NERVOUS COMMUNICATION BETWEEN GUT AND BRAIN

CHARACTERIZING THE FUNCTIONAL NATURE OF NERVOUS COMMUNICATION BETWEEN GUT AND BRAIN

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LAY ABSTRACT

The vagus nerve connects the brain and gut enabling the transfer of bidirectional nervous signals, of which 80-90% transmit towards the brain. Some of these vagal signals that travel from the gut to the brain can modify behaviour or mood. We investigated a potential mechanism by which vagal afferents change firing in response to stimuli inside the gut to communicate mood-altering information to the brain and explored whether vagal signaling is vulnerable to aging. We presented evidence that the antidepressant sertraline increased vagal firing by synaptic signaling between neurons in the gut wall and vagal afferents. We demonstrated that antidepressant agents produced a specific pattern of action potential firing that might encode mood-altering information to the brain. Lastly, we found that aging and Parkinson's disease decreased vagal afferent firing, but not insurmountably. This work identifies novel mechanisms by which intestinal vagal afferents signal the brain, which may have therapeutic applications.

ABSTRACT

Vagal afferents in the gut are polymodal for a multitude of chemical mediators, including beneficial and noxious sensory stimuli, and therefore must encode sensory information for the brain about the luminal environment. This sensory information has profound influence on related reflex pathways, gut function, and mood and behaviour via the gut-brain axis. Using an established mesenteric nerve recording protocol, we investigated how vagal afferents from the small intestine signal and encode information about luminal stimuli and somatic age to the brain. We investigated the role of an intramural sensory synapse between intrinsic primary afferent neurons (IPANs) of the enteric nervous system (ENS) and extrinsic vagal afferents in the gut wall in the excitatory response to luminal application of the vagus-dependent selective serotonin reuptake inhibitor (SSRI) sertraline. Vagal afferent excitation by sertraline was inhibited by intramural sensory synaptic blockade, indicating a potential role of IPAN to vagal crosstalk in the vagal response to sertraline. We examined patterns of vagal afferent firing produced by stimuli with opposing effects on behaviour to determine how the vagus encodes information pertaining to antidepressant stimuli. A distinct temporal pattern code of antidepressant vagal afferent signaling was identified that was different from the pattern code produced by non-antidepressant stimuli. Lastly, we examined how vagal afferent signaling to the brain differed in aged mice and in an aged Parkinson's disease (PD) model. There was a significant reduction in vagal afferent firing in old and PD model mice, but this reduction was partially reversed by treatment with the excitatory aminosterol squalamine. These studies

demonstrate that vagal afferent firing is critical to the communication of sensory information from the gut lumen to the brain and that this information is encoded in specific patterns of firing that are influenced by the type of stimulus and the welfare of the signaling pathway.

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less." – Marie Curie

(As quoted in Our Precarious Habitat (1973) by Melvin A. Benarde, p. v)

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TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

LIST OF ALL ABBREVIATIONS AND SYMBOLS

5-HT	5-hydroxytryptamine
5-MEOT	5-methoxytryptamine
Ach	Acetylcholine
AD	Alzheimer's disease
AH	Afterhyperpolarization
ANOVA	Analysis of variance
ANS	Autonomic nervous system
AP	Action potential
AREB	Animal Research Ethics Board
AUC	Area under the curve
BAC	Bacterial artificial chromosome
BD	Burst duration
CAP	Cholinergic anti-inflammatory pathway
CCK	Cholecystokinin
CGRP	Calcitonin gene-related peptide
CMMCs	Colonic migrating motor complexes
CNS	Central nervous system
CRF	Corticotropin-releasing factor
DCEBIO	5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazol-2-one
DMV	Dorsal motor nucleus of the vagus
DRG	Dorsal root ganglia
DSS	Dextran sodium sulfate
ECs	Enterochromaffin cells
EECs	Enteroendocrine cells
ENS	Enteric nervous system
ENT-01	Synthetic squalamine salt
FDA	Food and Drug Administration
FPO	Fecal pellet output
FST	Forced swim test
GABA	Gamma-aminobutyric acid
GD	Gap duration
GI	Gastrointestinal
HADS	Hospital Anxiety and Depression Scale
HPA axis	Hypothalamic pituitary adrenal axis
HRSD	Hamilton Rating Scale for Depression
$hSNCA^{A53T}$	Human A53T α-synuclein gene
IBI	Intraburst interval
ICC	Interstitial cells of Cajal
IGLEs	Intraganglionic laminar endings

IK_{Ca} channel	Intermediate-conductance Ca^{2+}-dependent K^+ channel
IL-1	Interleukin-1
IMAs	Intramuscular arrays
IML	Intermediolateral nucleus of the spinal cord
IPANs	Intrinsic primary afferent neurons
JB-1	*Lactobacillus rhamnosus* JB-1
LC	Locus coeruleus
LcS	*Lactobacillus casei* Shirota
LPS	Lipopolysaccharide
LR 6475	*Lactobacillus reuteri* ATCC PTA 6475
MANOVA	Multivariate analysis of variance
MDD	Major depressive disorder
MII	Mean interspike interval
MINI	Mini-International Neuropsychiatric Interview
MMC	Migrating motor complex
mPFC	Medial prefrontal cortex
mSnca	Mouse α-syn coding region
nAChR	Nicotonic acetylcholine receptor
NDRI	Noradrenaline dopamine reuptake inhibitor
non-Tg	Non-transgenic
NTS	Nucleus tractus solitarius
PBS	Phosphate-buffer saline
PCA	Principal component analysis
PCCs	Propagating contractile clusters
PD	Parkinson's disease
PNS	Peripheral nervous system
PrP	Prion protein promoter
PVN	Paraventricular nucleus
RM	Repeated measures
RMP	Resting membrane potential
RSA	Respiratory sinus arrhythmia
SEM	Standard error of the mean
sAHP	Slow afterhyperpolarization
SCFAs	Short-chain fatty acids
SEB	Staphylococcal enterotoxin B
SERT	Serotonin reuptake transporter
SNpc	Substantia nigra pars compacta
SSRI	Selective serotonin reuptake inhibitor
SW	Swiss Webster
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TRD	Treatment resistant depression

TRPV1	Transient receptor potential vanilloid 1
TST	Tail suspension test
VNS	Vagus nerve stimulation
WAS	Water avoidance stress
WT	Wild-type
α-syn	α-synuclein
η^2_p	Partial eta squared

DECLARATION OF ACADEMIC ACHIEVEMENT

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writing of the manuscript; Chapter 5: Experimental design, data collection (motility recordings and mesenteric nerve recordings), data analysis and interpretation, preparation and writing of the manuscript; and Chapter 6: Contributed to experimental design, data collection (some motility recordings and mesenteric nerve recordings), data analysis and interpretation, preparation and writing of the manuscript.

CHAPTER 1. INTRODUCTION

1.1. The Vagus Nerve as a Direct Route for Gut to Brain Communication

The gut-brain axis encompasses a complex bidirectional communication network that links, inter alia, the central (CNS) and enteric nervous systems (ENS). Through the gut-brain axis, the brain and spinal cord, enteric neurons within the gut wall, the hypothalamic pituitary adrenal (HPA) axis, and efferent and afferent nerves of the autonomic nervous system (ANS) are integrated via neuro-immuno-endocrine mediators and neuronal signaling mechanisms (Carabotti *et al.*, 2015). Emotion and cognition in the brain is influenced by intestinal function, and vice versa via these neural, endocrine, humoral, and immune signaling pathways (Rhee *et al.*, 2009). The gut-brain axis is also greatly influenced by the trillions of bacteria present within the gut (Sender *et al.*, 2016) which make up the intestinal microbiome; also referred to as the microbiota-gut-brain axis. Perturbation of the gut-brain axis or the microbiome have been implicated in the pathogenesis and pathophysiology of many diseases including neurodegenerative diseases, psychiatric and mental illnesses, and gastrointestinal (GI) disorders (Bonaz, 2003).

Afferent and efferent projections of the autonomic nervous system composed of the sympathetic and parasympathetic branches, regulate involuntary processes within the body. They mediate signaling between the CNS and the intestinal viscera and are involved in gut functions such as motility, secretion, and the mucosal immune response (Rhee *et al.*, 2009). The vagus nerve is a main component of the parasympathetic nervous system and plays a

critical role in interoceptive awareness of what occurs within the gut (Bonaz, 2003), relaying viscero-sensations or 'gut feelings' which have been likened to a sixth sense (Zagon, 2001). With a mixed nerve composition of roughly 80% afferent fibres and 20% efferent fibres (Agostoni *et al.*, 1957), the vagus is at the interface of afferent communication between the gut and brain. Originating from the medulla oblongata in the CNS, this tenth cranial nerve emerges from the jugular foramen and consists of two ganglia, the superior jugular ganglion and the inferior nodose ganglion, the latter of which contains the cell bodies of afferent neurons innervating the visceral organs (Stakenborg *et al.*, 2013). The cervical vagus then branches into the left and right cervical vagi and innervates the heart and lungs before wandering distally with the esophagus through the diaphragmatic hiatus, becoming the anterior and posterior vagus and innervating the visceral organs of the abdomen, including the stomach, liver, pancreas, and GI tract as far as the descending colon (Stakenborg *et al.*, 2013). In the abdomen, the anterior trunk (extending from the left cervical vagus), branches into the common hepatic, ventral gastric, and ventral coeliac branches supplying the i) pylorus, antrum, pancreas and proximal duodenum (common hepatic), ii) the ventral side of the stomach and proximal duodenum (ventral gastric), and iii) after joining the dorsal coeliac branch, innervate the small and large intestines following the superior mesenteric artery and its mesenteric tributaries (ventral coeliac branches) (Berthoud & Neuhuber, 2000).

The vagus is arguably one of the fastest routes of communication between gut and brain, given its direct anatomical connections between intestinal viscera and the CNS. Almost all subdiaphragmatic vagal sensory neurons are unmyelinated C-type fibres, which are

polymodal, meaning they respond to more than one type of stimulus (Waise *et al.*, 2018). The number of vagal axons in the rat abdomen was quantified at about 9500 and appears to increase allometrically with the size of the animal species, but the distribution of myelinated axons remains conserved at less than 1% (Gabella & Pease, 1973) even in humans (Keen, 1966). Unmyelinated C-fibres have a slow conduction velocity of less than 2 m/s, in comparison to other nerve types (Cervero & Sharkey, 1988). The conduction velocities of vagal afferents comprised of both C-type and Aδ fibres from the esophagus and stomach of rats were roughly 6 m/s on average, ranging from as low as 0.4 m/s to as high as 18.75 m/s (Page & Blackshaw, 1998). The pseudo-unipolar structure of vagal afferents allows them to detect peripheral signals and transmit them to the nucleus of the solitary tract (NTS) from which signals are relayed to the brain to induce the appropriate behavioural response (Browning *et al.*, 2017). Interestingly, ingestion of the psychoactive bacterium *Lactobacillus rhamnosus* JB-1 (JB-1) induced brain activation as measured by c-Fos immunoreactivity in several brain regions just 165 min after ingestion (Bharwani *et al.*, 2020). JB-1 increased vagal afferent firing following both intraluminal administration and 165 min post-ingestion and severing of the vagus by subdiaphragmatic vagotomy prevented c-Fos expression in most brain regions (Bharwani *et al.*, 2020).

In addition to modulation of the brain and behaviour by nervous connections to the nucleus tractus solitarius (NTS) and other brain regions (see 1.3.2 Vagus stimulation for treatment of depression), the vagus nerve also interacts with other signaling pathways along the gut-brain axis. The vagus serves as a hardwired link between the CNS and the immune system,

as immune cell-derived signals such as pro-inflammatory cytokines like interleukin-1 (IL-1) activate vagal afferents that terminate in the dorsal vagal complex (includes the NTS, area postrema, and dorsal motor nucleus of the vagus/DMV), ascending to the hypothalamus and preoptic area, central extended amygdala, and the visceral thalamo-cortical system (Goehler *et al.*, 2000; Breit *et al.*, 2018). These pathways are implicated in the experience of sickness including fever, neuroendocrine response, changes in behaviour, and protective reflexes to expel pathogens (Goehler *et al.*, 2000). The vagus participates in a neuro-immune axis to produce anti-inflammatory effects in two ways; via an afferent regulation of the HPA axis and via an efferent role involving the cholinergic anti-inflammatory pathway (CAP) (Bonaz *et al.*, 2017). The HPA axis is activated by stress and elevated pro-inflammatory cytokines (that can activate vagal afferents that project to the NTS (Bonaz *et al.*, 2017)) that trigger secretion of corticotropin-releasing factor (CRF) from the hypothalamus, leading to adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, and then release of the stress hormone cortisol from the adrenal glands (Breit *et al.*, 2018). The CAP is mediated by vagal efferents that suppress cytokine release from macrophages, which has been proposed to occur via alpha-7 subunit-containing nicotinic acetylcholine receptors (nAchRs) (Pavlov *et al.*, 2003).

1.2. **The Role of the Vagus in Gut Sensory Signaling**

GI function and sensation are controlled by intrinsic and extrinsic innervation of the gut wall. Generally, efferent neurons propagate information downstream of reflex and integrating centres, while afferent neurons deliver information to these centres, although neurons can

have multiple functions (Furness *et al.*, 2004). Intrinsic innervation comes from the ENS, which consists of ganglionic nerve plexuses that innervate the muscular layers of the gut wall and the submucosa and can function without input from the CNS (Furness *et al.*, 1995; Blackshaw *et al.*, 2007). These intrinsic neurons are called primary afferent neurons because they are the first neurons that encode chemical and physical information about the intestinal environment and convey this information to integrating centres to modulate gut function (Martin *et al.*, 1981; Furness *et al.*, 2004). Intestinal vagus innervation, paired with innervation from the spinal cord, comprise the extrinsic innervation of the gut responsible for connecting it to the CNS in order to regulate digestive function, relay information to modulate feeding and sickness behaviour, and perceive pain sensations (Beyak *et al.*, 2006). These vagal and spinal afferents serve primary afferent functions in the gut, although >2/3 of afferent vagal fibres innervating the small intestine act as interneurons receiving synaptic input from myenteric intrinsic primary afferent neurons (IPANs) (Perez-Burgos *et al.*, 2014).

Anatomically, extrinsic sensory afferents are distributed in three pathways to the CNS: vagal, thoracolumbar, and lumbosacral (Brookes *et al.*, 2013). Vagal afferents that innervate the intestine have cell bodies that lie in the nodose ganglia, which project to the NTS which then transmits signals to higher order neurons (Grabauskas & Owyang, 2017). Thoracolumbar and lumbosacral sensory afferents are spinal afferents whose cell bodies lie in dorsal root ganglia (DRGs) and innervate the esophagus, stomach, small intestine and proximal colon via the splanchnic nerves and for the distal colon and rectum via the pelvic nerves (Beyak *et al.*, 2006; Brookes *et al.*, 2013). Brookes et al. (2013) described five

structural types of sensory endings within the gut wall to characterize the afferent neurons in these three pathways, which include intraganglionic laminar endings (IGLEs), mucosal endings, muscular-mucosal endings, intramuscular endings, and vascular afferents. Of these five structural afferent types, most vagal afferents terminate as IGLEs, a minority are intramuscular endings (referred to as intramuscular arrays; IMAs), mucosal endings and less so as muscular-mucosal endings (esophagus only) (Berthoud & Powley, 1992; Brookes *et al.*, 2013; Wang *et al.*, 2020).

IGLEs which form at the surface of myenteric ganglia in the myenteric plexus were the first vagal visceral afferent ending in the GI tract to be described and are the most abundant (Phillips *et al.*, 2010; Forsythe *et al.*, 2014); they form reciprocal synapses with IPANs (Perez-Burgos *et al.*, 2014). Individual vagal IGLEs can innervate neighbouring myenteric ganglia by branching (Phillips *et al.*, 2010) and are believed to be associated with vagal afferent tone and emotional well-being (Forsythe *et al.*, 2014). The small intestine is estimated to contain the highest number of IGLEs, but the highest density is found in the stomach (Berthoud *et al.*, 1997). In contrast, IMAs are found predominantly in the stomach (Powley *et al.*, 2016). Mucosal afferents penetrate the lamina propria in the mucosal layer of the gut wall and are found throughout the esophagus, stomach and small intestine, but in smaller quantities (Wang *et al.*, 2020). These mucosal afferents are in contact with epithelial cells, but they do not have direct access to the intestinal lumen (Powley *et al.*, 2011). The specific structural subtypes of vagal afferents in the gut are related to the type of stimuli that activate them and the functional role they play in gut sensory signaling.

1.2.1. Mechanosensitivity and Related Functions

Mechanosensitive neurons and afferents respond to mechanical stimuli like distention and stretch and compression to activate reflex circuits and modulate peristaltic activity (Smid, 2009). Mechanical deformation of the mucosa stimulates the release of 5-HT from enterochromaffin cells (ECs) and the 5-HT can activate new pathways belonging to myenteric afterhyperpolarization (AH) neurons (IPANs), which make up about 30% of guinea pig myenteric neurons (Furness *et al.*, 1998; Blackshaw *et al.*, 2007). Mechanosensitive ion channels on IPANs allow them to respond to deformation of their processes caused by intestinal muscle contraction or stretching of the longitudinal muscle (Kunze *et al.*, 2000).

Vagal IMAs innervate the circular and longitudinal muscle and form parallel networks with smooth muscle nerve bundles (Berthoud & Powley, 1992). Active contraction or passive stretch of the intestinal muscle produces tension that activates vagal IMAs (Grundy, 2004). IGLEs are also mechanosensitive because they are found in the connective tissue of the myenteric plexus ganglia, between the longitudinal and circular muscle and thus exposed to muscle contraction (Grundy, 2004; Blackshaw *et al.*, 2007; Forsythe *et al.*, 2014). Vagal and spinal afferents in serosal and muscle layers of the GI wall also convey mechanosensory information in response to distention and contraction, but vagal afferents respond within physiological levels of distention, while spinal afferents respond both to physiological and noxious stimulation, supporting the role of spinal afferents in nociception (although not exclusively) (Grundy, 2002).

1.2.2. Chemosensitivity and Signaling Mechanisms

Chemosensing in the gut primarily occurs via interactions between endocrine cells in the epithelia that are able to 'taste' the intestinal lumen and release signaling molecules to afferent terminals in the lamina propria (Raybould, 2010). Enteroendocrine cells (EECs) make up 1% of intestinal epithelial cells and interact with vagal afferents through the direct release of serotonin or gut hormones like cholecystokinin (CCK), histamine, secretin, somatostatin, melatonin, uroguanylin, and CRF (Rhee *et al.*, 2009; Bonaz *et al.*, 2018). Luminal bacteria can communicate directly with EECs by the release of products like lipopolysaccharide (LPS) that bind to toll-like receptors (TLR) on the EECs and prompt the release of mediators which can then act on vagal afferents (Bonaz *et al.*, 2018). Chemosensitive vagal afferents are important for host defense against pathogens, by responding to immune cell-derived signals or as a primary afferent response to bacterial endotoxins (Goehler *et al.*, 2000). Additionally, chemosensitivity of vagal afferents is critical to gut-nutrient sensing and hormone-derived satiety/hunger signaling with the CNS and is modulated by various orexigenic and anorexigenic peptide receptors on vagal afferents, such as those for CCK and leptin (Waise *et al.*, 2018).

Although some mucosal vagal afferent fibres are present in the lamina propria of small intestinal villi or in crypts of Lieberkühn, they do not densely innervate the epithelial layer or have exposure to the luminal side of the intestine to directly respond to luminal contents (Forsythe *et al.*, 2014). Alternatively, sensory neurons and vagal afferents can be activated

via paracrine signaling by mediators released by neuroendocrine cells. This is sometimes referred to as "direct" vagal signaling because ECs (which make up the largest population of EECs) are in close proximity to vagal afferent terminals expressing 5-HT_3 serotonin receptors (Rhee *et al.*, 2009). These receptors are activated by the release of serotonin (5-HT) from EECs (Li *et al.*, 2000). Short-chain fatty acids (SCFAs) produced by bacteria, such as butyrate, can also act directly on vagal afferents, as opposed to longer chain fatty acids (>12 carbon acyl groups), like oleate, that have been shown to activate vagal afferents via a CCK-mediated mechanism, as this response was abolished by the CCK-A receptor antagonist Devazepide (Lal *et al.*, 2001). It is likely that luminal SCFAs are absorbed across the mucosal epithelium and directly activate vagal afferent arborizations around the crypts and lamina propria of villi and both response to butyrate and oleate are abolished by subdiaphragmatic vagotomy (Berthoud *et al.*, 1995; Lal *et al.*, 2001). Indeed, abnormal vagal afferent sensitivity may underlie various conditions including obesity and diabetes (Daly *et al.*, 2011; Kentish & Page, 2015; Grabauskas & Owyang, 2017).

1.2.3. The Intramural Sensory Synapse for IPAN to Vagus Neurotransmission

Another potential route through which molecules in the lumen may be able to activate vagal afferents is through interneuron synaptic signaling between enteric neurons in the gut wall and extrinsic sensory endings, forming a functional sensory synapse (Mueller *et al.*, 2009; Perez-Burgos *et al.*, 2014). IPANs in the intrinsic myenteric ganglia can respond to chemicals in the lumen or muscle tension consistent with their anatomy of a large, flat and

oval soma and multiple neurites (Dogiel type II morphology) which can terminate near the luminal surface of the intestine (Kunze & Furness, 1999). They can form synapses with other IPANs, enteric neurons or interneurons (Furness *et al.*, 2004) and lie in close proximity to vagal IGLEs that can cover and penetrate enteric ganglia (Brierley *et al.*, 2012). Functional and anatomic evidence of a nicotinic intramural sensory synapse between IPANs and vagal IGLEs has been previously reported (Perez-Burgos *et al.*, 2014). Pharmacological synaptic blockade of calcium dependent transmitter release (Meir *et al.*, 1999) decreased spontaneous mesenteric nerve firing in >60% of active vagal single units (Perez-Burgos *et al.*, 2014), indicating that roughly 2/3 of the signal occurs via this indirect signaling pathway. Furthermore, response to the psychoactive bacterium JB-1 which activates both vagal afferents (Perez-Burgos *et al.*, 2013) and IPANs (Mao *et al.*, 2013), was reduced in 60% of vagal afferents following total synaptic or nicotinic blockade (Perez-Burgos *et al.*, 2014).

1.3. **The Role of the Vagus in Mood and Affect**

1.3.1. Mood-Altering Vagus-Dependent Stimuli

Many microbes or substances ingested or injected into the abdomen exert psychoactive effects on the brain that influence mood, anxiety, and behaviour. Resident bacteria that make up the microbiome and ingested transient bacteria, including pathogens and probiotics, can modulate gut to brain signaling via endocrine, immune, and neural pathways, including the vagus nerve (Fulling *et al.*, 2019) and are described here. There is some evidence that the ENS and the vagus nerve are able to differentiate between pathogenic and non-pathogenic bacteria (Forsythe *et al.*, 2012). Several studies have identified neural, and specifically vagal,

mechanisms of action for ingested probiotic or pathogenic bacteria to exert behavioural effects on the brain, some of which are abolished if the vagus nerve is severed. The probiotic bacteria JB-1 stimulated vagal afferent firing *ex vivo* (Perez-Burgos *et al.*, 2013) and ingestion reduced stress-induced corticosterone and anxiety and depression-related behaviour (Bravo *et al.*, 2011). Behavioural changes were accompanied by regional-dependent alterations in $GABA_{B1b}$ and $GABA_{A\alpha2}$ mRNA expression in the brain and both behavioural and neurochemical effects were not observed if the subdiaphragmatic vagus was priorly severed (Bravo *et al.*, 2011). Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain and GABAergic dysfunction has been linked to psychiatric disorders, with animal models and mood disorder patients having reduced GABA levels and depression-like behaviour that may be improved by GABA agonists (Brambilla *et al.*, 2003). Similarly, the selective serotonin reuptake inhibitors (SSRIs) sertraline and fluoxetine had antidepressant behavioural effects in mice that were dependent on the intact subdiaphragmatic vagus, while the antidepressant effect of the noradrenaline dopamine reuptake inhibitor (NDRI) bupropion was not (McVey Neufeld *et al.*, 2019).

Another *Lactobacillus* strain, *Lactobacillus casei* Shirota (LcS), suppressed increases in plasma cortisol in medical students under academic stress and rats subjected to water avoidance stress (WAS), reduced physical symptoms including abdominal discomfort in the students, and reduced the number of CRF-expressing cells in the paraventricular nucleus (PVN) in rats (Takada *et al.*, 2016). Although these factors were not further examined following subdiaphragmatic vagotomy in the rats, intragastric administration of LcS

increased vagal afferent activity in the stomach, indicating a possible role for the afferent vagus in mediating the central effects of LcS (Takada *et al.*, 2016). In animals with dextran sodium sulfate (DSS)-induced chronic colitis and associated anxiety-like behaviour, *Bifidobacterium longum* (*B. longum*) administration reduced anxiety-like behaviour but did not affect the level of gut inflammation (Bercik *et al.*, 2011). Subdiaphragmatic vagotomy prevented the anxiolytic effects of *B. longum* (Bercik *et al.*, 2011). These studies demonstrate that the vagus nerve is critical for the transmission of anti-depressant or anxiolytic signals originating from bacteria and molecules in the intestine to the brain and therefore plays a crucial role in the modulation of behaviour and mood.

The vagus is also involved in the development of anxiety and depression-like behaviour following infection with pathogenic bacteria. One way that pathogenic gram-positive bacteria like *Escherichia coli* (*E. coli*) induce depression-like behaviour is the induction of NFκβ-dependent pro-inflammatory cytokines like IL-1 and tumor necrosis factor-α (TNF-α) mediated by their release of the endotoxin LPS (Ramana *et al.*, 2006). These proinflammatory cytokines induce sickness behaviour, which are changes in an ill patient's behaviour related to their motivational state and with interest to their survival (Dantzer, 2001), and true depressive behaviour (Dantzer *et al.*, 2008). One pathway through which this peripheral innate immune response may influence the brain is through activation of abdominal vagal afferents by cytokine production (Bluthe *et al.*, 1994; Watkins *et al.*, 1995) and systemic administration of LPS potently stimulates mesenteric afferent discharge (Liu *et al.*, 2007). There is evidence however, that LPS may also directly activate vagal neurons

via Toll-like receptor 4 (TLR-4) in the nodose ganglion (Hosoi *et al.*, 2005). Intraperitoneal administration of LPS depressed social exploration and induced pro-inflammatory cytokine (IL-1β) production, but only the behavioral effect was lost following prior subdiaphragmatic vagotomy, indicating a vagus-dependent mechanism (Bluthe *et al.*, 1994; Forsythe *et al.*, 2014). Furthermore, systemic administration of LPS or IL-1β induced fever and depression-like behaviour, the latter of which was blocked by vagotomy (Konsman *et al.*, 2000). In addition to depression-like behaviour and elevated cytokine response and spleen weight, LPS also downregulated synaptic proteins in the medial prefrontal cortex (mPFC) and altered gut microbial diversity (Zhang *et al.*, 2020). Subdiaphragmatic vagotomy attenuated the effects of LPS across all parameters, including cytokine response, in contrast to previous studies (Zhang *et al.*, 2020). Evidently there is a vagus-dependent mechanism whereby abdominal LPS administration elicits depression-like behaviour. Other pathogenic bacteria also produce depression or anxiety-like behaviour in the host organism. For example, *Campylobacter jejuni* (*C. jejuni*) or *Citrobacter rodentium* (*C. rodentium*) ingestion both produced anxiety-like behaviour and activation of vagal sensory neurons, as assessed by c-Fos expression, all in the absence of circulating cytokines (Goehler *et al.*, 2005; Lyte *et al.*, 2006).

1.3.2. Vagus Nerve Stimulation for Treatment of Depression

The role of the vagus in mediating depression and mood disorders has become an increasingly salient area of investigation. Mood and anxiety disorders afflicted an estimated 4 million Canadians in 2011 with the potential to reach 4.9 million by 2041 (Smetanin *et al.*,

2011) and an estimated 300 million people world-wide are living with major depressive disorder (MDD) (Akil *et al.*, 2018). Several treatment options exist for MDD including antidepressant therapy, psychotherapy, and brain stimulation techniques. However, the efficacy of these treatments in achieving remission for the patient after the first round is below 50% (Rush, 2007) and patients that fail to achieve remission after two or more cycles of antidepressant therapy may have treatment resistant depression (TRD) (McIntyre *et al.*, 2014).

Vagal tone refers to the constitutive firing of the vagus nerve and has been suggested as a physiological basis for regulating emotion (Diamond *et al.*, 2012). Vagal tone can be indirectly estimated by measuring a patient's respiratory sinus arrhythmia (RSA), which reflects the extent of parasympathetic vagus nerve activation on the heart (Chambers & Allen, 2002). Increasing vagal tone by stimulation of the afferent vagus is a promising approach for the treatment of depression and mood disorders (Carreno & Frazer, 2017). Patients who experienced greater changes in vagal tone in response to acupuncture for treatment of depression showed larger reductions in depression score as measured using the Hamilton Rating Scale for Depression (HRSD) (Chambers & Allen, 2002). Although originally approved by the Food and Drug Administration (FDA) in the mid-90s for the treatment of pharmaco-resistant epilepsy (O'Reardon *et al.*, 2006), vagus nerve stimulation (VNS) has since been approved as an adjunct therapy for treatment-resistant depression. Rats that received 30 min per day of continuous vagus nerve stimulation scored significantly better in the forced swim test (FST), a standard test for depression in animal models (Krahl

et al., 2004). In a 5-year observational study, patients receiving VNS showed enhanced antidepressant efficacy compared to those receiving pharmacological treatment alone (Aaronson *et al.*, 2017).

Current protocols for VNS involve stimulation of the left cervical branch of the vagus in the neck by an implantable device or stimulation of the ear by transcutaneous auricular VNS (Breit *et al.*, 2018; Kong *et al.*, 2018). Acute peripheral vagus stimulation in rats directly activated myelinated vagal afferents in the medial NTS and indirectly activated some second and higher order neurons (Beaumont *et al.*, 2017). From the NTS, vagal afferent stimulation can impact multiple regions of the brain via direct or synaptic connections that project upstream to other brain regions including, but not limited to, the locus coeruleus (LC), parabrachial nucleus, hypothalamus, thalamus, amygdala, hippocampus, anterior cingulate cortex, anterior insula and lateral prefrontal cortex, some of which are linked to depression via cortical-limbic-thalamic-striatal neural circuits (Kong *et al.*, 2018). One way that vagal stimulation can influence depression is by activation of noradrenergic neurons in the LC, which affects serotonergic signaling in the dorsal raphe nucleus (Fulling *et al.*, 2019). As discussed in the previous section, proinflammatory cytokines may contribute to the onset of depressive symptoms (Dantzer *et al.*, 2008; Dantzer, 2012) and thus it should be noted that some of the beneficial effect of VNS on depression may be due to suppression of inflammatory cytokines through the efferent cholinergic anti-inflammatory pathway (CAP) (Das, 2007; Ondicova *et al.*, 2010; Breit *et al.*, 2018).

Despite the growing popularity of VNS as described in the literature, there is still a lack of defined parameters for stimulating the vagus regardless of invasive or non-invasive technique. Programmable parameters of the implantable pulse generator used in cervical VNS include stimulus intensity, pulse duration, pulse frequency, and the stimulus on and off-time (Howland, 2014). It is likely that the parameters of stimulation may affect treatment outcome, and it has been suggested that different frequencies and patterns of electrical stimulation may produce different functional brain activity in certain areas of the brain (Zhang *et al.*, 2003). Indeed, neural signaling, such as that along the vagus between the gut and brain, encodes information in neuronal spike trains depending on the nature and magnitude of the stimulus (Furness *et al.*, 1999; Perez-Burgos *et al.*, 2013). If the neural code is a connectivity-based single line code then the message will be dependent on the type of vagal fibres that were stimulated, but if it is a temporal code, then the sensory information will be dependent on the specific pattern and timing of the spike train (Cariani, 1997). If the vagus nerve encodes sensory information along the vagus in a temporal pattern code, then the manner in which the vagus is stimulated may modulate the response elicited in the brain and thus warrants further investigation.

1.4. **Disintegration of Sensory Signaling: The Vulnerability of the Gut to Aging**

The extent to which the body is vulnerable to age, sometimes referred to as age-related frailty, depends on many factors including sociodemographic factors (age, gender, education), physical factors (weight), biological factors (hormone levels), lifestyle factors (diet, smoking and alcohol use), and psychological factors (depression) (Feng *et al.*, 2017).

A defining characteristic of aging and most age-related diseases is the presence of chronic low-grade inflammation, termed "inflammaging" (Franceschi & Campisi, 2014) and as the body ages, there is a decline in physiological function and ability to adapt to and overcome stressors (An *et al.*, 2018), to which the gut appears to be particularly vulnerable. GI disorders are a predominant cause of illness in elderly individuals and are often a result of impaired GI function resulting in disorders like dysphagia, reflux, constipation, fecal impaction, incontinence, delayed gastric emptying and impaired absorption (Saffrey, 2014). One of the most common of these morbidities is chronic constipation, which has a 30-40% incidence in people over age 65 and can lead to fecal impaction or incontinence (Saffrey, 2014). More significant changes in the gut to brain axis and GI function do not become apparent until after the age of 70 (Bitar *et al.*, 2011). The underlying causes of these GI disorders in elderly people are still under investigation but changes in intestinal motility (Hall, 2002; Orr & Chen, 2002; Saffrey, 2014), GI innervation (Phillips *et al.*, 2010), visceral sensation (Lasch *et al.*, 1997; Keating *et al.*, 2008), and sensory function (Yu *et al.*, 2015) have been observed in the aged GI tract of aged humans and animal models. Additionally, age-related pathological factors such as cancer, diabetes, pancreatic and liver disease, or drug-induced enteropathy could contribute to the incidence of GI disorders in older populations and thus physiological changes in the gut should ideally be studied in the absence of these factors (Drozdowski & Thomson, 2006).

Altered function along the alimentary tract appears to be region-specific, with more proximal regions, like the esophagus and stomach, and more distal regions like the colon and rectum

being more affected (Hall, 2002; Orr & Chen, 2002; Bitar & Patil, 2004; Drozdowski & Thomson, 2006; Saffrey, 2014). Generally small intestinal motility remains least affected but changes in bacterial overgrowth and malabsorption can occur (Fich *et al.*, 1989; Drozdowski & Thomson, 2006). However, while small intestinal motility patterns were maintained overall, the velocity of phase III of migrating motor complexes (MMC) was slower in a study of proximal small intestinal motility in patients over 80 years of age versus healthy young controls (Husebye & Engedal, 1992). Colonic motility was also slowed and may be a result of neuromuscular dysfunction in the elderly population, contributing to the incidence of chronic constipation and diverticulitis (Hall, 2002). Specifically, it may be attributed to reduced contractility of the smooth muscle due to a reduction in calcium and potassium channel currents (Xiong *et al.*, 1995) and inhibition of signal transduction pathways, as well as reductions in neurogenic and myogenic control via cholinergic neurotransmission or response of smooth muscle to neurotransmitters (Bitar & Patil, 2004). In addition, intraluminal colonic pressure is increased with age in both animal and human studies, and might be caused by an increase in non-peristaltic colonic contractions (Hall, 2002).

Impaired sensory signaling in the GI tract may underlie a reduced response to painful distention and interoceptive awareness (Khalsa *et al.*, 2009) of injury or disease in elderly individuals, contributing to the problem of chronic constipation and other GI disorders. In a study of rectal sensory thresholds and tone, subjects over age 80, had significantly higher thresholds, but rectal compliance was not different (Lagier *et al.*, 1999). Similar results were

reported for visceral pain threshold to esophageal distention in subjects over 65 (Lasch *et al.*, 1997). Mechanosensory afferent firing from the colon and jejunum of 24-month old C57BL/6 mice in response to ramp distention was reduced, particularly in high-threshold colonic fibres, and there was impaired chemosensitive function involving transient receptor potential vanilloid 1 (TRPV1) and serotonergic signaling pathways (Keating *et al.*, 2015). These studies demonstrate that there is reduced visceral afferent response to pain with age and that such sensory dysfunction may be related to loss of function in afferent signaling pathways (Keating *et al.*, 2015). Structural and morphological age-related changes have been characterized in vagal visceral afferents, such as the dilation or bulbous swellings of IGLEs, which changes are presumed to compromise afferent feedback and reflexes (Phillips *et al.*, 2010). Enteric neurons seem to be particularly vulnerable to age, but whether the number of neurons in the myenteric plexus are reduced (Santer & Baker, 1988) or preserved with age (Gamage *et al.*, 2013) is still under debate, and quantification of neurons over the lifespan may be variable and limited by the accounting method used (Saffrey, 2013). Additionally, neuronal loss occurs in specific subpopulations, affecting cholinergic neurons but not nitrergic neurons (Phillips *et al.*, 2003). Regardless of evidence for neurodegeneration with age, the gut maintains a remarkable capacity to function, albeit with some impairment.

GI disorders in elderly populations are often comorbid with age-related neurodegenerative diseases like Alzheimer's disease (AD), Parkinson's disease (PD), and other neurological conditions (Orr & Chen, 2002). There is also an increased prevalence of depression in the elderly, and aged mice over 18 months of age present with anhedonic-like traits and

behavioural despair (Malatynska *et al.*, 2012). This may be attributed in part to alterations in signaling along the gut-brain axis with age, including microbial dysbiosis and histopathological changes in the GI tract decades prior to neurodegenerative disease (Ambrosini *et al.*, 2019). The microbiome changes most significantly during the first three years of life, and in old age for which the microbiome is characterized by reduced microbial diversity, decreased *Bifidobacterium* and *Lactobacillus* species and an increase in *Enterobacteriaceae* (Kim & Benayoun, 2020). Alternatively, some probiotic bacteria have been identified that support longevity (Lee *et al.*, 2011; Matsumoto *et al.*, 2011; Donato *et al.*, 2017). It should also be noted that the intestinal microbiome of the elderly is more likely to be influenced by the sociodemographic and pathological factors outlined previously in this section (An *et al.*, 2018).

The "Prion-concept" is one hypothesis that describes how the gut-brain axis may be involved in the pathogenesis of neurodegenerative diseases (Goedert, 2015; Ambrosini *et al.*, 2019). Misfolded proteins such as those found in AD and PD may, much like typical prion pathology, interact with dendritic cells in the intestine and accumulate in the ENS before being trafficked trans-neuronally to the CNS (Ano *et al.*, 2009; Goedert, 2015; Ambrosini *et al.*, 2019). It has been hypothesized that the vagus nerve is a primary route where this occurs (Del Tredici & Braak, 2016). Consequentially, impaired function at the level of the gut with age may be both indicative of and result from comorbid neurodegenerative disease and gut dysfunction, which share common pathways in both healthy aging and neurodegenerative disease (Phillips & Powley, 2007).

1.5. **Clinical Relevance: The Vagus and Parkinson's Disease**

The vagus nerve has been implicated in the pathophysiology of PD since Braak et al. (2003) postulated the hypothesis that PD begins in the gut by the invasion of a pathogen through nasal and gastric routes that lead to the trafficking of alpha-synuclein (α-syn) from enteric neurons, to preganglionic parasympathetic motor neurons of the vagus nerve, and then finally the CNS (Braak *et al.*, 2003; Hawkes *et al.*, 2007). PD is associated with symptoms of motor dysfunction including hypokinesia, imbalance, rigidity, and tremor (Hawkes *et al.*, 2007) although cognitive, mood, circadian rhythm and hallucinatory disorders are also prevalent in PD to varying degrees (Hauser *et al.*, 2019). PD has two forms: familial, which is caused by genetic mutations in the gene for α-syn and sporadic, with both genetic and environmental factors that are unknown but postulated in Braak's hypothesis (Rietdijk *et al.*, 2017). The development of misfolded α-syn aggregates and Lewy bodies (aggregates that contain α-syn and other proteins and filaments) leads to neurodegeneration in select neuron types within the CNS and peripheral nervous system (PNS), including the ENS (Del Tredici & Braak, 2016). A key insight into the involvement of the gut-brain axis in the pathogenesis of PD is the high incidence of comorbid chronic constipation, present in about 2/3 of PD patients (Pedrosa Carrasco *et al.*, 2018), and sometimes preceding the onset of motor symptoms by as much as 20 years (Savica *et al.*, 2009). Indeed, a nigro-vagal pathway that controls gastric motility was identified whereby stimulation of the substantia nigra pars compacta (SNpc) increased gastric motility by activating receptors in the dorsal vagal complex, and this pathway was compromised by the paraquat-induced model of PD (Anselmi *et al.*, 2013). A recent study investigated Braak's hypothesis by injecting α-syn

preformed fibrils into the duodenal and pyloric muscularis layer of mice and observed α-syn pathology that spread via the vagus to the DMV, hindbrain, and other regions including the SNpc (Kim *et al.*, 2019). This α-synucleinopathy and associated neurodegeneration was not seen in mice that underwent truncal vagotomy or in α-syn knockouts (Kim *et al.*, 2019). Furthermore, there is some association between truncal vagotomy and reduced risk of subsequent PD later in life (Svensson *et al.*, 2015). Bilateral atrophy of unmyelinated fibres of the vagus have also been associated with PD, while spinal accessory or phrenic nerves were not affected, which may indicate that the vagus is more susceptible to α-synucleinopathies (Walter *et al.*, 2018).

The role of the vagus and the gut-brain axis in the pathophysiology of PD is corroborated by experimental evidence in the literature in support of Braak's hypothesis, as well as the development of enteric pathology and dysfunction that are characteristic of PD (Rietdijk *et al.*, 2017). Future studies on PD that target the ENS and α-syn aggregation that occurs here, may improve GI comorbidities including constipation (Barbut *et al.*, 2019). Additionally, therapeutic approaches that target the ENS and the gut-brain connection via the vagus may be useful in treating GI-related and additional comorbidities of aging and other neurodegenerative disease.

CHAPTER TWO. AIMS & HYPOTHESIS

Central Hypothesis: Mesenteric vagal afferents change firing within the gut to brain pathway to reflect variations in the luminal environment, which is influenced by the enteric nervous system.

Aim-Specific Hypotheses:

1. Stimuli with antidepressant properties trigger vagal afferent action potential discharge *in vitro* via the intramural sensory synapse.
2. Stimuli with antidepressant properties activate vagal fibre afferent firing with temporal firing patterns that are statistically different from non-antidepressant stimuli and intrinsic primary afferent neuron (IPAN) firing in the enteric nervous system is necessary for the existence of the vagal fibre antidepressant/depressant afferent code.
3. Vagal afferent firing pattern and rate will be altered to reflect the animal's somatic age and disease phenotype and can be functionally restored using a prokinetic aminosterol extracted from shark liver (squalamine) that stimulates IPAN activity.

These hypotheses will be tested using the following **three aims**:

1. Determine whether the vagal response to specific antidepressant stimuli is dependent on the intramural sensory synapse.

2. Identify unique patterned responses to antidepressant, pro-depressant or non-antidepressant stimuli and investigate the role of the IPAN to vagus intramural synapse in generating the afferent vagal antidepressant code.

3. Identify changes in gut to brain vagal afferent signaling rate as it relates to healthy aging and age-related neurodegenerative disease and whether this can be restored.

CHAPTER 3.

The selective serotonin reuptake inhibitor sertraline activates intestinofugal afferent vagal fibres via an intramural IPAN to vagus sensory synapse.

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Abstract

Extrinsic vagal afferents and intrinsic enteric neurons reside within the gut wall and respond to similar psychoactive stimuli. It is possible based on evidence from previous studies that crosstalk may occur between enteric neurons and vagal afferent intraganglionic laminar endings (IGLEs) through the release of chemical mediators or neurotransmitters. Indeed, the involvement of an intramural intrinsic primary afferent neuron (IPAN) to vagus sensory synapse has been documented in the vagal response to the psychoactive bacteria *Lactobacillus rhamnosus* JB-1. We have shown previously that, similarly to JB-1, the selective serotonin reuptake inhibitor (SSRI) sertraline excites both vagal afferents and IPANs. We therefore hypothesized that intraluminal sertraline may also activate vagal afferents via the intramural sensory synapse. We recorded from the jejunal mesenteric nerve bundle and selected vagal fibres responsive to CCK to avoid vagal afferents that may be directly stimulated via 5-HT_3 receptors. Synaptic blockade with the Ca^{2+} channel blocker ω-conotoxin increased the vagal interspike interval (reduced excitation) by 52% compared to the response to sertraline. Reduction of IPAN excitability using the IK_{Ca} channel opener DCEBIO increased the vagal interspike interval by 22% compared to sertraline. Since myenteric IPANs are cholinergic, the nicotinic acetylcholine receptor (nAchR) antagonist mecamylamine was tested and increased the vagal interspike interval by 78% compared to sertraline. Synaptic blockade, reduced IPAN excitability, and nicotinic blockade attenuated or abolished the previous response to sertraline in this population of vagal afferents. We conclude that in this population of vagal afferents, vagal afferent excitability to sertraline is modulated by a nicotinic intramural sensory synapse with myenteric neurons.

3.1 Introduction

Primary afferent neurons are the first to transmit both chemical and mechanosensory information about the tissues they innervate to control centres [1,2]. About 20-30% of ENS neurons are intrinsic primary afferent neurons (IPANs) with cell bodies (somata) within the gut wall, while extrinsic primary afferent neurons supplying the gut, such as vagal and spinal afferents, have somata outside the gut wall (in the nodose and jugular ganglia or dorsal root ganglia, respectively) [1]. Despite a primary afferent designation, the majority of vagal afferent fibres do not densely innervate the epithelial layer of the gut [3,4] and thus rely on paracrine and sensory signaling pathways and synaptic transmission to relay information upstream from the intestinal lumen. This is facilitated by multiple receptors for a range of mediators on vagal neuron cell bodies in the nodose ganglia and the afferent processes themselves, that can stimulate afferents in the gut wall via: (1) direct stimulation (opening of ion channels on nerve terminals), (2) sensitization (afferent hyperexcitability to chemical and mechanical stimuli), or (3) altering the afferent nerve phenotype (expression of mediators, channels, and receptors, ligand binding characteristics, or coupling of other receptors) [5].

We have recently shown that the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and sertraline activate mesenteric vagal afferent fibres *in vitro* when applied both acutely to the intestinal lumen or ingested for 14 days [6]. Primarily, we showed that the antidepressant behaviour produced by the SSRIs was no longer observed in the tail suspension test after the subdiaphragmatic vagus was severed. This lends a new perspective to the extensive and ongoing investigation into the mechanism of SSRI action, commonly

considered to prevent serotonin reuptake at presynaptic terminals in the brain, but which may have primary action in the gut and influence behaviour through peripheral vagal stimulation via the enteric nervous system (ENS) [6]. Indeed, the majority of serotonin or 5-HT in the body (80-90%) is produced, stored and released from enterochromaffin cells (ECs) in the mucosa of the intestine and mediates GI functions such as peristalsis, secretion, vasodilation, and pain perception [7]. The same serotonin reuptake transporter (SERT) in the brain is present in nearly all intestinal epithelial cells which rapidly removes 5-HT released by EC cells from the interstitial space. Remaining serotonin that enters circulation through the lamina propria is taken up by platelets expressing SERT [7]. Blockade of SERT in mucosal epithelial cells using the SSRI fluoxetine increased oxidation current, an indirect measure of 5-HT concentration, in adult guinea pig intestine [8]. In a hemi-dissection model of the myenteric plexus, adding sertraline to the mucosal epithelium, but not directly to exposed IPANs, significantly decreased the resting membrane potential and slow afterhyperpolarization of the IPANs [6]. It is possible that inhibition of SERT on mucosal epithelial cells by sertraline results in increased 5-HT leading to excitation of the IPANs.

In the GI tract, vagal afferents terminate in intraganglionic laminar endings (IGLEs) or intramuscular arrays (IMAs), which lie within the circular and longitudinal muscle layers, with IGLEs covering and sometimes penetrating enteric ganglia [9]. Using antero-retrograde tracing, the highest density of IGLEs was found in the stomach, with at least one IGLE innervating half to one-third of myenteric ganglia, although the highest total amount of IGLEs is likely found in the small intestine based on surface area [10,11]. What is sometimes referred to as direct vagal afferent activation occurs through the release of a mediator from

a primary cell, such as 5-HT and cholecystokinin (CCK) release from ECs and enteroendocrine cells (EECs), respectively, which stimulate afferent nerves in the lamina propria via paracrine signaling [5,12]. CCK-sensitive afferents are identified as exclusively vagal because subdiaphragmatic vagotomy and devazepide (CCK receptor antagonist) abolished response to CCK in mesenteric nerve bundles, of which 70% contained CCK-sensitive afferent fibres [13]. 5-HT released from ECs also stimulates both intrinsic and extrinsic afferent fibres within the lamina propria by binding to several types of 5-HT receptors [7]. The multitude of 5-HT receptors has allowed for the development of selective drugs that can modulate motility, secretion, and sensation in the gut [14].

Both CCK and 5-HT significantly activate small intestinal mesenteric afferent fibres upon systemic application [13,15]. CCK activates CCK1Rs expressed on vagal afferents whose peripheral terminals lie in close proximity to EECs within the lamina propria [16], while activation of vagal afferents by 5-HT occurs primarily via 5-HT_3 receptors [17]. These receptors have been identified on different sub-populations of rat vagal afferents and therefore CCK and 5-HT produced mutually exclusive activation in vagal afferent populations in this study [18]. It is therefore likely that increased 5-HT as a result of SERT inhibition in mucosal epithelial cells would also result in some direct activation of vagal afferents through binding to 5-HT_3 receptors. However, this only accounts for a distinct population of vagal afferents that respond directly to 5-HT and does not take into account the possibility of indirect vagal afferent stimulation through secondary activation of enteric neurons. Indeed, two separate afferent populations have been recorded whereby one

responded directly via 5-HT_3 activation and the other responded to 5-HT following 5-HT_{2A} mediated contractile activity [18].

It has been hypothesized that the close proximity of IPANs to extrinsic sensory afferent endings, particularly IGLEs, may allow for interneuron signaling between the two [2,19]. This signaling likely involves calcium-dependant transmitter release which occurs through N-type calcium channels [20]. IPANs are one of the first to respond to luminal stimuli and there is a high ratio of IPANs to extrinsic sensory fibres in the gut, about 2000:1 [21,22]. The response to the psychoactive bacteria *Lactobacillus rhamnosus* (JB-1) was previously evaluated to test this hypothesis [2]. Similar to the SSRIs tested in our previous study [6], it has been demonstrated that JB-1 produced vagal dependant antidepressant behavioural effects. Specifically, chronic feeding with JB-1 reduced anxiety and depression-related behaviour, which was not observed in vagotomized mice [23]. Furthermore, JB-1 increased excitability of IPANs even if synaptic intramural transmission is blocked [24]. JB-1 also activates vagal afferent fibres, increasing single and multiunit firing rate of the mesenteric nerve bundle, which is abolished by subdiaphragmatic vagotomy [25]. Blocking of the intramural sensory synapse with Ca^{2+} channel blockers reduced 66% of spontaneously firing afferent fibres, indicating that roughly 2/3 of the spontaneous firing signal occurs via an indirect signaling cascade [2]. Blocking of synaptic transmission or nicotinic receptor blockade reduced firing response to JB-1 in 60% of fibres that previously responded [2]. Increasing IPAN excitability using the IK_{Ca} channel blocker TRAM-34 increased vagal fibre discharge, but had no effect in the presence of synaptic blockade or after vagotomy [2].

We hypothesized that the SSRI sertraline may stimulate a population of vagal afferents through the same intramural sensory synapse pathway that is entrained by JB-1 (Figure 1A). To limit direct activation of vagal afferents by 5-HT binding to 5-HT_3 receptors, we specifically selected vagal afferents which responded to CCK [18]. We found few fibres that responded to 5-HT_3 agonists selectively in the mouse. We did, however, find a proportion of CCK-sensitive fibres that were also responsive to 5-HT_3 agonist. In a different study of vagal nodose neuronal firing in rats, high-dose (30 pmol) intra-arterial CCK-8 and intra-duodenal 5-HT (1 μM) increased neuronal firing in the same neuron [26]. Thus, we selected those fibres that clearly responded to CCK to investigate the involvement of IPAN to vagal neurotransmission in the response to sertraline. In the present study, we evaluated the effect of calcium channel blockade, IPAN silencing and nicotinic blockade on the response of CCK-sensitive vagal afferent fibres from the small intestine to sertraline using the previously tested mesenteric afferent nerve recording protocol [2,25].

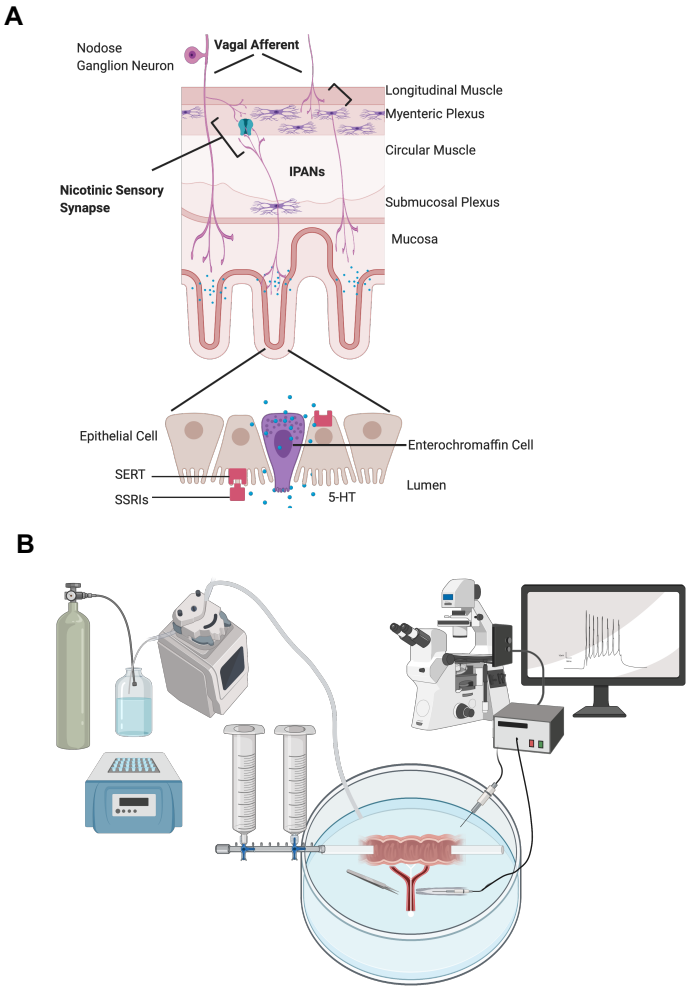

Figure 1: Potential 5-HT mediated IPAN to vagus intramural sensory synapse and schematic of mesenteric nerve recording experimental setup. (A) SSRIs like sertraline inhibit 5-HT reuptake by SERT on intestinal epithelial cells following release from ECs. Activation of the ENS stimulates vagal afferents through a nicotinic sensory synapse. (B) Intraluminal and serosal compartments are perfused separately, through direct cannulation of the intestinal lumen and via a pump in the petri dish surrounding the serosa. Afferent firing is recorded from attachment of a recording electrode within a glass micropipette to

the mesenteric nerve bundle. Tissue is kept viable by continuous perfusion of warmed, carbogenated Krebs. Figures created with BioRender.com.

3.2 Methods

3.2.1 Animals

6-8-week old male BALB/c mice were attained from Charles River Laboratories (Montreal, QB, Canada). Mice were allowed to acclimatize for minimum 1 week within the animal facility and provided food and water *ad libitum* on a 12h light/dark cycle. The BALB/c mouse was used because we used this strain in our previous investigation of the effect of SSRIs on vagal afferent firing [6] and because they represent an anxiety-like phenotype [27]. All experiments occurred *ex vivo* following cervical dislocation according to and approved by the McMaster University Animal Research Ethics Board (AREB) (permit 16-08-30).

3.2.2 Drugs and Solutions

Sertraline hydrochloride (Sigma Millipore) was dissolved and diluted in Krebs to a working concentration of 10 µM based on previous experiments [6]. ω-conotoxin GVIA (Alomone, Jerusalem, Israel) was initially dissolved in phosphate-buffer saline (PBS) and then diluted to a working concentration of 0.5 µM. 5,6-dichloro-1-ethyl-1,3-dihydro-2H- benzimidazol-2-one (DCEBIO; Sigma-Aldrich, Oakville, ON, Canada) was dissolved in DMSO before it was diluted to a working concentration of 5 µM in Krebs. Mecamylamine hydrochloride (Sigma-Aldrich) was dissolved in de-ionized water and diluted to a concentration of 50 µM in Krebs for use. All working concentrations of these drugs were based on our earlier

published experiments on mouse intestinofugal vagus action potential discharge [2]. Krebs buffer was prepared according to the following composition (mmol L^{-1}): 118 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 1.2 $MgSO_4$, 11.1 glucose, and 2.5 $CaCl_2$ bubbled with carbogen gas (95% O_2 and 5% CO_2) [28]. Nicardipine (3 μM) was dissolved in the Krebs solution perfusing the serosal compartment of the *ex vivo* preparation.

3.2.3 Mesenteric Nerve Recording

Mesenteric afferent nerve recordings were performed as previously described [2,25]. A short (2-3 cm) segment of jejunum and attached mesentery was dissected and removed from the mouse and immediately mounted on an agar-coated petri dish filled with carbogenated Krebs buffer and nicardipine. The tissue was cannulated at the oral and anal ends with silicone tubing and any luminal contents were gently flushed out using a Krebs filled syringe. The attached mesentery was pinned out flat and the mesenteric nerve bundle exposed by gently removing adipose tissue using fine-tipped forceps. The petri dish was then moved to an inverted microscope stage, where the exposed nerve bundle was sucked onto with a Krebs-filled glass recording electrode (Figure 1B). Control recordings, during which the gut lumen was gravity perfused with Krebs buffer, were made for 20-30 min. Then, sertraline in Krebs buffer (or another agent) was perfused into the lumen (treatment) followed by 10 min washout with Krebs buffer after intraluminal treatment was stopped. Intraluminal agents are applied by closing and opening 3-way stopcocks of a manifold containing three or more Mariotte bottles. The outflow of the manifold was attached to the oral cannula of the isolated segment. Intraluminal agents (treatments) were applied for a duration of 30 min. The nerve

preparation was kept viable with continuous perfusion of fresh carbogenated Krebs into the serosal compartment using a peristaltic pump. Drugs were applied to the serosal compartment of the petri dish. Multi-unit voltage spikes from the mesenteric nerve bundle were amplified using a Multi-Clamp 700B amplifier and converted to digital form with a Digidata 1440A signal converter [25]. Electrical signals were bandpass-filtered at 0.1–2 kHz, sampled at 20 kHz, and stored on a personal computer running pClamp 10 software (Molecular Devices) for later off-line data analysis. Post hoc identification of vagal afferent fibres was made from signals generated after control washout of the lumen by adding cholecystokinin (CCK, working concentration = 0.5 uM) into the serosal compartment, followed by 5-HT_3 receptor agonist 10 min later (SR 57227, working concentration = 0.1 mM). CCK selectively activates vagal but not spinal or myenteric intestinofugal fibres [18,29].

Off-line data analysis: Data recordings were imported into a program (Dataview) designed for extracellular spike analysis [30]. Multi-unit compound voltage spikes were separated into single unit activity using principal component analysis (PCA) of spikes to sort single fibre action potentials (single units) by their unique shape, size and duration [25]. Single units were identified as vagal fibres by their excitation in response to CCK, as described previously [31]. Each of the different single unit traces was then gated for control and treatment periods and the mean interspike interval between spike firing (the inverse of firing frequency) measured.

3.2.4 Statistical Analysis

All other statistical analysis was performed using GraphPad Prism software (Version 8). Percent difference in interspike interval was calculated using (treatment-control)/control. Experiments involving initial sertraline treatment, followed by an intramural sensory synapse modulator were analyzed by repeated measures (RM) 1-way ANOVA, followed by Tukey's post hoc multiple comparisons test. Combined treatment with sertraline and a modulator following Krebs control were analyzed by paired t-test, 2-tailed. Percent difference in interspike interval response to treatment was analyzed by ordinary 1-way ANOVA, followed by Holm-Sidak's multiple comparisons test. Outliers were removed using the recommended ROUT method (Q = 0.1-0.2%). All data were represented as mean ± SEM

3.3 Results

3.3.1 Vagal afferent response to sertraline was abolished by N-type calcium channel blockade

All afferent nerve recordings were done in the presence of nicardipine (3 μM), an L-type calcium channel blocker to paralyze smooth muscle preventing contractions, but which does not interfere with intramural synaptic transmission[32]. L-type calcium channels are involved in the excitation-contraction coupling process of smooth muscle in the intestine [33]. Similarly, when all voltage-gated calcium channels are blocked by cadmium, intestinal contractility is absent [19]. Synaptic transmission was inhibited by targeting and blocking N-type calcium

channels using ω-conotoxin GVIA because calcium entry through primarily these channels supports transmitter release [19,20]. Sertraline (10 μM) was initially perfused into the lumen for 30 mins following control Krebs perfusion and after which ω-conotoxin (0.5 μM) was perfused into the serosal compartment for an additional 30 mins while luminal sertraline perfusion was maintained; Figure 2A). A second experiment was performed in which a 30 min Krebs control period was recorded followed by simultaneous luminal sertraline (10 μM) and serosal ω-conotoxin (0.5 μM) (Figure 2B). Addition of intraluminal sertraline decreased the mean interspike interval by 28% from 1162 ± 148.6 ms to 834.7 ± 83.34 ms (P = 0.0414). Addition of ω-conotoxin to the serosal perfusate increased the mean interspike interval (1266 ± 178.5 ms) by 52% compared to the sertraline response (P = 0.0118). There was no difference in mean interspike interval between the Krebs control and the sertraline and ω-conotoxin combined (P = 0.5846). There was no effect of sertraline when it was added to the intestinal lumen simultaneously with ω-conotoxin (P = 0.9265) (Figure 2B).

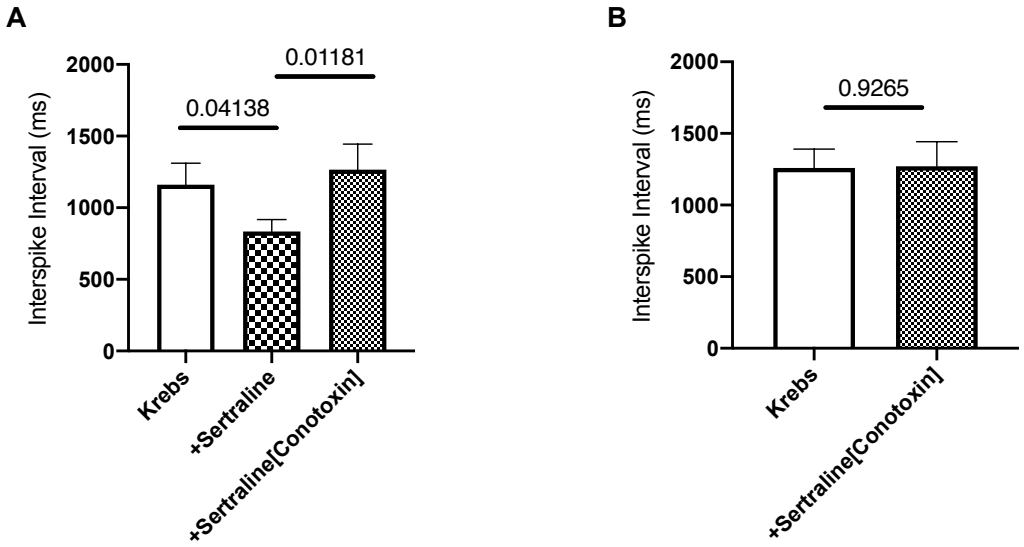

Figure 2: ω-conotoxin prevented the excitatory effect of sertraline on vagal afferent firing (A) Addition of intraluminal sertraline (10 μM) decreased the vagal interspike interval (P = 0.04138). Subsequent addition of ω-conotoxin (0.5 μM) increased the interspike interval back towards the Krebs control rate (P = 0.01181) (N= 4 mice:23 fibres, RM 1-way ANOVA with Tukey's multiple comparisons test). (B) Addition of combined intraluminal sertraline and serosal ω-conotoxin did not change the vagal interspike interval compared to Krebs control (P = 0.9265) (N= 3 mice:16 fibres, t-test paired 2-tailed). All data represented as mean ± SEM, *P < 0.05.

3.3.2 Silencing of IPANs with DCEBIO attenuated the vagal afferent response to sertraline

To test the involvement of IPAN firing in the vagal response to sertraline, we silenced IPANs by targeting their IK_{Ca} channels with DCEBIO. DCEBIO is an IK_{Ca} channel opener that increases the open probability of the channel, which is responsible for the prolonged post action potential slow afterhyperpolarization (sAHP). Because this channel is not fully closed in the absence of action potentials its background activity also contributes to the IPAN resting membrane potential (RMP) [34,35]. As the duration of the sAHP is increased, the refractory period is lengthened and the IPANs ability to fire a subsequent action potential after an initial burst remains diminished for longer period. Intraluminal sertraline decreased the mean interspike interval by 33% from 1479 ± 159.8 ms to 991.4 ± 86.57 ms (P = 0.0083). Subsequent addition of DCEBIO (5 μM) to the serosal compartment increased the mean

interspike interval response to sertraline by 22% to 1212 ± 131.4 ms (P = 0.0220). Addition of combined luminal sertraline and DCEBIO following Krebs control failed to increase vagal afferent firing rate and instead increased the interspike interval by 39% (P = 0.0103).

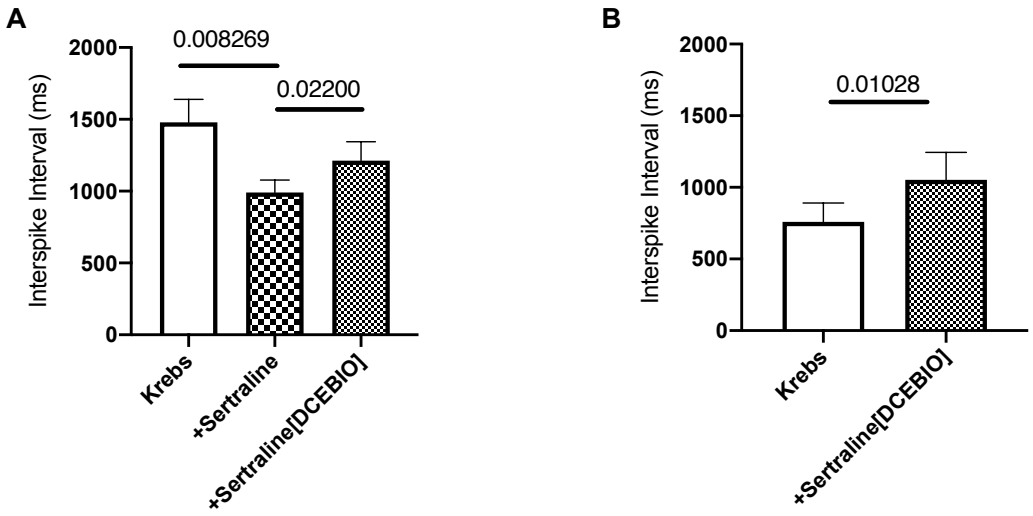

Figure 3: Reduction of IPAN firing ability using DCEBIO attenuated the excitatory effect of sertraline on vagal afferent firing (A) Addition of intraluminal sertraline (10 μM) decreased the vagal interspike interval (P = 0.008269). Subsequent addition of DCEBIO (5 μM) increased the interspike interval compared to sertraline, but not to the baseline rate (P = 0.02200) (N= 5 mice:27 fibres, RM 1-way ANOVA with Tukey's multiple comparisons test). (B) Addition of combined intraluminal sertraline and serosal DCEBIO increased the vagal interspike interval compared to Krebs control (P = 0.01028) (N= 2 mice:13 fibres, t-test paired 2-tailed). All data represented as mean ± SEM, *P < 0.05.

3.3.3 Nicotinic receptor blockade abolished the excitatory effect of sertraline on vagal afferent firing

Given the previous evidence that the intramural sensory synapse, when activated by *Lactobacillus rhamnosus* (JB-1) is cholinergic with nicotinic postsynaptic receptors (nAchR) [2], we tested whether nicotinic neurotransmission is also involved in our sertraline-evoked vagal single unit excitation. We repeated our sertraline experiment in the presence of the non-competitive nAchR antagonist mecamylamine. Intraluminal sertraline (10 μM) decreased the vagal single unit mean interspike interval by 33% from 2062 ± 339.1 ms to 1390 ± 166.2 ms (P = 0.0319) and subsequent addition of mecamylamine (50 μM) to the serosa increased the mean interspike interval by 78% compared to sertraline alone (P = 0.0514). There was no difference between mean interspike interval during Krebs control and after mecamylamine was added (P = 0.6059). Combined luminal sertraline and serosal mecamylamine increased the mean interspike interval by 46% above Krebs baseline (P = 0.0035).

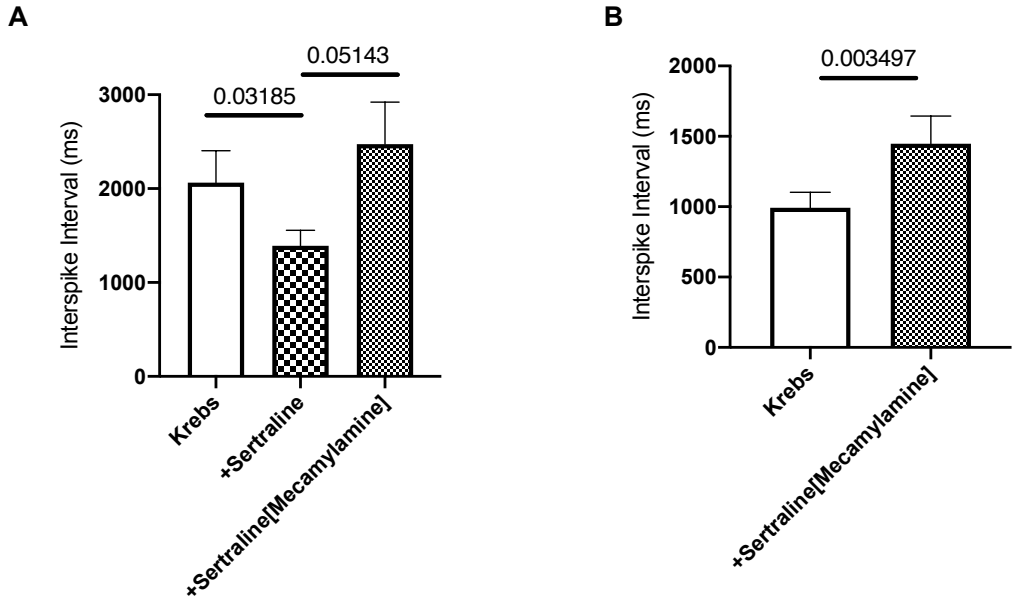

Figure 4: The excitatory effect of sertraline on vagal afferent firing was suppressed by mecamylamine (A) Addition of intraluminal sertraline (10 μM) decreased the vagal interspike interval (P = 0.03185). Subsequent addition of the nAchR antagonist mecamylamine (50 μM) increased the interspike interval above both sertraline and the control rate (P = 0.05143) (N= 2 mice:17 fibres, RM 1-way ANOVA with Tukey's multiple comparisons test). (B) Addition of combined intraluminal sertraline and serosal mecamylamine increased the vagal interspike interval compared to Krebs control (P = 0.003497) (N= 3 mice:22 fibres, t-test paired 2-tailed). All data represented as mean ± SEM, *P < 0.05.

3.3.4 Effect of intramural sensory synaptic blockade on excitatory vagal afferent firing in single units

To demonstrate the overall effect of synaptic blockade on the vagal afferent firing rate compared to baseline (Krebs) firing, we compared the percent difference in interspike interval between all treatments and Krebs. On average, between all trials, sertraline reduced the mean interspike interval between vagal afferent firing by 19.35 ± 4.224 % (Figure 5A). Following intraluminal sertraline, adding sertraline + ω-conotoxin together increased the mean interspike interval by 3.629 ± 6.284 % compared to Krebs control. Addition of sertraline and DCEBIO increased the mean interspike interval by 7.121 ± 14.64 % compared to Krebs control. Addition of sertraline and mecamylamine together increased the interspike interval by 34.20 ± 17.37 % compared to Krebs control. The effects of sertraline +DCEBIO and sertraline +mecamylamine on vagal afferent interspike interval at baseline were significantly different than the effect of sertraline (P = 0.0453 and 0.0004, respectively). The effect of sertraline +ω-conotoxin on baseline interspike interval was not statistically different (P = 0.0661) compared to the effect of sertraline. Under normal conditions sertraline excited an average of 71% of vagal afferent single units tested (18/27, 17/23, and 12/17 for Figure 5B,C, and D respectively). Addition of DCEBIO reduced the number of single units excited by sertraline to 26%, a reduction of 41% specifically, indicating that roughly 41% of the excitatory signal by sertraline was dependent on IPAN firing (Figure 5B). Addition of ω-conotoxin reduced the number of single units excited by sertraline to 22%, a reduction of 52% (Figure 5C) and addition of mecamylamine reduced the number of single units excited

by sertraline to 12%, a reduction of 58% (Figure 5D), which indicates that over 50% of the vagal excitatory response to sertraline was dependent on nicotinic neurotransmission.

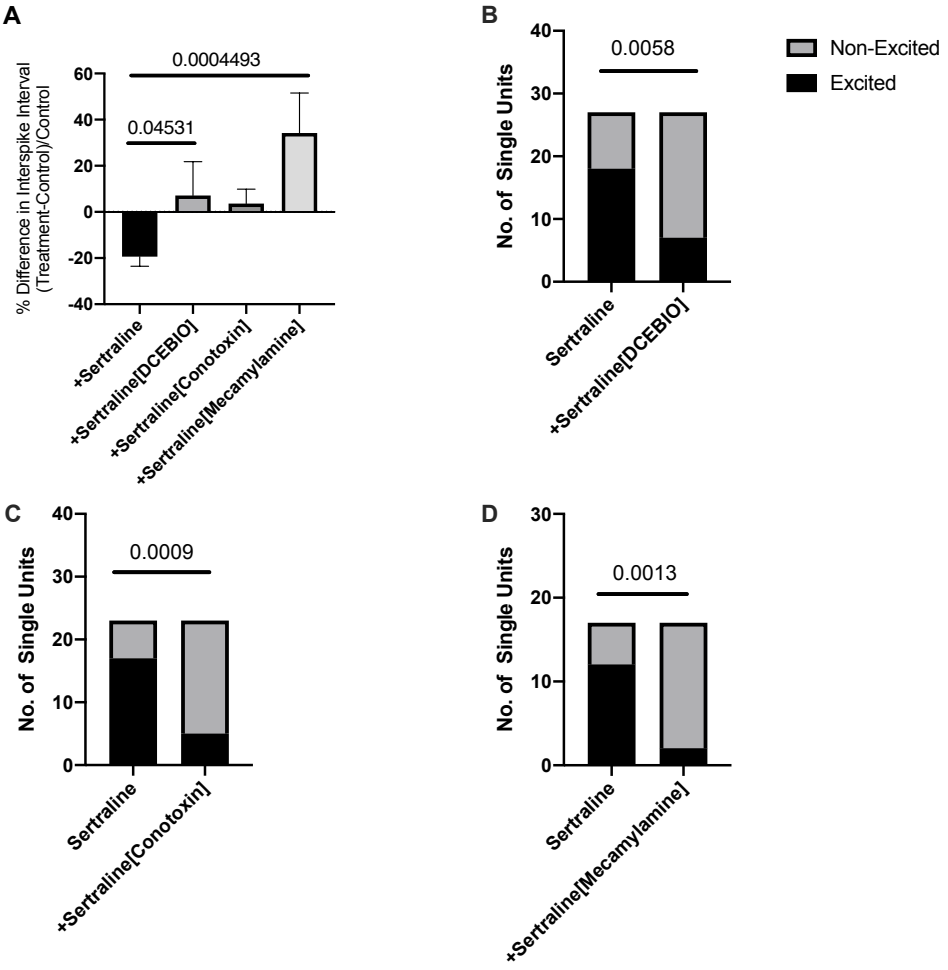

Figure 5: Intramural sensory synaptic blockade reduces the number of vagal single units excited by sertraline (A) Addition of intraluminal sertraline (10 μM) and serosal DCEBIO (5 μM) or mecamylamine (50 μM) increased the percent difference in vagal interspike interval between treatment and Krebs control. These effects were significantly different than the negative percent difference in vagal interspike interval produced by intraluminal sertraline (P = 0.04531 and 0.0004, respectively). Subsequent addition of ω-

conotoxin (0.5 μM) was not different from the percent difference in interspike interval produced by sertraline (N= 134 total fibres, 1-way ANOVA with Holm-Sidak's multiple comparisons test). Data represented as mean ± SEM, *P < 0.05. (B) (C) (D) The number of vagal single units excited by sertraline (black) was reduced by addition of DCEBIO, ω-conotoxin, and mecamylamine respectively (non-excited/grey) (Fisher's exact probability test, *P < 0.05).

3.4 Discussion

In the present study, we show that excitation of vagal afferents by sertraline is dependent, at least in part, on sensory synaptic signalling between IPANs and vagal afferents in the gut wall, and that this synapse may be nicotinic in nature. We verified that intraluminal sertraline increased vagal afferent firing rate [6]. In a population of vagal afferents, identified by their sensitivity to CCK, inhibition of synaptic transmission by blocking N-type calcium channels abolished the excitatory effects of sertraline. Correspondingly, addition of DCEBIO to silence IPANs reduced vagal afferent firing and partially reversed the excitation produced by sertraline. Nicotinic blockade with mecamylamine also abolished the excitatory effects of sertraline on vagal afferents. These results demonstrate that sertraline activates a population of vagal afferent fibres indirectly via relay by myenteric IPANs, and further supports the possibility that SSRIs have local intramural effects on the vagal gut-brain axis separate from entering systemic circulation.

Selection of CCK activated vagal afferents profoundly reduced the likelihood of observing direct activation of 5-HT_3 on vagal afferents by 5-HT. Hillsley et al. (1998)

demonstrated in anaesthetized rats that 5-HT activated two distinct populations of afferents within the mesenteric bundle: 1) an initial direct response via 5-HT_3 receptors, mimicked by the agonist 1-methyl-5-HT and abolished by the antagonist granisetron, and 2) a delayed, less intense but prolonged secondary response, mimicked by the 5-HT_4 agonist 5-methoxytryptamine (5-MEOT), attenuated by the 5-HT_{2A} antagonist ketanserin and coinciding with increased intrajejunal pressure [15]. This secondary response was attributed to mechanosensitive afferents as a consequence of 5-HT_{2A} activation on intestinal smooth muscle cells and was consequently attenuated by 50% by the L-type Ca channel inhibitor nifedipine [15]. However, 5-HT_{2A} receptors have been identified on enteric neurons and it has been suggested that a receptor from the 5-HT_2 family modulates acetylcholine (Ach) and neurotransmitter release from enteric nerves and their presence on axon terminals and synaptic junctions correlates with potential pre- and or post-synaptic effects [36]. In the present study, recordings were performed in the presence of the L-type Ca^{2+} channel blocker nicardipine to inhibit mechanical-induced afferent stimulation.

Serotonergic activation of IPANs can occur via several 5-HT receptors, with submucosal IPANs activated by 5-HT_{1P} receptors, causing the release Ach and calcitonin gene-related peptide (CGRP), which is amplified by $5HT_4$ receptors [14]. Myenteric IPANs on the other hand are activated by 5-HT_3 leading to Ach release [14]. Accordingly, both 5-HT_3 and 5-HT_4 agonists stimulate propulsion, secretion, and excitation of extrinsic afferent neurons [37]. Administration of a 5-HT_3 antagonist abolishes vagal afferent discharge from the intestine, which was inferred by electrophysiological recordings within the nodose ganglia after administration of luminal 5-HT [38]. Additionally, sertraline addition to the mucosal

epithelium, but not directly onto the myenteric neurons increased excitability of the IPANs [6]. This supports a potential role of 5-HT for epithelium to enteric nervous system signaling in the excitation of IPANs, as opposed to direct excitation of IPANs by luminal 5-HT. Overall these observations support the role of an intramural sensory synapse in the indirect excitation of vagal afferents following intraluminal sertraline administration. They are also consistent with the idea that the increased local 5-HT concentration following SERT inhibition by sertraline, leads to excitation of myenteric IPANs, which synapse with vagal afferent IGLE terminals (Figure 1A).

The use of ω-conotoxin allowed us to target Ca^{2+}-dependent neurotransmission by blocking N-type Ca^{2+} channels present at nerve terminals, which reduced the release of Ach and other neurotransmitters from postganglionic neurons [39]. In the rat ileum, ω-conotoxin GVIA blocked neural-mediated contractions [40]. A previous study showed that mesenteric afferent response to intraluminal 5-HT was unchanged by ω-conotoxin GVIA application [19], but this may have been a result of the addition of butyrate in their Krebs buffer because this volatile fatty acid can activate vagal afferents directly [2,41]. By selecting CCK-responsive afferents and using a butyrate-free buffer, we deduce that the inhibition of vagal afferent response to sertraline in the presence of ω-conotoxin indicates Ca^{2+}-dependent sensory cross-talk between intrinsic enteric neurons and extrinsic vagal afferents. It is also of importance for this interpretation to note that ω-conotoxin does not compromise Ca^{2+}-dependent receptor-mediated release of sensory mediators from luminal epithelial cells, for which release occurs via intracellular calcium stores in ECs [2,42].

We hypothesized that IPANs are the myenteric neurons involved in this cross-talk based on the presence of vagal IGLEs that terminate on these neurons. We targeted IPANs (AH cells) pharmacologically by exploiting the characteristic prolonged slow afterhyperpolarization present in these AH cells [34], which is generated by the opening of IK_{Ca} channels [35]. The IK_{Ca} channel opener DCEBIO increases the open probability of these channels and prolongs the post action potential relative refractory period (slow afterhyperpolarization) of the neuron, delaying a subsequent action potential. Mesenteric afferent firing rate was reduced by 26% by DCEBIO application in 13/19 single units in a previous study [2]. In our experiment, DCEBIO increased the vagal interspike interval by 22%, compared to the vagal interspike interval evoked by sertraline alone. The number of vagal single units excited by sertraline was reduced by DCEBIO from 18/27 to 7/27. The vagal interspike interval measured in response to DCEBIO was not different from the Krebs control, indicating that the excitatory effects of sertraline on vagal afferent firing were mediated by IPAN excitation. These results are consistent with our model that sertraline's excitatory effect on vagal afferents is mediated by activation of the IPAN to vagus intramural sensory synapse.

Interestingly, not only did the serosal addition of mecamylamine completely inhibit the excitatory effects of sertraline, it also increased the vagal basal interspike interval, decreasing firing frequency. However, in pilot experiments mecamylamine alone did not significantly affect baseline vagal firing (Figure S1), nor did it affect baseline firing in the previous study [2]. Mecamylamine [43] and other non-specific nAchR antagonists have been shown in both animal studies and clinical trials to produce antidepressant-like effects and

may be synergistically efficacious when paired with SSRIs or adjunct antidepressant therapy (reviewed in [44] and [45]). A subthreshold dose of 0.5 mg/kg, *in vivo*, of mecamylamine was ineffective on its own, but significantly reduced immobility time when paired with the tricyclic antidepressant amitriptyline [46]. This apparently contradictory effect may occur both because mecamylamine has been shown to block serotonin reuptake in the dorsal raphe nucleus of rats [47] and SSRIs and other antidepressants can act as non-competitive antagonists at nAchR [45]. Strain specific effects have also been reported as both nicotine and mecamylamine increased swim distance in the forced swim test in BALB/c mice, but only mecamylamine in NMRI mice and only nicotine in C57BL/6J were effective [48]. The nAchR partial agonist varenicline also had antidepressant-like activity in the forced swim test in both C57BL/6J and CD-1 mice and at low doses significantly improved the antidepressant effect of sertraline [49]. However, dexmecamylamine (the S isomer of mecamylamine) did not improve antidepressant scores adjunct to SSRI/SNRI treatment in patients with major depressive disorder (MDD) in two Phase III studies [50]. It is clear that when examined through a behavioural lens, mecamylamine and SSRIs may synergistically improve antidepressant activity *in vivo*. These synergistic behavioural considerations do not however apply to our *in vitro* pharmacological experimental design because mecamylamine cannot have central effects in these experiments.

However, in the context of this study, serosal mecamylamine combined with intraluminal sertraline did not excite vagal afferents such as was observed when sertraline alone was applied. This is interesting because the intact vagus nerve is necessary for the antidepressant behavioural effects of sertraline to be observed [6]. Within the intestine, both

myenteric IPANs and IGLEs contain nAchRs, and IGLEs surround myenteric neurons being anatomically positioned to form an IPAN to vagus nicotinic sensory synapse [2]. Additionally, 5-HT increases the release of Ach and CGRP from submucosal IPANs and Ach from myenteric IPANs upon binding [14]. Thus, addition of mecamylamine in our experiment likely prevented the binding of Ach, released from myenteric IPANs, to nAchRs on vagal afferent terminals. This hypothesis warrants further investigation and would require simultaneous recording from IPANs and mesenteric afferents which has so far proven to be technically challenging.

Ultimately the role of serotonergic signaling in the gut is complex and requires further investigation to identify the receptors involved in the transmission of the vagal afferent signal in this model. Future experiments should be undertaken to antagonistically target specific 5-HT receptors to determine if the luminal epithelial cell to IPAN signal is primarily serotonergic and to identify which 5-HT receptors are involved. Nevertheless, the results of the present study provide evidence that vagal afferent firing is indirectly stimulated following application of the SSRI sertraline through sensory synaptic transmission between IPANs and vagal afferents and that this pathway involves nicotinic cholinergic neurotransmission. This has possible therapeutic implications for the development of combinations of drugs that target specific 5-HT receptors to treat IBS and other GI disorders and for the investigation of chemotherapeutic approaches to treat certain psychiatric disorders.

3.5 Supplementary Figures

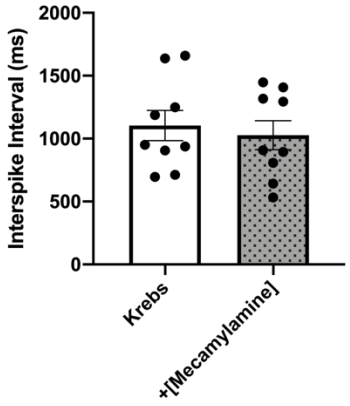

Figure S1: Pilot experiments showing addition of serosal mecamylamine (50 μM) did not affect baseline vagal afferent interspike interval. (N= 1 mouse:9 fibres, t-test paired 2-tailed). All data represented as mean ± SEM, $*P < 0.05$.

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CHAPTER 4.

Decoding vagal firing patterns in antidepressant gut to brain signaling

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Abstract

As an important pathway for gut to brain chemosensory signalling, vagal afferents can encode sensory information using a labelled-line code, where a single vagal afferent is finely-tuned to respond to one type of stimulus, or via an action potential interval code, where the nature of the stimulus is encoded in the timing and pattern of afferent spike firing. Several pro- or antidepressant agents, which when introduced into the intestinal lumen, excite vagal afferent firing and produce mood and behavioural changes that are dependent on an intact vagus nerve. We aimed to determine whether individual vagal afferents (single units) are specifically tuned to respond to pro-depressant or antidepressant substances or are broadly tuned, being excited by both pro- and antidepressant agents. If they are broadly tuned, as has been suggested by a previous study, we will determine whether an individual afferent encodes the antidepressant or pro-depressant signals in the timing and pattern of evoked firing responses. Specific mood altering vagal-dependent agents include the *Lactobacillus rhamnosus* JB-1 bacteria and the selective serotonin reuptake inhibitors (SSRIs) sertraline and fluoxetine that promote antidepressant behaviour upon ingestion; and the pro-depressant gram-negative bacterial endotoxin lipopolysaccharide (LPS). These were tested for effects on the intestinal afferent vagus in a well-established *ex vivo* model consisting of a mouse jejunal segment with an attached mesenteric neurovascular bundle that allows the recording of extracellular multiunit spikes as they leave the gut wall. JB-1, sertraline, and LPS all increased peak firing frequency in a vagal afferent single unit, suggesting that individual afferents are not specifically tuned to respond to either an antidepressant or a pro-depressant stimulus. We then characterized the afferent firing

response in individual vagal afferents to each of these substances by measuring following parameters: the overall rate of firing (mean interspike interval, MII), the time between bursting (gap duration; GD), the duration of spike bursting (burst duration; BD), and the time between spikes within a burst (intraburst interval; IBI). By comparing these firing parameters for each treatment agent using multivariate analysis of variance (MANOVA), we identified a unique antidepressant firing pattern that was different from the pro-depressant stimulus and different from stimuli that were not vagal dependent or had no psychoactive behavioural effects. Furthermore, this antidepressant firing pattern was preserved in mice that had been fed an SSRI for 14 days prior to *ex vivo* recording and across two different mouse strains. We show here, some of the first quantifiable evidence that vagal afferents encode information about the nature of mood-altering antidepressant stimuli in their temporal firing pattern. Such firing patterns may one day be utilized to screen for other bacteria, molecules, or agents with antidepressant properties or to develop a more targeted protocol for vagus nerve stimulation and electroceuticals used in the treatment of pharmaco-resistant depression and as an adjunct antidepressant therapy.

4.1 Introduction

The vagus nerve and various endocrine and immune signals are important messengers in gut to brain signaling [1-3], which influences brain function and behaviour. Of these afferent signalling routes, the vagal afferent pathway, which is continuously active, is the most rapid and direct. The abdominal vagus emerges from the dorsal and ventral coeliac

branches to innervate the small and large intestines terminating as mesenteric neurovascular bundles that innervate the gut wall in segments [4]. Vagal neurons innervating the intestines have cell bodies that lie in the nodose ganglia and project to the caudal part of the nucleus of tractus solitarius (NTS) [4]. The major cerebral targets from the NTS are to medullary motor nuclei in the midbrain, to the locus coeruleus and dorsal raphe, the reticular formation, and parabrachial nucleus, and to forebrain structures such as the amygdala, hypothalamus, insular cortex, and bed nucleus of stria terminalis [5].

Brookes et al. [6] divided intestinal extrinsic sensory neurons into 5 groups: neurons that innervate myenteric neurons via intraganglionic laminar endings (IGLEs), mucosal afferents, muscular-mucosal afferents, intramuscular arrays, & vascular afferents. Mucosal afferents do not penetrate the luminal epithelium but ramify in the mucosal lamina propria below the epithelial basement membrane where they can receive signals from enteroendocrine and epithelial cells, and luminal contents that penetrate the epithelial barrier [7]. Similar to the bidirectional synapse between intrinsic primary afferent neurons (IPANs) and IGLEs, extrinsic nerve afferents appear to form highly specialised reciprocal synapses with enteroendocrine cells of the intestinal epithelium [8]. Overall, 95% of sensory nerves supplying the epithelium arise from the enteric nervous system (ENS) as has been demonstrated by extrinsic denervation of the intestine because such denervation decreased sensory neuropeptide containing fibres within the gut mucosal layer by only 5% [9,10]. Thus, a major source of epithelial sensory neurites originates from intrinsic rather than extrinsic primary afferent neurons within the gut. Anterograde intra-axonal tracing studies demonstrate that the ENS in turn receives the densest and most abundant afferent innervation

from the vagus nerve [11] when compared to epithelial or muscular targets. Vagal IGLEs form functional intramural sensory synapses with IPANs [12] (Figure 1A).

Vagal chemosensory responses have long been recognized as being involved in behavioural and physiological regulation [13]. Such responses may involve substances that translocate across the epithelial barrier to stimulate vagal endings, mediators released by epithelial enteroendocrine cells [14] or transmitters released by IPANs of the enteric nervous system [12]. Irrespective of the pathway and intramural location of chemosensory transduction, all vagal afferent signalling must be conveyed to the brain via vagal fibres as they leave the intestine within the mesenteric nerve bundle.

Theoretically, vagal mesenteric afferent fibres could encode sensory information using a labelled line code where each single afferent or a group of similarly tuned afferents is selective for only one type of stimulus, either pro or antidepressant; or an interval code whose firing pattern in a single fibre contains the information as a unique identifier for the affective nature of the peripheral stimulus [15]. The tuning (modality and stimulus specificity) of individual vagal afferents supplying the intestine has not been completely elucidated. A small subset of vagal afferents has been identified as being finely tuned to specific sugars, amino acids, or fats [16-18]. However, the majority of individual chemosensory afferent fibres are thought to be broadly tuned (polymodal) group IV fibres responding to a variety of chemical and mechanical stimuli [4,11]. A recent comprehensive study [19] using *in vivo* calcium imaging of rat vagal neurons while substances were applied into the lumen has reported that vagal chemosensory neurons are polymodal responding to a broad range of behaviourally active substances.

A variety of pro- or antidepressant agents that are ingested or injected intra-abdominally appear to be vagus-dependent for the behavioural effects. Intra-abdominal injection of lipopolysaccharide (LPS) (*Escherichia coli* O127:B8, Sigma) dissolved in pyrogen-free saline has been shown to induce behavioural depression in rats which outlasted sickness behaviour and cytokine responses [20]. However, prior subdiaphragmatic vagotomy abolished the depressive response, although not levels of LPS-induced circulating cytokines [20]. The antidepressant behavioural effects of oral *Lactobacillus rhamnosus* JB-1 (JB-1) [21] or of the pharmacological antidepressant selective serotonin reuptake inhibitors (SSRIs) sertraline or fluoxetine are prevented by prior subdiaphragmatic vagotomy [22]. This effect of vagotomy was not observed for the noradrenaline dopamine reuptake inhibitor (NDRI) bupropion, which produced antidepressant behavioural effects regardless of prior vagotomy [22]. Interestingly, since the afferent vagus one of the most rapid routes for gut to brain signalling, activation of some regions of the brain occurred within 165 min of JB-1 reaching the small intestine after oral feeding; an effect not observed in some regions after vagotomy [23]. Furthermore, some bacteria do not appear to have mood-related effects; for example, *Lactobacillus reuteri* ATCC PTA 6475 (LR 6475) had no effect on behaviour as measured by the tail suspension test (TST) in pilot experiments (data not shown). Although the above research was performed using rodents, it has been asserted that subdiaphragmatic vagotomy in humans is associated with increased psychiatric disorders in humans [24].

There appears to be a fundamental gap in understanding pro- versus antidepressant mood altering signals encoded in the vagus. Both application of intraluminal LPS [25] (a pro-depressant stimulus) or antidepressant substances JB-1 [21], sertraline, and fluoxetine [22]

activate vagal afferent fibres. This raises the question as to whether the pro- and antidepressant stimuli act on the same individual afferent vagal axon or whether, despite the broad tuning attributed to vagal afferents, separate types of afferents are specifically tuned for pro- vs antidepressant luminal stimuli. In the present paper we aimed to determine whether individual vagal afferent fibres are broadly or specifically tuned in this respect; and if they are broadly tuned, whether individual afferents encode pro- or antidepressant signals in their action potential firing patterns. We addressed these questions using a well-established *ex vivo* mouse jejunal segment preparation to record afferent vagal activity using extracellular suction electrodes [22,26,27] while the lumen was exposed to mood-altering agents.

4.2 Results

4.2.1 JB-1, sertraline, and LPS all increase activity in a single vagal afferent

We first aimed to determine whether individual vagal afferents (single units) were broadly or specifically tuned to respond to antidepressant and pro-depressant stimuli. We recorded multi-unit afferent nerve firing from a mesenteric nerve bundle arising from the jejunum of a BALB/c mouse (Figure 1B). Principal component analysis (PCA) was performed using the Dataview program [28] to identify single units by matching near identical action potential waveforms originating from a single fibre (Figure 2A&B). Following intraluminal treatment and washout with Krebs buffer, vagal single units were identified from among the mesenteric nerve fibre multi-unit recording by their prominent excitatory response to exogenous CCK (Figure 2C). To determine whether pro- and antidepressant

stimuli act on the same nerve fibre, we applied two vagus-dependent antidepressant stimuli, JB-1 and sertraline [21,22], and the pro-depressant agent LPS [25], in succession to the jejunal mesenteric nerve bundle. JB-1, sertraline, and LPS all markedly increased vagal afferent firing rate, with no difference in peak frequency observed between agents in a single vagal afferent (Figure 2D&E). We concluded that vagal afferents are broadly tuned with respect to pro- and antidepressant luminal stimuli and hypothesize that afferent fibres encode information about the nature of the stimulus in the specific pattern of firing of the action potentials.

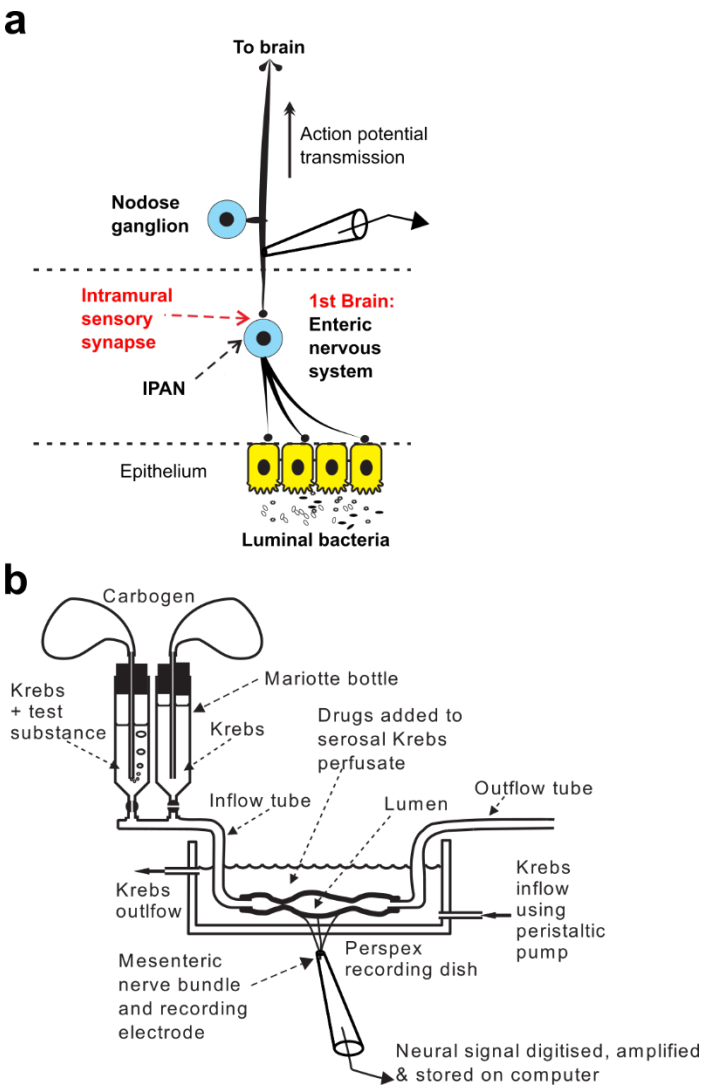

Figure 1: Gut to brain chemosensory vagal afferent signals were intercepted as the nerve fibres leave the intestine. a, the enteric nervous system (ENS), which first appeared in cnidarian, developed communication pathways with the brain in Bilateria including rodents. The visceral afferent vagus is an important pathway for gut to brain signals modulating mood and affect-related behaviour. **b**, afferent multiunit extracellular action potentials were recorded via a suction electrode from a jejunal mesenteric nerve bundle attached to a jejunal segment.

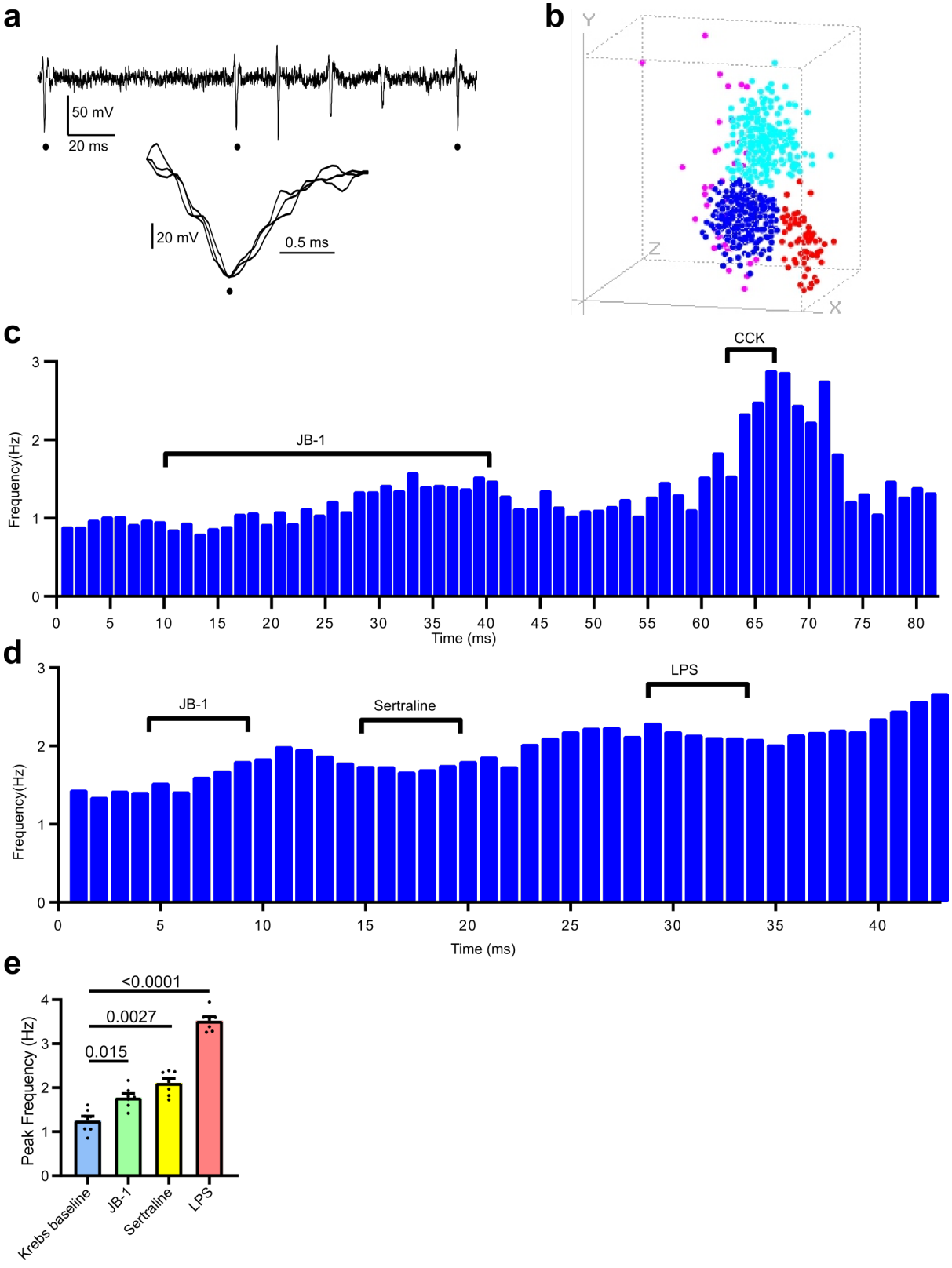
a
50 mV
20 ms
20 mV
0.5 ms
b
Y
Z
X
c
CCK
JB-1
Frequency(Hz)
Time (ms)
d
JB-1
Sertraline
LPS
Frequency(Hz)
Time (ms)
e
<0.0001
0.0027
0.015
Peak Frequency (Hz)
Krebs baseline
JB-1
Sertraline
LPS

Figure 2: Single unit analysis of multimodal vagal mesenteric afferent responses to intraluminal chemical stimuli. a, representative multiunit recording (from male BALB/c jejunal segments) showing identification of 3 single units with identical waveforms (peaks identified by filled circles). Insert shows 3 units aligned at their peak. **b**, individual single units were identified according to their shapes using the principal component analysis (PCA) algorithm in the Dataview program. PCA identified 4 shape clusters for the parent recording from which (**a**) was excerpted. Three clusters (red, blue & aqua) were fairly tight, identifying 3 different single units uncontaminated by temporally coincident spikes of a different shape. The single dispersed cluster (magenta) represents different spikes that were coincident so that their action potentials distort each other. The latter were not used for further analysis. **c**, sequential rate histogram showing single unit response to 30 min intraluminal application of *Lactobacillus rhamnosus* JB-1 in a fibre that was identified as being vagal afferent by its vigorous response to a brief application of cholecystokinin (CCK) to the serosal side of the jejunal segment. **d**, histogram illustrating the polymodal response to luminal stimuli for a vagus fibre single unit which was excited by 2 antidepressant agents (JB-1 or sertraline) and a pro-depressant agent (LPS). **e**, both antidepressants and LPS increased vagal single unit firing rate (n = 7); probabilities associated with no difference given by lines between bars (Holm-Sidak's multiple comparisons test). Data are mean ± SEM.

4.2.2 Acute treatment with vagus-dependent pro- and antidepressant stimuli, but not controls decreases mean interspike interval in vagal afferents

To quantitatively identify mesenteric afferent discharge patterns, we recorded the response to acute, luminal applications of our previously tested vagus-dependent antidepressants (JB-1, sertraline, fluoxetine), the pro-depressant LPS, vagus independent antidepressant bupropion and the non-antidepressant bacteria LR 6475 (Figure 3). Mean interspike intervals (MII) were measured, which represents the mean duration of time between vagal spike firing and is the reciprocal of firing frequency. All vagal-dependent stimuli JB-1, sertraline, fluoxetine, and LPS decreased MII ($\eta^2_p =$ 0.55, 0.29, 0.56, and 0.13, respectively), regardless of upstream effects on behaviour (Figure 3A-D). Bupropion, which did not have vagal-dependent antidepressant effects, increased MII ($\eta^2_p =$ 0.44). LR 6475 did not significantly affect MII ($\eta^2_p =$ 0.015). Effect sizes were calculated using the partial eta squared statistic η^2_p. According to Cohen's (1988) guidelines for interpreting η^2_p, the antidepressant agents, including bupropion, produced a large effect, while LPS produced a medium effect and LR 6475 produced a small effect [29]. Since vagus-dependent antidepressant agents and pro-depressant agents all decreased MII (increase firing rate) in vagal afferents, the information about the affective nature of the stimulus must be encoded in the 4 parameters (MII, GD, BD & IBI) defining the single unit firing patterns.

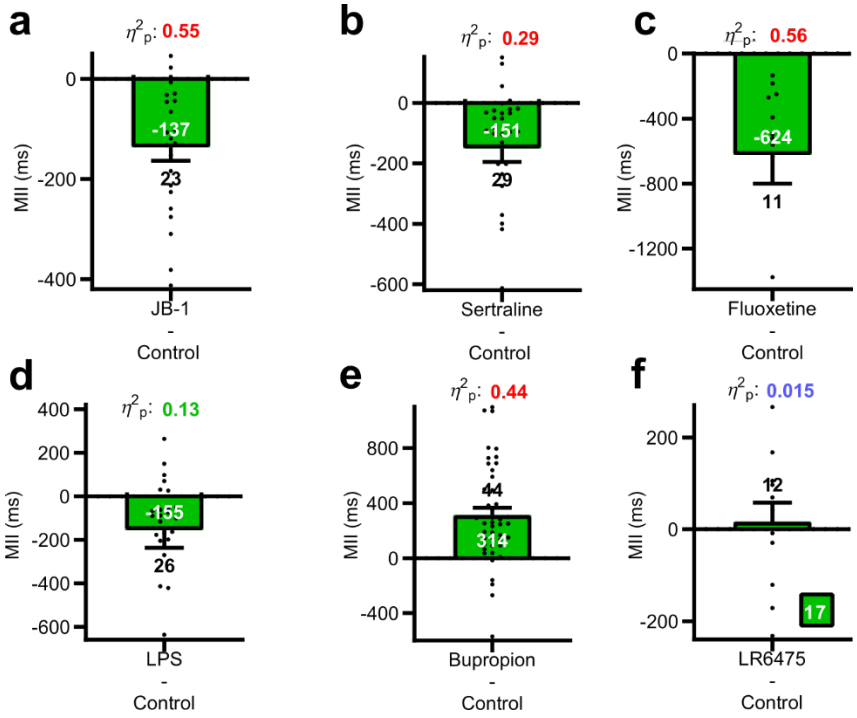

Figure 3: Paired differences graphs of mean interspike intervals before and after adding pro-, antidepressants or controls to the jejunal lumen. a-d, vagus-dependent antidepressant agents (JB-1, sertraline & fluoxetine) and LPS decreased mean interspike intervals (MII) compared to paired Krebs control recordings. **e-f**, vagus-independent antidepressant bupropion increased MII and the non-antidepressant bacteria *Lactobacillus reuteri* ATCC PTA 6475 increased or had little effect on MII. Effect sizes were calculated using the partial eta squared statistic η^2_p. Antidepressant agents produced a large effect, LPS a medium effect, and LR6475 had a small effect; see statistical analysis in Methods. Single unit numbers given above or below SEM, MII means given within solid bars.

4.2.3 Vagal-dependent antidepressants have a unique pattern of firing that is different from a pro-depressant stimulus

The MII, GD, BD and IBI for individual single units was measured while their firing was affected by the intraluminal agents tested (Figure 4A), in order to try to identify a temporal pattern code. A burst has been generally defined as the occurrence of many action potential spikes over a short time interval [30] and one technique for stochastically identifying bursts on neural spike trains employs the Poisson surprise method [31]. The temporal pattern code was calculated for each agent by determining the fractional difference (treatment-control)/control for each of the 4 parameters. A key discovery was that all of the vagus-dependent antidepressant stimuli exhibited the same unique pattern code that was statistically different from the pattern code produced by the pro-depressant LPS (Wilk's $\Lambda = 0.87729$, $P = 0.00011$). Although all four agents decreased MII, LPS increased BD and IBI to a larger degree, and increased GD, whereas the antidepressant agents decreased GD (Figure 4B). Group means and Bonferroni confidence intervals were used to determine which of these parameters account for the observed differences. Because the confidence intervals for BD and IBI did not overlap zero, the combination of these two parameters are responsible for the statistical difference between LPS and the vagus-dependent antidepressants (Figure 4C). We conclude that there is a unique firing pattern code for antidepressant agents that is not shared by a pro-depressant agent.

4.3.3 Vagal-dependent antidepressants have a unique pattern of firing that is different from a non-vagal dependent antidepressant stimulus

The vagus-independent antidepressant bupropion was then compared to the other antidepressant agents to determine if it shares the characteristic antidepressant pattern code. We did this because in the presence of a cause and effect relationship between the antidepressant code and antidepressant behavioural changes, an antidepressant whose effectiveness does not depend on the presence of an intact vagus might not evoke the antidepressant code in vagal afferent single units. Comparison of bupropion with the vagus-dependent antidepressant agents revealed a statistically different pattern of temporal firing for bupropion (Wilk's $\Lambda = 0.62627$, $P < 0.0001$). Bupropion increased MII, BD, and GD, but had a negligible effect (fractional difference of zero) on IBI (Figure 4D). This firing pattern was also statistically different from that observed by LPS (Figure 4B). The combination of MII and GD were responsible for the difference observed as revealed with the relevant Bonferroni confidence intervals (Figure 4E). The temporal pattern code characterized by decreased MII, increased BD, decreased GD & increased IBI fractional differences was a code uniquely associated with vagus-dependent antidepressant agents, but not LPS or bupropion.

4.3.4 Vagal-dependent antidepressants including Lactobacillus rhamnosus (JB-1) evoke a firing code that is different from that evoked by a non-antidepressant probiotic Lactobacillus reuteri 6475

The question might arise as to whether the antidepressant vagal code is inherent in *Lactobacillus* bacteria. We therefore tested a control probiotic bacterium (*Lactobacillus reuteri* 6475 (LR 6475)) that has no known effects on mood or related behaviours (non-psychoactive).We aimed to determine whether intraluminal applications of LR 6475 could evoke an afferent firing code similar to that of the antidepressant probiotic bacteria JB-1 (or the other vagus-dependent antidepressants). The non-psychoactive LR 6475 failed to replicate the antidepressant firing code. That is, rather than the antidepressant decrease, increase, decrease & increase in MII, GD, BD, & IBI fractional differences, LR 6475 increased MII and GD fractional differences with minimal effects on BD and IBI (Figure 4F) (Wilk's $\Lambda = 0.94806$, $P = 0.04987$). Evaluation of Bonferroni confidence intervals tells us that MII was responsible for this statistical difference (Figure 4G). The firing pattern produced by antidepressant agents remained statistically different from pro-depressant or non-psychoactive stimuli and was thus still limited to antidepressant vagus-dependent agents.

4.3.5 Antidepressant code generation is dependent on intramural IPAN to vagus sensory transmission

A proportion of the vagal afferent response to JB-1[12] and recently sertraline (manuscript in preparation/Chapter 3) has been shown to be dependent on synaptic transmission between IPANs in the myenteric plexus and vagal afferent fibres in the gut wall via an intramural nicotinic sensory synapse (Figure 1A). Through this pathway, IPANs respond to the luminal stimuli and synapse with abutting vagal afferents (IGLEs) to facilitate vagal single unit firing in response to JB-1 or sertraline. This pathway can be interrupted by blocking IPAN firing with the IK_{Ca} channel opener DCEBIO, blocking Ca^{2+}-dependent synaptic transmission using the N-type Ca^{2+} channel blocker ω-Conotoxin GVIA, or by blocking the nicotinic acetylcholine receptor (nAchR) with the nAchR antagonist mecamylamine [12]. We added each of the agents to the gut serosa in separate experiments, prior to luminal perfusion with sertraline, to determine whether generation of the antidepressant code was reliant on IPAN to vagus sensory transmission. Addition of DCEBIO, mecamylamine, or ω-Conotoxin GVIA disrupted the pattern of vagal afferent firing produced by sertraline in previous experiments. Intraluminal sertraline in the absence of the pathway blockers excited vagal afferent firing by the antidepressant code depicted in Figure 4B-F. In contrast, prior addition of any of the three blockers of the intramural sensory synapse visibly reversed this pattern (Wilk's $\Lambda = 0.5914$, $P < 0.0001$), increasing MII and GD and reducing BD and IBI (Figure 4H). The fractional change in MII, BD, and IBI was near zero for synaptic blockade with ω-conotoxin GVIA. According to the Bonferroni confidence intervals, the statistical differences could be attributed to alterations in MII, BD, and GD, but not IBI (Figure 4I). This indicates that generation of the antidepressant code

produced by sertraline was dependent on IPAN to vagus signaling and further supports the role of the intramural sensory synapse in the vagal response to luminal sertraline.

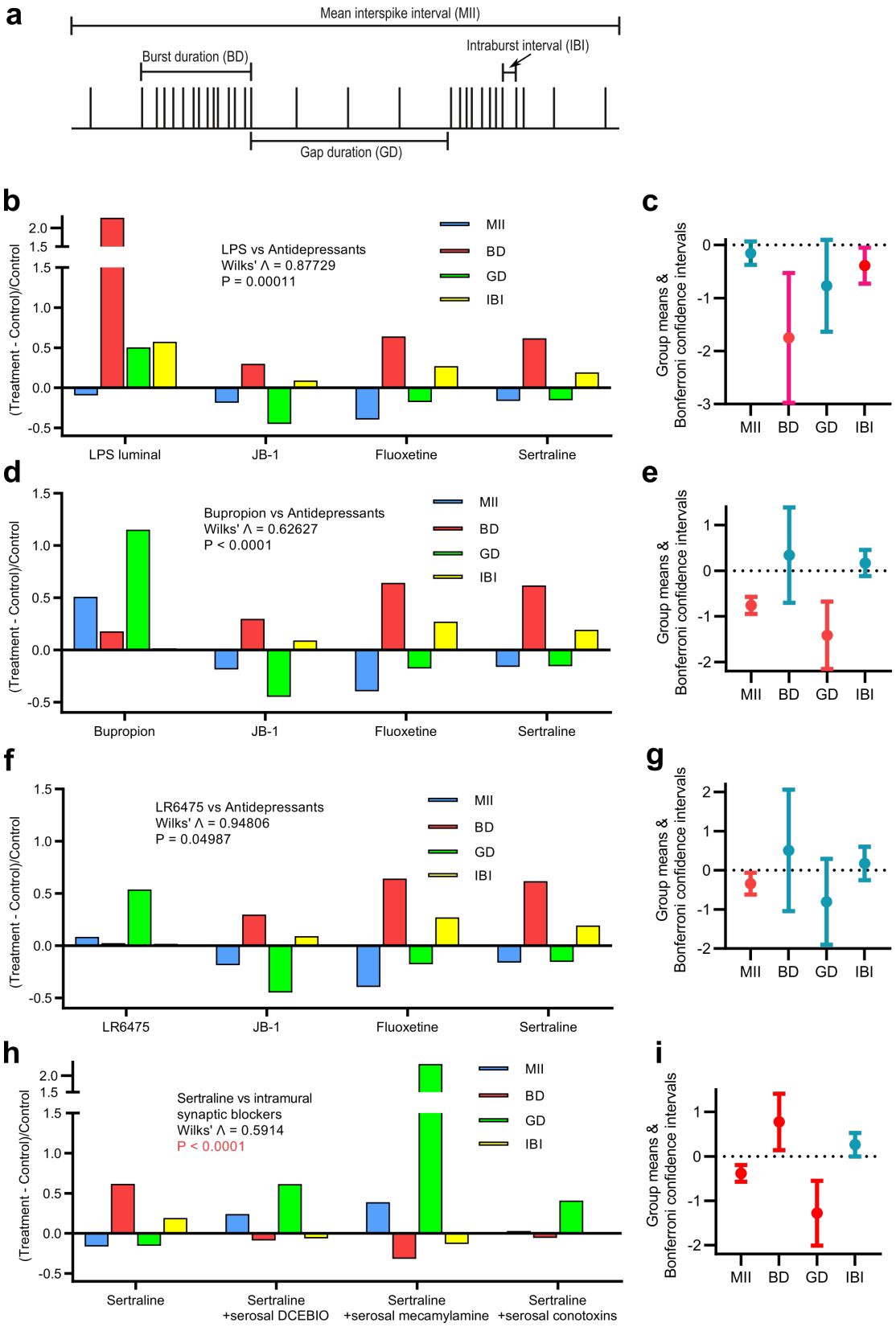
a
Mean interspike interval (MII)
Burst duration (BD)
Intraburst interval (IBI)
Gap duration (GD)
b
LPS vs Antidepressants
Wilks' Λ = 0.87729
P = 0.00011
MII
BD
GD
IBI
(Treatment - Control)/Control
LPS luminal
JB-1
Fluoxetine
Sertraline
c
Group means & Bonferroni confidence intervals
MII BD GD IBI
d
Bupropion vs Antidepressants
Wilks' Λ = 0.62627
P < 0.0001
Bupropion
e
f
LR6475 vs Antidepressants
Wilks' Λ = 0.94806
P = 0.04987
LR6475
g
h
Sertraline vs intramural synaptic blockers
Wilks' Λ = 0.5914
P < 0.0001
Sertraline
Sertraline +serosal DCEBIO
Sertraline +serosal mecamylamine
Sertraline +serosal conotoxins
i

Fig. 4 Vagus-dependent antidepressants had a unique spike firing pattern code that was disrupted by inhibition of IPAN to vagus synaptic transmission. a, diagram illustrating the 4 parameters measured to quantify vagal single unit firing patterns. **b**, fractional differences for the firing parameters showing a significant difference according to Wilk's statistic for LPS versus vagus-dependent antidepressants. Panel **c** gives group (for all treatments) means and their Bonferroni confidence intervals. Intervals for BD & IBI did not straddle 0 indicating that both parameters in combination were responsible for the statistical difference given by Wilk's statistic. **d**, significant difference between vagus-independent bupropion and vagus-dependent antidepressants. MII and GD in combination were responsible for this difference (**e**). **f**, non-antidepressant bacterium (LR 6475) had significantly different firing parameters than the antidepressants. Only the MII parameter was responsible for generating this difference (**g**). **h**, the intrinsic primary afferent neuron (IPAN) silencer DCEBIO (5 μM), the nicotinic receptor blocker mecamylamine (50 μM) or the ω-conotoxin GVIA (0.5 μM) disrupted the antidepressant code evoked by sertraline. **i**, MII, BD & GD together were responsible for the difference shown in **h**.

4.3.6 Feeding of SSRIs increased vagal firing parameters

Exposure to the gut to SSRIs by either feeding for 14 days in the drinking water or direct, acute exposure *ex vivo* increased the frequency of vagal afferent firing from the gut to the brain in a previous study [22]. We tested whether oral feeding of the SSRIs sertraline or fluoxetine for 14 days also recapitulated the vagal afferent firing code evoked by acute

intraluminal SSRI exposure *ex vivo* by measuring each of the four firing parameters, using plain drinking water as a control. Animals were sacrificed on day 15 and firing parameters of single unit vagal fibres were measured *ex vivo* in the absence of intraluminal SSRIs with only Krebs buffer perfusing the lumen. Both fluoxetine and sertraline notably decreased MII compared to mice who received only water (Figure 5A; $p = 0.0002$ and $p = 0.0031$, respectively). Fluoxetine and sertraline increased BD compared to the control (Figure 5B; $p = 0.040$ and $p = 0.0050$, respectively). Fluoxetine and sertraline significantly decreased GD compared to the control (Figure 5C; $p = 0.0032$ and $p = 0.0032$, respectively). Lastly, both fluoxetine and sertraline increased IBI (Figure 5D; $p = 0.010$ and $p = 0.010$, respectively). The fractional differences for each of the firing patterns for fed mice (Figure 5E) produced a firing code comparable to the code observed after acute SSRI administration (Figure 4B). Thus, the characteristic antidepressant firing code was replicated after feeding the SSRIs for 14 days.

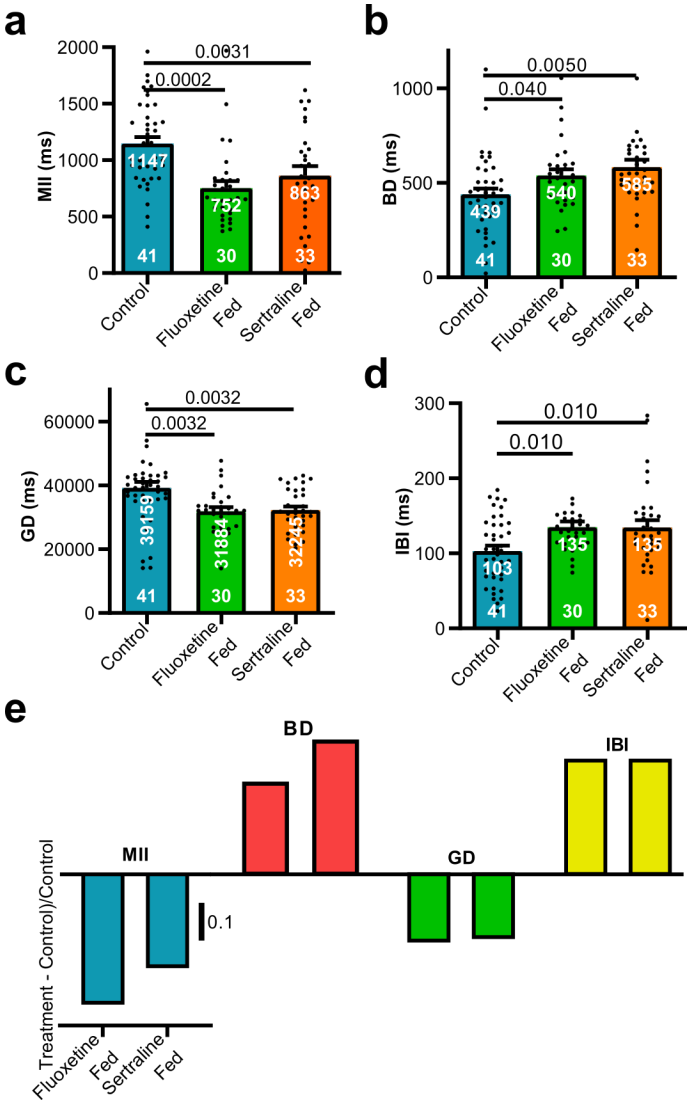

Figure 5: Prior feeding of sertraline or fluoxetine recapitulates the effects of their acute intraluminal application. 14-day feeding with either antidepressant (**a**) reduced, (**b**) increased, (**c**) reduced and (**d**) increased MII, BD, GD, & IBI respectively. Control animals were fed water only, while fluoxetine fed received 18 mg/kg added to water and sertraline fed received 6 mg/kg daily for 14 days. Animals were sacrificed on the 15th day for *ex vivo* measurements of resting afferent vagal discharge. Probabilities under the null hypothesis of no difference given above parallel lines according to Holm-Sidak's multiple comparisons

tests. Sample sizes are given at the bottom of filled bars. Data are means ± SEM. **e**, when the data were plotted as fractional differences similar firing codes to those observed after acute administration of the antidepressant drugs were evident (compare Figure 4b).

4.3.7 The antidepressant vagal firing code was conserved across two mouse strains

Mesenteric afferent nerve recordings were repeated in the Swiss Webster (SW) mouse strain at the same age as for BALB/c mice. The vagus-dependent antidepressant agents, fluoxetine, JB-1, sertraline, and the non-vagus-dependent antidepressant bupropion were tested intraluminally in *ex vivo* jejunal segments at the same concentrations used for BALB/c. Fluoxetine, JB-1, and sertraline all decreased MII, increased BD, decreased GD, and increased IBI in SW mice (Figure 6A). This was significantly different from the pattern of firing produced by bupropion, which promoted an increase in firing in all 4 parameters (Wilk's $\Lambda = 0.70783$, $P = 0.00031$). Bonferroni confidence intervals identified MII and GD as the parameters responsible for this difference (Figure 6B). Fractional differences for the firing parameters (x-axis) for each of the four treatment agents (y-axis) were contrasted for SW mice (Figure 6C) vs BALB/c mice (Figure 6D) using heatmaps. Blue represents a negative fractional change (-0.5), while red represents a positive fractional change (0.5). The heat maps were qualitatively similar between SW and BALB/c mice, indicating that the antidepressant firing code was essentially the same for these two different strains of mice.

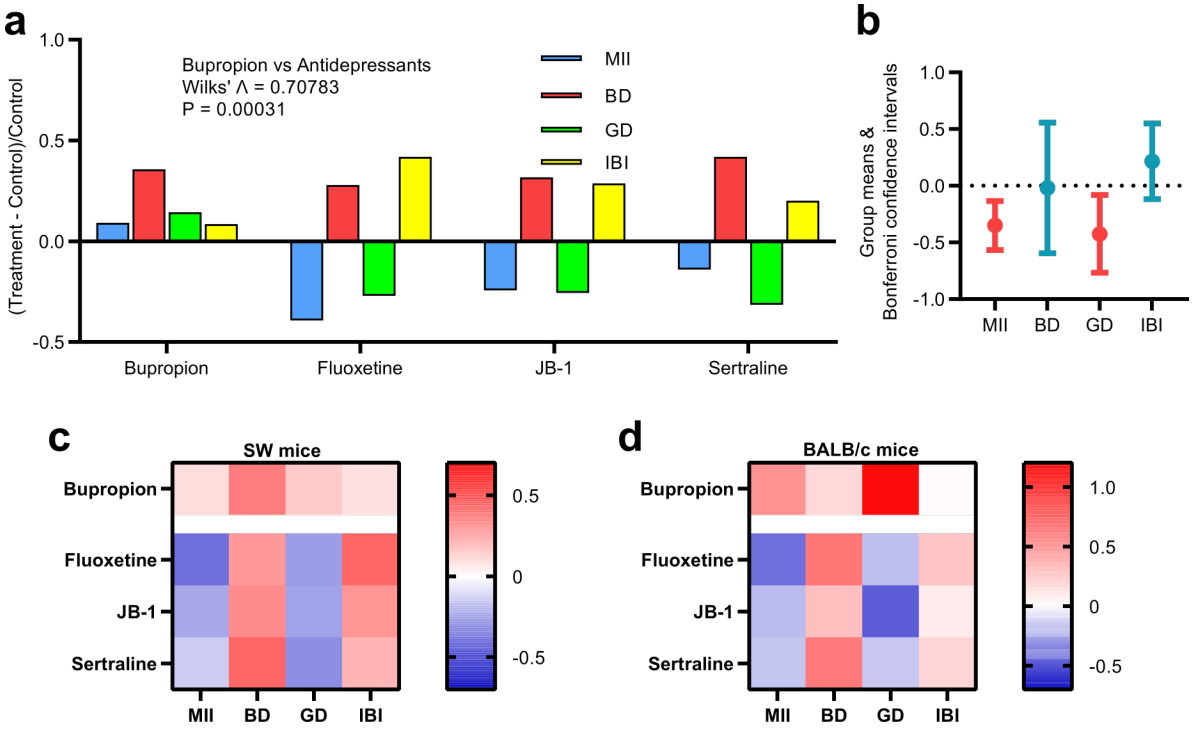

Figure 6: The vagal firing pattern code for antidepressants in SW mice is comparable to that for BALB/c mice. a, effects of adding vagus-independent (bupropion) and vagus-dependent antidepressants in acute before and after experiments for jejunal segments taken from male Swiss Webster (SW) mice. There was a significant difference (Wilk's Λ) in fractional differences for the 4 firing parameters between bupropion and the other antidepressants. **b**, as was the case for BALB/c mice, MII and GD in combination were responsible for this difference. **e** & **d**, heat maps of the fractional differences illustrating the similarity in the antidepressant codes between SW and BALB/c mice.

4.4 Discussion

In the present study we observed compelling evidence that vagal afferent sensory information in response to antidepressant mood-altering agents in the gut lumen is encoded in an antidepressant code represented by pattern and timing of single unit action potential firing.

Psychobiotics refer to probiotic bacteria, and prebiotic compounds, that influence microbiota-brain communication to exert behavioural benefits when consumed in sufficient amounts [32]. The psychophysiological effects they produce can be categorized in three ways: (1) psychological effects on emotion and cognition, (2) systemic effects on the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoid stress response, and inflammation, and (3) neural effects on neurotransmitters and proteins [33]. The psychobiotics could produce these effects via several pathways (reviewed in [33]), including direct modulation of the enteric nervous system [34,35], production of neurotransmitters [36,37], modulation of inflammation, such as increasing anti-inflammatory cytokines like interleukin-10 [38], and by direct vagal activation [21,34]. For JB-1 and *Bifidobacterium longum* NCC3001, the intact vagus was critical for the psychoactive effects as they were not observed after the vagus nerve was severed by prior vagotomy [21,34], as was also the case for antidepressant SSRI administration [22].

Likewise, ingestion of pathogenic bacteria, like *Campylobacter jejuni* (*C. jejuni*), *Citrobacter rodentium*, and *Escherichia coli*, can produce negative psychoactive effects such as increased anxiety-like behaviour [39-41] and depression [42]. Gram-negative bacteria like *E. coli* are enveloped in bacterial LPS, which is released into the circulation and triggers NF-

kB-dependent proinflammatory cytokine release that induces inflammation [43] and results in a depression-like phenotype [42]. Similarly, peripheral LPS administration induces depression-like behaviour [44]. However, depression-like behaviour following sub-diaphragmatic LPS administration can still be observed after the inflammation has resolved, outlasting sickness-behaviour and elevated cytokines[20]. This delayed effect occurs via a vagus-dependent mechanism because the depression-like behaviour was absent after LPS was injected following prior subdiaphragmatic vagotomy, but the elevated pro-inflammatory circulating cytokines are unaffected by the vagotomy [20]. Also, the anxiety-like behaviour observed following *C. jejuni* or *C. rodentium* ingestion was not accompanied by a systemic immune response but did induce c-Fos expression in vagal sensory neurons [41,45]. Therefore, while immune responses and other mechanisms of microbial modulation influence the brain and behaviour, it is necessary to characterize how the vagus differentiates and encodes information about mood-altering stimuli to elucidate the neural mechanisms underlying the behavioural effects of psychobiotics.

We investigated whether vagal afferents supplying the jejunum are specifically or broadly tuned to behaviourally active stimuli, and if they are broadly tuned, whether information about the nature of the stimulus is encoded in a specific vagal single unit firing pattern. We measured afferent discharge in single vagal afferents in response to intraluminal application of known vagus-dependent antidepressant agents JB-1 and sertraline [21,22] and a known vagus-dependent pro-depressant agent, LPS [20], to determine if vagal afferents were specifically tuned for pro- versus antidepressant stimuli. Both pro- and antidepressant stimuli increased vagal afferent firing frequency in a single fibre, consistent with broad tuning to

the behavioural stimuli. Not only did all three stimuli produce a response in a single vagal afferent, JB-1, sertraline, and LPS all significantly increased peak firing frequency (Figure 2E), thus it is unlikely that the psychoactive nature of the stimulus is encoded by a simple interspike interval or rate code [46]. Although stimulus intensity could also be a factor in such encoding, the polymodal chemosensitivity of vagal afferents to these and other stimuli that increase firing rate would make peak frequency ambiguous as a signal except in finely-tuned neurons, as was suggested to be the case for taste-responsive neurons [47].

Because of the above considerations, we hypothesized that the sensory response to the psychoactive agents is encoded in the timing and pattern of action potentials evoked by the stimulus and thus measured four parameters of firing that describe single unit firing patterns, namely, MII, BD, GD, and IBI, to decode the response to each agent. The specific pattern of firing that encodes the antidepressant response was different from either a vagus-dependent pro-depressant stimulus, a non-vagus dependent antidepressant, and a non-psychoactive probiotic stimulus. Temporal response patterns have been identified in the rat gustatory response to different tastes [48,49]. Stimulation of the rat chorda tympani nerve with a pulse train mimicking a taste stimulus produced orofacial reactions that corresponded with the stimulus and was different from a control pulse train [49]. Similarly, these temporal patterns can be played back by electrical stimulation delivered to the NTS *in vivo* and correspondingly affect licking behaviour when rats are confronted with a negative quinine-like stimulation versus a positive sucrose-like stimulation [48].

The question as to whether playback of our antidepressant temporal code is feasible is complicated. The antidepressant temporal pattern recorded by us at the intestinal

mesenteric nerve may be conserved as it is transmitted upstream to vagal neurons in the nucleus tractus solitarius (NTS) and eventually the brain or may be further processed within the NTS before it reaches the brain. The antidepressant effects of vagus nerve stimulation have also been suggested to be mediated by an anti-inflammatory efferent vagal pathway [50]. However, published studies demonstrating that pro- & antidepressant luminal stimuli exert the mood-altering actions independent of inflammation and cytokine levels would argue against this interpretation [40]. Current clinical vagus nerve stimulation (VNS) for treatment of pharmaco-resistant depression involves stimulation of the left cervical branch of the vagus (in the neck) or applying transcutaneous auricular VNS to the ear [51]. Acute peripheral vagus nerve stimulation has been shown to directly activate myelinated vagal afferents that project to second-order NTS neurons [52]. Whether playback of antidepressant or pro-depressant temporal codes via VNS promotes corresponding changes in behaviour and depression scores remains to be ascertained. So far our data only provides correlational evidence for an anti- and pro-depressant code, although the evidence was highly statistically significant and is capable of making important predictions. One prediction would be that vagus nerve stimulation using our antidepressant code would be more effective than stimulation using stochastically random stimulus pulses or the stereotyped simple burst patterns described for such stimulation in the literature. If it does, it would have important implications for the development of improved, targeted electroceutical antidepressant therapies.

The antidepressant code was not observed when IPAN to vagus transmission was disrupted. Both JB-1 and sertraline activate more than 2/3 of vagal afferents through sensory synaptic transmission from IPANs to vagal afferents at the intramural sensory synapse,

which also involves nicotinic cholinergic neurotransmission [12] (See Chapter 3). The intramural sensory synapse (Figure 1A) is formed by a close anatomical relationship between myenteric IPANs and the intraganglionic laminar endings (IGLEs) of vagal afferents that surround and abut them [53], facilitating reciprocal neuronal crosstalk [12,54]. Vagal afferents do not penetrate the epithelium, and although some mucosal afferents terminate as far as the lamina propria [7], the majority (>90%) of sensory innervation of the luminal gut epithelium comes from the ENS [9,10]. Because vagal afferents provide such a dense innervation of the rodent ENS [11], vagal sensing of ingested bacteria or mood-altering substances is likely to predominantly occur via release of transmitters from myenteric neurons onto vagal afferents [12]. This sensory transmission is dependent on transmitter release through N-type calcium channels [55] and is blocked by ω-conotoxin GVIA, as shown here and in previous experiments [12] (See Chapter 3). The intramural sensory synapse has been shown to be nicotinic in nature for responses to JB-1 [12] and similarly blockade of nAchRs using the non-competitive antagonist mecamylamine also prevented vagal activation and disrupted the generation of the antidepressant code by intraluminal sertraline. This sensory transmission is dependent on IPAN action potential generation because pharmacological silencing of IPANs using the IK_{Ca} channel opener DCEBIO, which hyperpolarizes IPANs by increasing background IK_{Ca} channel opening and which also prolongs the post action potential relative refractory period (slow afterhyperpolarization) [56,57], resulted in the obliteration of the SSRI induced antidepressant code. We conjecture that the behavioural response to antidepressant sertraline, and potentially other vagal-dependent antidepressant agents, encoded in vagal afferents, is mediated by IPAN to vagus neurotransmission.

Furthermore, the antidepressant firing code is conserved whether the antidepressants were applied acutely to the lumen or ingested for 14 days and was analogous for both SW and BALB/c mouse strains. This indicates that the antidepressant vagal pattern of firing may also be observed *in vivo*. Ongoing low-frequency stimulation of IPANs increases excitability of these myenteric AH neurons for up to 3.5 hours after the stimulus period [58]. Considering the critical role of the ENS in generating the antidepressant code herein, chronic oral administration of antidepressants may also elicit a period of prolonged, elevated excitability of IPANs and therefore generation of the antidepressant code for several hours post-stimulus. Furthermore, SW, unlike BALB/c mice, are not inbred to be anxiety and depression prone [59], yet intramural sertraline also evoked the antidepressant code in afferent vagal single units. This result strongly suggests that the difference between the vulnerability to depression between strains regulates within the brain rather than in the activity of peripheral afferents. This observation is analogous to the finding that the antidepressant probiotic JB-1 has no behavioural antidepressant effect in non-depressed healthy male humans [60]. Further studies will be needed to determine if the antidepressant firing code is conserved across other species including humans. Parenthetically, similar firing patterns across species in homologous regions of the brain have been documented for mice, rats, cats, and monkeys, which were specific to the brain regions recorded and the behavioural functions they support [61]. This similarity between brain firing patterns provide some hope that the mouse antidepressant code may, in essence, translate to humans, although it will also be important to replicate the present findings in female mice.

In conclusion we have quantified some of the first data, to our knowledge, identifying a specific antidepressant temporal firing pattern encoded by the vagus in response to luminal psychoactive agents. By characterizing the vagal afferent response to antidepressant stimuli, we may be able to apply the code to screen potential bacteria, molecules, and pharmaceuticals for vagus-mediated antidepressant effects. After further *in vivo* and translational studies, these findings may be applied to optimize vagus nerve stimulation protocols for patients seeking more targeted electroceutical antidepressant therapies.

4.5 Methods

4.5.1 Ex-vivo vagal nerve fibre recording.

BALB/c mice were used in this study because of their inherent predisposition to an anxiety-like phenotype [62] and their previous use in investigating the role of the vagus in response to clinical antidepressants [22]. Mice were purchased from Charles River (Montreal, QC, Canada) and acclimatized to the animal facility for minimum 1 week on a 12h light/dark cycle and food and water were provided *ad libitum*. Adult male mice were killed by cervical dislocation approved by and in accordance with the McMaster Animal Research Ethics (AREB) board (permit 16-08-30). Recordings and subsequent analysis were performed as described in the patent application by Bienenstock et al. (2019) (International Publication Number WO 2019/185735 A1) [63].

A small segment of jejunum (2-3 cm) and attached mesenteric tissue containing the neuromuscular bundle was excised and luminal contents were flushed using a plastic syringe

to perfuse Krebs buffer through the oral opening. The jejunal segment was placed into a polystyrene petri dish, previously lined with 1–2 mm deep with cured Sylgard 170 silicone elastomer (Sylgard, Dow Corning, MI, USA) and filled with carbogen (95% O_2, 5%CO_2) bubbled Krebs buffer. Krebs buffer was of the following composition: (mmol L^{-1}): 118 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4, 1.2 $MgSO_4$, 11.1 glucose [64]. The segment was cannulated at the anal end and the tissue and mesentery were fanned out and pinned using fine insect pins into the Sylgard lining. The nerve bundle was isolated from the mesenteric tissue under a stereomicroscope (Leika MZ6) by microdissection using forceps (Fine Science Tools, BC, Canada). Once dissected, the preparation was transferred to an inverted microscope (Nikon), and the oral end was cannulated with silicon tubing to several Mariotte bottles [65] attached to a plastic manifold to facilitate luminal gravity perfusion of (1 mL/min) room temperature carbogenated Krebs or treatments. The serosal compartment of the tissue was perfused with prewarmed (34° C) Krebs solution (5 mL/min) with 3 µM nicardipine hydrochloride to paralyze smooth muscle to isolate vagal chemosensory responses by preventing active muscle contractions [27].

A glass-recording pipette attached to a patch-clamp electrode holder was used to gently suck onto the cleaned, isolated mesenteric nerve bundle. Extracellular nerve recording of multiunit afferent firing were made using a Multi-Clamp 700B amplifier and Digidata 1440A signal converter (Molecular Devices) and bandpass filtered at 0.1-3 kHz, sampled at 20 kHz and stored on a computer using pClamp 10 software. Multiunit electrical activity was recorded for 15-20 mins with Krebs buffer in the lumen, after which it was switched to Krebs containing one of the test substances. The following psychoactive agents were tested:

luminal administration of 10^9 cfu/mL live *L. rhamnosus* (JB-1), 10 μM sertraline hydrochloride (Millipore Sigma, Burlington, MA, USA), 30 μM fluoxetine hydrochloride (Millipore Sigma), 10 μM bupropion hydrochloride (Millipore Sigma)[22], or 1 mM LPS dissolved in pyrogen-free saline (*Escherichia coli* O127:B8, (Millipore Sigma)[20]. Pilot experiments showed that these concentrations excited vagal fibres by at least 20% above baseline firing rates. Test substances were perfused for 30-40 mins after which the lumen was washed out with Krebs. Cholecystokinin (CCK) was pipetted onto the serosa 10 min after the cessation of treatment and Krebs washout to allow for identification of vagal fibre single units from all other single units during post-hoc computer analysis. Lastly, the tissue was distended by raising the intraluminal pressure to 14 hPa to demonstrate that single units were still responsive and not subject to rundown.

4.5.2 Single unit identification using principal component analysis and measurement of parameters

Multi-unit recordings were sorted post-hoc to identify single units using the principal component analysis (PCA) algorithm in the Dataview program for extracellular action potential analysis [28] (Figure 1A&B). Single units were discriminated by their unique action potential, shape, amplitude, and width [26]. Delineated clusters of units distinguished by a single colour represented single unit types and single units from each cluster were converted to single event point processes and displayed as sequential rate histograms used in subsequent analysis. Single unit events were separated and colour-coded in point process event channels where each coloured channel represented a different single unit. Each

coloured single unit channel was subdivided and gated into control (Krebs) and treatment (intraluminal agent) periods using event timing markers applied during recording. Vagal single units were identified as belonging to an individual vagal fibre and separated based on an excitatory response (increased firing rate) to serosal CCK during recording. Each event channel in Dataview was displayed as probability density functions (√Y) vs. log(time) using the "Event Parameter Histogram" plot option. A single exponential fit to this plot gave the mean interspike interval (MII).

The "Poisson surprise" method [31] was used to identify Event Bursts for each single unit. A surprise is defined as –log10(p), where p is the probability of a set of events (such as a burst of spikes) occurring this close together by chance. Bursts were detected using a Poisson surprise value of 2, which reflects a p value of 0.01. Using the gated control and treatment periods measured in Dataview, the "Event analyse: Histogram/statistics" option calculated the gap (GD) and burst (BD) durations.

To measure the intraburst intervals (IBI) for each different single unit point process, the control and treatment burst event channels that were created by the Poisson surprise method were logically combined using the AND gate function. Thus, only the bursts were extracted from the point process events. Then, the burst channel for a given single unit was selected as the gate channel and the "Event analyse: Histogram/statistics" option gave the IBI for either the control or treatment channels.

4.5.3 Statistical Analysis.

MII for each independent variable (drug) was analyzed by paired student's t-test in GraphPad Prism (Version 8.3, GraphPad Software, San Diego, USA). Effect sizes were given by the partial eta squared statistic η^2_p for paired differences calculated in the t-test module using GraphPad Prism (Version 8.3) and interpreted as per Cohen's guidelines, where 0.01 indicates a small, 0.06 a medium, and 0.14 a large effect [29]. Fed data was analyzed by one-way ANOVA and Holm-Sidak's multiple comparisons test in GraphPad Prism (Version 8.3).

Interval and burst data (MII, BD, GD, and IBI) collected for each single unit and for control and treatment conditions was pasted in an Excel 2016 spreadsheet and converted into fractional differences (treatment – Krebs)/Krebs. Single factor multivariate analysis of variance (MANOVA) was performed for each of the treatments using the **Real Statistics** Resource Pack software (Release 7.2) Copyright (2013 – 2020) Charles Zaiontz, www.real-statistics.com. Multivariate calculations gave the Wilk's Lambda test statistics for the contrasts between a single luminal agent and several other agents. To determine which of the firing parameter(s) the difference can be statistically attributed, Bonferroni confidence intervals were calculated for all of the four parameters. The Bonferroni confidence interval is equal to the (mean - standard error)/critical t-value for 0.05 significance for the lower bound, and (mean + standard error)/critical t-value for the upper bound. Parameters for which confidence intervals did not span 0 therefore contributed to the statistical differences between the luminal agents.

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CHAPTER LINK.

In the previous two chapters we investigated how vagal afferent information is transmitted from the gut lumen in response to antidepressant stimuli and how this mood-altering signal is encoded within the pattern and frequency of action potentials elicited by the stimulus. We demonstrated that the excitatory vagal afferent response to sertraline was dependent on IPAN to vagus synaptic transmission via a nicotinic intramural sensory synapse (Perez-Burgos *et al.*, 2014). We then demonstrated in Chapter 4 that sertraline and other antidepressant agents produced a specific pattern of vagal afferent firing that was statistically different from the pattern of firing produced by the pro-depressant agent, LPS. This antidepressant pattern code was also dependent on the nicotinic intramural sensory synapse, because it was abolished by synaptic blockade. After having presented this data that demonstrated a critical role of the ENS in vagal afferent signaling, we used two mouse models of aging and neurodegenerative disease in which GI function was compromised to investigate whether vagal afferent signaling from the gut was altered. In the following two chapters, we measured vagal afferent firing and intestinal motility in old versus young mice and in PD versus control mice to determine if impaired GI function is accompanied by impaired vagal afferent firing in these models and whether these changes were recoverable.

CHAPTER 5.

Colonic motility and jejunal vagal afferent firing rates are decreased in aged adult mice and can by restored by an aminosterol

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Abstract

There is a general decline in gastrointestinal function in old age including decreased intestinal motility, sensory signaling, and afferent sensitivity. There is also increased prevalence of significant constipation in aged populations. We hypothesized this may be linked to reduced colonic motility and alterations in vagal-gut-brain sensory signaling. Using *in vitro* preparations from young (3 months) and old (18–24 months) male CD1 mice we report functional age-related differences in colonic motility and jejunal mesenteric afferent firing. Furthermore, we tested the effect of the aminosterol squalamine on colonic motility and jejunal vagal firing rate. Old mice had significantly reduced velocity of colonic migrating motor complexes (MMC) by 27% compared to young mice (p = 0.0161). Intraluminal squalamine increased colonic MMC velocity by 31% in old mice (p = 0.0150), which also had significantly reduced mesenteric afferent single unit firing rates from the jejunum by 51% (p < 0.0001). The jejunal vagal afferent firing rate was reduced in aged mice by 62% (p = 0.0004). While the time to peak response to squalamine was longer in old mice compared to young mice (18.82 ± 1.37 min vs. 12.95 ± 0.99 min; p = 0.0182), it significantly increased vagal afferent firing rate by 36 and 56% in young and old mice, respectively (p = 0.0006, p = 0.0013). Our results show for the first time that the jejunal vagal afferent firing rate is reduced in aged-mice. They also suggest that there is translational potential for the therapeutic use of squalamine in the treatment of age-related constipation and dysmotility.

Keywords: aging, vagal afferent, motility, constipation, squalamine

5.1 INTRODUCTION

Old age is associated with increased incidence of chronic constipation, which increases in prevalence with age (Higgins and Johanson, 2004; De Giorgio et al., 2015; Ranson and Saffrey, 2015). In addition, aging is also associated with behavioral depression (Malatynska et al., 2012). Age-related changes in the gut and gut to brain nervous signaling via the vagus nerve may underlie many of these problems in the geriatric age group, although psychosocial and economic factors may play a role (Strawbridge et al., 1996). Old animals show delayed gastric emptying, slowed colonic transit and reduced fecal output (Smits and Lefebvre, 1996). For example, 2-year-old mice deliver fewer fecal pellets than 3-month-old ones, and when epoxy coated pellets are introduced into the colon of old mice they move with decreased velocity in the oral to anal direction (Patel et al., 2012).

Whether age-related changes in intestinal propulsion are due to alterations of smooth muscle function or cells that coordinate or pace contractions such as neurons or interstitial cells of Cajal (ICC), is unclear (Saffrey, 2014). However, while reductions in the number of ICC and enteric glial cells have been reported in the aged gut (Saffrey, 2014) the enteric nervous system (ENS) is also vulnerable to age-related damage. Generation and propagation of colonic migrating motor complexes (CMMCs) in mice are generated by activity of the ENS (Fida et al., 1997; Roberts et al., 2007; Spencer et al., 2018), recorded *in vitro* (Wang et al., 2010a,b; Wu et al., 2013) and are absent if the ENS is missing or destroyed as in Hirschsprung's or Chagas' diseases (Furness, 2006, p. 157). Indeed peristalsis, but not ICC dependent slow wave related contractions, is abolished by tetrodotoxin (Wu et al., 2013;

Delungahawatta et al., 2017). In fact, neurogenic migrating motor complexes still occur in mutant mice lacking pacemaker-type ICC and slow waves in the small intestine (Spencer et al., 2003).

The myenteric plexus of the ENS is essential for normal MMCs to occur in the colon (Fida et al., 1997; Roberts et al., 2007; Wang et al., 2010b; Spencer et al., 2016, 2018). Intrinsic primary afferent neurons (IPANs) represent the class of myenteric neurons most affected by degenerative changes in old age (Wade, 2002; Wade and Cowen, 2004) and MMCs are absent if they are selectively silenced (Howe et al., 2006). However, the ENS appears to be more susceptible to age-related degeneration than other nervous systems (Saffrey, 2013). While some animal studies suggest that there may be reductions in the number of myenteric neurons in old age (El-Salhy et al., 1999; Phillips et al., 2004; Phillips and Powley, 2007; Zanesco and Souza, 2011), it is probable that myenteric neuron numbers are actually maintained, but an increasing proportion show structural degenerative changes with increasing old age (Gamage et al., 2013; Saffrey, 2013).

We are not aware of extant data on age-related functional changes in vagal nerves, but vagal afferents in aged rats have swollen varicosities in fibers innervating the myenteric plexus, smooth muscle and mucosa (Phillips and Powley, 2007). There is no information available whether there is an actual decrease in the number of vagal fiber endings supplying the myenteric plexus. However, dystrophic changes including dilations and swellings of the intraganglionic laminar endings (IGLEs) in the NIH Fisher 344 rat model of aging have been described and the extent of the terminal arbors is also reduced compared to young rats

(Phillips et al., 2010). A previous study showed that aged mice had attenuated colonic and jejunal afferent mechanosensitivity and suggested that the loss or decrease of this sensory innervation or sensitivity may be linked to the reduced awareness of constipation in the elderly (Keating et al., 2015).

In the present paper we report the effects of old age on colon motility and jejunal vagal afferent firing using *in vitro* preparations from male CD1 mice. Squalamine is a prokinetic aminosterol originally synthesized by the liver of the dogfish shark (Zasloff et al., 2011), and it has previously been shown to stimulate colonic motility in a 1-year-old mouse and loperamide model (Kunze et al., 2014). Here we explore in detail the effects of old age (2-year) on colon motility and constitutive vagal afferent firing rates from the jejunum, and whether these functions might be restored to youthful levels by the aminosterol squalamine.

5.2 MATERIALS AND METHODS

5.2.1 Animals

Young (3 months) and old (18–24 months; retired breeder) male CD-1 mice from Charles River Laboratories (Quebec, Canada) were used for all portions of the study. Experiments were performed *in vitro* following cervical dislocation in accordance with the Animal Research Ethics Board (AREB) of McMaster University (permit 16-08-30). Mice were housed on a 12-hour light/dark cycle, food and water were provided *ad libitum*, and mice were allowed 1-week acclimatization following arrival. In view of the current debate about sex differences in mammalian nervous systems (O'Connor and Cryan, 2014), we plan to

conduct future studies with female CD1 mice when these become available after an 18–24-month aging period.

5.2.2 Colonic Motility

The colonic motility recordings were performed as described previously (West et al., 2017). The whole colon was extracted from young (3 months) and old (18–24 months) mice, flushed with Krebs, and cannulated with silicon tubing at the oral and anal ends within a heated, Krebs-filled tissue flotation bath. Krebs was prepared with the following concentrations (mmol L^{-1}): 118 NaCl, 4.8 KCl, 25 $NaHCO_3$, 1.0 NaH_2PO_4 , 1.2 $MgSO_4$, 11.1 glucose, and 2.5 $CaCl_2$, bubbled with carbogen gas (95% O_2 and 5% CO_2) and heated to 37˚C (Wu et al., 2013). The inflow (oral end) and outflow (anal end) tubes were adjusted in height to create an intraluminal pressure difference of 2–3 hPa during perfusion of the lumen. Gravity-evoked contractions were recorded by a Microsoft LifeCam 3000 web camera positioned 8 cm above the tissue. The serosal compartment of the bath was constantly perfused with fresh oxygenated Krebs. Squalamine (10 μM) was applied luminally by opening and closing the stopcocks of the Mariotte tubes at the oral end [see Figure in Wu et al. (2013)].

Intestinal contractions were recorded during a 20 min Krebs control and 20 min treatment period. Spatiotemporal diameter maps were developed from the motility video recordings as described previously (Wu et al., 2013; West et al., 2017). Alternating dark and light bands indicate contraction and relaxation along the gut representing migrating motor complexes (MMCs). MMC velocity was measured as the slope of the large dark contractions. MMC

frequencies were determined by measuring the number of MMCs over a given time interval and amplitude was measured as a function of the gut diameter at peak contractions.

5.2.3 Mesenteric Nerve Recordings

The mesenteric nerve bundle is a mixed nerve containing populations of vagal and spinal fibers (Perez-Burgos et al., 2013). Jejunal mesenteric nerve recordings were performed as described previously (Perez-Burgos et al., 2013). A 2–3 cm segment of mouse jejunum with attached mesentery was excised and mounted on an agar-coated petri dish filled with oxygenated Krebs buffer and nicardipine (3 μM) to paralyze smooth muscle. The oral and anal ends of the tissue were cannulated with silicon tubing and the luminal contents were flushed using Krebs. The remaining mesentery was pinned out and the mesenteric nerve bundle was dissected using fine-tipped forceps. The petri dish was then mounted on an inverted microscope and the nerve bundle was sucked onto with a glass micropipette attached to a microelectrode. The nerve preparation was continuously perfused with fresh oxygenated Krebs in the serosal compartment using a pump. Multi-unit electrical activity was recorded using a Multi-Clamp 700B amplifier and Digidata 1440A signal converter (Perez-Burgos et al., 2013). Control periods were recorded for 15–30 min during luminal Krebs perfusion. Intraluminal squalamine (10 μM) was perfused following the control (Krebs) for a duration of 30 min. Cholecystokinin (CCK) was applied 10 min after the cessation of treatment and Krebs washout to allow for identification of vagal fibers during *post hoc* computer analysis as vagal fibers respond potently to CCK, while spinal fibers do not (Richards et al., 1996; Hillsley and Grundy, 1998). Lastly, $5HT_3$ agonist was applied as

it activates a small population of vagal afferent fibers not activated by CCK (Hillsley and Grundy, 1998) and a 37 hPa distention for 1 min tested response to painful distention or a high-threshold stimulus (Perez-Burgos et al., 2015).

Multi-unit electrical activity was analyzed for single unit activity using principal component analysis (PCA) and spike waveform analysis in the DataView program (Heitler, 2007). Each single unit fiber has a unique action potential spike that is distinguished from other single fibers by its shape, size and duration (Heitler, 2007; Perez-Burgos et al., 2013). Once sorted into single units, vagal fibers were identified by response to CCK, as described previously. Single unit vagal activity was gated for control and treatment periods and the mean interval between spike firing (the inverse of firing frequency) was measured. Decreases in the interspike interval are described as increased afferent firing rate and vice versa.

5.2.4 Statistics

Researchers were not blinded to experimental groups (young vs. old mice). Percent difference was calculated by (treatment- control)/control for paired before and after treatment or (old- young)/young for age comparisons. Data are presented as mean ± SEM. *N* represents number of mice. Where multiple afferent fibers are measured from one animal, *N* is represented as *N* = # of mice (# of fibers). Statistical comparisons were performed using paired or unpaired, two-tailed *t*-tests using GraphPad Prism software (Version 7.0). Any outliers were identified or removed using Grubbs' test (a = 0.05) or the ROUT Method (Q = 1%).

5.3 RESULTS

5.3.1 Colonic Contractile Motility Is Reduced in Aged Mice

Colonic contractile motility was assessed in a total of 32 male CD1 mice; 22 old mice (18–24+ months) and 10 young mice (3 months). The whole length of the colon was excised and the colonic MMCs were video recorded in our gut motility apparatus during Krebs luminal perfusion for later measurements of MMC velocity, frequency and amplitude. Means for MMC velocity in old CD-1 mice during Krebs control were significantly reduced compared to young mice controls. Mean MMC velocity in old mice controls was 0.948 ± 0.09 mm/s, 27% slower than young mice controls, 1.31 ± 0.10 mm/s (p = 0.0161) (**Figure 1A**). MMC frequency and amplitude were not significantly affected by age. Mean frequency was 7.2% smaller in old mice controls (0.007 ± 0.001 Hz) compared to young mice controls, 0.008 ± 0.001 Hz (p = 0.5639) (**Figure 1B**). Mean MMC amplitude in old mice was 0.601 ± 0.062 cm, 4.4% smaller than for young mice, 0.628 ± 0.116 cm (p = 0.8356) (**Figure 1C**). Based on these findings there was a reduction in contractility with age in the colon, with the greatest effect being a reduction in MMC velocity.

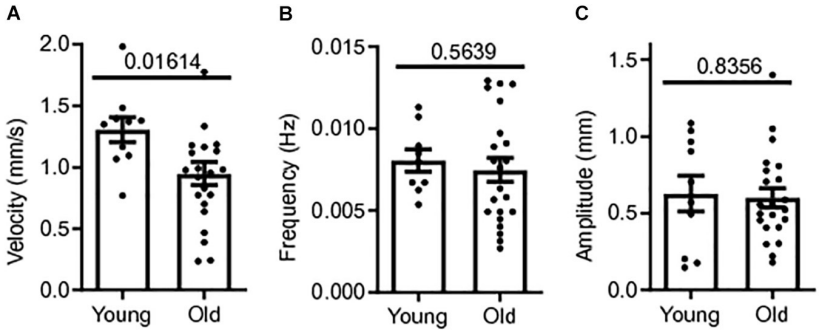

FIGURE 1 | Colonic contractile motility is reduced in aged mice. (A) Sample MMC velocity was decreased in old mice (N = 22) compared to young mice controls (N = 10). **(B)** Sample MMC frequency in old mice compared to young mice controls. **(C)** Sample MMC amplitude in old mice compared to young mice controls. Data represented as mean with SEM, t-test unpaired, two-tailed, with Welch's correction.

5.3.2 Squalamine Restores Colon Motility in Aged Mice

Squalamine (10 μM) was added *in vitro* to the lumen of colon segments taken from old mice (N = 9), following a 20-minute period of control recording with only Krebs in the lumen. Changes to MMC parameters before and after adding squalamine were measured from spatiotemporal maps. Intraluminal squalamine significantly increased mean velocity by 31% from 0.99 ± 0.06 mm/s during Krebs control to 1.3 ± 0.11 mm/s (p = 0.0150) (**Figure 2A**). Intraluminal squalamine increased frequency 26% from 0.007 ± 0.001 to 0.009 ± 0.001 Hz (p = 0.1964), but not within statistical significance (**Figure 2B**). Amplitude was significantly increased 65% from 0.50 ± 0.07 to 0.83 ± 0.15 mm (p = 0.0100) (**Figure 2C**). Spatiotemporal heat maps of MMCs demonstrate a loss of contractile motility and regularity in old (**Figure 2E**) compared to a young mouse (**Figure 2D**). Propulsive contractility was restored in the old mice following application of intraluminal squalamine (**Figure 2F**).

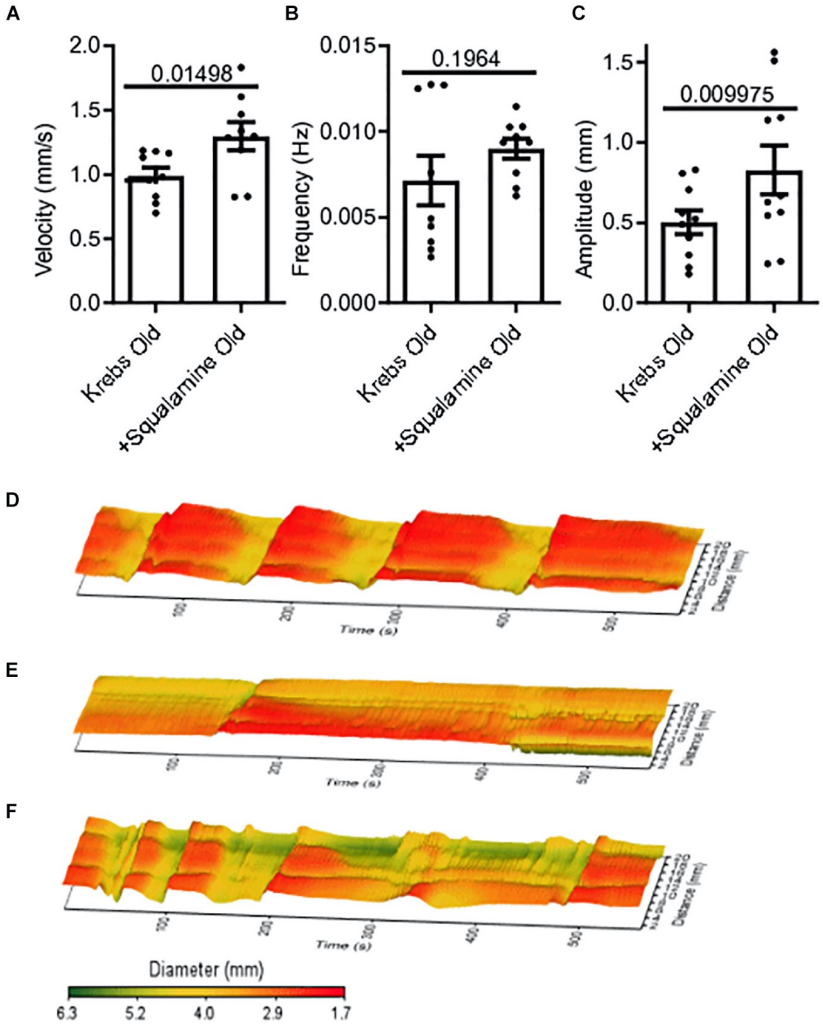

FIGURE 2 | Reduced colonic motor activity could be partially restored by intraluminal application of the aminosterol squalamine. (A) Intraluminal squalamine (10 μM) increased sample colonic MMC velocity in aged mice ($N = 10$). **(B)** Intraluminal squalamine and colonic MMC frequency in aged mice. **(C)** Intraluminal squalamine increased colonic MMC amplitude in aged mice. 3D spatiotemporal heat maps display MMC contractions over time (*x*-axis) and distance (*y*-axis). Areas of contraction or small diameter are red, while areas of relaxation or larger diameter are yellow to green for **(D)** a young mouse during

Krebs control, **(E)** an old mouse during Krebs control, and **(F)** an old mouse after intraluminal treatment with squalamine. Data represented as mean with SEM, paired *t*-tests, two-tailed.

5.3.3 Single Unit Firing from the Mesenteric Afferent Nerve Bundle Is Reduced in Aged Mice

Baseline multiunit mesenteric nerve afferent firing in old ($N = 9$) or young mice ($N = 10$) was measured to determine the effect of aging on afferent discharge, *in vitro*. Afferent firing was measured as mean interspike intervals, in which a decrease in the mean interval between spikes indicates an increase in the firing frequency of the fiber. The multiunit interspike interval of 81.9 ± 19.4 ms for old mice was 55% longer than that for young mice (52.9 ± 6.72 ms) but did not reach statistical significance ($P = 0.1881$) (**Figure 3A**). Single unit firing from individual afferent fibers in the mesenteric nerve bundle was identified from multi-unit recordings using DataView as described in the section "Materials and Methods" (Heitler, 2007). The mean interspike interval from all single unit fibers was 51% longer in old mice, 1676 ± 117.8 ms [$N = 9(121)$], compared to young mice, 1111 ± 63.36 ms [$N = 10(149)$], ($P < 0.0001$) (**Figure 3B**). A representative trace of multi-unit afferent firing from a young mouse is shown in **Figure 3C**. **Figure 3D** shows the same obtained using an old mouse.

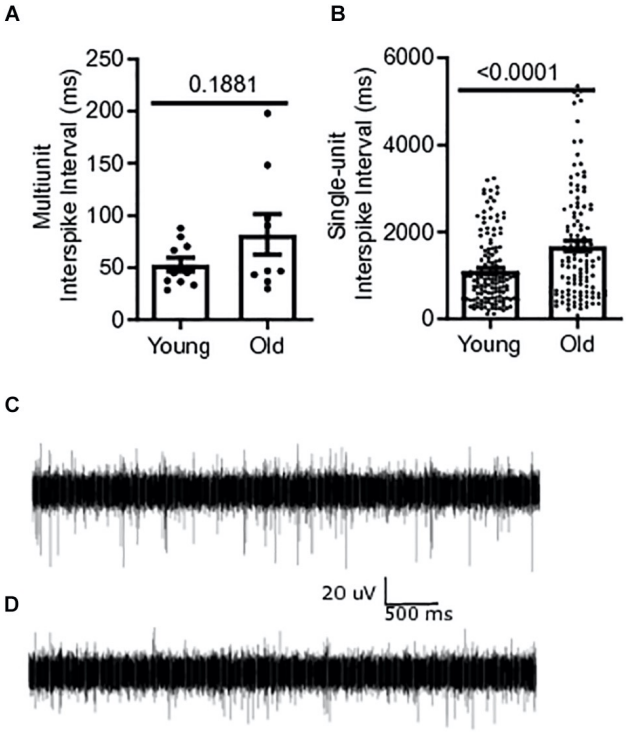

FIGURE 3 | Mesenteric single unit afferent sensory signaling is decreased in aged mice. **(A)** Multi-unit mesenteric afferent firing from the mesenteric nerve in old mice compared to young mice (*N* = 10 mice). **(B)** Single unit mesenteric afferent firing from the mesenteric nerve had longer interspike intervals, indicating decreased afferent firing in old mice (*N* = 121 and 149 fibers). **(C)** Representative traces of mesenteric afferent firing for young mice, and **(D)** mesenteric afferent firing for old mice. Data represented as mean with SEM, *t*-test unpaired, two-tailed, with Welch's correction.

5.3.4 Single Vagal Fiber Firing Rate Is Decreased in Aged Mice but Can Be Rescued by Squalamine

Single unit afferents within the multiunit mesenteric nerve bundle recordings were identified as vagal afferents based on their response to CCK, which selectively stimulates vagal fibers (Grundy et al., 1998). Single unit vagal afferent firing rate was assessed from the mesenteric nerve bundle of the jejunum of young and old mice. Mean vagal single unit interspike intervals were 62% longer for old mice, 1886 ± 176.9 ms [N = 10(65)], compared to young mice, 1166 ± 86.22 ms [N = 9(83)], (p = 0.0004) (**Figure 4A**).

The ability of squalamine to stimulate vagal fibers was first tested in young mice. Squalamine (10 μM) decreased the mean intervals between vagal spikes by 36% from 1118 ± 117 ms to 718 ± 45.2 ms in young mice [N = 3(38), p = 0.0006)] (**Figure 4B**). We then tested whether squalamine could increase the vagal firing rate recorded from the *in vitro* mesenteric nerve preparation taken from old mice. For old mice intraluminal squalamine (10 μM) decreased the mean interval between vagal spikes by 56% from 2509 ± 396 ms to 1093 ± 105 ms [N = 4(22), p = 0.0013] (**Figure 4C**). Thus, the reduction in vagal afferent firing for old mice was at least partially reversed by intraluminal squalamine, and squalamine increased vagal firing rate in old mice more strongly than in young mice, 56 vs. 36% increases over the resting firing rates recorded with only Krebs in the lumen.

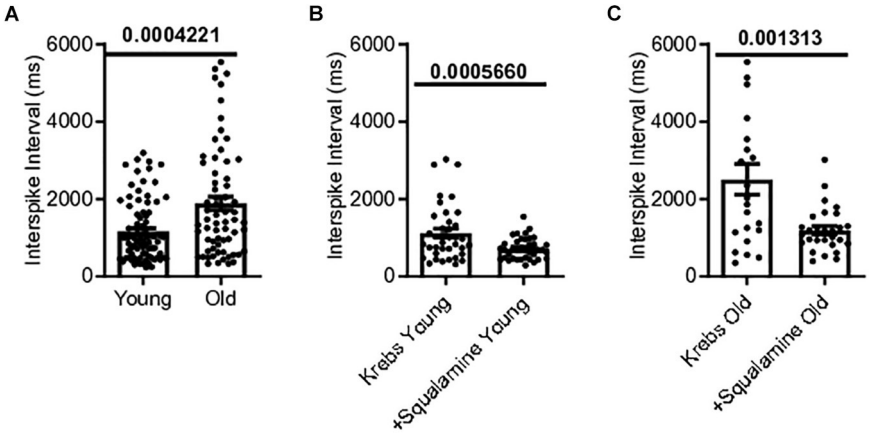

FIGURE 4 | Single vagal afferent firing was reduced in aged mice but could be rescued. **(A)** Mean interval between spikes of vagal afferents were longer in aged mice, indicating a reduction in vagal afferent firing rate in the aged mice (N = 148 fibers). **(B)** Intraluminal squalamine (10 μM) reduced the mean interval between vagal spikes in young mice (N = 38 fibers). **(C)** Intraluminal squalamine (10 μM) reduced the mean interval between vagal spikes in aged mice, increasing firing frequency toward that of the young controls (N = 22 fibers). Data represented as mean with SEM, two-tailed t-test unpaired, with Welch's correction and paired, two-tailed t-tests.

5.3.5 Onset to Peak Response of Squalamine Is Longer in Old Mice

The onset to peak response of squalamine was measured in single unit vagal afferents of young mice [N = 3(38)] and old mice [N = 4(22)]. Onset to peak response was measured from the time of addition of squalamine to the lumen to the time that squalamine increased vagal firing rate to peak. The onset to peak response following luminal addition of

squalamine was significantly longer for old mice ($P = 0.0182$) (**Figure 5A**). The onset to peak response in young mice was 12.95 ± 0.99 min compared to 18.82 ± 1.37 min in old mice. Representative frequency histograms over the course of the experiment demonstrate the squalamine onset to peak response to increase vagal afferent firing between young and old mice (**Figures 5B,C**). We hypothesize that vagal afferent firing is stimulated via an intramural sensory synapse between intrinsic primary afferent neurons (IPANs) of the enteric nervous system and vagal afferent endings (Perez-Burgos et al., 2014). A longer onset to peak response in aged mice would be consistent with a decrease in the excitability of these IPANs in old mice and is discussed later.

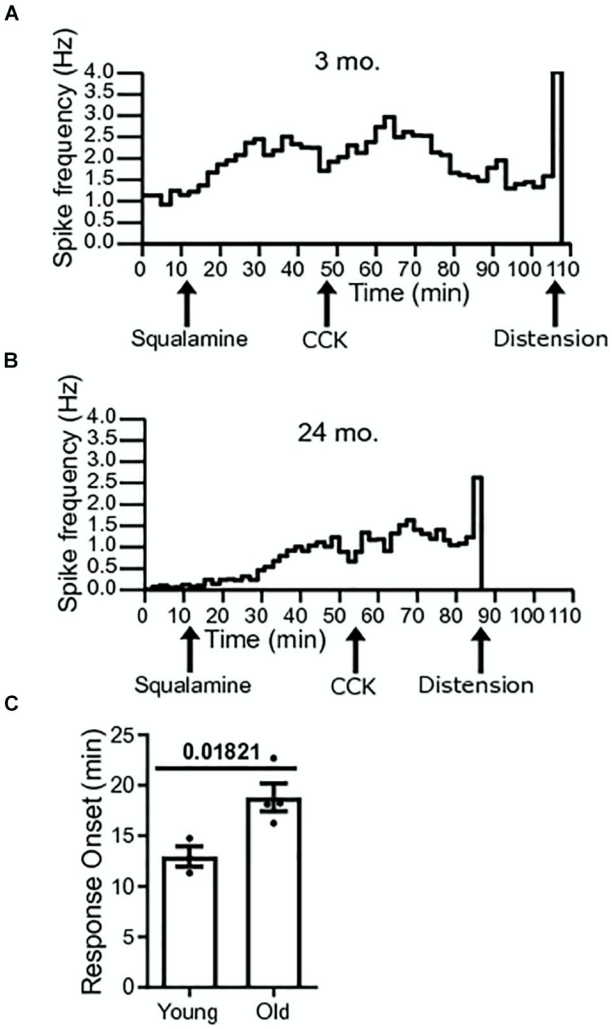

FIGURE 5 | Response onset to squalamine is longer in old mice. (A) Spike frequency histograms show changes in spike frequency over the course of the experiment. Onset to peak squalamine response in vagal afferent fibers in a young mouse after addition of squalamine at 12 min. **(B)** Response onset to squalamine in vagal afferent fibers in an old mouse after addition of squalamine. **(C)** Response onset to squalamine on vagal afferent firing rate was longer in old mice [N = 4(22)] compared to young mice [N = 3(38)]. Data represented as mean with SEM, t-test unpaired, with Welch's correction, two-tailed.

5.4 DISCUSSION

In the present study, we used *in vitro* preparations to measure male CD1 mouse colon motility and single unit vagal afferent spike firing rates from the jejunal mesenteric nerve. We showed that motility and spike firing are reduced for old compared to young mice. We also demonstrated that acute intraluminal application of squalamine could partially reverse the effects of old age on motility and constitutive vagal single unit firing rates. The stimulatory effect of squalamine on vagal firing rates had a longer onset latency to peak response in old compared to young mice.

Constipation and decreased gastrointestinal motility disproportionately affect old humans and animal models (Higgins and Johanson, 2004; De Giorgio et al., 2015; Ranson and Saffrey, 2015). For example, aged 2-year-old mice had reduced total fecal output compared to their younger 3-month- old counterparts (Patel et al., 2012); in particular, there was a decrease in velocity of epoxy coated pellet movement in the colon and an increase in impaction (Patel et al., 2012). Despite the large range of laxatives including prokinetics and secretagogues that are available, a significant number of constipated patients are dissatisfied with treatment results (De Giorgio et al., 2015; Sbahi and Cash, 2015). The present work is the first complete report, except for a pilot data abstract in a different mouse model (Kunze et al., 2014), showing that squalamine can partially reverse the old age-related decrease in MMCs velocity in otherwise healthy mice.

Squalamine is a cationic amphipathic sterol with broad antibacterial and antiviral properties (Zasloff et al., 2011) that was first isolated from tissues of the dogfish shark *Squalus*

acanthias (Moore et al., 1993). As well as having broad-spectrum anti- microbial activity, squalamine may have potential for treating a variety of diseases such as cancers, age-related macular degeneration and obesity (Brunel et al., 2005). Squalamine has also been shown to restore motility in a *C. elegans* model of Parkinson's disease (Perni et al., 2017). We have previously reported in abstract form the results of a pilot study showing that the firing rates of vagal afferent fibers are decreased in old compared to young mice and that squalamine increases this function (West et al., 2018). Our present study shows that squalamine can restore colonic motility and vagal afferent firing rates back toward young controls, following a decrease in function with age.

The vagus nerve exhibits anatomic degenerative changes in older animals displaying swollen varicosities (Phillips and Powley, 2007), yet there has so far been little evidence of a reduction in vagal firing in old age. Intestinal mesenteric nerve discharge has been reported to be decreased for old compared to young humans (Yu et al., 2016) and mice (Keating et al., 2015). However, the intestinal mesenteric nerve is a mixed nerve and it is not clear whether the reduction recorded was from vagal, spinal or other fibers. Heart rate variability indices obtained from power spectral density and time domain analysis showed a significant decrease in vagal activity in the elderly (68–85 years) compared to matched young (21–34 years) adults. There was no indication in this study whether afferent, efferent or both types of vagal fibers innervating the heart were involved (Junior and Oliveira, 2017). Of the vagal fibers innervating the gastrointestinal tract we have selected those activated by CCK. We believe our present results are the first to demonstrate reduced constitutive afferent vagal discharge for old compared to young animals. Although the *in vitro* vagal nerve recording

methodology is commonly employed, a potential limitation could be that the full scope of sensory signals available *in vivo* may not be available *in vitro*.

Although the vagus can modulate small and large intestinal motility (Collman et al., 1983, 1984; Raybould and Tache, 1988; Gustafsson and Delbro, 1994; Tong et al., 2010), it is not known whether or to what extent reduced vagal firing rates contribute to a decrease in propulsive peristalsis in old mice *in vivo*. Chronic extrinsic denervation of the intestine allowing extrinsic nerve fibers to fully degenerate does not abolish or alter peristalsis reflexes (Furness et al., 1995) further emphasizing that MMCs are generated by the ENS. On the other hand, propulsive peristalsis directly reflects intrinsic primary afferent neuron (IPAN) functioning (Howe et al., 2006) and the same IPANs transmit to vagal afferent endings (Perez-Burgos et al., 2014) suggesting that compromised IPAN functioning may be an important determinant in reduced propulsion and vagal firing in old age. Therefore, it may be more likely that reduced colonic motility and reduced vagal afferent firing are not completely causally linked to each other. Because squalamine stimulates colonic motility in the absence of extrinsic innervation, it is likely that squalamine stimulated IPAN firing and that this may produce improvements in both the aged colon motility and vagal afferent firing.

The increased onset latency to peak response of squalamine on afferent vagal firing in tissue taken from old compared to young mice may be interpreted by the effects of old age on the enteric nervous system. Ninety to 95% of sensory neuron processes innervating the intestinal epithelium arise from the ENS, with the rest originating from neurons whose somata are located outside the intestine (Keast et al., 1984; Ekblad et al., 1987). In agreement with this

anatomical data, is our recent discovery that more than two thirds of vagal afferent signals evoked by a luminal commensal bacterium is relayed to the vagus via the enteric neurons (Perez-Burgos et al., 2014). Neuroactive luminal molecules first excite juxtaepithelial neurites belonging to IPANs whose cell bodies are located within the ENS. The excited IPANs release acetylcholine and perhaps other neurotransmitters to activate vagal IGLEs which closely surround and abut the IPANs (Berthoud et al., 1997; Perez-Burgos et al., 2014). The IPAN to IGLE nicotinic sensory synapse we have described (Perez-Burgos et al., 2014), is perfectly positioned to act as a gatekeeper to regulate gut to brain signaling. Accordingly, the amount of information transmitted to the brain via the vagus would be markedly influenced by whether IPANs are refractory or readily responsive to luminal stimuli, and by the density of IPAN sensory innervation of the epithelium. Given the important role of enteric IPANs in gut to brain signaling (Perez-Burgos et al., 2014), the vulnerability of the ENS to old age in terms of numbers and degeneration could have a significant impact on the amount, quality and latency of signals reaching the brain from the gut. Thus, even if the numbers of vagal afferent fibers are not appreciably reduced in old age there could be decreased and delayed vagal afferent responses to luminal stimuli.

Functional GI disorders are often comorbid with mood and anxiety disorders (Ballou et al., 2019). In a study of 54 constipated patients with motility disorders, 22.2% showed depression using the Hospital Anxiety and Depression Scale (HADS) and 31.5% on the Mini-International Neuropsychiatric Interview (MINI) (Hosseinzadeh et al., 2011). As previously mentioned, constipation incidence and prevalence increase with age (Higgins and Johanson, 2004; De Giorgio et al., 2015; Ranson and Saffrey, 2015) and depression is

common in old age (Beekman et al., 1999; Djernes, 2006; McCall and Kintziger, 2013). Clinically relevant depressive symptoms may, for example, reach a prevalence of up to 49% in institutionalized elderly Caucasians (Djernes, 2006). Indeed, in a cross-sectional study between psychiatric diagnoses and constipation, inpatients older than 60 years had a significantly increased risk of constipation (odds ratios 3.38–6.52) (Jessurun et al., 2016). Increased depression in old age may be related, at least in part, to alterations in vagus nerve activity (Forsythe et al., 2014) and successful treatment of depression may also relieve the associated constipation. Vagus nerve stimulation is FDA approved, and shows promise as an essential or adjunct antidepressant treatment in humans (Carreno and Frazer, 2017; Johnson and Wilson, 2018). Indeed, a 5-year observational follow up of vagus nerve stimulation in depression has shown very promising results (Aaronson et al., 2017).

Activation of the visceral afferent vagus appears to have antidepressant behavioral and mood-altering effects in mice. Indeed, the antidepressant effects of certain neuroactive microbes, which increase discharge frequency in mesenteric vagal afferents (Perez-Burgos et al., 2013), depend on the presence of an intact vagal nerve since subdiaphragmatic vagotomy abrogated both the antidepressant effects and regional changes in GABA receptor expression in the brain (Bravo et al., 2011).

Our results show that vagal afferent firing is reduced in old age, but these changes are not permanent regardless of causation since these effects can be restored to within range of the young mouse controls. Future studies should seek to evaluate the effect of reduced vagal afferent firing in old age on depression and also GI function as a whole. Additionally,

experiments should be repeated *in vivo* where possible to test for translatability of the *in vitro* results. The marked improvement induced by squalamine in the disordered motility of aged mice may have a translatable clinical use in treating old age-related constipation and awaits clinical testing.

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CHAPTER 6.

Squalamine restores the function of the enteric nervous system in mouse models of Parkinson's disease

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Abstract and Keywords

BACKGROUND: Parkinson's disease (PD) is a progressive neurodegenerative disorder thought to be caused by accumulation of α-synuclein (α-syn) within the brain, autonomic nerves, and the enteric nervous system (ENS). Involvement of the ENS in PD often precedes the onset of the classic motor signs of PD by many years at a time when severe constipation represents a major morbidity. Studies conducted *in vitro* and *in vivo*, have shown that squalamine, a zwitterionic amphipathic aminosterol, originally isolated from the liver of the dogfish shark, effectively displaces membrane-bound α-syn.

OBJECTIVE: Here we explore the electrophysiological effect of squalamine on the gastrointestinal (GI) tract of mouse models of PD engineered to express the highly aggregating A53T human α-syn mutant.

METHODS: GI motility and *in vivo* response to oral squalamine in PD model mice and controls were assessed using an *in vitro* tissue motility protocol and via fecal pellet output. Vagal afferent response to squalamine was measured using extracellular mesenteric nerve recordings from the jejunum. Whole cell patch clamp was performed to measure response to squalamine in the myenteric plexus.

RESULTS: Squalamine effectively restores disordered colonic motility *in vivo* and within minutes of local application to the bowel. We show that topical squalamine exposure to intrinsic primary afferent neurons (IPANs) of the ENS rapidly restores excitability.

CONCLUSIONS: These observations may help to explain how squalamine may promote gut propulsive activity through local effects on IPANs in the ENS, and further support its possible utility in the treatment of constipation in patients with PD.

KEY WORDS:

Parkinson's disease, squalamine, intestinal motility, intrinsic primary afferent neuron, electrophysiology, gut-brain axis, vagus

6.1 Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease associated with the accumulation of the protein α-synuclein (α-syn) within the peripheral and central nervous systems (CNS) [1]. Diagnosis of PD is primarily based on the presence of a combination of motor and non-motor symptoms [2]. The latter include severe neuropathic constipation which presents an important therapeutic challenge [3]. Lewy bodies containing α-syn are a pathognomonic sign of PD and are classically found in the CNS. However, they can also be detected within the enteric nervous system (ENS) of patients with sporadic disease [4, 5] and have been detected in the ENS before the clinical diagnosis was made [6]. In a majority of people with PD, constipation occurs many years before the onset of motor symptoms [7-9].

According to Braak's hypothesis PD may begin peripherally with the development of misfolded α-syn aggregates (Lewy bodies) in the olfactory nerves, submandibular gland and ENS [4, 5], then traffic to the CNS. In the case of the gut they move by vagal preganglionic fibres that arise from viscero-motor projection neurons of the dorsal motor nucleus (DMV) [1]. This hypothesis has been supported recently by experiments in rodents. α-syn from PD patient brain lysate injected into the intestine progressed to the brainstem [10]. α-syn preformed fibrils injected into the duodenal and pyloric muscularis layer were

observed in the DMV and then dispersed caudally in the hindbrain, eventually reaching the basolateral amygdala, dorsal raphe nucleus, and substantia nigra pars compacta [11]. Truncal vagotomy prevented gut to brain spread of α-syn pathology [11]. In another study, α-syn preformed fibrils, injected into the duodenal wall of bacterial artificial chromosome (BAC) transgenic rats, propagated to the DMV as well as via a sympathetic route to the celiac ganglia and the intermediolateral nucleus of the spinal cord (IML) [12]. Additionally, low-dose, chronic administration of the pesticide rotenone in mice induced α-syn aggregation in the ENS, DMV, intermediolateral nucleus of the spinal cord, and the substantia nigra, reproducing PD pathological staging [13]. This progression was preventable by vagotomy [14]. Clinically Lewy bodies have been detected in the vagus of patients with PD [15] and a single large-scale retrospective epidemiological study of vagotomy in humans showed a reduced risk of development of PD in those patients who received truncal vagotomy [16]. However, some evidence suggests that ENS spread of α-syn pathology to the CNS after exposure may not be sufficiently sustained and may require other factors [17], although this may be influenced by differing experimental methodologies including type and quantity of α-syn and location of injection [11].

Ninety to 95% of the sensory neurites that innervate the intestinal epithelium originate from the ENS, with the rest arising from cell bodies outside the intestine [18, 19]. Intrinsic primary afferent neurons (IPANs) are ENS sensory neurons in the myenteric plexus that are essential for normal gut propulsive peristalsis and indeed life itself as their selective silencing in transgenic mice leads to death shortly after weaning [20]. We have shown recently that two thirds or more of intestinal vagal afferent signaling is relayed via IPANs

through an IPANs-to-vagus intramural sensory synapse [21]. If myenteric IPANs' excitability is compromised in the hSNCA^{A53T} model used in the present report, constitutive afferent vagal firing rates could be reduced due to decreased activity at the intramural sensory synapse. To test this, we have measured vagal afferent firing rates from the mesenteric nerve bundle of the small intestine of the hSNCA^{A53T} model mice and controls. Furthermore, we have examined these rates before and after adding squalamine to the gut lumen.

Squalamine is a natural zwitterionic amphipathic aminosterol with a net positive charge [22], produced in the liver of the dogfish shark and secreted along with bile salts into the proximal duodenum [23]. The synthetic squalamine salt (ENT-01), inhibits the formation of neurotoxic α-syn aggregates in a *C. elegans* model of PD, both *in vitro*, and *in vivo* [24]. We have recently demonstrated that acute intraluminal administration of squalamine reverses the reduced colonic propulsive motility and decreased constitutive vagus nerve firing observed in aged (≥ 2 y) compared to young (3 month) male mice [25]. The clinical prokinetic effect in gut motility appears to occur through local action of the compound on the ENS, since squalamine is not significantly absorbed into the systemic circulation [26].

In the present studies we explore the electrophysiologic effect of squalamine on the gastrointestinal (GI) tract of both normal mice and two mouse models of PD that express the highly aggregating A53T mutant form of human α-syn. The first model used was a mouse α-syn knock-out/knock in model that expressed four copies of human A53T α-syn (hSNCA^{A53T}) driven by the endogenous α-syn promoter but lacking the murine α-syn coding sequence (mSnca). The control is represented by a strain engineered to express two copies

of the normal human α-syn protein (hSNCA) but also lacking the endogenous mouse α-syn coding region (mSnca) [27]. This model (dbl-PAC-Tg ($SNCA^{A53T}$), will be referred to as $hSNCA^{A53T}$ in the subsequent text and in corresponding figures. The $hSNCA^{A53T}$ strain exhibits a constipation phenotype that is more severe than that observed for the corresponding control strain [27]. These animals represent an early model of familial PD, as they display ENS dysfunction by 3 months, progressing in severity to 6 months, in the absence of CNS pathologies such as olfactory dysfunction, dopaminergic deficits, Lewy body inclusions, and neurodegeneration [27, 28]. A recent study by the same group demonstrated that the translational inhibitor of α-syn, Posiphen, normalized colonic dysmotility in the $hSNCA^{A53T}$ strain [28].

The second model, homozygotic A53T human α-syn overexpressing mice (7 months) [29] and their non-transgenic (non-Tg/WT) littermate controls (7 months), were compared to assess the effect of α-syn aggregation on colonic transit in another PD model. In this engineered mouse model, human A53T ($hSNCA^{A53T}$) expression is driven by a mouse prion promoter (PrP) resulting in the progressive accumulation of aggregates of A53T α-syn throughout the nervous system [29]. These mice are referred to as PrP-A53T and their controls as non-Tg in the following text and figures. When the homozygotes (PrP-A53T mice) reach an age of 7-8 months they begin to develop progressive impairment of motor function so severe that they are eventually unable to support themselves to feed and succumb at about 16 months [29]. Unlike the $hSNCA^{A53T}$ model, PrP-driven human A53T α-syn expression results in accumulation of filamentous α-syn aggregates in the CNS leading to

late-onset neurodegeneration (> 6 months) and mimicking human familial PD neuropathology [29].

We show here that colonic motility is impaired in both mouse models of PD, accompanied by reduced firing rate in vagal nerve fibres of the mesenteric nerve bundle and electrical behavior of IPANs in the hSNCA^{A53T} model. Application of squalamine rapidly restores the normal firing rate and electrical behavior together with promotion of propulsive activity.

6.2 Materials and Methods

All animal studies were approved by and performed in accordance with the Animal Research Ethics Board (AREB) of McMaster University (permit 16-08-30) and of the Florey Institute of Neuroscience and Mental Health (approval 16-029).

hSNCA^{A53T} mice and their controls (endogenous promotor) used for motility (Figure 1), mesenteric nerve recording (Figure 3), and electrophysiological recordings (Figure 4&5) were obtained from Jackson Laboratories (Maine, USA). 13-16 male PAC-Tg (SNCA^{WT}) (Stock No. 010710; Control) and (dbl-PAC-Tg (SNCA^{A53T}) (Stock No. 010799; PD mice) were aged 8-9 months prior to experiments. Researchers were blinded to animal groups.

7-month old male and female PrP-A53T human α-syn overexpressing transgenic mice (prion promotor) and their non-Tg littermate controls (25-35g) [29] were reared in a breeding colony at the Florey Institute. A total of 30 mice were used for *in vitro* colonic motility (Figure 2A). For the dose ranging *in vivo* fecal pellet output test, a total of 100 male mice, or 5 sets of non-Tg (N= 10) and PrP-A53T (N = 10) mice aged 7-months were used.

All mice were housed 3-5 per cage on a 12 h light/dark cycle with food and water provided *ad libitum* and allowed a 1-week acclimation period after arrival.

6.2.1 Squalamine dilactate

Squalamine dilactate was donated by Dr Michael Zasloff, Georgetown University (Washington, DC, United States). Squalamine dilactate powder was dissolved in 90% ethanol to make a stock solution, then aliquoted and stored at -20˚C until use. Stock solution was diluted in Krebs buffer to a working concentration of 30 μM for *in vitro* experiments, as determined in a dose-ranging pilot experiment [30]. Squalamine was diluted to 20, 40, 80, or 120 mg/kg for oral gavage (2% ethanol, included in the control gavage), in order to study *in vivo* fecal pellet output.

6.2.2 In vitro colon motility

The *in vitro* colonic segment experiments were performed with only minor differences in equipment/protocol and as described previously [31-35]. The colon was excised and placed within an organ-bath perfusion chamber filled with warmed, oxygenated Krebs buffer or physiological saline (35˚C, 95% O_2, 5% CO_2). The colon was flushed and cannulated at the oral and anal ends to a manifold and syringe to allow inflow of oxygenated Krebs buffer (or physiological saline) or Krebs and squalamine and to maintain intraluminal pressure. Recordings in the first portion of the study (data in Figure 1) were measured at a mechanical threshold causing a pressure differential of 2 hPa. The height of the inflow tube at baseline measurements (data in Figure 2A) was parallel to the height of the colon in the organ bath (1.1 cm). Mechanical threshold was defined as an inflow pressure great enough to generate a contraction in under 30 sec (1.8 cm). Motor patterns were recorded using a

Microsoft LifeCam 3000 web camera (Figure 1) positioned 7-8 cm above the tissue. Videos were recorded during a 20-minute Krebs control and a 20-minute Krebs plus squalamine period in which solutions were added to the inflow syringe.

6.2.3 In vivo fecal pellet output (FPO)

PrP-A53T and non-Tg controls were subjected to the FPO test 1 day prior to the start of dosing (day 0). Mice were fasted for one hour and then given access to food one hour before testing. Baseline values were obtained on day 0, following oral gavage with vehicle (sterile water). On days 1-5 mice were fasted for one hour prior to oral gavage with vehicle or 20, 40, 80, or 120 mg/kg squalamine. Oral gavage occurred between 10:00 to 11:00 am daily. On day 5, the FPO test was performed 1 hour after the final dose was administered. Total number of stool pellets produced in the first 15 min and over a 60 min period was measured in each group.

6.2.4 Mesenteric nerve recording

Jejunal mesenteric nerve recordings were performed as described previously [21]. A segment of hSNCA^{A53T} mouse jejunum (2-3 cm) was mounted on an agar-coated petri dish filled with oxygenated Krebs buffer and nicardipine (3 μM) and the attached mesentery was pinned out. The luminal contents of the tissue were flushed with Krebs and the oral and anal ends of the tissue were cannulated with silicon tubing. The mesenteric nerve bundle was exposed using fine-tipped forceps by gently removing excess mesentery around the mesenteric arteries. The petri dish nerve preparation was then placed on an inverted microscope stage and perfused with fresh oxygenated Krebs in the serosal compartment using a pump. The nerve bundle was sucked onto a glass micropipette attached to a

microelectrode to record multi-unit electrical activity recorded using a Multi-Clamp 700B amplifier and Digidata 1440A signal converter. Baseline afferent firing was recorded for 20-30 min during luminal Krebs perfusion. Intraluminal squalamine (30 μM) was perfused following the control (Krebs) for a duration of 30 min. Single unit activity was sorted from multi-unit recordings using principal component analysis (PCA) and spike waveform analysis for spike shape, size and duration in the Dataview program [36]. Vagal fibres were identified by response to cholecystokinin (CCK) applied to the serosa 10 min following the cessation of treatment [37, 38]. The mean interspike interval between vagal afferent spike firing (the inverse of firing frequency) was measured during Krebs and squalamine treatment periods for control and hSNCA^{A53T} mice.

6.2.5 Whole-cell patch clamping

Whole-cell patch clamp was performed on a myenteric plexus preparation as previously described [39]. The mucosa, submucosa, and circular muscle were removed to expose the myenteric plexus over about half the length of the preparation while the adjacent epithelium remained intact. The preparation was dissected in a carbogen-bubbled recording dish filled with Krebs buffer. A single myenteric ganglion was cleaned for patching by application of 0.02% protease type XI (Sigma-Aldrich, Oakville, Canada) dissolved in Krebs for 10-15 min followed by washout with perfused Krebs for 5 min. Only whole-cell recordings greater than 4 GΩ were used. IPANs' excitability parameters were measured as described previously first with perfusion with Krebs buffer for 20 min followed by perfusion with Krebs buffer containing squalamine for 15-30 min. The patched cells were confirmed to be IPANs at the end of the recording by injection of Neurobiotin and confirming the

morphology of the cell was that of a Dogiel type II neuron [40]. The neurons were ionophoretically filled with Neurobiotin, from the intracellular patch pipette saline and then fixed overnight at 4°C in Zamboni's fixative (2% vol/vol picric acid, 4% paraformaldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH =7.0) [41]. Neurons were subsequently washed using three 10-min washes of DMSO and then PBS and then visualized with fluorescence epi-illumination after exposure to streptavidin-Texas Red (Vector, http://www.vectorlabs.com), diluted 1:50, revealing Neurobiotin [41] [42].

6.2.6 Statistical Analysis

Effects of squalamine on IPANs' excitability in control and PD model mice were assessed in paired experiments following Krebs control and subsequent squalamine exposure. Percent difference was calculated by (treatment-control)/control. Data are presented as mean ± SEM *In vitro* statistical comparisons were performed using paired or unpaired, two-tailed t-tests or 1 or 2-way ANOVA using GraphPad Prism software (Version 7.0). *In vivo* studies were analysed using 1-way and 2-way ANOVA. Statistical significance was determined when $P < 0.05$.

6.3 Results

6.3.1 Squalamine increased colonic motor activity in hSNCA^{A53T} mice, in vitro

Colonic segments from the homozygotic A53T human α-syn overexpressing mice (hSNCA^{A53T}) (8-9 months) and their controls (8-9 months) were compared to assess the effect of α-syn aggregation on colonic motility. The velocity of propagating contractile clusters (PCCs) was not significantly different in colonic segments from control mice

compared with hSNCA^{A53T} mice (N = 12-13 mice/group) (P = 0.0946) (Figure 1A). According to the 2-way ANOVA, there would be 9.5% chance of observing the present effect if the animal model had no effect at all, therefore the observed effect of the animal model was not quite significant. Addition of intraluminal squalamine (30 μM) increased PCC velocity by 20% in the control (P = 0.044) and by 38% in hSNCA^{A53T} mice (P < 0.0001), compared to Krebs. In contrast neither the frequency nor force (amplitude) of the PCCs differed significantly between control and hSNCA^{A53T} intestinal preparations, nor did squalamine increase either parameter in control or hSNCA^{A53T} intestinal segments (Figure 1B&C). In addition, while the colonic segment from the control animals exhibited a regular period pattern of peristaltic waves, the colonic segments from the hSNCA^{A53T} model exhibited an irregular peristaltic wave pattern characterized by discontinuous contractions, as shown in the spatiotemporal maps (Figure 1D&E, top). Addition of intraluminal squalamine stimulated regular peristaltic waves that travelled the full length of the colonic segment from the oral to anal direction (Figure 1D&E, bottom).

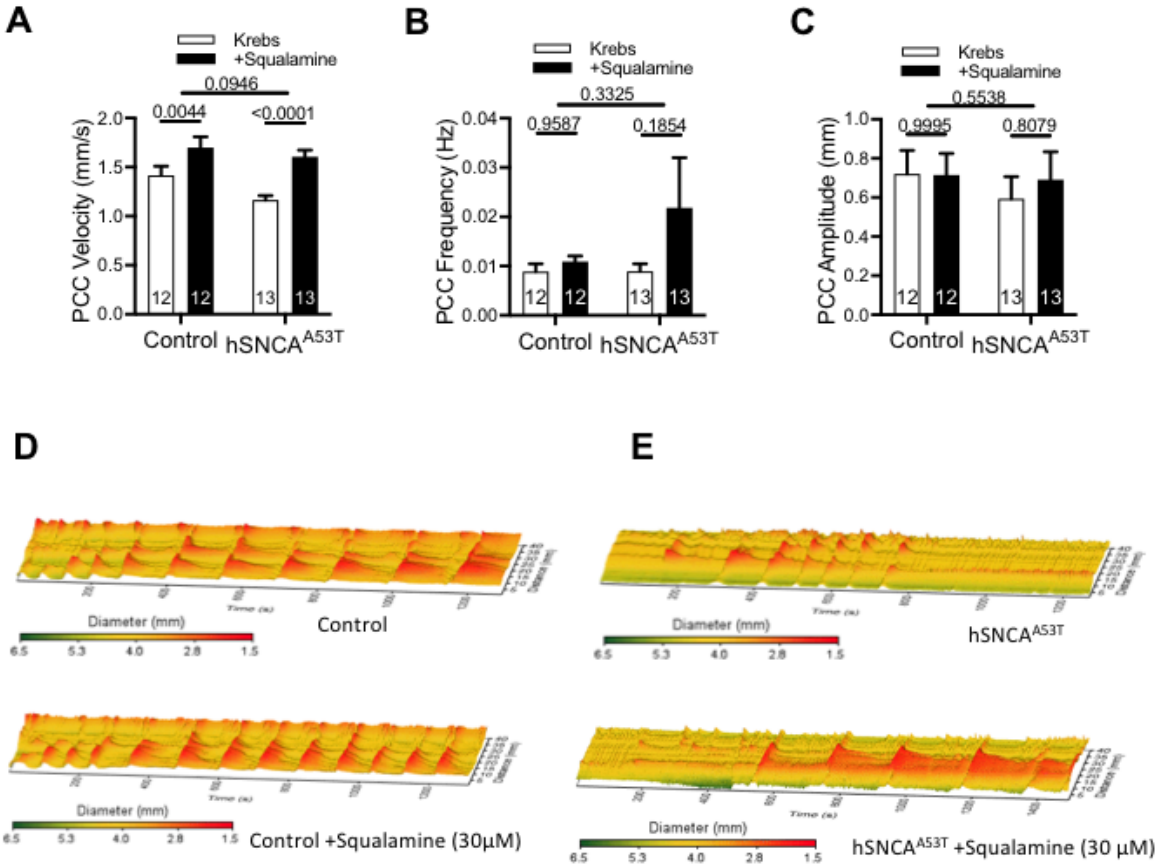

Figure 1. Colonic motor activity was increased with intraluminal squalamine in both hSNCA^{A53T} (endogenous promoter) mice and control mice (**A**) hSNCA^{A53T} mice did not have reduced PCC velocity compared to control mice (P = 0.0946). Intraluminal squalamine (30 µM) significantly increased colonic PCC velocity in control and hSNCA^{A53T} mice (N= 12-13 mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (**B**) hSNCA^{A53T} mice did not have reduced colonic frequency compared to control, nor did squalamine produce an effect on colonic frequency (N= 12-13 mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (**C**) hSNCA^{A53T} mice did not have reduced colonic amplitude compared to control, nor did squalamine produce an effect on colonic amplitude (N= 12-13

mice/group, 2-way ANOVA with Sidak's multiple comparisons test). (**D**) Control mice had more uniform and consistent colonic contractions during Krebs control compared to (**E**) hSNCA^{A53T} mice who had infrequent shorter contractions as represented in the 3D heat maps. Intraluminal squalamine increased colonic contractions, particularly in hSNCA^{A53T} mice. Contractions (red) and relaxation (yellow-green) are depicted across time and distance along the colon. All data represented as mean ± S.E.M, **P* < 0.05.

6.3.2 Colonic motor activity is reduced in a second mouse model (PrP-A53T), in vitro

We evaluated the total number of contractions in non-Tg and PrP-A53T mice at baseline and during pressure-induced distention (threshold) (Figure 2A). We also evaluated the effect of intraluminal squalamine on PCCs when colon segments were not distended (baseline) and during pressure induced distension (Figure 2B). The total number of contractions was significantly reduced between PrP-A53T and non-Tg mice both at baseline (1.3 ± 0.4 mm/s compared to 4.7 ± 0.9 mm/s, P < 0.01) and at threshold (5.5 ± 1.6 mm/s compared to 13.4 ± 1.4 mm/s, P < 0.01) (Figure 2A) Threshold significantly increased the total number of contractions compared to baseline in both groups (P < 0.0001 in non-Tg and P < 0.05 in PrP-A53T). There was no difference in PCC velocity for segments from PrP-A53T (prion promotor) mice compared to non-Tg controls (N = 6-12 mice/group) at both baseline (1.2 ± 0.2 mm/s compared to 1.7 ± 0.3 mm/s) and upon distention (1.6 ± 0.3 mm/s compared to 3.0 ± 0.7 mm/s) (Figure 2B). Intraluminal squalamine (30 μM) increased baseline PCC velocity from 1.2 ± 0.2 mm/s to 2.3 ± 0.4 mm/s for PrP-A53T mice (P < 0.05)

and from 1.7 ± 0.3 mm/s to 2.8 ± 0.4 mm/s in non-Tg mice ($P < 0.05$) (Figure 2B). Upon colonic distension, squalamine had no effect on PCC velocity in PrP-A53T mice (from 1.6 ± 0.3 to 2.1 ± 0.3 mm/s) or non-Tg mice (from 3.0 ± 0.7 to 2.4 ± 0.3 mm/s) ($P > 0.05$). These experiments demonstrate that colonic motility was impaired *in vitro* in both hSNCA^{A53T} and PrP-A53T models and that squalamine increased PCC velocity for both control and hSNCA^{A53T} and PrP-A53T mice.

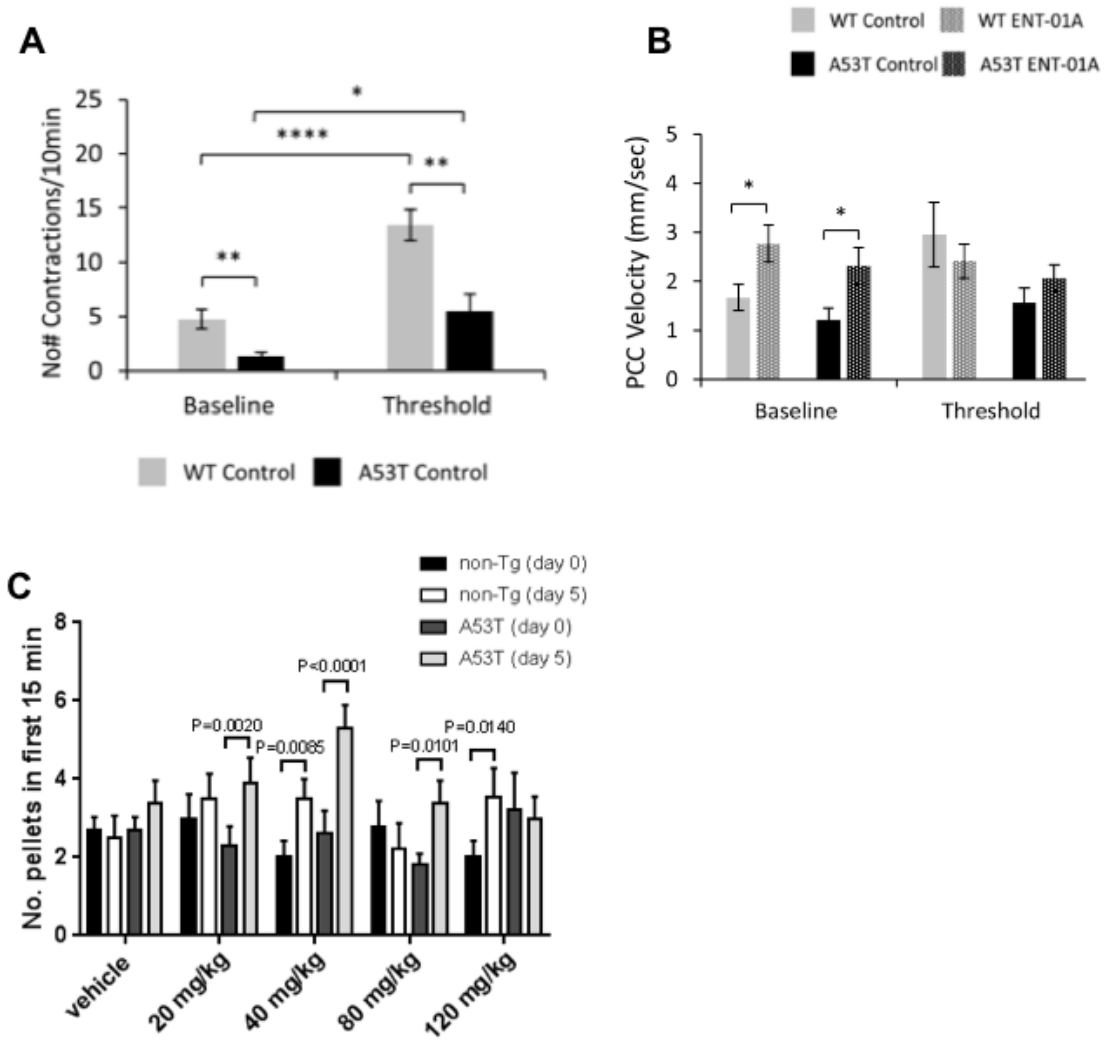

Figure 2. A53T (prion promoter) mice had reduced colonic motor activity compared to non-Tg control mice but improved fecal pellet output with intraluminal squalamine. (**A**) PrP-A53T mice (black) had a significantly reduced total number of contractions compared to non-Tg (gray) at baseline and threshold. Threshold significantly increased the total number of contractions in both non-Tg and PrP-A53T mice (N= 6-12 mice/group, 1-way ANOVA). (**B**) There was no difference in PCC velocity between PrP-A53T mice (black) compared to non-Tg (gray) at baseline and threshold. Intraluminal squalamine (30 μM) significantly increased colonic PCC velocity in non-Tg (gray patterned) and A53T (black patterned) at baseline (N= 6-12 mice/group, 1-way ANOVA). (**C**) Feeding of squalamine for 5 days increased fecal pellet output in non-Tg and A53T mice at several doses. (N = 10 mice/group/dose, 2-way ANOVA). All data represented as mean ± S.E.M, $*P < 0.05$.

6.3.3 Oral administration of squalamine increased colonic transit in the PrP-A53T mice strain in vivo

Fecal pellet output following oral gavage with squalamine was measured in the PrP-A53T mice to assess repeated oral administration of squalamine in a dose ranging study on defecation, *in vivo* (Figure 2C). Pellet output within the first 15 minutes following oral gavage provided an estimate of colonic motility, since gastric feeding stimulates colonic peristalsis, resulting in the discharge of fecal pellets. Both PrP-A53T and non-Tg mice (N = 10 mice/group/dose) were administered vehicle only on Day 0 to establish each animal's baseline pellet output. From Day 1 to Day 5 the animals received vehicle only or squalamine orally by gavage at a range of daily doses from 0 (vehicle only), 20, 40, 80 and 120 mg/kg. Pellet output within the first 15 minutes at Day 5 were compared to baseline values measured at Day 0. We could not detect a significant difference in the fecal pellet output between non-Tg and PrP-A53T mice in the groups receiving only vehicle ($P > 0.05$), possibly due to the poor sensitivity of this assay when compared to measuring PCC velocity and frequency, *in vitro*. However, following gavage of squalamine on Day 5, a treatment associated increase was observed in non-Tg mice dosed with 40 and 120 mg/kg ($P < 0.01$ and $P < 0.05$, respectively) as well as in PrP-A53T mice dosed with 20, 40, and 80 mg/kg ($P < 0.005$, $P < 0.0001$, and $P < 0.01$, respectively). These experiments demonstrated that oral administration of squalamine stimulated colonic motility in both non-Tg and PrP-A53T mice. Effect of squalamine on wet weight, dry weight, % water content of stool, and % change in stool output/body weight are presented in Supplementary Figure 1.

6.3.4 Squalamine significantly increased vagal afferent firing frequency in hSNCA^{A53T} mice in vitro

Vagal afferent firing rates were measured by extracellular action potential (multiunit) recording from a mesenteric nerve bundle attached to segments of the jejunum excised from hSNCA^{A53T} mice and controls. The interspike interval of vagal single units were measured and these represent the reciprocal of their firing rate. Mean afferent vagal firing rate was significantly reduced in the hSNCA^{A53T} mice compared to the control group ($p = 0.0375$). The mean interspike interval between vagal spikes was shorter in control mice compared to hSNCA^{A53T} mice, indicating a lower firing rate in the latter (2389 ± 230 ms compared to 3088 ± 392 ms), (Figure 3A). Squalamine had strong effects on vagal firing rates in both groups of mice (Figure 3B&C). Intraluminal addition of squalamine (30μM) decreased the interspike interval between vagal afferent spikes in the control mice by 63% to 874 ± 60 ms ($p < 0.0001$). Acute treatment with squalamine also decreased the interspike interval between vagal afferent spikes in the hSNCA^{A53T} model mouse by 47% to 1646 ± 168 ms ($p < 0.0001$). These results demonstrate that the vagal afferent firing rate is reduced in the hSNCA^{A53T} mice and is potently stimulated by the addition of intraluminal squalamine in both the control and hSNCA^{A53T} model mice.

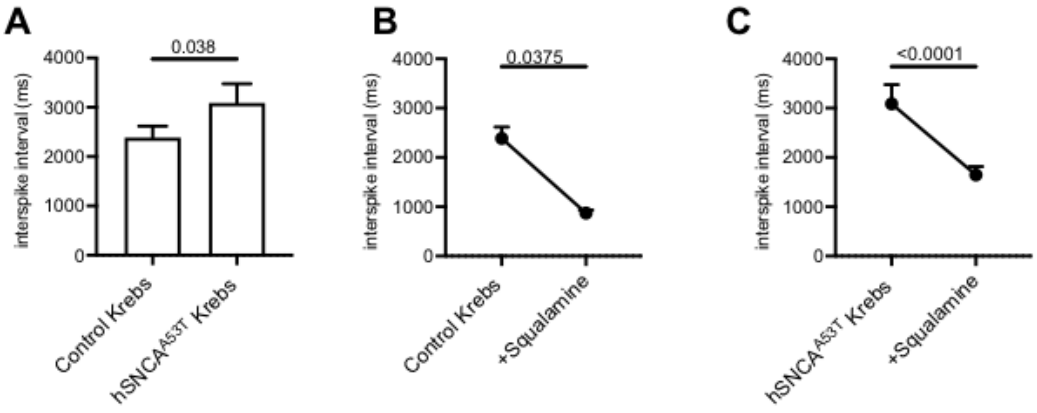

Figure 3. Intraluminal squalamine significantly increased vagal afferent firing (reduced interspike interval) in both control and hSNCA^{A53T} mice. (**A**) The interspike interval was significantly longer in hSNCA^{A53T} mice than control mice (N= 13-14 mice/group). (**B**) Intraluminal squalamine (30 μM) significantly reduced the vagal afferent interspike interval for control mice (N = 13 mice). (**C**) Intraluminal squalamine (30 μM) significantly reduced the vagal afferent interspike interval for hSNCA^{A53T} mice (N = 14 mice). 1-way ANOVA, Holm-Sidak's multiple comparisons test. All data represented as mean ± S.E.M, *$P < 0.05$.

6.3.5 hSNCA^{A53T} model mice have reduced myenteric intrinsic primary afferent neuron (IPANs) excitability

To help elucidate the mechanism by which squalamine stimulated intestinal motility we conducted electrophysiological studies on single neurons within the intact myenteric plexus of the hSNCA^{A53T} mice [27] and corresponding control animals using published methods [18]. IPANs [40] in the myenteric plexus have neurites that project to the epithelial

layer where they respond to molecules present in the gut lumen and then conduct action potentials to the myenteric plexus [39, 43, 44]. IPANs excitation generates PCCs that move luminal contents in the oral to anal direction [44]. We investigated whether IPANs' excitability was reduced in the hSNCA^{A53T} mice compared to the control mice and whether squalamine administration to intestinal segments taken from hSNCA^{A53T} mice could facilitate IPANs' excitability.

Using whole-cell patch pipette recordings from IPANs, we measured the threshold for action potential generation in response to intracellular injection of square depolarising current pulses (AP threshold), the number of action potentials (AP) generated in response to current injection of 2x threshold intensity (No. AP 2x threshold), the area under the curve for the slow after-hyperpolarisation generated by 3 action potentials (sAHP AUC), and the resting membrane potential. In this experiment, 14 IPANs from 14 hSNCA^{A53T} mice and 9 IPANs from 9 control mice were successfully studied (Figure 4). The AP threshold (mean ± SEM) was 32.2 ± 20.0 pA for the control compared to 59.2 ± 46.1 pA for the hSNCA^{A53T} IPANs (P =0.036) (Figure 4E). The number of action potentials produced by a current 2x the threshold intensity was 3.9 ± 5.1 for the control versus 1.6 ± 0.6 for the hSNCA^{A53T} IPANs (P = 0.06) (Figure 4F). The area under of the curve for the sAHP was -49.5 ± 63.7 mV s for the control versus -85.5 ± 78.2 mV s for the hSNCA^{A53T} cells (P= 0.17) (Figure 4G). The resting membrane potential was -56 ± 10 mV for control IPANs versus -62 ± 6 mV for those from the hSNCA^{A53T} model (P=0.034) (Figure 4H). Thus, IPANs from the

hSNCA^{A53T} mouse strain exhibited a reduced excitability compared to those from the control animals.

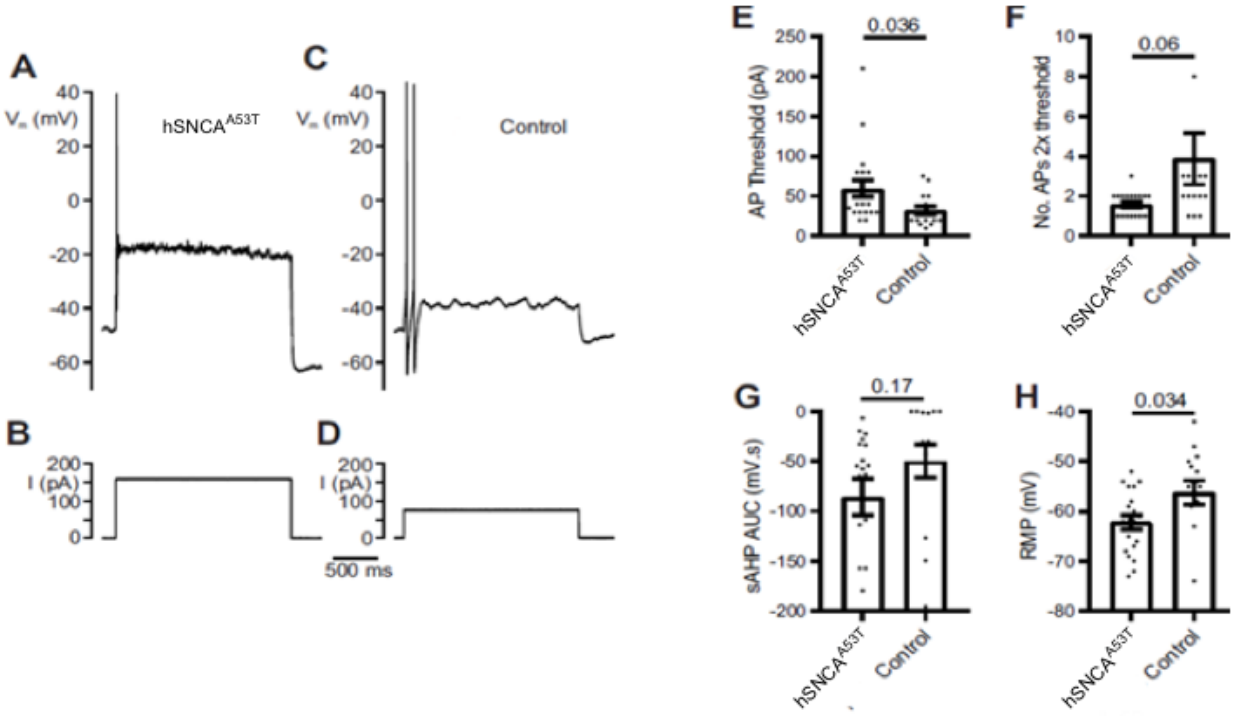

Figure 4. hSNCA^{A53T} (endogenous promoter) mice had decreased intrinsic excitability of myenteric intrinsic primary afferent neurons compared to control mice. (**A**) Representative action potential firing response to injected depolarizing square wave current stimulus (**B**) of 2x threshold intensity (hSNCA^{A53T}). (**C**) Representative action potential firing response to current stimulus (**D**) of 2x threshold intensity (control). (**E-H**) Probabilities under the null hypothesis of no difference given above dot plots, mean values given by open bars, error bars represent SEM. (**E**) The threshold intracellular current (AP threshold) required to evoke a single action potential (AP) was larger for hSNCA^{A53T} (N = 20 recordings) than for control mice (N = 16 recordings) (t = 2.2, t-test unpaired 2-tailed).

(**F**) The number of action potentials evoked at 2 times threshold current intensity (No. APs 2x threshold) was greater for control (N = 19 recordings) than for hSNCA^{A53T} mice (N = 16 recordings) (t = 1.9, t-test unpaired 2-tailed). (**G**) The slow action potential after hyperpolarisation area under the curve (sAHP AUC) showed little difference between hSNCA^{A53T} (N = 19) and control mice (N = 14) (t = 1.4, t-test unpaired 2-tailed). (**H**) The resting membrane potential was more hyperpolarized for hSNCA^{A53T} (N = 20) than for control mice (N = 16 recordings) (t = 2.2, t-test unpaired 2-tailed).

6.3.6 Squalamine excites IPANs

We tested for the effect of squalamine on the excitability of an isolated colonic segment from the hSNCA^{A53T} (endogenous promoter) mouse using preparations in which myenteric neurons are exposed on one half of a small intestinal segment [39]. In this experiment we asked whether squalamine influenced the activity of the IPANs through direct interaction with the neuron or indirectly, by stimulating release of epithelial mediators that influenced IPANs' behaviour. Addition of squalamine (30 μM) to the Krebs buffer in either the epithelial or the myenteric plexus compartments of the preparation increased IPANs' excitability (Figure 5). Adding squalamine to the luminal side of the hSNCA^{A53T} mouse colon (N = 15 cells) decreased the mean AP threshold from 63.7 ± 50.4 to 35.7 ± 22.3 pA (P=0.037) (Figure 5C) and increased the number of APs produced by a current 2x the threshold intensity from 1.6 ± 0.6 to 3.1 ± 0.7 (P=0.0016) (Figure 5A, 5C). Addition of squalamine decreased the area under the curve of the sAHP from 86.8 ± 88.2 to 20.3 ± 25.3 mV s (P=0.0035) (Figure 5C) and depolarized the resting membrane potential from -62 ± 7 to -54 ± 6 mV (P< 0.001) (Figure 5C). Adding squalamine directly to the myenteric plexus of the hSNCA^{A53T} mouse colon (N = 5 cells) (Figures 5D, 5E) did not significantly decrease mean AP threshold from 46.0 ± 31.3 to 29.0 ± 10.1 pA (P=0.058) and increase the number of APs produced by a current 2x the threshold intensity from 1.4 ± 0.5 to 4.4 ± 2.8 (P=0.095). Squalamine significantly depolarized the resting membrane potential from -63 ± 4 to -55 ± 6 mV (P= 0.0067), but did not significantly decrease the area under the curve of the sAHP (-71.9 ± 60.1 to -9.6 ± 15.1 mV s (P=0.099)), when added to the myenteric plexus of the hSNCA^{A53T} mouse. These experiments demonstrated that squalamine can augment the

reduced excitability of the IPANs in tissue taken from hSNCA^{A53T} mice and that squalamine can act directly on the IPANs, rather than indirectly through release of a chemical such as a neurotransmitter from a luminal epithelial cell, like an enteroendocrine cell.

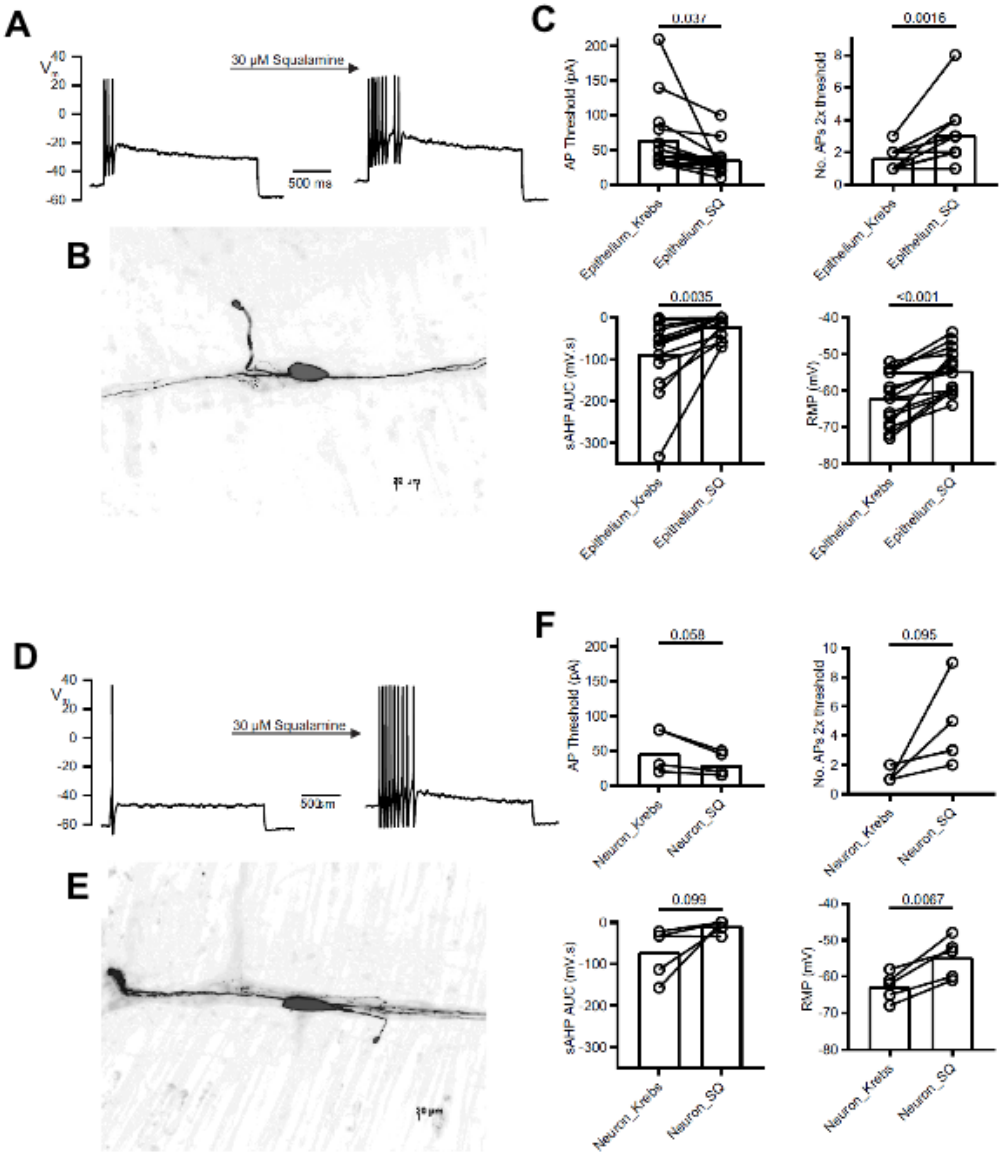

Figure 5. Application of squalamine onto the intestinal epithelium or directly onto the exposed myenteric plexus increased excitability of intrinsic primary afferent neurons

(IPANs) in hSNCA^{A53T} mice. (**A**) Representative action potential firing increase to injected square wave current stimulus after acute application of 30 µM squalamine onto the *intestinal epithelium* using the divided hemidissection preparation. (**B**) Texas Red fluorescence image of neuron recorded from in (**A**) after tissue fixation revealing flattened oval soma and circumferentially directed neurites (Dogiel type II morphology) characteristic of chemosensitive myenteric intrinsic primary afferent neurons. (**C**) Addition of squalamine to the epithelial layer decreased the action potential firing threshold (AP Threshold) (t = 2.3, t-test paired 2-tailed), increased the number of action potentials discharged (No. AP 2x threshold) (N = 15 recordings, t = 4, t-test paired 2-tailed), decreased the area under the curve for the post action potential slow after hyperpolarisation (sAHP AUC) (N = 14 recordings, t = 3.6, t-test paired 2-tailed) and depolarized the neuron membrane potential (N = 15 recordings, t = 5.9, t-test paired 2-tailed). (**D**) A representative action potential firing increase in response to an injected square wave current stimulus after application of 30 µM squalamine onto the myenteric plexus. (**E**) Texas Red fluorescence image of neuron recorded from in (**D**) reveals Dogiel type II morphology. (**F**) Addition of squalamine to the myenteric plexus decreased AP threshold (N = 5 recordings, t = 2.6, t-test paired 2-tailed), increased No. AP 2x threshold (N = 5 recordings, t = 2.2, t-test paired 2-tailed) decreased the post action potential sAHP AUC (N = 5 recordings, t = 2.1, t-test paired 2-tailed), and depolarized resting membrane potential (N = 5 recordings, t = 5.2, t-test paired 2-tailed). (**C** & **F**) Probabilities under the null hypothesis of no difference given above individual value barbell plots, mean values given by open bars, error bars represent SEM.

6.4 Discussion

The purpose of the present study was to investigate the ability of squalamine, a zwitterionic amphipathic aminosterol that has previously been shown to improve age-related gut dysmotility [25, 30] and inhibit the formation of α-syn aggregates [24], to improve colonic motility and constipation in two mouse models of Parkinson's disease (PD). We demonstrate in this report that squalamine can restore gastrointestinal motility in mouse models of PD.

Constipation in PD presents a significant challenge in the management of the disease and often precedes the onset of motor symptoms by years or decades [9]. It has been recently reported that α-syn expression is induced in the ENS in response to viral, bacterial and fungal infections [45] and that excessive intraneuronal accumulation of α-syn promotes formation of toxic aggregates [24]. Because of the normal trafficking of α-syn aggregates from the ENS to the central nervous system (CNS) via the vagus [10, 16], neurotoxic aggregates accumulate progressively within the brainstem and more rostral structures. Additionally, there is potential for bidirectional spread of α-syn between the ENS and CNS via efferent vagal fibres, as suggested by Di Monte [46]. α-syn overexpression in the DMV and vagal ganglia was efficiently trafficked via vagal afferents and efferents to the stomach wall [46]. It would be logical to suggest therefore that targeting α-syn aggregation in the ENS may be beneficial in the treatment of PD constipation [47].

The two models of PD used in this particular study both expressed the human A53T α-syn autosomal dominant mutation, one being driven by a prion promoter (PrP-A53T) [29], the other by the endogenous mouse sequence ($hSNCA^{A53T}$) [27] and both exhibit GI

dysmotility. Overexpression of normal human α-syn in the ENS of the control strain was not associated with apparent gastrointestinal dysfunction, nor were α-syn aggregates seen on pathology, in contrast to the strain engineered to overexpress A53T (hSNCA^{A53T}) [27]. Addition of squalamine increased colonic PCC velocity in both strains and FPO in the PrP-A53T strain. It is notable however, that squalamine also produced prokinetic effects on motility in control strains in the absence of α-syn pathology, which likely occurs via stimulation of the ENS. These observations support the hypothesis that the beneficial effect of squalamine in PD model mice with, respect to motility, may in part involve displacement of membrane disruptive aggregates within the enteric neurons.

Indeed, squalamine has been shown to have two beneficial properties of relevance to PD. By displacement of monomeric α-syn from a membrane, squalamine reduces the surface concentration of the protein, reducing the probability that neurotoxic aggregates will form. In addition, squalamine can also displace aggregates that have already formed on the membrane, restoring membrane function, and releasing the aggregate into the cytoplasm for subsequent digestion. Upon entry into a cell, or potentially a primary afferent neuron [48], squalamine is electrostatically attracted to the negatively charged phospholipid head groups that comprise the cytoplasmic surface of the plasma membrane thereby neutralizing its negative electrostatic surface potential [48, 49]. In a *C. elegans* model of PD, in which the animal has been engineered to express human α-syn in its muscles, orally administered squalamine inhibits formation of membrane disruptive intracellular aggregates and prevents the onset of disordered motility and paralysis [24]. In addition, *in vitro*, squalamine prevents the binding of membrane disruptive α-syn aggregates to the surface of neuronal cells,

protecting them from loss of viability [24]. We believe a similar effect occurs when squalamine enters the enteric neuron of the hSNCA^{A53T} mouse. Our studies demonstrate, as well, that the enteric nerves in the hSNCA^{A53T} mouse models are not irreversibly damaged, since squalamine restored the disordered GI motility in both models of PD. Orally administered squalamine also increased FPO in A53T mice at the mid-range doses of 20, 40, and 80 mg/kg and at the doses of 40 and 120 mg/kg in the non-Tg controls, which may represent a bell-shaped dose response curve for squalamine, with an optimal range of 40 mg/kg. Additionally, chronic administration may produce significant effects on α-syn aggregates by day 5. Indeed, in a recent Phase 2a clinical study of 50 PD patients with severe constipation, orally administered ENT-01 reversed constipation dose dependently in most patients some suffering for 60 years [26].

As predicted in the introduction, constitutive vagal afferent firing rates were decreased (increased interspike intervals) for hSNCA^{A53T} model animals compared to controls (Figure 3). Squalamine (30 μM) potently increased vagal fibre firing rates when it was added to the Krebs buffer perfusing the lumen for both controls and hSNCA^{A53T} animals. Given the important role of enteric IPANs in gut to brain signaling [21], the vulnerability of the ENS to misfolded α-syn in terms of reduced function or IPANs excitability would have a significant impact on the amount and quality of information reaching the brain from the gut. If behavioural or mood-related Parkinsonian symptoms are mediated, in part, by reduced afferent vagal discharge, the excitatory action of squalamine on IPANs and the vagus may serve to reverse the symptoms.

We have shown here that IPANs from animals in which A53T is overexpressed ($hSNCA^{A53T}$) exhibit reduced excitatory activity compared with IPANs from controls. Reduced excitability was characterized by a more hyperpolarized resting membrane potential in the $hSNCA^{A53T}$ mouse, requiring a larger threshold current for action potential generation. The number of action potentials produced by a current 2x the threshold intensity was much lower for the $hSNCA^{A53T}$ mouse, which correlates with the larger area under the curve of the slow after-hyperpolarization (sAHP). These results demonstrate that the IPANs from the $hSNCA^{A53T}$ mouse requires a greater stimulus to fire an action potential and takes longer to repolarize before it can fire a subsequent action potential (AP) than the control mouse and is thus less excitable.

Subsequent squalamine exposure restores normal excitatory responses of the colonic IPANs within minutes of local application to preparations from $hSNCA^{A53T}$ animals; the resting membrane potential is more depolarized. In this model less current is required to reach threshold and generate an AP, and the sAHP area under the curve (AUC) is smaller, indicating faster repolarization and the ability to respond and produce a subsequent AP sooner. It is possible that the effects on PD model mice are not α-syn specific, given the prokinetic effects of squalamine in young and old wild-type mice [25] and in the controls. In the setting of the GI tract, we hypothesize that squalamine can be readily transported across the intestinal epithelium and, as described in this report, stimulate the activity of local IPANs and, in turn, GI motility in a healthy animal. It is also possible that IPANs are stimulated via a sensory transduction cascade mediated by intestinal epithelial cells but given that squalamine increases IPAN excitability when applied luminally or directly to the

myenteric plexus, we suggest the former. Interestingly, squalamine does not seem to enter the systemic circulation so that the effects of oral administration are focused in the gut [26].

Locally applied squalamine can stimulate the peristaltic activity of both WT and A53T mice minutes after exposure of the colonic lumen to the compound. Similarly, squalamine restores normal excitatory responses of the colonic IPAN within minutes of local application to preparations from both control and A53T animals. These observations are consistent with a mechanism in which the squalamine ion increases the excitatory status of the IPAN, regardless of the presence of functionally disruptive α-syn aggregates. A reduction in the negative surface charge on intracellular membranes could both influence the electrical behavior of the neuron as well as displace membrane associated aggregates of α-syn. Because squalamine can displace proteins that are bound electrostatically to intracellular membranes, it rapidly activates the AMPA glutamate receptor in primary cortical neurons by displacing the inhibitory TARP regulatory protein from the cytoplasmic face of the plasma membrane [48]; similarly the squalamine ion inhibits the sodium hydrogen exchanger (type 3) by displacing its positively charged carboxyl-terminus from the cytoplasmic face of the plasma membrane [50]. The mechanism by which squalamine alters the electrical excitability of the enteric neuron , independent of the presence of α-syn, is under investigation.

The action of squalamine on IPANs to increase their excitability is entirely consistent with its prokinetic effects since propulsive motility throughout the small and large intestines is critically dependent on normal IPANs function and excitability [20, 51]. Silencing of IPANs by inhibition of protein kinase A activity produces lethal pseudo-obstruction in the

murine intestine [20], and the pharmacological inhibition of IPANs excitability by application of 5,6-Dichloro-1-ethyl-1,3- dihydro-2H-benzimidazol-2-one to the Krebs buffer superfusing *ex vivo* segments of mouse intestine reduces and then abolishes all propulsive PCCs [51]. Manipulation of the current underlying the AHP by calcium-activated, intermediate-conductance potassium channels (IK or IKCa) block or activation has similar effects in the small intestine [52]. Because of their role in generating the propulsive peristaltic reflex, action at myenteric IPANs provides a cellular explanation for the increased propulsive motility and reduced constipation in the hSNCA^{A53T} and PrP-A53T animals caused by squalamine. We found evidence for the direct action of squalamine on the IPANs as demonstrated by the increased excitability of the neuron when squalamine was applied directly to the myenteric plexus.

In conclusion, we have shown that squalamine, locally administered to the lumen of the GI tract of hSNCA^{A53T} mice, can restore intestinal motility and enteric sensory neuron function, suggesting that the ENS is not irreversibly damaged, at least in murine models of PD.

6.5 SUPPLEMENTARY FIGURES

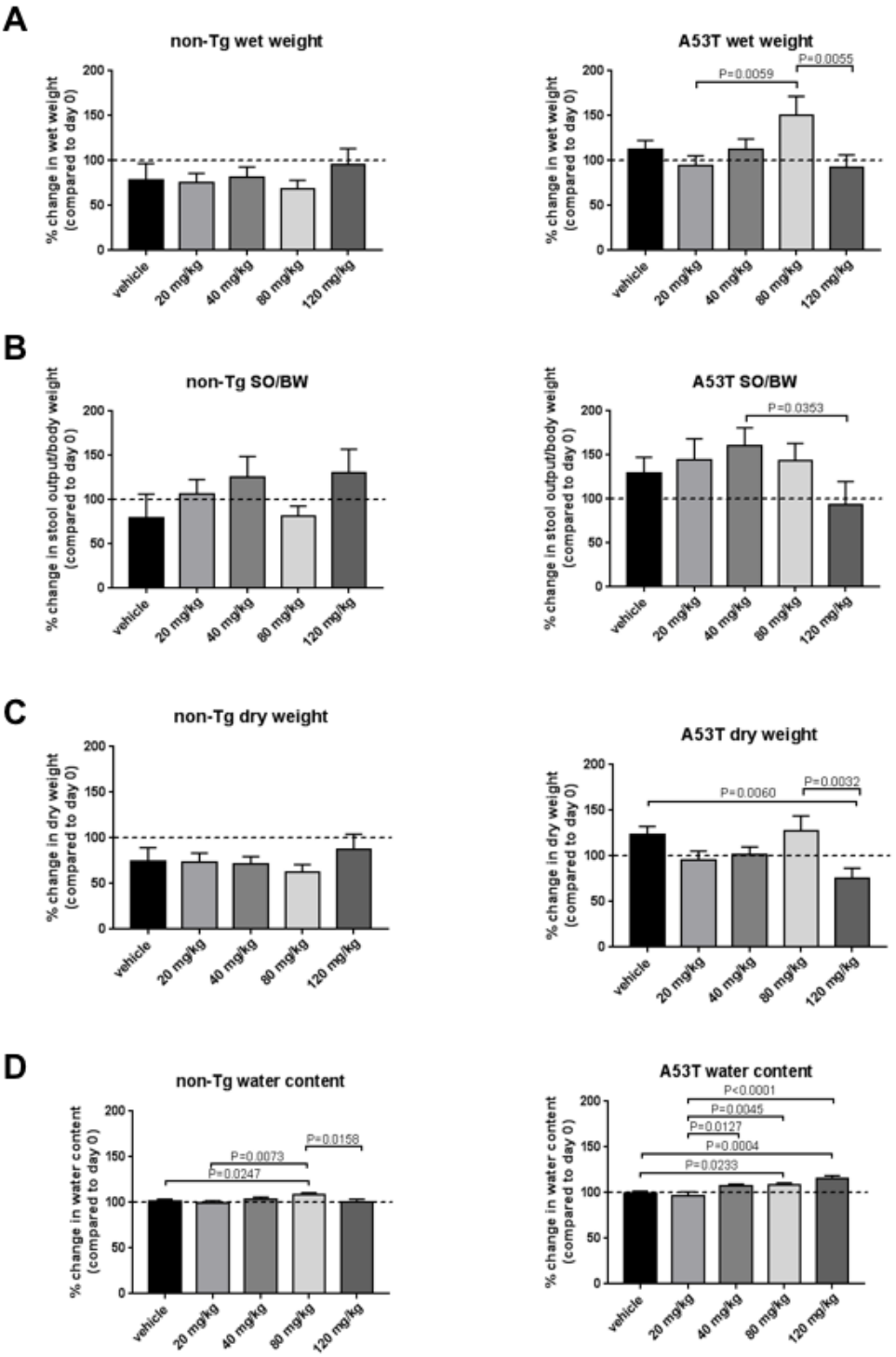

Figure S1. Effect of increasing doses of squalamine (ENT-01) on stool wet and dry weight in non-Tg and PrP-A53T mice. (**A**) Squalamine did not affect stool wet weight in the non-Tg or A53T mice when the % change in wet weight between day 5 and 0 was compared to vehicle-treated mice. High dose squalamine (80 mg/kg) increased wet weight compared to 20 mg/kg and decreased between 80 and 120 mg/kg in A53T mice, but not compared to vehicle. (**B**) Squalamine had no effect on dry stool weight in non-Tg mice when the % change in dry weight between day 5 and 0 was compared to the vehicle-treated mice. Dry stool weight was decreased at 120 mg/kg in the A53T mice compared to vehicle-treated and 80 mg/kg treated mice. (**C**) Squalamine increased % water content between day 5 and 0 at 80m/kg in non-Tg mice when compared to vehicle-treated mice, but this effect was not seen at 120mg/kg. Squalamine increased % water content at 80 and 120 mg/kg in A53T mice when compared to vehicle-treated mice. (**D**) Squalamine had no effect on the % change in stool output/body weight between day 5 and 0 in non-Tg or A53T mice compared to vehicle-treated mice. (N = 10 mice/group/dose, 1-way ANOVA). All data represented as mean ± S.E.M, $*P < 0.05$.

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CHAPTER 7. DISCUSSION

7.1 Summary of Findings

This dissertation examined the complex role of peripheral vagal afferent signaling in gut to brain neurotransmission. This was investigated in two parts: i) mechanistically, by examining how vagal responses to intraluminal stimuli are encoded at the level of the intestine (Chapter 3&4) and ii) functionally, by examining changes in vagal neurotransmission in models of aging and disease (Chapter 5&6). Operationally, we measured changes in vagal afferent firing rate using a before and after treatment paradigm and a validated *ex vivo* mesenteric nerve recording protocol with post hoc computer analysis that enables the separation and identification of single unit vagal afferents by CCK response. Given the ever-growing body of evidence on the role of the vagus in mediating mood and affect (O'Reardon *et al.*, 2006; Carreno & Frazer, 2017; Breit *et al.*, 2018), we used the excitatory afferent response to mood-altering, vagus-dependent stimuli to investigate a potential pathway of neurotransmission from the ENS to vagus and to characterize unique encoded responses to antidepressant or pro-depressant stimuli as they travel upstream to the central nervous system. This work is secondary to that of McVey-Neufeld et al. (2020), in which we demonstrated that the behavioural effect of the SSRIs sertraline and fluoxetine was vagal dependent, whereas the effect of the NDRI bupropion was not.

In Chapters 3 and 4, we used the BALB/c mouse strain which is genetically predisposed to anxiety-like behaviour (Crawley, 2008) to optimize behavioural responses to mood-altering stimuli. In Chapter 3, we showed that the vagus-dependent antidepressant sertraline excited a proportion of vagal afferents through a nicotinic intramural sensory synapse between IPANs and vagal IGLEs. Specifically, blockade of Ca^{2+}-dependent synaptic signaling, IPAN firing, or nicotinic acetylcholine receptors all diminished the excitatory response of sertraline on vagal afferents. Furthermore, we showed in Chapter 4 that generation of the antidepressant code was also dependent on the IPAN-to-vagus intramural sensory synapse as the pattern of firing elicited by sertraline was abolished by the same pharmacological blockades. In Chapter 4, we demonstrated that the pattern of firing produced by intraluminal sertraline was unique to several vagus-dependent antidepressants and distinct from the vagus-dependent pro-depressant LPS. This was consistent with the hypothesis that sensory information about the nature of a mood-altering stimulus in the intestinal lumen is encoded in the specific pattern and timing of spike firing transmitted by the vagal afferents it stimulates. We also observed that this antidepressant code was conserved when the antidepressants were orally fed for 14 days and when applied intraluminally in a separate mouse strain that does not demonstrate inherent anxiety- or depressive-like phenotypes.

In the publications comprised in Chapters 5 and 6, we investigated whether the mean firing rate of sensory information trafficked to the CNS by vagal afferents was altered in mouse models of aging and Parkinson's disease and whether this could be surmounted by squalamine. In Chapter 5, we demonstrated that vagal afferent firing rate was diminished in

old (1.5-2 years) compared to young mouse controls (3 months) and that colonic motility in the aged model was characterized by reduced velocity of contractions (MMCs) (West *et al.*, 2019). Intraluminal application of the prokinetic aminosterol squalamine acutely restored vagal afferent firing rate and colonic motility to within range of young mice. Analogously, in Chapter 6, we demonstrated that colonic motility and vagal afferent firing rate, as well as IPAN excitability, were also diminished in the PD model of aging and neurodegenerative disease (West *et al.*, 2020). Both colonic and vagal function were acutely restored by intraluminal squalamine, as was excitability of IPANs in the ENS. This supports that although neuromuscular and neurosensory function is diminished with age and disease, functional capacity remains and demonstrates that it can be at least partially restored or surmounted with intervention. Reduced excitability of IPANs with age and reduced vagal firing rate may in turn contribute to a reduction in vagal sensory information transmitted to the CNS, as investigated via the intramural sensory synapse in Chapters 3 and 4. This is perhaps some of the first evidence of reduced vagal function in aged mice recorded from vagal single units and may play a role in the prevalence of comorbid mood-related disorders in the elderly demographic.

7.2 The function of the ENS is critical for vagal neurotransmission to the CNS

The ENS is composed of complex neural circuits of enteric neurons and enteric ganglia that form networks within the myenteric plexus and submucosal plexus and are capable of producing local and autonomic functions without input from the CNS (Costa *et al.*, 2000). The ENS is sometimes referred to as the second or little brain on account of this capacity for

autonomous function, and an argument can even be made that the ENS is actually the first brain when considering that even animals that lack a CNS, such as hydra, echinoderms, and hemichordates, must have an ENS (Furness & Stebbing, 2018; Annahazi & Schemann, 2020). The densest and most abundant afferent innervation of the ENS comes from the vagus (Powley *et al.*, 2011) and these structural networks of vagal innervation connect the brain and nodose ganglia to the enteric plexuses facilitating bidirectional neural communication with the CNS (Powley, 2000). However, extrinsic denervation of the intestine only decreases mucosal sensory neuropeptide containing fibres by 5%, indicating that the majority of these fibres originate within the gut wall (Keast *et al.*, 1984; Ekblad *et al.*, 1987). The ENS retains function, including intestinal contraction, after extrinsic denervation as demonstrated in the intestinal motility paradigm used to record colonic contractions in Chapters 5 and 6 and *in vivo* following vagotomy. However, the loss of the ENS in diseases and illnesses such as Hirschsprung's disease and intestinal pseudo-obstruction can be fatal (Breit *et al.*, 2018). Therefore, the ENS and vagus are heavily integrated and transmit sensory information about the state of the intestine to the CNS and while the ENS can function in the absence of the vagus, we propose that intestinofugal vagal neurotransmission to the CNS is heavily dependent on ENS sensory function.

In models where ENS integrity and function are compromised, such as in the aging and PD models examined in Chapters 5 and 6 of this thesis, the ability to traffic sensory afferent information from the intestinal epithelium to the CNS may be impaired. While we do not currently have the technical ability to perform electrophysiological recordings of the ENS in

series with vagal afferent activity in a combined tissue preparation, this information would better address the extent to which pharmacological blockade or damage to the ENS modulates vagal afferent activity. Presently, the pharmacological blockades employed in Chapter 3 and 4 and in previous studies (Perez-Burgos *et al.*, 2014) to block IPAN to vagus transmission through the nicotinic intramural synapse provide insight into this mechanism. It is apparent in the results of these chapters that IPAN firing and Ca^{2+}-mediated synaptic signaling was critical to vagal afferent excitation following intraluminal sertraline application. Furthermore, generation of the antidepressant code in response to sertraline was IPAN-dependent, which suggests that ENS to vagus synaptic transmission plays a role in encoding information about the stimulus in the vagal afferent signal to the brain. Therefore, loss of IPAN excitability that may occur with aging and that was observed in the PD model (Chapters 5 and 6) would also reduce activation of vagal afferents through the intramural sensory synapse (Perez-Burgos *et al.*, 2014). Reduction in overall vagal tone in these models may be related to the increased incidence of depression and psychiatric comorbidities in older populations (Malatynska *et al.*, 2012). This may also impair perception and ability to encode the appropriate response to both beneficial and pathogenic stimuli in these models.

7.3 Implications for temporal pattern coding in the vagus

The processing and integration of countless inputs of sensory information is integral to the initiation of an appropriate behavioural response and the overall survival of an organism. The challenge in understanding and deciphering how information is encoded in sensory systems lies in understanding two concepts; i) the relationship between the stimulus and the

neural activity it evokes and ii) the relationship between the neural activity and perception of the stimulus (Johnson, 2000). At its core, sensory processing is an exchange of information between neural circuits, whereby electrical information in the form of action potential spikes that form trains, termed a neural code, is propagated between neurons and their targets, including other neurons (Gerstner *et al.*, 1997). Functionally, changes in neural activity defined by the neural code represent changes in information from the senses used to modify behaviour (Cariani, 2004).

The best way to approach deciphering how a change in behaviour across the gut-brain axis is influenced by a peripheral stimulus is through reverse engineering: beginning with the primary afferent response to the stimulus, which in our model, begins at the level of the gut. In Chapter 4 of this thesis, we use the documented vagal response to mood-altering stimuli (Konsman *et al.*, 2000; Bravo *et al.*, 2011; Perez-Burgos *et al.*, 2013; McVey Neufeld *et al.*, 2019) to dissect the pattern and timing of vagal afferent action potentials leaving the gut through the mesenteric nerve bundle. While some vagal afferents have been identified in the literature as being selective for certain micro and macromolecules (Mei, 1978; Jeanningros, 1982; Lal *et al.*, 2001), the majority of vagal afferents are thought to be polymodal (Berthoud & Neuhuber, 2000; Powley *et al.*, 2011). This is important because it allows for a high degree of redundancy, whereby fibres respond to many kinds of stimuli and instead encode information in the pattern of firing. Indeed, in Chapter 4 single vagal afferents responded to both a pro-depressant stimulus and antidepressant stimuli, and both caused increases in mesenteric vagal afferent firing frequency, despite having opposing central effects on

behaviour in the literature (Konsman *et al.*, 2000; Bravo *et al.*, 2011; McVey Neufeld *et al.*, 2019). This is a critical finding, as it suggests that information regarding the nature of the stimulus is neither dependent on the type of vagal afferent activated nor the change in mean firing rate, otherwise known as a rate code (Gerstner *et al.*, 1997). Instead, we discovered the existence of a specific temporal pattern code for antidepressant stimuli that differs from the temporal firing elicited by a pro-depressant stimulus.

Few efforts have been made to decipher patterns of coding in the vagus, however one study suggests that afferent signals from the cervical vagus encode cytokine-specific information that discriminate between cytokine-specific signals (Zanos *et al.*, 2018). We believe this study is the first to quantify a conserved temporal pattern code from vagal afferents arising from the GI tract that is specific to antidepressant stimuli. Furthermore, we showed that this antidepressant pattern code was conserved across two mouse species and replicated in mice that were fed antidepressants for two weeks. Given our findings, we propose that playback of this antidepressant code by electrically stimulating the vagus nerve may also produce antidepressant effects, as has been achieved with neural codes for pleasant and repellent tastes and their associated behavioural responses in rats (Di Lorenzo & Hecht, 1993). Such specificity in programming of vagus nerve stimulation devices may be more efficacious in eliciting an antidepressant behavioural response than current parameters.

Lastly, the identification of a vagal antidepressant temporal code from mesenteric fibres leaving the gut raises more important questions and possibilities. While we have

characterized the temporal code of firing from intestinal vagal afferents, it is unclear whether this temporal code is conserved upstream. One possibility is that the temporal pattern code may convey information about the stimulus that is integrated at the brain, leading to region-specific changes in brain activity that correspond to a change in behaviour. Acute administration of live JB-1 induced c-Fos expression in several brain regions also activated by VNS and which was abolished in vagotomized mice (Bharwani *et al.*, 2020), which indicates that antidepressant stimuli and electrical stimulation have shared targets in the brain. Furthermore, it has been suggested that different pathogenic stimuli may produce differential patterns of activation in the brain as observed in *Staphylococcal* enterotoxin B (SEB) and LPS-induced c-Fos expression in the rat brain (Goehler *et al.*, 2000). It is possible that when the temporal code reaches the brain, the pattern is changed post-synaptically or the code signals a coordinated release of specific neurotransmitters. Even if this were the case, subdiaphragmatic electrical stimulation with our vagal antidepressant code parameters should mimic the intraluminal antidepressant agents. Future experiments should determine whether the antidepressant code is conserved at the level of the NTS and identify what regions of the brain are affected.

7.4 Age and PD-associated decline in gastrointestinal neuromuscular function is not insurmountable

Aging is associated with the structural and functional decline of the body's tissues, and thus efforts to halt or even reverse this process are central to longevity. Research on aging has become more popular over time in an effort to increase the average human lifespan and as a

result of a greater strain on our health system due to a larger elderly population (Weinert & Timiras, 2003). Given that the probability of developing chronic and debilitating illnesses increases with age, of which many (Parkinson's disease, stroke, etc.) have accompanying GI complications, it is also important that research into longevity also considers the aging population's quality of life (Orr & Chen, 2002). GI disorders are prevalent in the elderly, often as a result of impaired GI function related to changes in intestinal motility and neural control (Hall, 2002; Orr & Chen, 2002; Saffrey, 2014). We showed in Chapters 5 and 6 that the velocity of colonic motor contractions was decreased in old mice (18-24 months) and in aged PD model mice. We also showed that the vagal afferent firing rate was reduced in both models of aging. Furthermore, in Chapter 6, we showed that excitability of IPANs in the myenteric plexus was reduced. Although we did not measure IPAN activity in the old mice experiments in Chapter 5, we hypothesize that IPAN excitability would be similarly decreased in this model. Indeed, excitability of hippocampal CA1 pyramidal neurons is reduced due to increased and prolonged Ca^{2+}-dependent, K^{+}-mediated afterhyperpolarization (IK_{Ca}) in aged rats, which may be a result of increased intracellular Ca^{2+} with age (Landfield & Pitler, 1984; Kumar & Foster, 2007). The prolonged post action potential slow afterhyperpolarization that is characteristic of IPANs is also governed by IK_{Ca} (Kunze *et al.*, 2000; Nguyen *et al.*, 2007) and thus, excitability may be similarly affected by aging in myenteric neurons.

Reducing excitability by prolonging the afterhyperpolarization and increasing the length of the refractory period can prevent a subsequent action potential from firing and thus silence

the IPAN for a time (Nguyen *et al.*, 2007). We showed in Chapters 3 and 4 that silencing of IPANs using the IK_{Ca} channel opener DCEBIO to prolong the afterhyperpolarization reduced the excitatory vagal afferent response to sertraline and abolished the antidepressant pattern code. By silencing IPANs, there was less vagal activation through the intramural sensory synapse. Therefore, we hypothesize that reduced IPAN excitability with age and in PD would reduce vagal afferent firing rate as was observed in these models in Chapters 5 and 6. We also propose that temporal coding in vagal afferents would be affected by reduced IPAN excitability in these aged models as we showed that the antidepressant code was dependent on IPAN firing in Chapter 4. There is also evidence that there is a reduction in acetylcholine-release from the myenteric plexus and in the contractile response of smooth muscle in the rat associated with age (Roberts *et al.*, 1994; Bitar & Patil, 2004). Furthermore, neuronal loss in the myenteric plexus with age occurs in cholinergic neurons, but not nitrergic neurons (Phillips *et al.*, 2003). Given that IPANs are cholinergic (Li & Furness, 1998) and that the intramural sensory synapse appears to be nicotinic in nature according to previous work (Perez-Burgos *et al.*, 2014) and our results using mecamylamine in Chapters 3 and 4, it is also possible that reduced cholinergic neurotransmission in the gut with age could contribute to reductions in motor function and ENS to vagal synaptic transmission.

One of the most striking results in Chapters 5 and 6 was that the reduced colonic motility and vagal afferent firing in these aged models was acutely increased by intraluminal squalamine. Squalamine is a cationic amphipathic aminosterol first isolated from the dogfish shark and has both antibacterial and antiviral properties (Moore *et al.*, 1993; Zasloff *et al.*,

2011). In Chapter 6, squalamine increased IPAN excitability in the PD model mouse, which would have a prokinetic effect on motility as intestinal motility is dependent on IPAN function (Howe *et al.*, 2006; Wang *et al.*, 2010). Correspondingly, we propose that vagal afferent firing would also increase through the intramural sensory synapse. Squalamine has been demonstrated to inhibit formation of intracellular α-synuclein aggregates that cause disordered motility and paralysis in a *C. elegans* model of PD (Perni *et al.*, 2017). While it might be possible that squalamine could displace α-syn aggregates to increase IPAN excitability and improve colonic motility and vagal afferent firing rate, this would have to be tested in future experiments. The results in Chapters 5 and 6 reveal that reductions in GI motility, vagal afferent firing and ENS function with age and neurodegenerative disease may not be permanent. Despite changes in morphological structure in vagal afferents (Phillips *et al.*, 2010) and myenteric neurons (Saffrey, 2013), the gut and its signaling pathways maintain a remarkable capacity to function, and we show here that losses in function can be restored briefly. It is important to note however, that these experiments only demonstrate an acute prokinetic response to squalamine and cannot be used to conclude that damage that may have occurred with physiological or pathophysiological aging has been permanently restored. We do show that these losses in function are not insurmountable, and future studies should seek to evaluate the effect of chronic squalamine administration on vagal function in models of aging and PD.

7.5 Conclusions

The role of the vagus in the gut-brain axis has justifiably received a lot attention in recent years and the body of research in the literature continues to grow. The results in this thesis

contribute to the literature by presenting several novel findings regarding signaling mechanisms of the intestinal afferent vagus and functional changes that occur with age and PD. We have followed up the work by McVey-Neufeld et al. (2019) showing that the antidepressant effect of SSRIs was vagus dependent (McVey Neufeld *et al.*, 2019) by showing in Chapter 3 that intraluminal sertraline activated a population of vagal afferent fibres through the intramural sensory synapse. In Chapter 4, we presented novel research showing that information from a stimulus that causes antidepressant behavioural effects was encoded by vagal afferent fibres in a specific temporal antidepressant pattern code. This has several implications, whereby this antidepressant pattern code could potentially be used to screen for other possible antidepressant agents that share the same pattern of firing. Additionally, other temporal pattern codes may exist for other types of intraluminal stimuli that stimulate vagal afferents, including a pro-depressant code. In Chapter 5, we show for the first time, to our knowledge, that vagal afferent firing rate was reduced in old mice. We also demonstrate that vagal afferent firing rate was reduced in an aged PD model mouse in Chapter 6, but more importantly, that vagal afferent firing rate can be restored again in both models. Future work should continue to investigate the extent of IPAN-to-vagus crosstalk in the gut and how temporal coding in the vagus mediates nervous communication from the gut to the brain to modulate behaviour and influence overall health. The widespread functions of the vagus along the gut-brain axis and its interactions with other signaling pathways makes it clear that “what happens in vagus” (Fulling *et al.*, 2019), does not stay in vagus.

CHAPTER 8. REFERENCES FOR INTRODUCTION AND DISCUSSION

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