding to mucosal stroking and circular tension (tension receptors) as reported previously [46].

Mechanical thresholds of all types of fibre were determined using calibrated von Frey hairs. The most reproducible, stimulus dependent response of these afferents to mucosal stimuli was evoked when the probe was moved at a rate of $5mms^{-1}$ across the receptive field rather than being stationary. A single test at each intensity of calibrated von Frey hair is prone to error due to such small sizes of receptive field, we therefore minimised experimental error by measuring mean response to 10 standard strokes given at 1 second intervals, omitting the first and last from analysis because of increased associated error. A bent von Frey hair (10-1000mg) stroking over the preparation meant that an even force was distributed over the receptive field during stroking stimulus. This protocol was found to give reproducible data and was therefore used to assess the effects of agonists and antagonists of iGluR and mGluR on vagal afferents.

Tension response curves were also obtained for afferent fibres responsive to circular tension. Tension receptors also responded to von Frey hair stroking, however were distinguished from mucosal receptors by their clear responsiveness to circular tension which was slowly adapting. Tension stimuli was used in combination with von Frey hair stroking to determine whether the receptive fields of fibres were located in the mucosa, the muscle layer or both.

Tension stimuli were applied via a thread attached to an unpinned point adjacent to the receptive field. The thread was attached to a cantilever and pulley system close to the preparation, with reference standard weights applied to the opposite side of the cantilever. Each weight was applied in a step wise manner and maintained for duration of 1 minute, with a 1 minute rest between weight applications. The response was measured as the mean discharge evoked, and subsequent responses during drug exposure are expressed as the percentage of the maximum control response (without drug addition), so that increases and decreases in responsiveness may be directly compared. The tension response curves were produced by applying weights to the cantilever system in a range of (1-5g) as previously described was sufficient to evoke a maximum response without tissue damage.

Effect of GluR agonists and antagonists on mechanosensitivity of vagal afferents

After the mechanical sensitivity of gastro-oesophageal vagal afferents had been established under control conditions, the effects of various agonists and antagonists on mechanical sensitivity were determined. Kynurenate (0.1–1μM) was added to the superfusing Krebs' solution and was allowed to equilibrate for 20

minutes in order to ensure complete penetration of the drug into all layers of the tissue. Tension response and stroke stimulus response curves were then re-determined. The agonists NMDA (0.1– 1μM), AMPA (10–100μM), and SYM 2081 (1–10μM), were added to the superfusing solution. The antagonists AP-5 (1–10μM), NBQX (1–10μM), and UBP 302 (0.1–10μM) were added to a ring placed directly over the receptive field in the preparation. Concentrations used were based on previous published potencies [247, 251-254].

Prior to this work, 6 pilot studies were performed in which the non-selective iGluR antagonist Kynurenate was tested at concentrations varying between 10^{-7} – 10^{-4}M. These experiments demonstrated concentration dependent effects and ultimately complete abolition of vagal afferent discharge; ranges around the subsequent EC_{50} were chosen for this study.

Drug addition to the ring was followed by a twenty minute equilibration period before mechanical stimulus curves were once again established immediately after removal of the ring. An equilibration period was essential for complete penetration of the drug into all layers of tissue. Preliminary data has indicated that drug effects are observed for approximately 10 min after removal of drug. Time control experiments (n=9) were performed prior to the study and showed there to be no significant change in mechanical sensitivity responses over a comparable time period.

Five of nine experiments involving NMDA were performed in the absence of Nifedipine and all other experiments were performed in the presence of nifedipine (1μM), in order to limit the effects of drugs secondary to smooth muscle responses.

Three additional experiments were performed with suspended, circular orientated rings, to investigate length tension relationships. No change in these was seen with any of the agonists used at any concentration. Also no effect was seen on smooth muscle responses to electrical field stimulation in separate experiments (O'Callaghan, unpublished observations). Statistical analysis of mechanical stimulus response curves was by two-way ANOVA. Results were considered to be significant where $p < 0.05$.

Data Recording and Analysis

Afferent impulses were amplified with a biological amplifier (world Precision Instruments, Sarasota, FL, DAM 50) and scaling amplifier (JRAK, Melbourne, Australia), filtered (CWE Inc., Ardmore, PA band-pass filter-932) and monitored using an oscilloscope (DL 1200A; Yakogawa, Tokyo, Japan). Single unit action potentials were discriminated on the basis of size and shape using spike II software (Cambridge Electronic Design, Cambridge, UK).

Statistical Analysis: All data were recorded and analysed off-line using a personal computer. All data were expressed as mean ± SE with n = number of individual afferents in all instances. Statistical difference between stimulus response curves were evaluated using a two-way ANOVA performed using graphpad Prism (Version 3.00 for Windows, Graphpad Software, San Diego, California, USA). Differences were considered significant where $p < 0.05$.

Drugs

Stock solutions of all drugs were kept frozen (-80°C) and diluted to their final concentration in Krebs' solution on the day of the experiment.

NMDA, AP-5, NBQX, UBP-302 and SYM-2081, were all obtained from Astrazeneca (Astrazeneca, Molndal, Sweden). Kynurenate and AMPA were obtained from Sigma (Sigma, Castle Hill, NSW, Australia).

RESULTS

Expression of glutamate receptor subunits in vagal (nodose) cell bodies

We were able to identify the expression of a number of transcripts corresponding to iGluR subunits and mGluR in mouse nodose ganglia. RT-PCR and gel electrophoresis confirmed the presence of amplified products of predicted sizes generated by primers specific for all the NMDA receptor subunits (NMDAR1, NR2A, NR2B, NR2C, NR2D and NR3A; Figure 1A). RT-PCR also revealed the expression of all AMPA receptor subunits (GluR1, GluR2, GluR3, GluR4; Figure 1B), and all Kainate receptor subunits (KA1, GluR5, GluR6, GluR7; Figure 1C). Expression of mGluR5 was also evident (Figure 1D) in mouse nodose ganglion. Isolation of single bands of correct predicted transcript size was achieved by UV analysis of the gel. Where template RNA was substituted for sterile water, no evidence of amplified products was observed. Expression of the reference β-actin gene was observed in all cases using template RNA.

Electrophysiological Studies

A role of peripheral iGluR and mGluR in modulation of mechanosensitivity was determined by examining responses of single afferent fibres using an *in vitro* gastro-oesophageal vagal afferent preparation. The effects of specific agonists and antagonists directed at iGluR and mGluR are illustrated in Figures 2 – 6. Single afferent fibres were classified as either mucosal or tension depending on their

response to mechanical stimulation. All afferents had their corresponding receptive fields located either in the distal oesophagus or on the oesophageal sphincter. As previously described mucosal receptors are distinct from tension receptors in their response to circular tension where mucosal receptors are unresponsive, however both types of afferents respond to mechanical stimulus by mucosal stroking with calibrated von Frey hairs (10 – 1000mg) in a graded manner [46]. Tension receptors elicited a graded increase in firing of action potentials in response to applied load (1-5g).

Effects of non-selective iGluR blockade on vagal afferents

The effect of Kynurenate on both mouse mucosal and tension receptors is illustrated in Figure 2. Kynurenate (0.1–1μM) was shown to significantly attenuate both mucosal and tension receptors sensitivity to mechanical stimulus in a concentration dependent manner with maximal effect at (1μM). These data prompted further investigation of the role of endogenous glutamate at subtypes of ionotropic glutamate receptors.

Effects of NMDA receptor ligands on vagal afferents

The effect of NMDA receptor agonist, NMDA, on mouse mucosal and tension receptor responses to mechanical stimulation is illustrated in Figure 3 A & B. Whilst a trend of potentiation of mechanical sensitivity was observed, NMDA (0.1-1μM) did not significantly alter the mechanical sensitivity of tension receptors to applied circular tension. Individual analysis of experiments revealed a clear increase in sensitivity of tension receptors where responses returned to original values after 10-20 minute wash-out of the drug from the preparation, possibly

suggesting sub-groups of responders and non-responders. These may be comparable to two populations of tension-sensitive fibres found in ferrets which showed differing pharmacology [121]. Effects of NMDA on responses to tension were similar to their responses to local stimulation by von Frey hair stroking in two additional experiments (Data not shown).

NMDA demonstrated significant potentiation of mechanical sensitivity of mucosal receptors response to mucosal stroking with calibrated von Frey hairs (Figure 3B; $p < 0.001$). These effects of NMDA were also reversible upon washout with normal Krebs solution. A subset of experiments observing the effects of NMDA on mechanical sensitivity of vagal afferents was performed in the absence of nifedipine however all other experiments were performed in the presence of nifedipine (1μM) in order to block smooth muscle contractions and therefore remove any effects of drugs on smooth muscle. The effects of NMDA were unaffected by this treatment indicating a direct effect on vagal nerve endings.

The effects of the competitive NMDA receptor antagonist AP-5 is illustrated in Figure 3C & D. AP-5 (1-10μM) significantly and dose dependently reduced the sensitivity of mucosal receptors to mucosal stroking with calibrated von Frey hairs ($p < 0.001$) and of tension receptors to circular tension ($p < 0.001$).

Effects of AMPA/Kainate receptor ligands on vagal afferents

The effect of AMPA, the selective AMPA receptor agonist, on mouse mucosal and tension receptors is illustrated in Figure 4 A and B. AMPA (10-100μM) demonstrated significant and concentration dependent potentiation of mechanical sensitivity of tension receptors response to circular tension ($p < 0.001$), whilst also significantly increasing the effect of mucosal receptors response to

mucosal stroking by calibrated von Frey hairs ($p < 0.001$), in a similar dose dependent manner. These effects of AMPA were shown to be washed out over an equilibration period with discharge returning to near baseline levels.

Selective Kainate receptor agonist SYM-2081 at concentrations that had been previously effective in other systems (1-10μM) [252], was shown to have no significant effect on mechanical sensitivity of tension receptors response to circular tension nor mucosal receptors response to mucosal stroking, however there was some indication of slight potentiation of mucosal receptors to mucosal stroking. The kainite receptor antagonist UBP 302 (0.1-10μM) also failed to have any significant effect on mechanical sensitivity of either mucosal nor tension receptors sensitivity to mechanical stimulation (Figure 5C and D). The AMPA/Kainate receptor antagonist NBQX (1-10nM; Figure 4C and D) was shown to significantly attenuate sensitivity of tension response to circular tension ($p < 0.0001$), but failed to have a significant effect on mucosal receptors sensitivity to mucosal stroking.

Effects of mGluR5 antagonist on vagal afferents

The effects of the selective mGluR5 antagonist, MTEP on mouse mucosal and tension receptors is illustrated in Figure 5 A and B. MTEP (1-30μM) significantly inhibited sensitivity of mucosal receptors to mucosal stroking (10 - 1000mg; $p<0.01$ at 30μM). MTEP (1-30μM) also significantly inhibited mechanical sensitivity of tension receptors to circular tension in a concentration-dependent manner ($p<0.0001$ for all concentrations). Potency of MTEP on mucosal receptors appeared less than on tension receptors as significance was only seen at (30μM, $p<0.01$), the highest concentration applied.

DISCUSSION

This current investigation demonstrates a number of significant advances. The first is the demonstration that NMDA, AMPA and mGluR 5 are involved in, and are coupled to excitatory modulation of peripheral gastro-oesophageal vagal afferent mechanosensitivity. These findings complement the established effects glutamate in the synaptic transfer of chemo- and mechanosensitive input from peripheral gastro-oesophageal vagal afferents to the CNS.

Secondly, in addition to potentiation of mechanosensitivity by iGluR agonists, a role for iGluR in endogenous glutamatergic modulation at a peripheral site is evidenced by attenuation of mechanosensitivity by several iGluR antagonists.

Third, we have previously demonstrated glutamate to play an inhibitory role via group III mGluR [121]. The current study demonstrates there to be a "counter-balance" of excitatory and inhibitory effects of glutamate where by specific iGluR (AMPA and NMDA) and mGluR (mGluR 5) oppose the effects of inhibitory group III mGluRs.

Fourth, whilst this study clearly demonstrates involvement of NMDA, AMPA and mGluR 5 receptors in glutamatergic modulation of vagal afferent sensitivity, there was no observable effect of a specific kainite receptor agonist or antagonist, indicating no functional involvement of kainite receptors on gastro-oesophageal vagal afferent mechanosensitivity. However, receptor subtype mRNA expression was observed in the nodose ganglion indicating that receptor expression does not always correlate with a functional role.

Fifth, our data demonstrates each receptor subtype to have a slightly different functional role on the two different subtypes of primary afferents.

Sixth, these data indicate that at least part of the effect of MTEP to cause inhibition of transient lower oesophageal sphincter relaxations in response to gastric distension may be mediated peripherally. This may correspond to a peripheral target for the therapeutic treatment of gastro-oesophageal reflux disease.

Finally these results suggest a similar role for glutamate in vagal afferent transmission similar to that in spinal transmission where it is evident that they may underlie behavioural change in response to colonic distension. Each of the above concepts is addressed below.

Peripheral role for excitatory glutamate receptors

Excitatory glutamate receptors are expressed in the caudal brainstem, and have been shown to play a specific role in transmission from peripheral gastro-oesophageal afferents to the NTS, particularly implicated in signalling of gastric distension, swallowing, transient lower oesophageal sphincter relaxations, emetic signalling and satiety [269-272]. Whilst establishing an important role for these receptors in peripheral vagal afferent modulation, in addition to their involvement in central transmission, these findings further add to and complement previous findings in rat and ferret studies, that demonstrate an excitatory role for glutamate receptors on vagal afferents [137, 273]. In contrast, *in vitro* investigations in the guinea pig have been unable to identify functional glutamate and GABA receptors on afferent endings. [30, 255].

Previous studies have demonstrated expression of iGluR and mGluR subtypes in vagal afferent cell bodies of several species, with these studies in addition demonstrating bi-directional axonal transport of iGluR and mGluR on peripheral and central branches of the vagus nerve [121, 256-260], implicating a

peripheral role for these receptors. Glutamate receptors are also expressed and function in peripheral terminals of sensory spinal afferents innervating the skin [276-279], and may be involved in hyper-algesia associated with inflammation [280, 281]. NMDA receptors have been implicated in signalling painful distension of the colon, as peripheral administration of an ionotropic receptor antagonist, memantine, caused a reduction in afferent and behavioural responses to colorectal distension [139]. However memantine is a non-selective ligand and may have effects other than at NMDA receptors.

Positive and negative modulation of mechanosensitivity by glutamate

Our previous *in vitro* studies in ferrets identified a possible counter-balance of excitatory and inhibitory glutamatergic signalling from peripheral vagal afferents whereby glutamate acts simultaneously at iGluR and mGluR [121]. The potent inhibitory effect of glutamate, an effect mimicked by selective group II and III agonists, on vagal afferent mechanosensitivity, was only revealed after the addition of the non-specific iGluR antagonist kynurenate to the preparation. *In vivo* observations in the rat indicated similarly, that iGluR may be involved in excitatory modulation of vagal afferents [120], however which subtypes of iGluR were involved, and in addition, the potential for involvement of excitatory group I mGluR, remained undetermined. The current data clearly indicate a counter balance of excitatory modulation of vagal afferent mechanosensitivity by iGluR and mGluR 5, and inhibitory modulation by group II and III mGluR. More specifically, agonist and antagonist data of both AMPA and NMDA receptors indicate these two receptors to play a major role in excitatory balance of modulation of

mechanosensitivity with kainite receptors having little if any effect under the current conditions.

Endogenous glutamate plays a role in modulation of vagal afferents

The inhibitory effects of exogenous iGluR and mGluR antagonists, particularly to NMDA, AMPA and mGluR5 receptors, indicate endogenous glutamate to exert an ongoing tonic stimulation of mechanosensitivity. The question arises as to the likely source of endogenous glutamate. Glutamate is detected in approximately 60% of nodose ganglion cells and on vagal afferent fibres originating from the nodose ganglion [261]. The vesicular glutamate transporter VGLUT2 is widespread in the enteric nervous system as well as being contained within peripheral endings of vagal afferents and is necessary for glutamate release [262, 263]. We therefore speculate vagal afferents themselves are a potential source, likely to release glutamate both centrally and peripherally. There are also separate sources of endogenous glutamate other than vagal afferents where glutamate is in close proximity with vagal afferents. Studies have shown that whilst almost 40% of ingested glutamate is pooled in skeletal muscle, a significant rise in circulating plasma and intraluminal glutamate may both represent candidate sources of glutamate causing the observed effects on vagal afferents [264]. An excess of endogenous glutamate may account for the lack of effect on mechanosensitivity of NMDA receptors of exogenous glutamate, in contrast to the potent inhibition of mechanosensitivity by a NMDA receptor antagonist. Excess endogenous glutamate may also provide an explanation for why a kainite receptor agonist had little effect, and why a group I mGluR agonist had no effect in our previous study [121]. On the

other hand, endogenous activation of AMPA receptors may be overcome by exogenous AMPA.

Glutamate receptor subtypes have varying effects on subtypes of primary afferents

Selective agonists and antagonists of both iGluR and mGluR5 receptors were effective on responses of both tension sensitive and mucosal receptors to mechanical stimulation. In general, ligands had highly potent effects on vagal afferents, however, discrepancies as to the effectiveness of ligands on specific afferent types differed. NMDA exerted a more potent modulation of mucosal receptors, whilst MTEPs effect on tension receptors was more powerful, suggesting NMDA receptors may be more highly expressed by mucosal receptor cell bodies, and mGluR5 receptors may be the predominant receptor expressed on tension receptors. However localization of receptors on individual afferent populations ending in different layers of the gut is yet to be investigated.

All Subtypes of GluR are expressed in the nodose ganglion

Expression of mRNA encoding all mGluR and some subtypes of iGluR in nodose ganglia has been reported previously in several species [121, 257, 259, 260]. This study confirms the expression of all iGluR and mGluR5 receptor subunits in mouse nodose ganglion, which from previous observations in the ferret we would expect to be transported to peripheral endings.

All NMDA receptors function as tetrameric structures composed of multiple subunits including an obligate NR1 subunit required for glycine occupation and trafficking of the heteromeric assembly to the plasma membrane [117].

Functionality of NMDA receptors depends on their permeability to Ca^{++}, affinity for glutamate as well as Mg^{+} block, all which are dictated by composition of receptor subunits and may be important in altering conductance of sensory neurones, and thus influence nociception and mechanosensitivity. NMDA receptors must contain at least one NR2, and may contain both NR2 and NR3 subunits to be functional [265, 266]. Sub-unit composition of NMDA receptors in sensory neurones has been previously described, those expressing NR1 subunits conjugated with NR2B subunits which are present in both A and C fibres, and those with NR1 conjugated with NR2D subunits, present only on A fibres. This study confirmed the expression of all subunits in vagal afferent cell bodies in the nodose ganglion, however the assembly of sub-units in individual vagal afferent neurones is beyond the scope of the current investigation.

AMPA receptors also likely function as tetramers and are composed of a four subunit family (GluR1-4). The GluR2 subunit plays a critical role in determination of the permeability of heteromeric receptors to calcium ions and thus, AMPA receptors lacking GluR2 are impermeable due to voltage dependent block of the ion channel [103]. Whilst all AMPA receptors subunits are not required for full function, previous studies of AMPA receptors in certain brain regions have found that AMPA receptors contain the full complement of receptor subunits [247].

All AMPA receptor subunits were found to be expressed in the nodose ganglion including GluR2, providing a consistent molecular correlate with functional evidence of the AMPA receptor agonist and antagonist data gained in this study. All subtypes of kainate receptors were found in the nodose ganglion, however we identified little evidence to suggest a functional role of this receptor in the modulation of mechanosensitivity of gastro-oesophageal vagal afferent endings.

It may be possible that kainate receptors play a role in other organs innervated by the vagus nerve or other regions of the gastro-intestinal tract. These results may also indicate that whilst a receptor is expressed in the nodose ganglion, their protein products may exhibit poor functional coupling on peripheral vagal afferent endings.

Actions on vagal afferents may underlie the potential therapeutic effects of mGluR5 antagonists in reflux disease

Transient lower oesophageal sphincter relaxations triggered by gastric distension are the most important underlying mechanism of gastro-oesophageal reflux in normal patients as well as patients with gastro-oesophageal reflux disease (GORD) [202, 267]. We have demonstrated an mGluR5 antagonist to inhibit transient LOS relaxations in response to gastric distension in conscious ferret and dog studies [122, 123]. This inhibition was correlated with inhibition of gastro-oesophageal reflux, highlighting the potential for therapeutic intervention with mGluR5 in the treatment of GORD. Given the importance of glutamatergic signalling at central synapses [122, 123], a peripheral mechanism of action is ideal to minimise central side effects of therapeutic intervention, however, previous findings have not determined whether the action of MTEP observed is mediated via a central or peripheral afferents, or both. The current data indicate that at least part of the effect is mediated peripherally by reducing sensitivity of gastro-oesophageal vagal afferents to distension. These results therefore provide good rationale for mGluR5 antagonists that are restricted to the peripheral compartment.

Roles for glutamate receptors in the vagal and spinal sensory system

Functional GluR are present throughout the CNS where they modulate neuronal excitability and synaptic transmission. Anatomical studies in skin have demonstrated the expression of GluR on afferent endings, including mGluR5 and NMDA receptors [265, 268-270]. Functional studies have demonstrated NMDA and mGluR5 to be involved in nociceptive primary afferent signalling. Afferent and behavioural studies demonstrate an effect of these receptors on sensory afferents whereby they were responsible for increased signalling in response to an algesic stimulus. This effect is reflected in our studies of primary vagal afferent which demonstrate similarly, excitatory coupling of primary afferent signalling in sensory neurones. A similar finding was made in the viscera, whereby the non-selective ionotropic antagonist memantine reduced afferent and behavioural responses to colorectal distension [271]. The functional role of iGluR receptor subtypes in spinal afferents is unclear, and also if there are opposing influences of positively- and negatively- coupled receptors. Therefore similarities between vagal and spinal afferents in the actions of glutamate are possible. An interesting difference between vagal and spinal afferents however, is the presence of VGLUT2 in vagal cell bodies and endings [262, 272], which was not observed in their dorsal root ganglion counterparts [273], which may in turn, be suggestive of a greater role for activation of glutamate auto-receptors on vagal afferents than on spinal afferents.

CONCLUSION

In conclusion, this study in conjunction with previous findings by our group, further clarifies the influence of all major GluR subtypes on vagal afferent mechanosensitivity. There is clearly scope for both excitatory and inhibitory

modulation of afferent sensitivity by glutamate from both exogenous and endogenous sources, providing a balance to achieve normal vagal afferent function. This balance is clearly possible to manipulate pharmacologically. How it may be altered in disease states we hope will be the subject of our continuing investigations.

Receptor	Sequence	Predicted Product size (bp)
GluR1	Fwd: GGACCACAGAGGAAGGCATGATC Rev: CAGTCCCAGCCCTCCAATC	358
GluR2	Fwd: TGTGTTTGTGAGGACTACGGCA Rev: GGATTCTTTGCCACCTTCATTC	226
GluR3	Fwd: GCAGAGCCATCTGTGTTTGTGAGTT Rev: AGTTTTGGGTGTTCTTTGTGAGTT	472
GluR4	Fwd: GCAGAGCCGTCTGTGTTCACTAG Rev: CGGCAAGGTTTACAGGAGTTCTT	220
GluR5	Fwd: GCCCCTCTCACCATCACGTAT Rev: TGGTCGATAGAGCCTTGGGCA	358
GluR6	Fwd: TTCCTGAATCCTCTCTCCCCT Rev: CACCAAATGCCTCCCACTATC	259
GluR7	Fwd: GCAGAGTCAGGCCTGCTGGA Rev: ACTCCACACCCCGACCTTCT	300
KA1	Fwd: CCCATCGAGTCTGTGGATGA Rev: CTGTGGTCCTCCTCCTTGGG	434
NMDAR1	Fwd: GCTGTACCTGCTGGACCGCT Rev: GCAGTGTAGGAAGCCACTATGATC	210
NR2A	Fwd: GCTACGGGCAGACAGAGAAG Rev: GTGGTTGTCATCTGGCTCAC	257
NR2B	Fwd: GCTACAACACCCACGAGAAGAG Rev: GAGAGGGTCCACGCTTTCC	314
NR2C	Fwd: AACCACACCTTCAGCAGCG Rev: GACTTCTTGCCCTTGGTGAG	464
NR2D	Fwd: CGATGGCGTCTGGAATGG Rev: AGATGAAAACTGTGACGGCG	265
NR3A	Fwd: CCGCGGGATGCCCTACTGTTC Rev: CCAGTTGTTCATGGTCAGGAT	417
mGluR5	Fwd: GTCCTTCTGTTGATCCTGTC Rev: ATGCAGCATGGCCTCCACTC	118
β-ACTIN	Fwd: ATCATGTTTGAGACCTTCAACAC Rev: TCTGCGCAAGTTAGGTTTTGTC	830

Table 1

Nucleotide primer sequences for RT-PCR identification of ionotropic receptor subunit and mGluR5 transcripts.

Figure 1

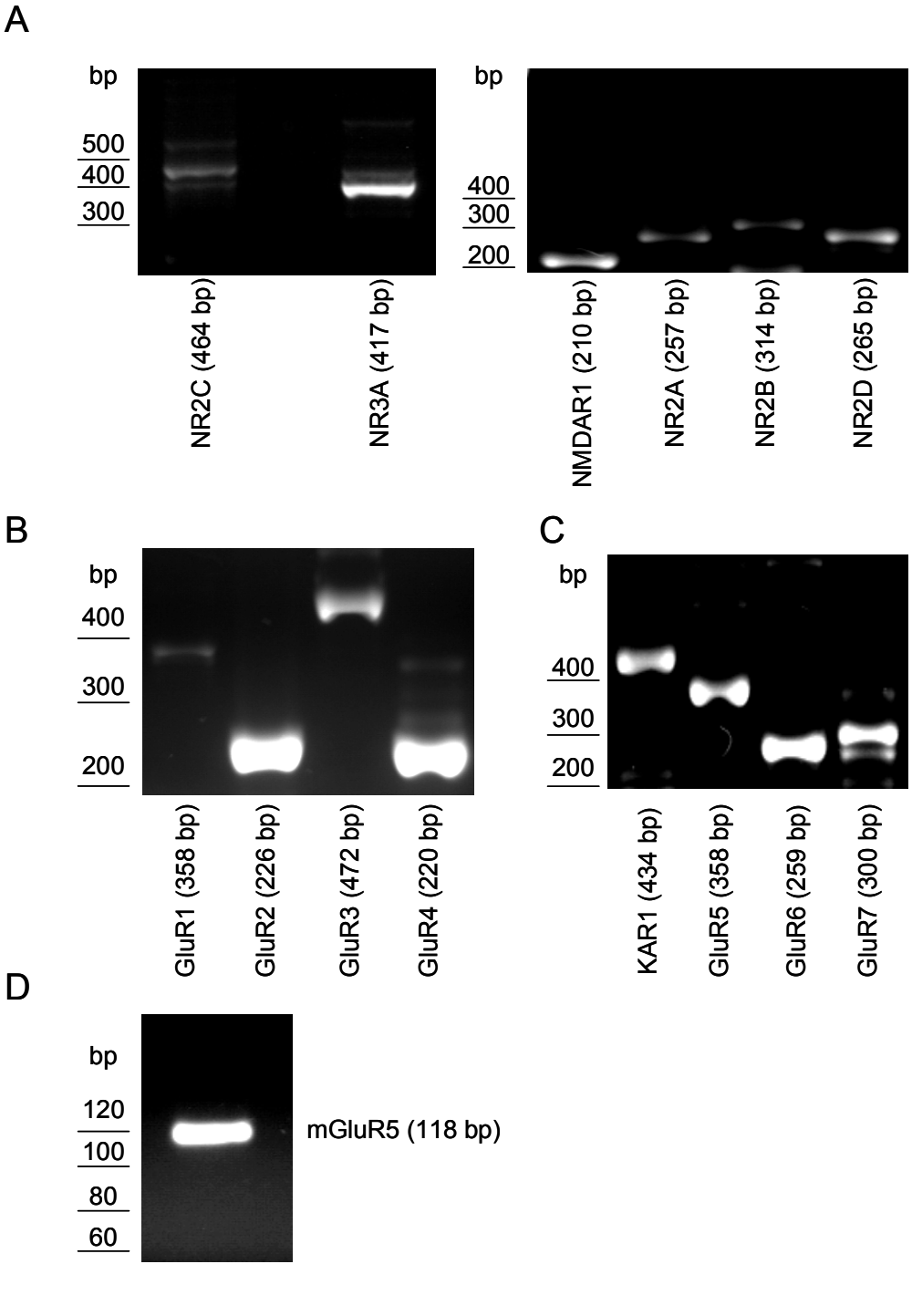

A
bp
500
400
300
NR2C (464 bp)
NR3A (417 bp)
bp
400
300
200
NMDAR1 (210 bp)
NR2A (257 bp)
NR2B (314 bp)
NR2D (265 bp)
B
bp
400
300
200
GluR1 (358 bp)
GluR2 (226 bp)
GluR3 (472 bp)
GluR4 (220 bp)
C
bp
400
300
200
KAR1 (434 bp)
GluR5 (358 bp)
GluR6 (259 bp)
GluR7 (300 bp)
D
bp
120
100
80
60
mGluR5 (118 bp)

Figure 1.

Detection by RT-PCR of iGluR and mGluR subunits in mouse nodose ganglion. A. All six NMDA receptor subunits (NMDAR1, NR2A-D, and NR3A) were detected with the correct product size for the target. B, All subunits of the AMPA receptor, GluR1-4, were detected, with strong signals for GluR2 and GluR4. C, All subunits of the kainate Receptor (KA1, GluR5-7) were detected, along with mGluR5 (D). See Table 1 for primers used to detect GluR subtypes.

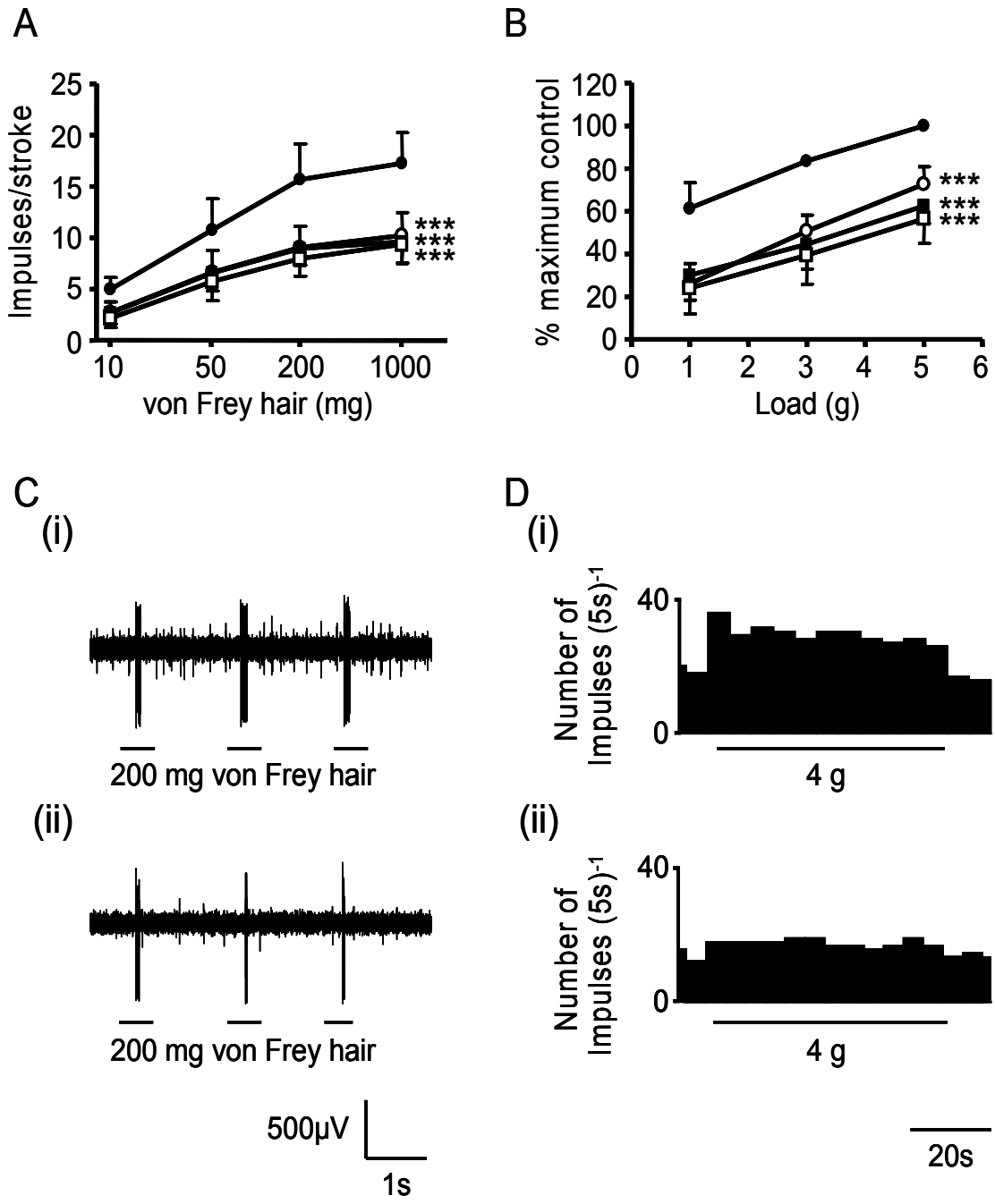

A
Impulses/stroke
25
20
15
10
5
0
10
50
200
1000
von Frey hair (mg)
***
***
***
B
% maximum control
120
100
80
60
40
20
0
0
1
2
3
4
5
6
Load (g)
***
***
***
C
(i)
200 mg von Frey hair
(ii)
200 mg von Frey hair
500μV
1s
D
(i)
Number of Impulses (5s)-1
40
0
4 g
(ii)
Number of Impulses (5s)-1
40
0
4 g
20s

Figure 2.

The effect of Kynurenate on mouse gastro-oesophageal vagal afferents.

Stimulus-response functions of mucosal (A, n=5) and tension receptors (B, n=6) to mucosal stroking and circumferential tension respectively. The responses are before (●) and after exposure to Kynurenate (0.1µM (○), 0.3µM (■) & 1µM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (***$p<0.001$). C, original recording of a mucosal receptor response to mucosal stroking with a 200mg von Frey hair before (C i) and after exposure to Kynurenate (1 µM, C ii). D, original recording of a tension receptor response to circumferential tension using a 4g weight before (D (i)) and after (D (ii)) exposure to Kynurenate (1 µM).

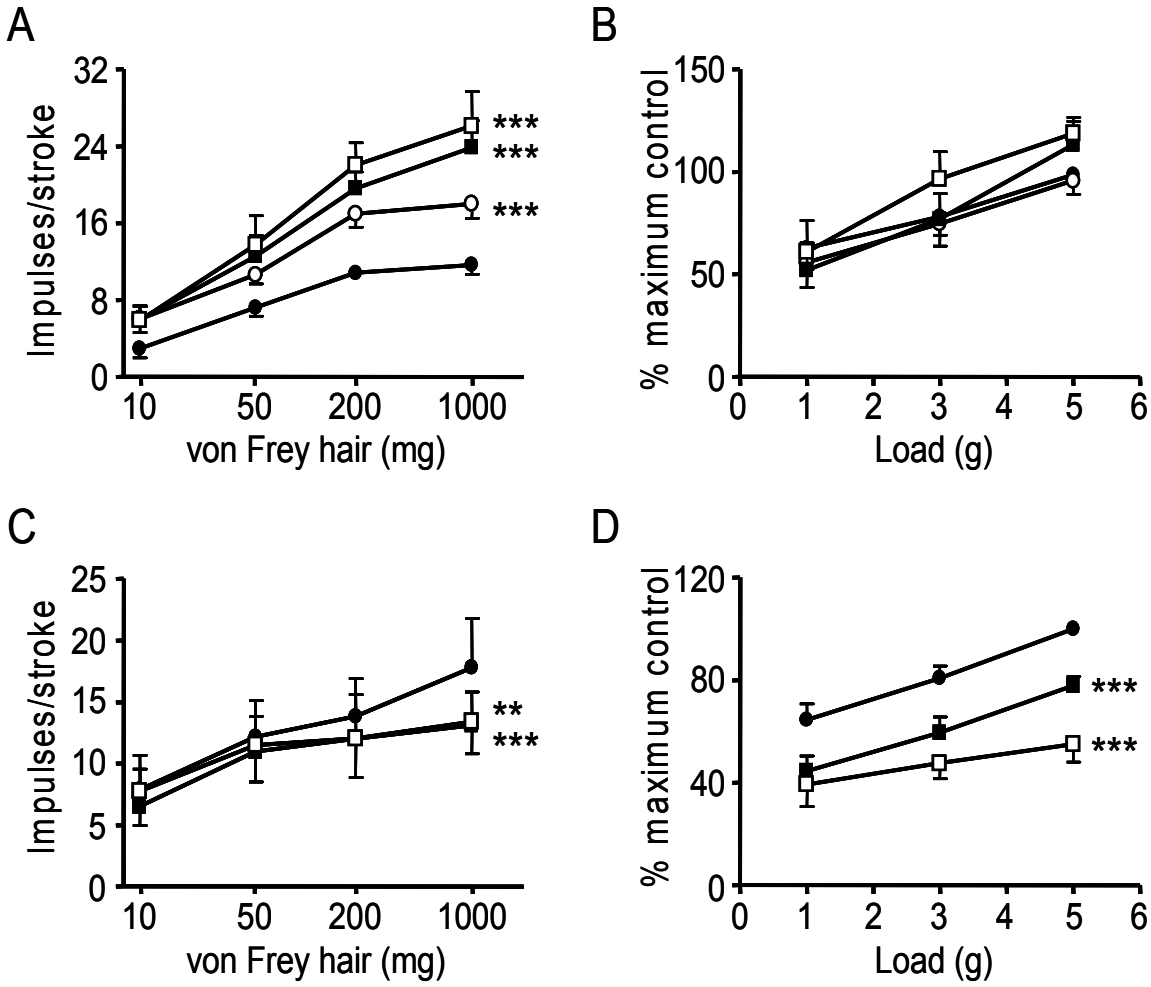

A
Impulses/stroke
32
24
16
8
0
10
50
200
1000
von Frey hair (mg)
***
***
***
B
% maximum control
150
100
50
0
0
1
2
3
4
5
6
Load (g)
C
Impulses/stroke
25
20
15
10
5
0
10
50
200
1000
von Frey hair (mg)
**
***
D
% maximum control
120
80
40
0
0
1
2
3
4
5
6
Load (g)
***
***

Figure 3.

The effect of NMDA and AP-5 on mouse gastro-oesophageal vagal afferents. Stimulus-response functions of mucosal (A, n=6) and tension receptors (B, n=9) to mucosal stroking and circumferential tension respectively. The responses are before (●) and after exposure to NMDA (0.1μM (○), 1μM (■) & 10μM (□)). Stimulus-response functions of mucosal (C, n=5) and tension receptors (D, n=6) before (●) and after exposure to AP-5 (1μM (■) & 10μM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (**p<0.01, ***p<0.001).

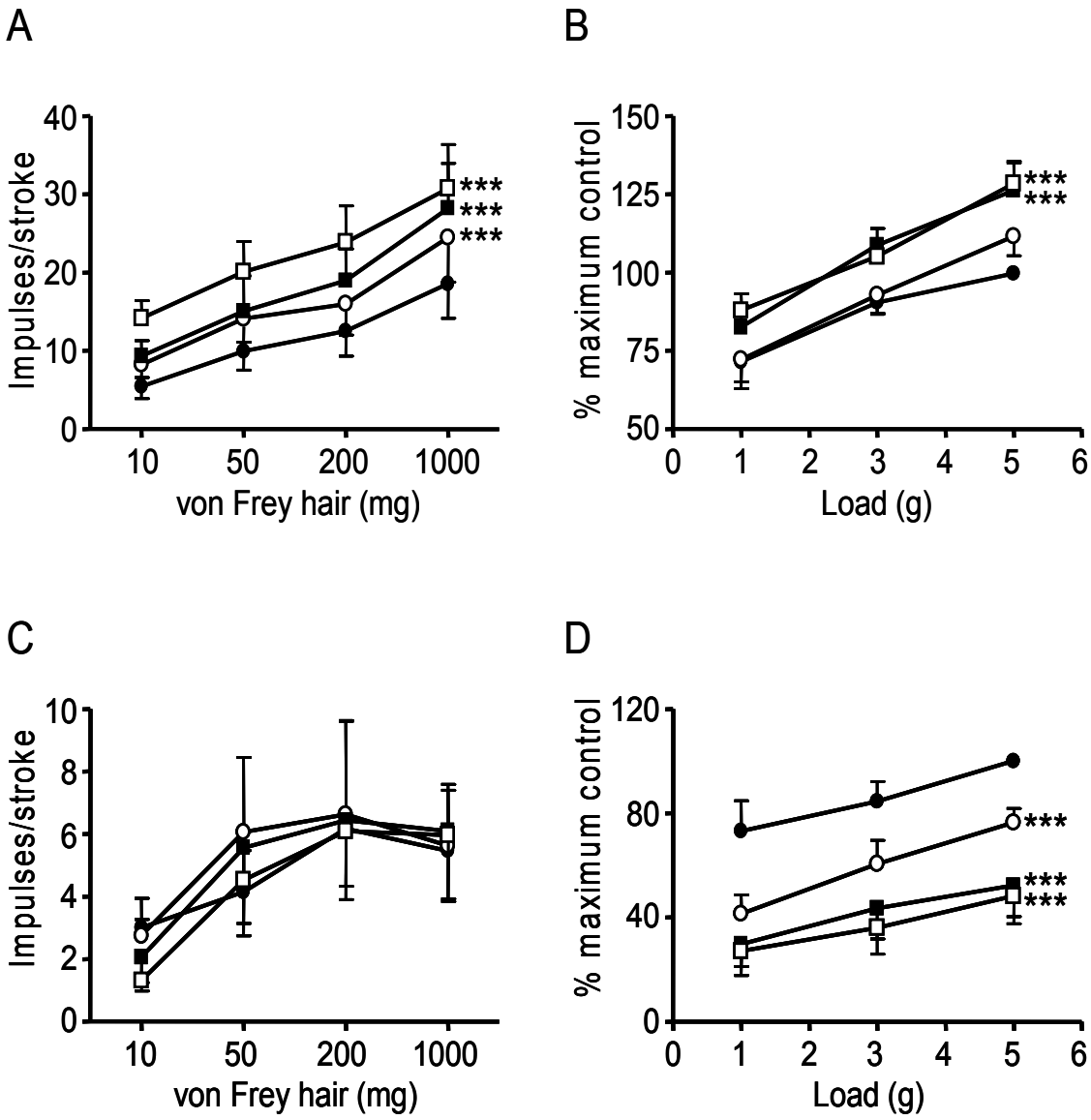

A
B
C
D
Impulses/stroke
% maximum control
von Frey hair (mg)
Load (g)
***
***
***
***
***
***
10
50
200
1000
0
1
2
3
4
5
6
0
10
20
30
40
50
75
100
125
150
0
2
4
6
8
10
0
40
80
120

Figure 4.

The effect of AMPA and NBQX on mouse gastro-oesophageal vagal afferents. Stimulus-response functions of mucosal (A, n=5) and tension receptors (A, n=7) to mucosal stroking and circumferential tension respectively. The responses are before (●) and after exposure to AMPA (10μM (○), 30μM (■) & 100μM (□)). Stimulus-response functions of mucosal (C, n=4) and tension receptors (D, n=5) before (●) and after exposure to NBQX (1nM (○), 3nM (■) & 10nM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (***$p<0.001$).

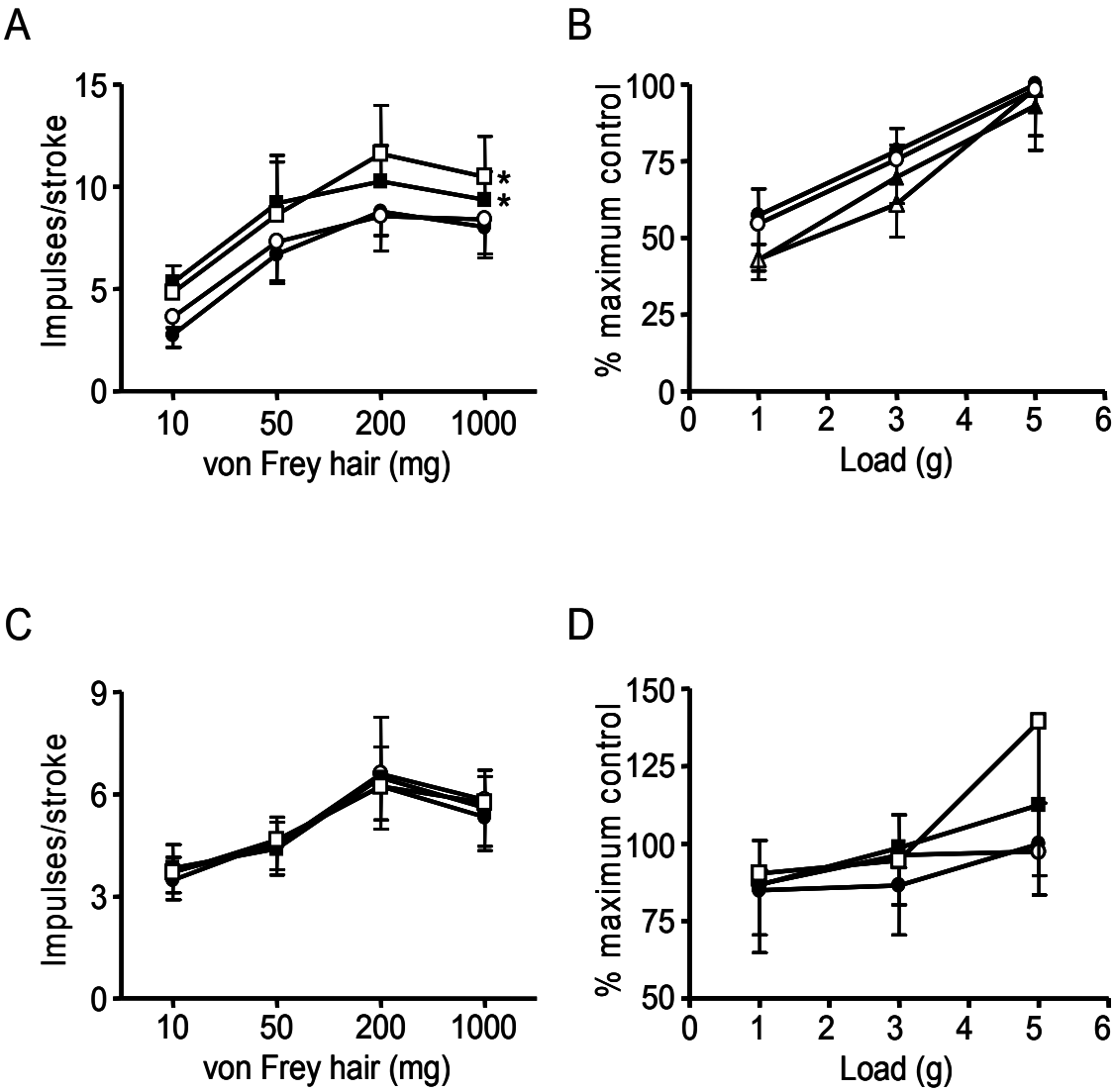

A
B
C
D
Impulses/stroke
von Frey hair (mg)
% maximum control
Load (g)
10
50
200
1000
0
1
2
3
4
5
6
*
*

Figure 5.

The effect of SYM 2081 and UBP 302 on mouse gastro-oesophageal vagal afferents.

Stimulus-response functions of mucosal (A, n=5) and tension receptors (B, n=5) to mucosal stroking and circumferential tension respectively. The responses are before (●) and after exposure to SYM 2081 (1μM (○), 3μM (■) & 10μM (□)). Stimulus-response functions of mucosal (C, n=5) and tension receptors (D, n=5) before (●) and after exposure to UBP 302 (0.1μM (○), 1μM (■) & 10μM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (***$p<0.05$).

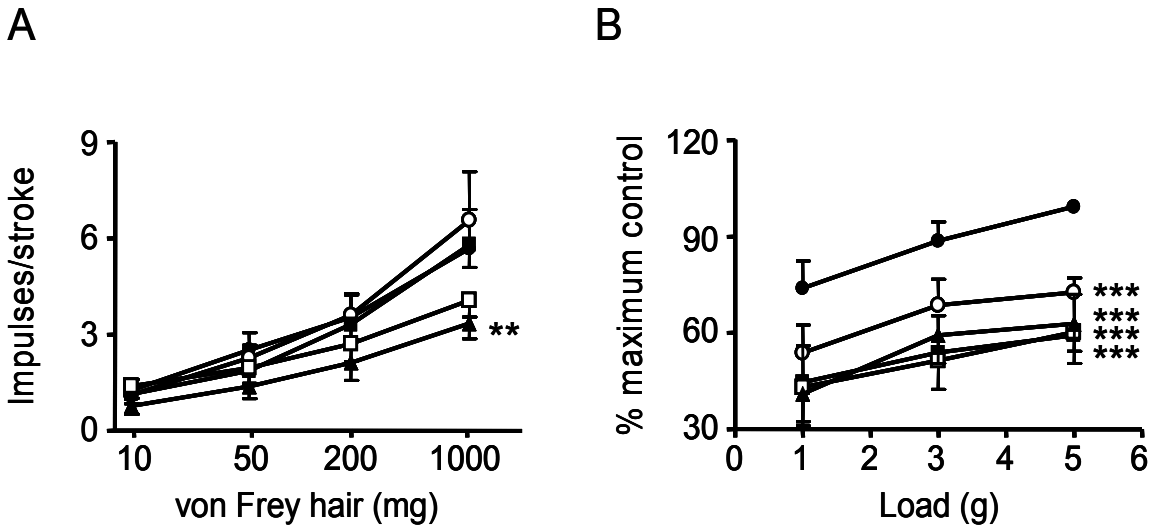

A
B
Impulses/stroke
9
6
3
0
10
50
200
1000
von Frey hair (mg)
**
% maximum control
120
90
60
30
0
1
2
3
4
5
6
Load (g)
***
***
***
***

Figure 6.

The effect of MTEP on mouse gastro-oesophageal vagal afferents.

Stimulus-response functions of mucosal (A; n=9) and tension receptors (B; n=5) to mucosal stroking and circumferential tension respectively. The responses are before (●) and after exposure to MTEP (1μM (○), 3μM (■), 10μM (□) & 30 μM (▲)). Asterisks indicate significant difference from control using a two-way ANOVA (**p<0.01; ***p<0.001).

CHAPTER 3: Ghrelin Selectively Reduces Mechanosensitivity of Upper Gastrointestinal Vagal Afferents

SUMMARY

Ghrelin, a circulating peptide, predominantly released from endocrine cells of the gastric fundus exerts its orexigenic effects via a vagal pathway. An *in vitro* preparation enabled the responses of gastro-oesophageal afferents to graded mechanical stimulation and the effect of ghrelin on these peripheral endings to be determined. Three types of vagal afferent were recorded from two animal models, namely the ferret and mouse, they include tension receptors responding to circumferential tension, mucosal receptors responding only to von Frey hair mucosal stroking, and a third afferent subtype specific to the ferret, tension/mucosal (TM) receptors located in the ferret oesophagus which responded to both stimuli. In the mouse, ghrelin significantly reduced the responses of tension receptors to circumferential tension ($p<0.005$; two-way ANOVA) by up to 40%, an effect reversed by a specific ghrelin receptor antagonist [D-Lys-3]-GHRP-6. Responses of mucosal receptors to mucosal stroking with calibrated von Frey hairs were however not significantly affected. In the ferret, ghrelin significantly reduced the responses of mucosal and TM receptors to mucosal stroking with calibrated von Frey hairs, however ghrelin did not have a significant effect to reduce the response to circumferential tension in both tension and TM receptors. RT-PCR analysis demonstrated the expression of both ghrelin and its receptor in vagal afferent cell bodies in mouse nodose ganglia. In conclusion, ghrelin was shown to selectively inhibit subpopulations of mechanically sensitive gastro-oesophageal vagal afferents, however its effects were not conserved across species. Expression of both peptide and its receptor indicates the potential for release from vagal afferents. These findings implicate a possible role for ghrelin in mediation of satiety signalling via vagal afferent mechanoreceptors.

***I would like to acknowledge Dr Amanda Page, Tracey O'Donnell, Camilla Dorian, Rhianna Laker and Catherine Milte who contributed electrophysiological ferret studies that were included in this chapter.**

INTRODUCTION

Originally identified for its role in growth hormone (GH) secretion, ghrelin is a 28 amino-acid acylated peptide found mainly in the stomach, and is an endogenous ligand for the growth hormone secretagogue receptor 1a (GHS-R1a) [274-276].

It is localised in distinct cells of the gastric mucosa, predominantly distributed in the mid portion of the oxyntic gland characterised by P/D1 granules in man and X/A like granules in rodents [275-278]. The extensive endocrine and extra-endocrine biological activities of ghrelin are evidenced by the widespread distribution of the peptide and expression of its receptor [275].

The ghrelin cells which synthesize round electron dense granules containing ghrelin proteins, represents the second most frequent endocrine cell type after the enterochromaffin-like cells in gastric oxyntic mucosa, indicating a relevant role in physiological processing at the level of the stomach [275, 279-282].

Ghrelin is predominantly concentrated in the upper gastrointestinal tract, but is still present distally where ghrelin concentration gradually diminishes form the duodenum to the colon [283].

Ghrelin is one of several brain-gut neuroenteric peptides that regulate food intake. To date, the most important role of ghrelin appears to be stimulation of appetite and regulation of energy homeostasis where it favours adiposity [274]. Secretion of ghrelin is up-regulated and causes increased food intake and body weight during conditions of negative energy balance such as starvation, insulin-

induced hypoglycaemia, cachexia and anorexia nervosa, and is down regulated during conditions of positive energy balance such as feeding, hyperglycaemia, and obesity [277].

Ghrelin, which is negatively regulated by leptin and IL-beta, is secreted by the stomach and increases arcuate neuropeptide NPY expression, which in turn acts through Y_1 receptors to increase food intake and decrease energy expenditure [284]. Additional roles for ghrelin have been identified including alteration of gastric motility as well as affecting gastric acid secretion implicating a role in gastroprotection [274]. Ghrelin is structurally related to motilin and therefore may play the role of motilin in rodents as rodents are natural motilin null mutants [275]. Neuronal and hormonal signals produced in the gastrointestinal tract transmit messages of satiety and starvation to the brain via the afferent limb of the vagus nerve or via circulation in an endocrine manner.

Ghrelin appears to be an endocrine signal however studies have demonstrated ghrelin to exert its effect in the periphery via neural pathways, with the vagus nerve being implicated in conveying ghrelinergic signal from the periphery to the CNS [275, 277, 279].

An effect of ghrelin acting via vagal pathways has been demonstrated previously whereby ghrelin stimulated gastric acid production via vagal activation of gastric parietal cells [274]. In addition, recent work has demonstrated ghrelin is the first neuro-enteric peptide that can stimulate appetite via an effect on the vagus nerve in the periphery [277]. In addition, these findings demonstrated both central and peripheral neuronal pathways to be responsible for the effect of ghrelin on appetite [277].

Vagal afferent fibres have a continuous low-frequency spontaneous discharge that is modulated by sensory inputs [285, 286]. Ghrelin, at a dose required to stimulate feeding and GH secretion, suppressed the resting discharge of whole vagus nerve resting discharge in both mouse and rat models [277, 279]. This is in contrast to other anorexigenic peptides that transmit satiety signals via vagal afferents such as CCK, which increase signalling to the nucleus of the solitary tract via the vagus [277, 287, 288].

Involvement of vagal pathways were confirmed when lesion of gastric vagal afferent fibres was also shown to inhibit appetite-stimulating actions of ghrelin [277, 279]. Morphological evidence has shown ghrelin receptors are synthesised in vagal afferent cell bodies and are transported to the periphery, and thus there exists a close proximity between ghrelin producing cells and vagal afferent terminals in the stomach [277, 289].

Recent studies have demonstrated ghrelin to have an effect on mechanosensitivity of vagal afferents innervating the GIT whereby ghrelin was shown to modulate mechanosensitivity of rat jejunal afferents. An effect on vagal afferents confirmed by vagotomy and selective ghrelin receptor antagonist studies [150].

Together, these results provide functional and molecular evidence for a role of ghrelin acting on ghrelin receptors at peripheral vagal afferent endings.
In humans and animals, a major source of satiety signal from the stomach is gastric filling and distension [290, 291]. These gastric distension-induced satiety signals are transferred to the CNS via vagal afferent mechanisms [292]. To date, there is a gap in our knowledge of this system as there is no direct information on the effect of ghrelin on signalling of distension by gastro-oesophageal vagal afferent fibres.

We used an *in vitro* gastro-oesophageal vagal afferent preparation to study accurately different populations of mechanosensory afferent fibres [45, 46]. Using this preparation, we investigated the effect of ghrelin on afferent response to mechanical stimulation. We studied all of the mechanosensitive gastro-oesophageal vagal afferent subtypes to determine if ghrelin actions were selective for a particular type of mechanosensitive afferent fibre. We also investigated the effect of ghrelin in two species, namely mice and ferrets to determine if the effects are conserved across species, which may be important since the vast majority of evidence for a role of ghrelin so far originates from rodent studies.

MATERIALS AND METHODS

All studies were performed in accordance with the guidelines of the Animal Ethics Committees of the Royal Adelaide Hospital and Institute for Medical and Veterinary Science, Adelaide, Australia. These committees followed the Australian code of practice for the care and use of animals for scientific purposes, 6th edition 1997. Every attempt was made to minimise the number of animals required and to minimise their suffering.

In Vitro ferret and mouse gastro-oesophageal afferent preparations

Female ferrets (0.5-1kg; N=21) were deeply anaesthetized by sodium pentobarbitone (50mg.Kg, i.p), with the thorax and abdomen opened by midline incision. Ferrets were then exsanguinated by cardiac puncture. Female C57 mice (20-30g; N=37) were killed via CO_2 inhalation. Both ferret and mouse *in vitro* gastro-oesophageal afferent preparations were carried out in a similar manner with the exception of the size of the organ bath.

The stomach, oesophagus and attached vagus nerves were then removed and placed in a modified Krebs' solution of the following composition (mM): 118.1 NaCl, 4.7 KCl, 25.1 $NaHCO_3$, 1.3 NaH_2PO_4, 1.2 $MgSO_4$.7H2O, 1.5 $CaCl_2$, 1.0 Citric acid and 11.1 glucose, bubbled with 95% O_2-5% CO_2.

The preparation temperature was maintained at 4°C during dissection to preserve tissue integrity and prevent metabolic degradation. The peripheral organs, major blood vessels and diaphragm were removed by further dissection, leaving the stomach oesophagus and attached vagus nerves intact. The preparation was opened longitudinally and pinned out flat mucosa side up in a perspex organ chamber perfused at a rate of 12mL/min with Krebs' bicarbonate buffer solution maintained at 34°C. The vagus nerves were pulled into a separate isolate recording chamber which was filled with paraffin oil. Under a dissecting microscope, the vagus sheath was removed exposing the underlying nerve fibres, which were teased into small bundles and placed on platinum recording electrodes. Nifedipine (1μM) was also added to the Krebs superfusate in order to prevent smooth muscle contraction. Previous preliminary studies have demonstrated nifedipine to have no effect on the mechanical sensitivity of gastro-oesophageal vagal afferents [148].

Similar to the ferret preparation, female C57 mice (20-30g; N=37) were killed via CO_2 inhalation, and the thorax was opened by midline incision. The stomach and oesophagus were then placed in an organ bath. This preparation has been described in detail previously in previous chapters.

Characterisation of gastro-oesophageal vagal afferent properties

Corresponding receptive fields of all types of afferent fibre were located by mechanical stimulation throughout the preparation with a brush. Accurate

quantification of mechanical responses was performed differently according to the primary adequate stimulus for the type of fibre. The mechanical sensitivity of afferents was characterised by mucosal stroking with calibrated von Frey hairs (10mg-1000mg) and applied circular tension (0.5-7g).

In the ferret, three distinct types of afferent were recorded: those responding to circular tension but not to low intensity mucosal stimuli (tension receptors), those responding only to mucosal stroking (mucosal receptors) and those responding to mucosal stroking and circular tension (tension/mucosal (TM); only found in the ferret. These three types of afferent endings have been previously described in detail [45, 146]. In the mouse preparation two types of mechanosensitive afferent were studied, those responding to mucosal stroking but not to circular tension (mucosal receptors) and those responding to mucosal stroking and circular tension (tension receptors) as reported previously [146].

Mechanical thresholds of all types of fibre were determined using calibrated von Frey hairs. The most reproducible, stimulus dependent response of these afferents to mucosal stimuli was evoked when the probe was moved at a rate of $5mms^{-1}$ across the receptive field rather than being static. Because receptive fields are small ($1\text{-}3mm^2$), a single test at each intensity is prone to missing the centre of the receptive field on occasions. In order to minimise experimenter error, we measure the mean response to the middle 8 of 10 standard strokes given at 1s intervals. A bent von Frey hair (10-1000mg) stroking over the preparation meant that an even force was distributed over the receptive field during stroking stimulus. This protocol was found to give reproducible data and was therefore used to assess the effects of ghrelin on vagal afferents.

Tension response curves were also obtained for all afferent fibres, which were used in combination with von Frey hair stroking to determine whether the receptive fields of fibres were located in the mucosa, the muscle layer or both.

Tension stimuli were applied via a thread attached to an unpinned point adjacent to the receptive field. The thread was attached to a cantilever and pulley system close to the preparation, with reference standard weights applied to the opposite side of the cantilever. Each weight was applied in a step wise manner and maintained for duration of 1 minute. Sensory receptors response to circular tension was recorded as mean discharge evoked over this period. Because all responses to tension were similarly slowly adapting, this method of assessment was considered representative of physiological responsiveness to distension. The tension response curves were produced by applying weights to the cantilever system in a range of (1-5g) and (0.5-7g) for mice and ferrets respectively as previously described [46, 211], to evoke a maximum response without tissue damage. A recovery period of at least 1 minute was allowed between each tension stimulus.

Effect of ghrelin on mechanosensitivity of vagal afferents

After the mechanical sensitivity of gastro-oesophageal vagal afferents had been established under control conditions, the effect of ghrelin on mechanical sensitivity was determined. Ghrelin (1nM) was added to the superfusing Krebs' solution and was allowed to equilibrate for 20 minutes in order to ensure complete penetration of the drug into all layers of the tissue. Tension response and stroke response curves were then re-determined. The procedure was repeated for ghrelin at increasingly greater doses (3-10nM). Time control experiments had previously been

performed demonstrating no significant change in mechanical responses over a comparable duration (see Chapter 1, Fig 6).

In a separate series of experiments in mice, an excess of the ghrelin antagonist [D-Lys-3]-GHRP-6 (30-100μM) was used to reverse the effects of ghrelin (3nM). After mechanical sensitivity of the gastro-oesophageal afferent had been established, ghrelin (3nM) was added to the superfusing Krebs' solution and allowed to equilibrate for 20 min. Mechanical response curves were then re-established. [D-Lys-3]-GHRP-6 (30μM) was then added to the superfusing Krebs' solution along with ghrelin (3nM), and again this was allowed to equilibrate for 20 min before mechanical response relationships were re-determined. The time frame as outlined for ghrelin experiments was carried out for higher doses of [D-Lys-3]-GHRP-6 (100μM). Therefore any reversal of effect of ghrelin by [D-Lys-3]-GHRP-6 is unlikely to be due to desensitisation to ghrelin. There was no obvious difference in the effect of ghrelin on oesophageal and gastric afferents, and therefore data from these afferents were pooled.

Effect of [D-Lys-3]-GHRP-6 on mechanosensitivity of vagal afferents

The ghrelin receptor antagonist [D-Lys-3]-GHRP-6 was used to determine the involvement of endogenous ghrelin on mechanosensitivity of gastro-oesophageal vagal afferents. After mechanical sensitivity of the mouse tension sensitive gastro-oesophageal vagal afferent had been determined, the effect of [D-Lys-3]-GHRP-6 on mechanical sensitivity was determined. [D-Lys-3]-GHRP-6 (0.1μM) was added to the superfusing Krebs' solution and allowed to equilibrate for 20 min. after which time the tension-response and stroke-response curves were re-determined. The concentration of [D-Lys-3]-GHRP-6 used was markedly lower

than that used in attempts to reverse the effects of exogenous ghrelin. [D-Lys-3]-GHRP-6 had a different effect on gastric and oesophageal tension receptors and so the afferents were compared according to location.

Data Recording and Analysis

Afferent impulses were amplified with a biological amplifier (World Precision Instruments, Sarasota, FL, DAM 50) and scaling amplifier (JRAK, Melbourne, Australia), filtered (CWE Inc., Ardmore, PA band-pass filter-932) and monitored using an oscilloscope (DL 1200A; Yakogawa, Tokyo, Japan). Single unit action potentials were discriminated on the basis of size and shape using spike II software (Cambridge Electronic Design, Cambridge, UK).

Statistical Analysis: All data were recorded and analysed off-line using a personal computer. All data were expressed as mean ± SE with n = number of individual afferents in all instances. Statistical difference between stimulus-response curves were evaluated using a two-way ANOVA performed using graphpad Prism (Version 3.00 for Windows, Graphpad Software, San Diego, California, USA). Differences were considered significant where $p < 0.05$.

Drugs

Stock solutions of all drugs were kept frozen (-80°C) and diluted to their final concentration in Krebs' solution on the day of the experiment. Ghrelin was obtained from AUSPEP Pty Ltd (Victoria, Australia) and [D-Lys-3]-GHRP-6 was obtained from Sigma (Sydney, Australia).

Nodose Ganglia Dissection and RNA extraction for RT-PCR and Quantitative RT-PCR

Nodose ganglia were removed bilaterally from five male or female C57 mice (20-30g). Nodose ganglia were pooled in RNAlater® (QIAGEN, NSW, Australia) at -20°C for subsequent RNA extraction. RNA isolation involved homogenisation with a sterile glass pestle, of the nodose ganglia, after being snap frozen using liquid nitrogen. A total of 1ml of TRIzol reagent (Invitrogen, Vic, Australia) was added before the homogenised material was added to Qiashredders and centrifuged (12,000 rpm) at room temperature. Tubes containing homogenate were capped after Qiashredders were removed, vortexed and allowed to stand at room temperature for 15 minutes in order to promote dissociation of nucleoprotein complexes. After which 0.2 ml of chloroform was added, tubes were vortexed and left to stand at room temperature for a further 15 minutes. The samples were then cold centrifuged (12,00 rpm, 4°C) for 15 minutes with the upper aqueous layer (containing RNA substrate) being removed and transferred to a sterile eppendorf tube followed by isopropanol precipitation of RNA, by which 0.5 ml isopropanol was added cold centrifuged for 10min (38, 47). The upper aqueous phase was discarded leaving an RNA pellet to which 1 ml of 75% ethanol was added. A 1 min vortex and 4 min cold centrifuge and then supernatant was removed with the pellet being re-suspended in 100µl RNAase free sterile water and heated for 2 min at 60°C. RNA was separated into 5µL aliquots and stored at -80°C

RNA quantification was determined by measuring the absorbance at 260nm (A260) using a spectrophotometer (Biorad, Sydney, Australia). RNA quality was estimated by the A260: A280 nm ratio.

Determination of ghrelin and ghrelin receptor transcript expression in Nodose Ganglia using RT-PCR

Reverse transcription (RT) and polymerase chain reaction (PCR) was performed using a Qiagen® one-step RT-PCR kit, with QuantiTect Primer Assays (Qiagen) optimised for the detection of the known sequence of mouse ghrelin, ghrelin receptor and β-actin. RT-PCR master-mix was prepared as follows: for each reaction 25μL RT-PCR Master Mix, 10μL RNAse free water, 10μL of 5x QIAGEN One-Step RT-PCR Buffer, 2μL dNTP Mix (containing 10 mM of each dNTP), 2μL QIAGEN One-Step RT-PCR Enzyme Mix and), 1μL of RNAse inhibitor (Ambion).

RT–PCR reactions were performed using an alpha unit block for PTC DNA engine systems (MJ Research) attached to a PTC-200 Peltier thermal cycler (MJ Research). The following amplification program was applied: Reverse Transcription: 50°C for 30min, Initial PCR activation: 95°C for 15 min, PCR cycles: denaturing: 94°C for 1 min, annealing: 47°C for 1 min and extension: 72°C for 1 min repeated for 40 cycles followed by a final extension step of 72°C for 10 min.

For each reaction 25μL of Master Mix was added to 0.2mL clear thin walled PCR tubes (Axygen) with 10μL (3μM) forward primer (for a final concentration of 0.6 μM), 10μL (3μM) backward primer (for a final concentration of 0.6μM) and 5μL of 50ng template RNA. For each reaction, RNA template was substituted with 5μL distilled RNA-free water as controls. Amplified products (5μL PCR

product/2μL loading buffer) were resolved by 1.5-3% agarose gel electrophoresis and visualised by ethidium bromide staining.

Determination of Relative ghrelin and ghrelin receptor transcript expression using Quantitative RT-PCR

Quantitative reverse transcription polymerase chain reactions (QRT-PCR) were performed using a Qiagen QuantiTect® SYBR® green RT-PCR one step RT-PCR kit, using QuantiTect® primer assays (Qiagen) optimized for the detection of the known sequence of mouse ghrelin, ghrelin receptor and β-actin transcripts contained in the NCBI reference sequence database. Real-time PCR master mix was prepared according to product manufactures specifications as follows: for each reaction 24.5μL of 2x QuantiTect SYBR Green RT-PCR Master Mix, each master mix containing HotStarTaq® DNA polymerase, QuantiTect® SYBR green RT-PCR buffer, dNTP Mix including dUTP, SYBR® Green I (Fluoresces in presence of double stranded DNA), ROX (passive reference dye), 5mM $MgCl_2$, and 0.5μL of QuantiTect® RT mix (Omniscript® Reverse Transcriptase and Sensiscript® Reverse transcriptase).

For each reaction 25μL of the master mix, 10μL (3μM) forward primer, 10μL (3μM) backward primer and 5μL of 50ng of template RNA were added in MJ White PCR tube (MJ Research). RNA template was replaced by RNAase free, sterile water in control samples.

QRT-PCR reactions were performed using a Chromo4 (MJ research, Biorad) real-time instrument (designed to detect SYBR green fluorescence, therefore amplified cDNA products) attached to PTC-200 Peltier thermal cycler (MJ Research) and analysed using Opticon Monitor Software (MJ Research).

The following amplification program followed: Reverse Transcription: 50°C for 30mins; Initial PCR activation: 95°C for 15 min; PCR cycles; denaturing: 94°C for 15 sec, annealing: 47°C for 30 sec and extension: 72°C for 30 sec repeated for 44 cycles with final extension step of 72°C for 10 min. A melting curve program was performed to verify the specificity and identity of the RT-PCR products, whilst all other reactions were verified using gel electrophoresis. Assays were run in triplicate in separate experiments. The size of the amplified products (5μL PCR product + 2μL loading buffer) was confirmed by 1.5-3% agarose gel electrophoresis and visualised by ethidium bromide staining.

Results for QRT-PCR were viewed via Opticon software and were represented as cycle threshold (CT) required to achieve a certain level of fluorescence (amount of double stranded PCR product). In all experiments CT was obtained from the exponential/linear join of the PCR fluorescence curve. As CT is proportional to the logarithm of total DNA in sample, comparing CT enabled determination of the relative expression of transcripts.

Titration curves were obtained to confirm amplification efficiencies and therefore the validity of the comparative cycle threshold. Template RNA of known concentration from nodose ganglia was used to create a five log dilution series of RNA. Equal amplification was indicated by the linearity of the plots assay over a range of input RNA concentrations for β-actin, ghrelin and ghrelin receptor. These curves demonstrated the efficiencies of targets and references were equal.

The relative concentration of a sample can be determined by the number of cycles required to reach a certain fluorescence level when compared with other samples. The β-actin sample was the first sample to reach CT and thus ghrelin and

ghrelin receptor transcripts expression values were referenced relative to β-actin. The CT for each was compared with that of β-actin using the equation of CT of target transcript – CT of β-actin. To determine relative expression of these transcripts in whole nodose ganglia, the ΔΔCT was calculated using the formula: ΔΔCT= ΔCT [ghrelin receptor] - ΔCT [ghrelin] and the relative fold differences calculated using the formula $2^{-\Delta\Delta CT}$. CT values for β-actin were highly reproducible between samples and between methods of RNA preparation. Quantitative data are expressed as mean ± SD, and significant differences in transcript expression determined by a Mann-Whitney test. Significance considered where $p < 0.05$.

RESULTS

RT PCR localisation of ghrelin and ghrelin receptor transcripts

Using RT-PCR and gel electrophoresis we were able to confirm the presence of amplified products generated by primers specific for ghrelin, ghrelin receptor, and β-actin. Isolation of single bands of correct predicted transcript size was achieved by UV analysis of the gel. Bands of correct predicted product size for both ghrelin and ghrelin receptors was observed in mouse nodose ganglia. Where template RNA was substituted for sterile water, no evidence of amplified products was observed. Expression of the reference β-actin gene was observed in all cases using template RNA.

Quantitative RT-PCR comparing relative transcript expression

Using gel electrophoresis we confirmed the size of amplified products generated by the QuantiTect® Primer Assays specific for ghrelin, ghrelin receptor

and β-actin, showing intense single bands corresponding to predicted sizes of ghrelin, ghrelin receptor and β-actin transcripts (Figure 4A.).

Separate quantitative RT-PCR experiments were performed to compare expression between ghrelin and its receptor. Using β-actin as a reference CT value, QRT-PCR analysis of whole nodose ganglia revealed the ghrelin receptor to have significantly greater transcript expression (2.33 fold) than ghrelin ($p < 0.05$, Figure 4B).

Electrophysiology

Effect of ghrelin on the mechanosensitivity of gastro-oesophageal vagal afferents

Mouse

The effect of ghrelin (1-10nM) on the mechanosensitivity of mouse gastro-oesophageal vagal afferents is illustrated in figure 1. Ghrelin (1 – 10nM) did not significantly affect the responses of 9 mucosal receptors to mucosal stroking throughout range of stimuli (Calibrated von Frey hairs; 10-1000mg; Fig 1A). However, ghrelin (3-10 nM) did significantly reduce the response of mouse tension receptors (n = 11) to circular tension (1-5g; Fig 1B & D).

The spontaneous activity of mouse tension receptors was 11.54 ± 2.54 impulses/ sec. In the presence of ghrelin (3 & 10nM) the spontaneous activity was significantly reduced (table 1). Mouse mucosal receptors tend to have no spontaneous activity, thus it is not surprising that ghrelin (1 -10nM) had no significant effect on the spontaneous activity of mouse mucosal receptors (Data not illustrated).

The effect of ghrelin (3nM) and the ghrelin antagonist [D-Lys-3]-GHRP (30-100μM) on mouse tension receptors (n=7) is illustrated in figure 1C. Ghrelin alone significantly reduced the response curves of tension receptors to circular stretch (1-5g; Figure 1C.). [D-Lys-3]-GHRP was added in addition with ghrelin (3nM) to the Krebs' superfusate and was shown to reverse the inhibitory effect of ghrelin (Figure 1C.). [D-Lys-3]-GHRP (100μM) demonstrating complete reversal of the inhibitory effects of ghrelin back to control levels. [D-Lys-3]-GHRP, however, had no significant effect on the baseline discharge of tension sensitive afferents (data not shown).

Effect of specific ghrelin receptor antagonist D-Lys-3]-GHRP-6

The ghrelin receptor antagonist [D-Lys-3]-GHRP-6 (0.1μM) alone on mouse tension receptors as illustrated in figure 3. It significantly potentiated the response of gastric tension receptors to circular tension (Fig 3A), but did not have a significant effect on the response of oesophageal tension receptors to circular tension (Figure 3B.). The antagonist had no significant effect on the spontaneous activity of tension sensitive afferents (data not shown).

Ferret

The effect of ghrelin on the mechanical sensitivity of ferret gastro-oesophageal vagal afferents is illustrated in figure 2. Ghrelin (3 - 10 nM) significantly reduced the response of a subpopulation of mucosal receptors to mucosal stroking with calibrated von Frey hairs (10 – 1000mg). The mechanosensitivity of three, from a total of eight mucosal receptors studied was

reduced in presence of ghrelin (3-10 nM). This reduction of mechanosensitivity was reversed upon washout of ghrelin from the Krebs' superfusate (Figure 2D). Ghrelin (1–10 nM), did not significantly alter the response of tension receptors (n = 7) to circular tension (1-5g; Figure 2B.). The effect of ghrelin (1–10 nM) on the mechanosensitivity of TM receptors (n = 11) is illustrated in Figure 1C. Ghrelin (1–10 nM) significantly reduced the response of TM afferents to mucosal stroking with calibrated von Frey hairs (10 – 1000mg) (Figure 2C (i)), whilst it had no effect on the response of the same TM receptors to circular stretch (1 -5g; Fig 2C (ii)). Ghrelin (1-10 nM) did not significantly affect on the spontaneous activity of ferret mucosal (data not illustrated due to the fact that these receptors are generally silent), TM, or tension receptors (Table 1).

DISCUSSION

The current study, investigating the effects of ghrelin on the sensitivity of vagal afferent mechanoreceptors across separate species, provides the first evidence that ghrelin receptors are involved in transduction of mechanical stimuli by the gastro-oesophageal vagal afferents. In particular ghrelin is coupled to inhibition of mechanotransduction in a subset of primary sensory endings. This study identified ghrelin, the endogenous ligand for the growth hormone secretagogue receptor, to inhibit ascending vagal afferents signalling in both mice and ferret models. This was reversed by a ghrelin receptor antagonist [D-Lys-3]-GHRP-6, confirming its selectivity for this receptor.

Our data revealed important differences between species in the afferent subtypes influenced by ghrelin. In mice, the inhibitory effect of ghrelin was specific to tension receptors. This is in contrast to ferret studies where ghrelin had no effect

on the responses of tension and TM receptors to circumferential tension, however it inhibited afferent responses to mucosal stroking of TM and mucosal receptors. These results indicate the possibility of specific roles for ghrelin on specific afferent pathways, which are discussed in detail below.

Vagal afferents provide the main neuro-anatomical link between the brain and the upper GIT and are involved in the triggering of behavioural responses [41, 44], reflexes controlling digestive function [293], and motor patterns leading to gastro-oesophageal reflux [143, 168]. Several physiological and pathophysiological processes can be triggered by upper gastrointestinal vagal afferents. These include retching, vomiting, transient lower oesophageal sphincter relaxations (proposed mechanism of gastro-oesophageal reflux disease), secondary peristalsis, relaxation of the proximal stomach, gastric emptying and most importantly to the current investigations, sensations associated with satiety, nausea or discomfort. Modulation or alteration of this pathway may have important implications in a number of pathological states. Our data implicate ghrelin acting at ghrelin receptors located on vagal afferent endings to exert an endogenous influence on this pathway. Exogenous infusion of ghrelin has been shown previously to enable larger meal sizes [294]. This may correspond with our finding that ghrelin reduces the response of tension receptors to circular stretch. Therefore, it would take more distension (i.e. larger meal size) to give the same degree of inhibition of food intake.

Vagal afferents provide a means of signalling of satiety and influence food intake via multiple mechanisms including meal related metabolites, monoamines, peptides as well as mechanical and chemical stimuli [295-297]. Distension of the stomach has been shown to inhibit feeding via stimulation of vagal afferent

mechanoreceptors [290]. In addition, the gastrointestinal tract releases various hormones that regulate short-term food intake via vagal afferent neurones [298]. For example leptin and cholecystokinin (CCK) receptors are expressed in nodose ganglia and vagal afferents transmit leptin or CCK signals from the gastrointestinal tract to the CNS [103, 299, 300]. Therefore vagal afferents play a key role in the inhibition of food intake [301]. Distension sensitive vagal afferents in both stomach and duodenum have been shown to be activated upon application of CCK, demonstrating integration of signals elicited by CCK and gastric loads at the level of the vagal afferent fibre, but are controversial as to their effects on single sub-types of vagal afferents [47, 104, 111, 302, 303]. Our findings indicate ghrelin should be considered as a potential mediator of modulating mechanical activation of gastric vagal afferents, with clear implications for satiety signalling from the gastro-intestinal tract.

It has been proposed previously that ghrelin increases food intake by suppressing the discharge of vagal afferents [277, 284]. Whether this effect is a direct action on vagal nerves or other vagal responses has not been clarified.

Murray, et al (2006)[150], have since demonstrated modulation of mechanosensitivity of vagal afferents innervating the rat jejunum in response to distension. Interestingly though, this group demonstrated augmentation of afferent response to distension and no effect on resting discharge by ghrelin in contrast to previous studies in stomach [277]. Whilst this study indicates actions of ghrelin may differ in different segments of the gastro-intestinal tract, they do demonstrate subdiaphragmatic vagotomy and a selective ghrelin receptor antagonist to prevent these effects, once again highlighting an action of ghrelin receptors located on vagal afferent terminals [150].

In contrast to previous work where whole nerve afferent discharge was recorded [277, 284], the *in vitro* preparation used in this study enabled us to isolate and record specific subtypes of vagal afferent receptor discharge and to characterize the effects of exogenously applied to ghrelin on each. The importance of this was reflected in the fact that ghrelin had selective effects of different receptor subtypes.

The current study demonstrated ghrelin to selectively reduce mechanosensitivity, as well as inhibit basal discharge of mouse tension receptors, whilst having no effect on the basal discharge of other types of mouse and ferret afferents. We therefore propose the decrease in basal discharge observed in multi-unit recordings in mice and rats *in vivo* [277, 284], may be due to a decrease in discharge of tension receptors, or a possible inhibition of ongoing mechanical activation. We propose a mechanism, whereby ghrelin inhibits signalling of mechanical activation from distension or contraction of vagal afferents from the upper gastro-intestinal tract, occurring continually in both fed and fasted states that may correlate with effects on food intake.

Endogenous ghrelin requires an n-octanylation for endogenous functionality, however is rapidly degraded from its functional state to functionally inert des-n-octanyl ghrelin by plasma esterases [304]. Therefore due to a limited half life (10 minutes) the concentration of active ghrelin is greatest closer to site of release in the stomach, thus implicating vagal afferent endings in the stomach as a probable site of action for ghrelin. Previous studies have isolated ghrelin in the nodose ganglion [277], as well as morphological evidence of the existence of ghrelin receptor producing cells in the nodose ganglion, with ghrelin receptors being produced and transported to peripheral vagal afferent endings [277]. We were also able to isolate ghrelin and its receptor in nodose ganglion. Quantitative measurements revealed

relatively low levels of ghrelin compared with its receptor, but nevertheless it raises the possibility of an auto-regulatory role of ghrelin released from vagal afferent endings, in addition to the role of ghrelin released from gastric endocrine cells. This may be of more importance in oesophageal afferents where there is less ghrelin in the tissue surrounding the afferents than in the stomach [305].

We investigated a role for endogenous ghrelin on mouse tension receptors using the ghrelin receptor antagonist [D-Lys-3]-GHRP-6. We observed [D-Lys-3]-GHRP-6 to have an effect specifically on gastric tension receptors whilst it had no effect on oesophageal tension receptors. These findings suggest an endogenous source of ghrelin in the stomach but not the oesophagus, however, argues against an auto-regulatory role of ghrelin in the oesophagus.

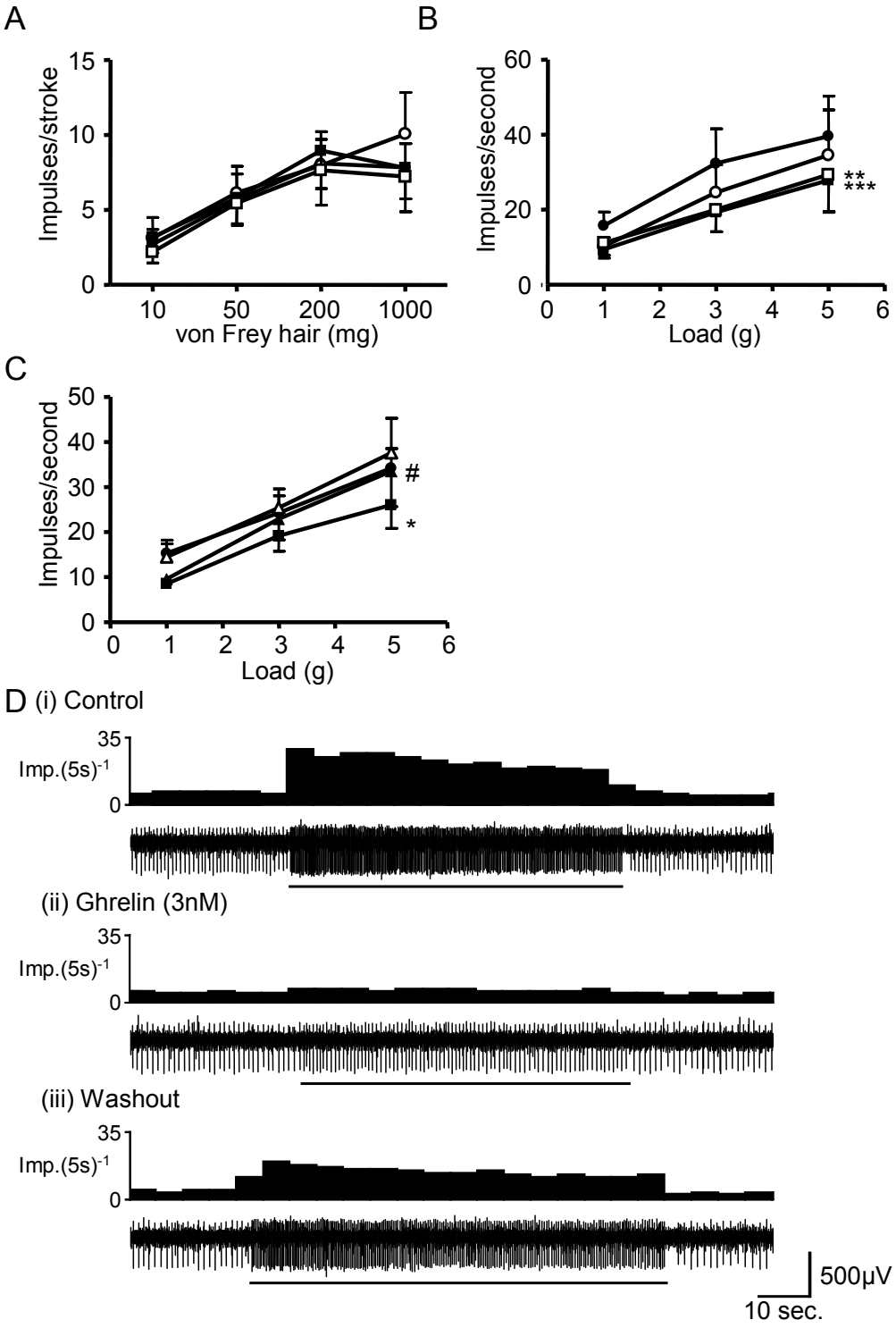
In our previous studies we identified a role for endogenous glutamate in afferent modulation at both gastric and oesophageal sites, where glutamate was most likely derived from the afferents themselves [306]. Together, these findings are suggestive of the gastric mucosa as the primary source of endogenous ghrelin acting on vagal afferents.

The current study demonstrates ghrelin to reduce the response to mechanical stimulation of gastro-oesophageal vagal afferents in two species. More specifically, responses of tension sensitive afferents to circumferential stretch in the mouse, and mucosal sensitive afferents to mucosal stoking in the ferret. It is possible that this discrepancy between mice and ferrets reflects the inability of rodents to vomit and to exhibit gastro-oesophageal reflux [45], whereas ferrets, like humans, are able to vomit and do exhibit gastro-oesophageal reflux [45]. Ghrelin has previously been found to show anti-emetic effects in the ferret [307]. Emesis is commonly linked with activation of mucosal receptors in the stomach and small intestine [6, 308-

311], which agrees with our finding of selective inhibition of mucosal receptors in the ferret. In mice which are unable to vomit, the ability of ghrelin to act on vagal tension receptors in the periphery may play a more important role in food intake.

CONCLUSION

In conclusion, the current study provides the first direct evidence for the inhibitory modulation of primary vagal afferent mechanosensitivity by the gastric peptide ghrelin. These results implicate the ghrelin receptor (GHS-R) as a possible target for the treatment of obesity, and possibly other disorders of upper gastro-intestinal dysfunction. This study also provides greater insight into the underlying mechanisms of action of ghrelin.


A
B
Impulses/stroke
15
10
5
0
10
50
200
1000
von Frey hair (mg)
Impulses/second
60
40
20
0
0
1
2
3
4
5
6
Load (g)
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C
Impulses/second
50
40
30
20
10
0
0
1
2
3
4
5
6
Load (g)
#
*
D (i) Control
Imp.(5s)-1
35
0
(ii) Ghrelin (3nM)
Imp.(5s)-1
35
0
(iii) Washout
Imp.(5s)-1
35
0
500μV
10 sec.

Figure 1.

The effect of ghrelin on mouse gastro-oesophageal vagal afferents.

Stimulus-response functions of mucosal (A, n=9) and tension receptors (B, n=11) to mucosal stroking and circumferential tension respectively. The response are before (●) and after exposure to ghrelin (1nM (○), 3nM (■) & 10nM (□)). Stimulus-response functions of tension receptors (C, n=7) before (●) and after exposure to ghrelin (3nM (■)) and ghrelin (3nM) along with the ghrelin receptor antagonist [D-Lys-3]-GHRP-6 (30μM (▲) and 100μM (△)).*P<0.05, **p<0.01, ***p<0.001 compared to control, #p<0.05 compared to responses obtained in the presence of ghrelin (3nM) alone (two-way ANOVA). Bonferoni post hoc tests revealed that the significant differences occurred at larger tension stimuli (not shown), which may be due to decreasing variance in responses at higher loads. D, original recording of a tension receptor response to circumferential tension with a 3g weight before (Di), during exposure (Dii) and after washout (Diii) of ghrelin (3nM).

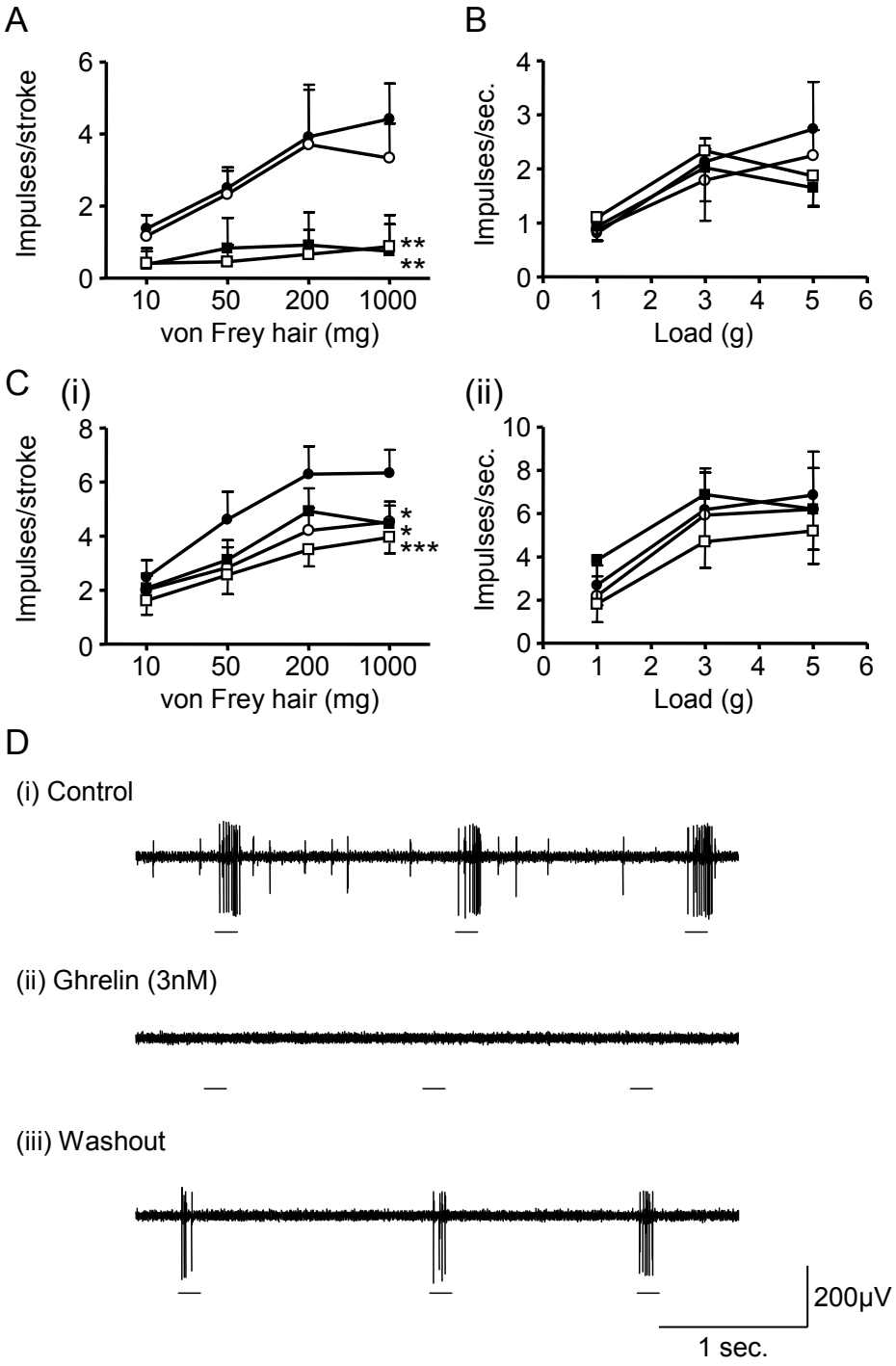
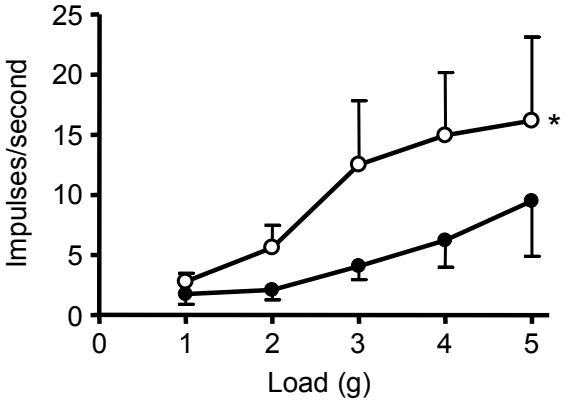

A
Impulses/stroke
6
4
2
0
10
50
200
1000
von Frey hair (mg)
**
**
B
Impulses/sec.
4
3
2
1
0
0
1
2
3
4
5
6
Load (g)
C
(i)
Impulses/stroke
8
6
4
2
0
10
50
200
1000
von Frey hair (mg)
*
*
***
(ii)
Impulses/sec.
10
8
6
4
2
0
0
1
2
3
4
5
6
Load (g)
D
(i) Control
(ii) Ghrelin (3nM)
(iii) Washout
200μV
1 sec.

Figure 2.

The effect of ghrelin on ferret gastro-oesophageal vagal afferents.

Stimulus-response functions of mucosal (A, n=3), tension (B, n=7) and tension/mucosal receptors (C, n=11) to mucosal stroking (A & Ci) and circumferential tension (B & Cii). The responses are before (●) and after exposure to ghrelin (1nM (○), 3nM (■) & 10nM (□)). Asterisks indicate significant difference from control using a two-way ANOVA (*$p<0.05$, **$p<0.01$ & ***$p<0.001$). D, original recording of a mucosal receptor response to mucosal stroking with a 50mg von Frey hair before (Di), during exposure (Dii) and after washout (Diii) of ghrelin (3nM).

A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20
15
10
5
0
Impulses/second
*
0
1
2
3
4
5
Load (g)

B

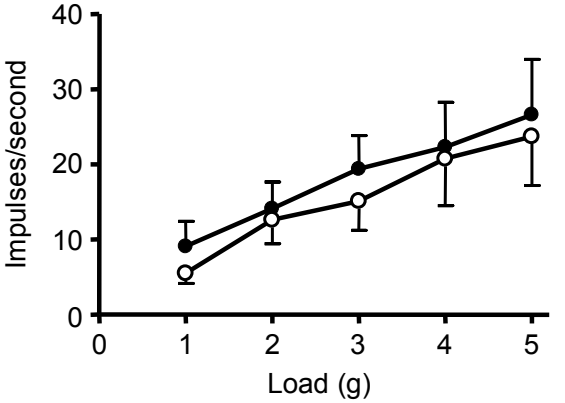
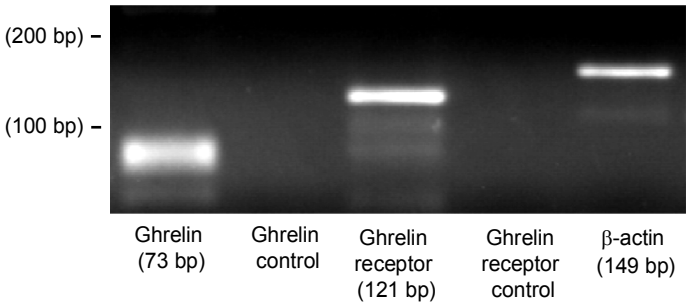

40
30
20
10
0
Impulses/second
0
1
2
3
4
5
Load (g)

Figure 3.

The effect of [D-Lys-3]-GHRP-6 on mouse gastro-oesophageal vagal afferents.

Stimulus-response functions of gastric (A, n=6) and oesophageal (B, n=4) tension receptors to circumferential tension. The responses are before (●) and after (○) exposure to [D-Lys-3]-GHRP-6 (0.1μM). *$p<0.05$ compared to control (two-way ANOVA).

A) Transcript expression



B) Relative expression

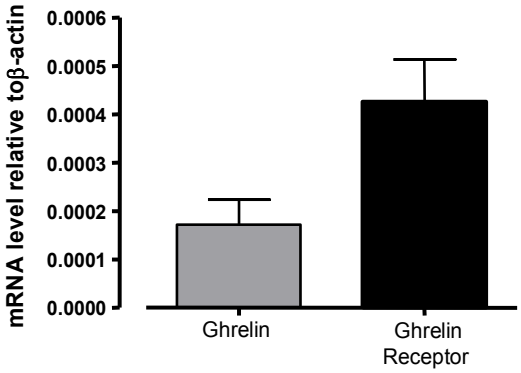


Figure 4.

Transcript expression and relative expression of ghrelin and ghrelin receptor in whole mouse nodose ganglia.

A) PCR products of quantitative RT-PCR experiments separated on a 3% agarose gel to confirm the presence of ghrelin receptor and ghrelin transcripts. The sizes of the amplified products were confirmed, showing intense single bands, corresponding to the predicted sizes of ghrelin, ghrelin receptor and β–actin transcripts. In addition this validated the products obtained during quantitative analysis. B) Quantitative RT-PCR data revealed that the ghrelin receptor had a significantly greater transcript expression than ghrelin ($P < 0.05$; Mann-Whitney test). Experiments were performed in at least triplicate. Fold differences are calculated relative to β–actin mRNA levels.

	Mouse	Ferret	
	Tension receptor	**Tension receptor**	**TM receptor**
n	11	11	7
Control	11.54 ± 2.54	0.31 ± 0.18	1.77 ± 0.95
Ghrelin (1nM)	8.42 ± 2.11	0.57 ± 0.35	1.39 ± 0.79
Ghrelin (3nM)	7.46 ± 1.88*	0.57 ± 0.25	2.96 ± 1.91
Ghrelin (10nM)	8.73 ± 2.59**	0.92 ± 0.54	1.32 ± 0.68

Table 1.

Spontaneous activity of gastro-oesophageal afferents sensitive to tension.

The spontaneous activity of mouse tension and ferret tension and tension/mucosal receptors in the absence and presence of ghrelin (1, 3 and 10nM). Values are impulses/second ± S.E. Ghrelin significantly reduced the spontaneous activity of mouse tension sensitive receptors but did not significantly affect the spontaneous activity of ferret tension and tension/mucosal receptors. *$p<0.05$, **$p<0.01$ compared to control (paired t-test).

CONCLUSIONS

Modulation of vagal afferent mechanoreceptor signalling to the CNS has been well established as a novel therapeutic target for the treatment of upper gastrointestinal tract disorders such as disordered food intake, functional dyspepsia and most importantly to the current investigation, gastro-oesophageal reflux disease (GORD).

GORD is one of the most common clinical problems of our society, affecting up to 20% of the population [162]. Conventional therapies aimed at reducing gastric acid secretion, thus limiting acid content of the refluxate have been successful in a significant proportion of patients. However, up to 30% of patients are refractory to this treatment, or suffer from gastro-duodenal or bile reflux which is also damaging to the oesophagus [179, 180].

Transient lower oesophageal sphincter relaxations (TLOSRs) triggered by activation of distension sensitive afferents in the proximal stomach and underly the major pathophysiological mechanism of reflux disease. TLOSRs mediated by a vagal pathway involving activation of proximal gastric tension receptors, which in turn, activate a program generator in the brainstem. Output of this generator leads to brief activation of vagal motorneurones connected to inhibitory pathways in the LOS causing relaxation [168].

Intense interest in management of reflux disease has seen pharmacological modulation of the TLOSR pathway at several points [187, 188, 193, 195, 312, 313]. In recent years it has become apparent that modulation of peripheral vagal afferent mechanoreceptors innervating the gastro-oesophageal region correlates with a novel therapeutic approach to reflux disease via reduction in gastric distension evoked TLOSRs and gastro-oesophageal reflux episodes.

Emerging evidence has shown metabotropic receptors to the amino acids GABA and glutamate, are able to modulate triggering of TLOSRs, corresponding to reduction in gastro-oesophageal reflux [122, 123]. Importantly, this is corroborated by our observations that $GABA_B$ and mGluR5 receptors are expressed in vagal sensory ganglia [145, 148], and both $GABA_B$ agonists and mGluR5 antagonists reduce responses to distension of gastro-oesophageal vagal afferents when applied peripherally both *in vitro* and *in vivo* [145, 146, 191, 306]. These studies confirmed signalling from vagal mechanoreceptors is crucial in the initiation of this motor pattern [144]. The most accessible target for selective modulation of the neuronal pathway mediating TLOSRs is via the vagal afferent terminal mechanoreceptors. Such peripheral targets offer advantages by minimising interference with motor pathways that may produce unwanted side effects elsewhere in the GI tract as well as in other target organs of the vagus [198].

The success of $GABA_B$ agonists and mGluR agonists and antagonists to reduce TLOSRs as well as gastro-oesophageal reflux provide the best example of G-protein coupled receptors (GPCR) as therapeutic targets in the management of reflux disease.

There are three families of GPCR that respond to stimuli as diverse as light, odour, taste, calcium, and a wide range of chemical mediators, but few have been explored in the sensory system. Whilst our interest initially was focussed on inhibitory neuronal GPCR including galanin and ghrelin receptors given the therapeutic success of previous GPCR, the excitatory glutamatergic counterbalance observed in our mGluR studies highlighted the need for comprehensive evaluation of mechanisms of glutamatergic modulation of vagal afferents and therefore,

excitatory coupled iGluR and mGluR were an obvious addition to those targets mentioned above.

The effect of galanin to potently modulate vagal afferent mechanosensitivity raised the possibility that there were other features of vagal afferents that may provide alternative, possibly more selective G-Protein coupled receptor targets [148].

Galanin is a 29-30 amino acid peptide found throughout the central and enteric nervous system, known to have either inhibitory or excitatory effects on motor function including gastric smooth muscle activity [205], and neuronal excitability including transmission in the spinal cord [204], and signalling in central gastric vagal pathways [206, 207].This study replicated the divergent effect of galanin in our *in vitro* gastro-oesophageal vagal afferent preparation, demonstrating galanin to exert a predominantly inhibitory effect. Three receptors for galanin have been cloned, Gal-R1, -R2 and -R3. We confirmed using GalR1-null mutant mice, and receptor specific ligands, that the inhibitory effect of galanin on mechanosensation is mediated via the GalR1 receptor located on vagal afferents innervating the upper gastrointestinal tract. In addition, GalR2 was implicated in the mediation of excitatory effects of galanin, previously observed. A minor but significant potentiation in mechanosensitivity by galanin was seen in GalR1-/- mice, an effect that was reproduced by a selective GalR2 agonist. We were unable to identify a functional role for GalR3 on gastro-oesophageal vagal afferents and as yet a physiological role for GalR3 is still undetermined. In contrast to these findings, all three galanin receptors were identified in vagal sensory ganglia and relative expression of all three receptors was similar. Other findings have demonstrated

gastric projecting vagal neurones to contain much less GalR3 than other subtypes so the possibility of a role for GalR3 in other organs innervated by the vagus still exists.

The ghrelin receptor or growth hormone secretagogue receptor 1a (GHSR1a), is also an inhibitory brain-gut GPCR which has widespread distribution but is particularly localised in the upper GI tract [275, 279-283]. Ghrelin signalling to the CNS is predominantly mediated via the vagus nerve and electrophysiological recordings have demonstrated ghrelin to modulate whole vagus afferent discharge from the stomach and jejunum [150, 277].

Other studies have identified ghrelin and its receptors located in the vagus nerve, where they are transported peripherally [277]. We were able to isolate ghrelin and its receptors in whole nodose ganglia and demonstrate relative expression of ghrelin is low compared with its receptor.

We provided the first evidence that ghrelin is involved in signalling of mechanical stimuli via vagal gastro-oesophageal afferents. In particular ghrelin is coupled to inhibition of mechanotransduction in a subset of primary sensory endings. Important differences between species were noted however. Ghrelin was shown to inhibit mucosal receptors in the ferret, having no significant effect on tension or TM receptors, whilst it was shown to inhibit tension receptors in the mouse, having no significant effect on mucosal receptors in the mouse. These results suggest there may be specific roles for ghrelin in specific pathways. Ghrelin is a known satiety signal, an effect mediated peripherally, and is dependent on the vagus nerve. The fact that ghrelin had no significant effect on ferret tension receptors probably rules out a therapeutic role for ghrelin in terms of inhibiting

TLOSRs however, vagal afferents provide a means of signalling of satiety via multiple mechanisms including mechanical and chemical stimuli. Distension of the stomach inhibits feeding via stimulation of vagal afferent mechanoreceptors. These results therefore implicate ghrelin to affect satiety and possibly TLOSRs via modulation of peripheral vagal mechanoreceptors.

Glutamate exerts its effects at central synapses via ionotropic (iGluR-NMDA, AMPA & Kainate) and metabotropic (mGluR) receptors. Group I mGluR are coupled to excitatory, whilst group II and III mGluR are coupled to inhibitory mechanisms. Previous *in vitro* work in the ferret identified a possible counter-balance of excitatory and inhibitory glutamatergic signalling from peripheral vagal afferents whereby glutamate acts simultaneously at iGluR and mGluR [121]. The potent inhibitory effect of glutamate, an effect mimicked by selective group II and III agonists, on vagal afferent mechanosensitivity was only revealed after the addition of the non-specific iGluR antagonist Kynurenate to the preparation. *In vivo* observations in the rat indicated similarly that iGluR may be involved in excitatory modulation of vagal afferents [120]. The current data indicate AMPA, NMDA and mGluR5 receptors to play a major role in the excitatory balance of modulation of mechanosensitivity with kainate receptors having little if any effect under the current conditions. Also identified was that the role of each differs according to afferent subtypes. NMDA was more potent on mucosal receptors, and MTEP was more potent on tension receptors, suggesting NMDA receptors may be more highly expressed by mucosal receptor cell bodies, and mGluR5 may be expressed more by tension receptors. These results clearly indicate a counter balance of excitatory modulation of vagal afferent mechanosensitivity by iGluR and mGluR 5, and

inhibitory modulation by group II and III mGluR. The inhibitory effects of exogenous iGluR and mGluR antagonists indicate glutamate to exert an endogenous action on mechanosensitivity, with vagal afferents themselves, contracting smooth muscle, luminal and circulating glutamate as candidates for an endogenous source. In conjunction with previous findings by our group, this study further clarifies the influence of all plausible major GluR subtypes on vagal afferent mechanosensitivity, as well as confirming previous findings in rat and ferret, which suggest an excitatory role for glutamate receptors on vagal afferents. There is clearly scope for both excitatory and inhibitory modulation of afferent sensitivity by glutamate from both exogenous and endogenous sources, providing a balance to achieve normal vagal afferent function. These data were reinforced by the finding of a complete complement of subtypes of all receptors located in the nodose ganglion, therefore implicating each receptor as a possible candidate for neuromodulation.

Results from this thesis confirm further contenders in an expanding list of modulators of vagal afferent mechanosensitivity, and thus establishing possible future novel therapeutic targets for the treatment of disordered upper gastrointestinal vagal afferent signalling.

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