ANTIBIOTICS AND THE GUT-BRAIN AXIS

ACUTE EFFECTS OF ANTIBIOTICS ON GUT MOTILITY AND GUT-BRAIN NEURONAL SIGNALLING

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TITLE: Acute Effects of Antibiotics on Gut Motility and Gut-Brain Neuronal Signalling

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Lay Abstract

Little is known about the mechanisms by which high-dose antibiotics produce changes in gut-brain signalling to negatively affect brain functions and behaviour. Although the general consensus is that these changes are caused by antibiotic-mediated perturbations of the gut microbiota, whether high-dose antibiotics also act directly on the nervous system remains a topic of debate. I have hypothesized that high-dose antibiotics, as used in experimental models associating pathophysiological outcomes to gut microbial changes, also produce adverse effects by direct modulation of enteric neuronal circuits. Indeed, our findings suggest that high-dose antibiotics directly signal to enteric neurons, which locally regulate gut motility reflexes and can transmit that information further to vagal neurons, to influence homeostatic regulation of brain functions and behaviour. This work offers novel therapeutic potential for antibiotics and advises careful interpretation of studies that have attributed effects of high-dose antibiotics solely to alterations in the gut microbiome.

Abstract

Associations between the use of antibiotics and altered brain function and mental illness are now well evidenced from animal models and clinical trials. Based on these findings, emerging research efforts have largely focused on how high-dose antibiotic-mediated perturbations of the gut microbiota result in altered neurophysiological and behavioural outcomes. However, these studies have not investigated whether antibiotics also act directly on the host nervous system. My central hypothesis is that high-dose antibiotics, as used in experimental models testing the modulatory role of the gut microbiome, can induce pathophysiological outcomes by direct interaction with enteric neuronal circuits.

I designed two sets of experiments to characterize the acute effect of high-dose antibiotics on gut motility and gut-brain neuronal signalling. The first experimental study aimed to determine whether acute exposure of the gastrointestinal tract to high-dose antibiotics directly modulates enteric neurons, with consequences for gut motility. To test this, I used enteric nervous system dependent motility reflexes, *ex vivo*, as an index of putative effects on the intestinal nervous system. The results of these experiments have shown that luminal antibiotics alter oral to anal propulsive peristalsis in a system where such motility is dependent on the enteric nervous system. The second study aimed to test whether these local effects modulate brain function and behaviour by altering responses of vagal afferent pathways. I performed single-unit recordings from the mesenteric nerve bundle in *ex vivo* preparations to test this research question. The results suggest that antibiotics can increase activity of extrinsic vagal afferent neurons largely through cholinergic synapses with myenteric IPANs.

The present work offers significant therapeutic implications, although its main relevance is in the interpretation of the experimental use of high-dose antibiotics on animal models and where effects on behaviour and the nervous system are attributed solely to alterations in the microbiome.

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First, I would like to thank my supervisor, Dr Wolfgang Kunze, for his continued guidance and support throughout this research project. From the very beginning, Dr Kunze has made every effort to understand and appreciate my goals and interests and to help me achieve greater motivation, self-confidence, and independence. Dr Kunze was available to discuss problems, to provide knowledge and advice, whenever needed. Dr Kunze has also provided me with several opportunities to attend and present my work at national and international conferences, allowing me to make invaluable connections with well-established researchers in the field and to strengthen my communication skills. For these reasons, Dr Kunze has not only drawn me to the field of gastroenterology but has also taught me to think critically, communicate my ideas clearly and be open-minded to diverse viewpoints.

Secondly, I would like to thank my committee members Dr Paul Forsythe and Dr Luke Janssen for their support and feedback throughout the progress of my project. The questions and suggestions they have shared have greatly encouraged me to expand my knowledge and to attempt novel approaches in interpreting my data. Dr Forsythe has played an enormous role in advising me on my experimental design. He has always been present at lab meetings to share his expertise and offer any suggestions on what next steps I can take to achieve my goals. Dr Janssen has also been an excellent mentor. He has dedicated a lot of time to carefully review my work, to discuss new ideas and approaches, and to ask challenging questions – all of which have definitely made my research stronger.

I would also like to thank my fellow lab members for without them I would not have been able to complete my experiments in a timely manner and would not have enjoyed working in the lab as much as I have. I must especially thank Christine West for teaching me how to perform the gut motility and mesenteric nerve recordings. Christine has always been very patient and willing to assist. She has helped me to overcome many

difficulties I have encountered during my lab work. Jessica Amin has also been a tremendous support. She has helped me perform several gut motility experiments and having managed to always bring positive energy to the lab, she has always made me feel more motivated. I would also like to thank our lab technician, Dr Yu-Kang Mao, for helping me to understand and troubleshoot any issues that would arise.

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List of All Abbreviations and Symbols

The following abbreviations were used throughout the thesis, in addition to those conventionally defined for units of measurement.

ANOVA	Analysis of variance
β-lactam	Beta-lactam
BDNF	Brain-derived neurotrophic factor
Ca^{2+}	Calcium ion
CCK	Cholecystokinin
$CdCl_2$	Cadmium chloride
CNS	Central nervous system
Dmap	Spatiotemporal diameter map
EC	Enterochromaffin
EPAN	Extrinsic primary afferent neuron
ENS	Enteric nervous system
GABA	Gamma-aminobutyric acid
GLP-1	Glucagon-like peptide-1
HPA	Hypothalamic pituitary adrenal
IBD	Inflammatory bowel disease
IL-1β	Interleukin-1-beta
ICC	Interstitial cells of Cajal
IGLE	Intraganglionic laminar ending
IPAN	Intrinsic primary afferent neuron
IBS	Irritable bowel syndrome
NK-1	Neurokinin-1
PCC	Propagating contractile cluster
SW	Slow wave
TTX	Tetrodotoxin
5-HT	5-hydroxytryptamine

Declaration of Academic Achievement

Dr Wolfgang Kunze and I designed all the experiments with input from Dr Paul Forsythe and Dr Andrew Stanisz. I performed and analyzed all the data with assistance from fellow lab members, Jessica Amin and Christine West. Jessica Amin assisted in the collection of data pertaining to the organ bath motility experiments conducted from September 2016 to May 2017. This data has been written as a manuscript and published in the journal Frontiers in Neuroscience in 2017. I am the primary author. I have adapted much of the figures and discussion presented in this publication in the following chapters of this thesis: Chapter 1 (subsection 2.3: Antibiotics and the Gut Microbiome, subsection 3.1: Antibiotic Mediated Changes in Gastrointestinal Motility: Mode of Action?), Chapter 2 (section 2: Ex Vivo Gut Motility Recordings, section 4: Luminal Stimuli), Chapter 3 (section 1: Impact of Antibiotics on *Ex Vivo* Measures of Gut Motility and associated figures), Chapter 4 (section 1: Direct Effect of Antibiotics on Propagating Contractile Clusters). Finally, Christine West helped with performing the mesenteric nerve recordings conducted from September 2017 to February 2018.

Chapter 1: General Introduction and Literature Review

1. The Rise and Fall of Antibiotics

Antibiotics are routinely prescribed to treat or prevent bacterial infections ("Antibiotics", 2016; "Be Antibiotics Aware", 2017). While the host immune system normally detects and defends against infectious bacteria, in some cases, activated immune responses may be insufficient to effectively manage the spread of the bacteria. In these situations, antibiotics can be used to quickly eliminate the bacteria or inhibit their growth.

Antibiotics can be administered through several different methods to optimize clinical efficacy. The safest and simplest route of administration is achieved by oral tablets or capsules (Cyriac & James, 2014). These formulations are effective in the treatment or prevention of mild to moderate bacterial infections within the body (Erbil et al., 2014; Legnani, 1997; O'Toole et al., 2014). Topical antibiotics in the form of creams, sprays or drops are more effective in treating skin infections (Eady & Cove, 1990). Lastly, for severe bacterial infections, antibiotics can be administered either intravenously (Shiu, Wang, Tejani, & Wasdell, 2013) or intraperitoneally (Morse, Apicella, & Walshe, 1988) directly into blood or muscle circulation.

1.1. Types of Antibiotics

The efficacy of antibiotics further depends on their pharmacokinetics and spectrum of antimicrobial activity. Based on structural and biological characteristics, antibiotics can be broadly classified into the following five groups: beta-lactams, aminoglycosides, tetracyclines, macrolides and fluoroquinolones. A brief summary of these classes of antibiotics is provided here.

The class of beta-lactam antibiotics includes compounds such as penicillins and cephalosporins (Holten & Onusko, 2000). The basic molecular structure of penicillins consists of a thiazolidine ring, a side chain, and most distinguishably the four-membered

beta-lactam ring (Bentley, 2004). This latter moiety enables binding of beta-lactam antibiotics to the active site of the DD-transpeptidase enzyme to prevent the formation of bacterial peptidoglycan cross-links (Ghuysen et al., 1996; Holten & Onusko, 2000; Sykes, Nagle, & White, 2014; Yocum, Waxman, Rasmussen, & Strominger, 1979). Since regulated hydrolysis of these cross-links is maintained, the rate of cell wall breakdown exceeds its synthesis (Sykes et al., 2014). As a result, the integrity of cell walls is weakened, and cellular lysis ensues. In this manner, penicillins defend against many gram-positive and gram-negative bacterial infections of the respiratory tract and soft tissues (Holten & Onusko, 2000; Sykes et al., 2014). The antimicrobial action of benzylpenicillin (Penicillin G) was first identified from the mould *Penicillinum notatum*, by Alexander Fleming in 1928 (Holten & Onusko, 2000). Further purification and mass production quickly led to its widespread use in the 1940s. Benzylpenicillin and most other penicillins are taken orally and can reach peak serum levels at least 60 minutes after administration (Holten & Onusko, 2000; McDermott & Bunn, 1946). About one-fifth of a single oral dose is absorbed, primarily from the upper regions of the gastrointestinal tract with quantities absorbed diminishing significantly towards the large intestine (McDermott & Bunn, 1946). Once absorbed, penicillins are largely excreted as intact molecules through the kidney. Metabolism by the liver is negligible. However, there are now many isolated natural penicillins differing in their side chains, which has led to variations in bioavailability, antimicrobial spectrum, stability and tolerance. For instance, penicillin V, the potassium salt of benzylpenicillin, has been shown to be better absorbed orally, more acid-stable, and less active against gram-negative bacteria (Bunn, Caldwell, Adair, Lepper, & Dowling, 1950; Heatley, 1956; McDermott & Bunn, 1946; Wright, Kirshbaum, Arret, Putnam, & Welch, 1955). Penicillin V is frequently prescribed for the treatment of group A streptococci induced pharyngitis (Portier et al., 2002; Schwartz, Marcy, Phillips, Gerber, & Dowell, 1998). Cephalosporin antibiotics share structural composition and mode of action with penicillins but differ slightly in the spatial arrangement of atoms connected to the beta lactam ring (Abraham & Newton, 1967). In comparison to penicillins, cephalosporin antibiotics therefore encompass a broader

antibacterial spectrum, less susceptibility to beta-lactamases and evoke fewer hypersensitivity reactions (Holten & Onusko, 2000; Neu, 1980; Rodman, McKnight, & Anderson, 1994; Stohlmeyer & Kraus, 1996). As a result, cephalosporins are now a favoured antibiotic in the treatment of pulmonary, urinary, surgical, bone or orthopaedic infections caused by *Streptococcal, Pneumococcal, Staphylococcal, Klebsiella*, *Escherichia coli* and *Proteus* bacteria (Neu, 1980).

In contrast, aminoglycoside antibiotics such as gentamicin and neomycin, are mainly used in the treatment of very severe bacterial infections caused by gram-negative and to a lesser degree gram-positive bacteria (e.g. septicaemia) (Wright, Berghuis, & Mobashery, 1998; "Antibiotics", 2016). Doses are generally administered intravenously or intramuscularly due to poor bioavailability after oral ingestion (Edson & Terrell, 1991). The core molecular structure consists of several aminated sugars attached through glycosidic linkages to a dibasic cyclitol (Mingeot-Leclercq & Tulkens, 1999; Wright et al., 1998). Following energy-dependent aminoglycoside transport across the bacterial membranes (Vaara, 1992), the dibasic cycitol allows binding of aminoglycosides to the 30S subunit of ribosomes in the cytosol (Bryan & Kwan, 1983; Mingeot-Leclercq & Tulkens, 1999). The result is inaccurate mRNA translational proofreading and consequently inhibition of bacterial protein synthesis (Melancon, Tapprich, & Brakier-Gingras, 1992)

Tetracycline antibiotics, including compounds like doxycycline and minocycline, are broad-spectrum antibiotics that exhibit antibacterial activity against a wide range of infections (Chopra & Roberts, 2001). They are identified chiefly by their linearly fused tetracyclic rings. Tetracyclines differ in the functional groups attached to the tetracyclic nucleus, however, 6-deoxy-6-demethyl tetracycline has been shown to be the minimum molecular structure needed for antibacterial function (Chopra & Roberts, 2001). Similar to aminoglycosides, tetracyclines eliminate infectious bacteria by inhibiting protein synthesis (Schnappinger & Hillen, 1996). They are transported across gram-positive and

gram-negative bacterial membranes through porin channels and energy-dependent mechanisms (Nikaido & Thanassi, 1993; Schnappinger & Hillen, 1996). In the cytosol, binding of tetracyclines to the 30S ribosomal subunit prevents the attachment of aminoacyl-tRNA to the acceptor site on the ribosome (Brodersen et al., 2000; Geigenmüller & Nierhaus, 1986; Maxwell, 1967). Moreover, tetracyclines are well absorbed from the stomach and small intestine following oral administration (Chopra & Roberts, 2001). However, absorption is largely limited by the presence of and type of food in the gastrointestinal tract. For instance, in the presence of calcium, tetracyclines can form nonabsorbable chelates.

Macrolides are another widely used group of antibiotics. The core structure consists of a 14-, 15- or 16-membered lactone ring to which sugar moieties and other functional groups are attached (Gaynor & Mankin, 2003; Mazzei, Mini, Novelli, & Periti, 1993). The first macrolide to be identified was erythromycin from the strain *Saccharopolyspora erythraea*, in 1949 (Bradley & Sauberan, 2012). Like all other macrolides, erythromycin is a protein synthesis inhibitor of gram-positive (e.g., *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes*) and limited gram-negative bacteria (Gaynor & Mankin, 2003). They are thought to do this by binding to the large 50S ribosomal subunit and preventing function of peptidyl transferase in the catalysis of peptide bond formation between the growing peptide chain and the nascent amino acid (Gaynor & Mankin, 2003; Mao & Robishaw, 1972). However, this natural compound had high acidic instability and poor oral bioavailability, requiring multiple doses daily to maintain therapeutic serum levels (Gaynor & Mankin, 2003; Kaneko, Dougherty, & Magee, 2007; Mazzei et al., 1993). Moreover, the antibiotic was subject to metabolism in the liver which resulted in metabolites with significantly reduced antibacterial properties (Kaneko et al., 2007). Elimination of erythromycin and these metabolic by-products largely involved secretion into bile. Acknowledgement of such problems underlying the clinical use of erythromycin, prompted the development of second-generation derivatives (e.g. clarithromycin and azithromycin) with greatly improved pharmacokinetic properties

(Kaneko et al., 2007; Mazzei et al., 1993). These new compounds are comparably effective when administered through the oral, topical and intravenous routes (Kaneko et al., 2007). The antimicrobial spectrum of second-generation macrolides has also been enhanced and is now greater than that of penicillins, making these macrolides the drug of choice for patients with a penicillin-related allergies (Farrington, 2012).

Fluoroquinolones are another class of broad-spectrum antibiotics frequently used to treat a wide range of respiratory and urinary tract infections from gram-positive (e.g., *Staphylococci, Streptococcus pneumoniae, Enterococcus faecalis*) and gram-negative (e.g., *Neisseria meningitides, Haemophilus influenzae, Pseudomonas aeruginosa*) bacterial species ("Fluoroquinolones", 2014). Their core structure is comparable to the compound 4-quinolone with the addition of a fluorine atom and a carboxylate substituent (Wolfson & Hooper, 1985). Due to their small molecular size, fluoroquinolone antibiotics largely utilize porins to pass through bacterial membranes. Subsequently, they inhibit DNA synthesis by antagonizing bacterial DNA gyrases (Hawkey, 2003; Wolfson & Hooper, 1985). These compounds are usually administered orally as they have shown high absorption and tolerance, and low rates of adverse side effects through this route ("Fluoroquinolones", 2014; Hooper & Wolfson, 1985).

1.2. Overuse of Antibiotics Can Negatively Impact Brain Function and Behaviour

When antibiotics were first developed the observable side effects were minor, leading to their mass production and widespread use in the treatment of bacterial infections acquired by World War II soldiers and patients in hospital and outpatient settings (Fielding & Tyrer, 1980). There is now accumulating data that demonstrates how overuse of antibiotics may not only foster resistant strains of bacteria (FAO/WHO, 2001; Légaré et al., 2012), but also shifts the gut microbial community to favour development of chronic health conditions such as obesity, type 1 diabetes and allergies (Baldo, Zhao, & Pham, 2008; Blaser & Falkow, 2009; Cho et al., 2012; Davey, 1988; Droste et al., 2000;

Hoban, 2003; Iwata & Akita, 1996; Livanos et al., 2016). More recently, detrimental effects of antibiotic use on brain neurochemistry and behaviour have been reported (for review see Forsythe, Kunze, & Bienenstock, 2016). For instance, macrolide antibiotics have been correlated with cases of mania, agitation and delirium (Bichler et al. 2016). Similarly, β-lactam antibiotics, particularly broad-spectrum penicillins, have been recognized as a likely trigger of convulsions (Bichler et al., 2017; Silber & D'Angelo, 1985; Tsuda et al., 1994; Wallace, 1997) and coagulopathy (Fass et al., 2018). Further, administration of fluoroquinolones has resulted in onset of nausea, dyspepsia and vomiting, and CNS effects such as dizziness, insomnia and headache (Davey, 1988; Norrby, 1991). Despite this and the development of evidence-based guidelines, overuse and misuse of antibiotics continues to be a problem (Louie & Bell, 2002). In the year 2010, it was found that 30% (47 million) of antibiotic prescriptions provided in the United States were unjustified (Fleming-Dutra et al., 2016). In fact, a large percentage of these prescriptions were written for viral infections like common colds, viral sore throats and bronchitis – illnesses that antibiotic use cannot improve. These patients are thus placed at unnecessary risk of developing severe systemic pathologies.

2. Antibiotics and the Gut-Brain Axis

The gut-brain axis consists of hard-wired bi-directional connections between the microbiome, ENS, CNS, autonomic nervous system and the HPA axis (Carabotti, Scirocco, Maselli, & Severi, 2015; Forsythe, Kunze, & Bienenstock, 2016). Essentially, luminal signals are transmitted through enteric, vagal and spinal neuronal circuitry to the CNS. From the CNS, the sympathetic and parasympathetic divisions of the autonomic nervous system and the HPA axis can be activated to then transmit the appropriate efferent signals to regulate internal body processes (Tsigos & Chrousos, 2002). Thus, the gut-brain axis is a highly integrated network of neurons, hormones and humoral components that work in concert to not only maintain gastrointestinal homeostasis but can also influence emotional well-being, behaviour and cognition (Carabotti et al., 2015; Forsythe et al., 2016). The simultaneous presence of psychiatric symptoms in patients

with IBD or IBS demonstrates how abnormal functioning of one limb of the gut-brain axis can impact others (Bienenstock, Kunze, & Forsythe, 2016; Cryan & O'Mahony, 2011). Moreover, ever since oral antibiotics were shown to manage hepatic encephalopathy (and its spectrum of neuropsychiatric abnormalities) through modulation of the gastrointestinal microbiota (Ahluwalia et al., 2014; Bajaj et al., 2013; Bass, 2006; Morgan, 1991), there has been interest in establishing a mechanistic basis for the effects of antibiotics on gut-brain signalling.

2.1. Functional Significance of the Gut Microbiome

The human gastrointestinal tract encompasses trillions of commensal bacteria (Carabotti et al., 2015). Although there is inter-individual variation, the composition and distribution of prominent bacterial species remains fairly similar amongst healthy individuals. For instance, about 75% of the human bacterial community is comprised of bacteria from the phyla *Firmicutes* and *Bacteroides* (Eckburg et al., 2005). It is therefore reasonable to infer that a stable gut microbiome – maintenance of bacterial community structure – holds importance for normal metabolic and physiological functioning (Carabotti et al., 2015). Indeed, several studies have shown that commensals are necessary for normal development of the ENS and CNS (Barbara et al., 2005; Bienenstock et al., 2016). Germ-free animals devoid of gut microorganisms have shown changes to concentrations of enteric and central neurotransmitters (Barbara et al., 2005; Clarke et al., 2013; Heijtz et al., 2011), enteric neuron density and function (Collins, Borojevic, Verdu, Huizinga, & Ratcliffe, 2014; Neufeld, Mao, Bienenstock, Foster, & Kunze, 2013), microglial morphology and development (Erny et al., 2015), hippocampal neurogenesis (Ogbonnaya et al., 2015) and blood–brain barrier permeability (Braniste et al., 2014). In adult germ-free animals, intestinal motility and transit time changes have been observed (Abrams & Bishop, 1967; Caenepeel, Janssens, Vantrappen, Eyssen, & Coremans, 1989; Husebye, Hellström, Sundler, Chen, & Midtvedt, 2001). Furthermore, germ-free animals have been characterized with displaying an anxiolytic behavioural phenotype (Clarke et al., 2013; Heijtz et al., 2011; Neufeld, Kang, Bienenstock, & Foster,

2011; Neufeld, Kang, Bienenstock, & Foster, 2011; Nishino et al., 2013), with exaggerated HPA axis stress responses (Neufeld et al., 2011; Sudo et al., 2004). Microbial colonization of these germ-free animals was needed to restore homeostatic set-points (Collins et al., 2014; Neufeld et al., 2013; Sudo et al., 2004).

The gut microbiome also plays a role in regulating the production of several metabolites such as the short-chain fatty acids and neurotransmitters like 5-HT, which can then influence intestinal metabolism (De Vadder et al., 2014), immunity (Smith et al., 2013), brain function and behaviour (De Vadder et al., 2014; Louis & Flint, 2009; Schroeder, Lin, Crusio, & Akbarian, 2007). Many research efforts have focused for instance on the bioactivity of the short chain fatty acid butyrate, on colonic mucosal functions (for review see Hamer et al., 2008). Butyrate has been shown to regulate size and function of colonic regulatory T cells (Smith et al., 2013), inhibit colonic intrinsic sensory neurons (Kunze et al., 2009), activate intestinal gluconeogenesis (De Vadder et al., 2014), and promote anti-depressant effects through the inhibition of histone deacetylase (Schroeder et al., 2007). In addition, commensals like the *Clostridium* species can regulate EC biosynthesis of 5-HT, and thus influence host serotonergic pathways (Yano et al., 2015). This has important implications for regulation of gastrointestinal motility and secretion, and systemic platelet function.

2.2. Probiotics and the Gut Microbiome

The Food and Agricultural Organization of the United Nations and the World Health Organization have defined probiotics as "living microorganisms, which when administered in adequate amounts confer health benefits on the host" (FAO/WHO, 2001). In general, probiotics and their metabolites are theorized to restore composition and function of the microbial community by competing with other intestinal microorganisms (Hemarajata & Versalovic, 2013). It was Elie Metchnikoff who first introduced the idea of 'useful microbes' by demonstrating how consumption of fermented milk products with *Lactobacilli* improved health and longevity (Metchnikoff, 1907). Metchnikoff

speculated that ingestion of probiotics offset the effects of toxins produced from intestinal cell putrefaction. Since then, the beneficial effects of probiotics have been widely explored.

Bacteria from the genera *Lactobacillus* and *Bifidobacterium* are the most common strains found in probiotic formulations (Rolfe, 2000). These groups are favoured in the treatment of gastrointestinal disorders such as IBD or IBS, where colonized levels of *Lactobacillus* and *Bifidobacteria* were greatly reduced (Madden & Hunter, 2002; Swidsinski et al., 2002). Once ingested, they are able to survive the acidic gastric environment, colonize the intestine, and inhibit the growth of pathogenic bacteria (e.g., *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens*, *Clostridium difficile*) (Hemarajata & Versalovic, 2013; McFarland et al., 1994; Meurman, Antila, Korhonen, & Salminen, 1995; Silva, Jacobus, Deneke, & Gorbach, 1987).

Strain specific benefits on gut epithelial, immune and enteric function have also been discussed. Firstly, several probiotic strains such as *Lactobacillus plantarum* (Anderson, Cookson, McNabb, Kelly, & Roy, 2010; Karczewski et al., 2010; Qin, Zhang, Hang, & Jiang, 2009), *Lactobacillus rhamnosus R0011* and *Lactobacillus helveticus R0052* (Zareie et al., 2006), *Escherichia coli Nissle 1917* (Ukena et al., 2007), *Bifidobacterium longum SP 07/3* and *Lactobacillus rhamnosus GG* (Ghadimi, De Vrese, Heller, & Schrezenmeir, 2010), have been shown to improve tight-junction function. These protein structures are a key innate defense barrier as they selectively regulate the flux of fluid, macromolecules and leukocytes between the intestinal lumen and the lamina propria (Hemarajata & Versalovic, 2013; Ulluwishewa et al., 2011). Moreover, most *Lactobacillus* and *Bifidobacteria* can modulate immune signalling pathways (e.g., NFκB, MAPKs, PI3K/Akt) to some degree in order to maintain immune homeostasis (Thomas & Versalovic, 2010). Alterations to these signalling pathways have resulted in increased epithelial mucin production (Mack, Ahrne, Hyde, Wei, & Hollingsworth, 2003; Mack,

Michail, Wei, McDougall, & Hollingsworth, 1999), macrophage mediated reduction of pro-inflammatory cytokine secretion (Fitzpatrick et al., 2008), dendritic cell maturation, survival and increased anti-inflammatory IL-10 production (Hoarau et al., 2008), and up-regulation of regulatory T cells (Kwon et al., 2010). Finally, strains of *Lactobacilli* including *L. rhamnosus JB-1* and *L. reuteri DSM 17938* have shown strain- and region-specific effects on neuronally dependent parameters of intestinal motility (Mao et al., 2013; Wang et al., 2010; West et al., 2016).

If the host microbiome plays a role in gut-brain signalling, then luminal exposure to probiotics may also have beneficial effects on CNS function and behaviour (Bienenstock et al., 2016; Messaoudi et al., 2011). Several recent studies have provided evidence in support of this concept. For example, in a study conducted by Bravo et al. (2011), chronic treatment with *L. rhamnosus JB-1* induced region specific alterations in GABA B1b and Aα2 receptor mRNA in the mouse brain. Changes to central GABAergic neurotransmission in this manner has been associated with pathological anxiety states (Nuss, 2015). Accordingly, authors also found reduced anxiety and stress-induced plasma corticosterone levels in these mice. In another study testing the effect of 2-week administration of the probiotics *L. helveticus R0052* and *B. longum R0175* in combination, demonstrated reduced anxiety-like behaviour in rats as measured by the conditioned defensive probe burying test (Messaoudi et al., 2011). In a follow-up double-blind, placebo-controlled, randomised parallel group study, authors tested the health outcomes of the same probiotic combination when administered to healthy human participants for 30 days. Results showed improvement of psychological distress and reduced serum corticosterone as evaluated by behavioural tests (Hopkins Symptom Checklist, the Hospital Anxiety and Depression Scale, the Perceived Stress Scale, and the coping checklist) and 24 h urinary free cortisol. Moreover, in a randomized, double-blind, placebo-controlled pilot study, the effects of 2-month *Lactobacillus casei Shirota* treatment in patients with chronic fatigue syndrome, a condition where patients often present feelings of anxiety, was assessed (Rao et al., 2009). The probiotic treatment was

able to increase both *Lactobacillus* and *Bifidobacteria* species in stool samples and decrease anxiety symptoms as measured by the Beck Depression and Beck Anxiety Inventories. More recently, a randomized, controlled, parallel-arm single centered clinical trial tested the outcomes of 4-week consumption of a cocktail consisting of a fermented milk product with probiotics (*Bifobacterium animalis Lactis, Streptococcus thermophiles, Lactobacillus bulgaricus, and Lactococcus lactis Lactis* combination) in healthy women (Tillisch et al., 2013). As observed with functional magnetic resonance imaging, probiotic treatment produced a reduction in the reactivity of the primary interoceptive and somatosensory regions and the periaqueductal gray, to emotional attention tasks.

2.3. Antibiotics and the Gut Microbiome

Many prior studies on the role of the gut microbiome have also measured modulation by antibiotics (Verdú et al. 2006; Bercik et al. 2011; Cryan et al. 2012; O'Mahony et al. 2014; Aguilera, Cerdà-Cuéllar, and Martínez 2015; Desbonnet et al. 2015; Lurie et al. 2015; Fröhlich et al. 2016; Möhle et al. 2016; Rogers et al. 2016; Tochitani et al. 2016). In studies employing chronic, high-dose antibiotic treatments, the depletion of the gut microbiota has been attributed to several physiological anomalies including changes in key inflammatory and neuro-modulatory substances (Aguilera et al., 2015; Verdú et al., 2006). For example, in a study conducted by Verdu et al. (2006), a 10-day treatment with the antibiotics, bacitracin and neomycin in combination with the antifungal agent primaricin, perturbed the microbial community, increased myeloperoxidase and substance P immunoreactivity in the colon, and evoked visceral hypersensitivity. Secondly, antibiotics have also been shown to produce cognitive deficits through depletion of microbial abundance and diversity (Cryan et al., 2012; Desbonnet et al., 2015; Fröhlich et al., 2016; Lurie et al., 2015; Möhle et al., 2016; Rogers et al., 2016). Desbonnet el al. (2015), tested the cognitive abilities of adult mice treated with a cocktail of ampicillin, vancomycin, neomycin, metronidazole, and the antifungal agent amphotericin B, from weaning (post-natal day 21) onwards. Results not only demonstrated alterations to the gut microbial community, but also impaired learning and recognition memory, as mice were

less able to identify a novel object that had previously been exposed to a cage mate. Interestingly, authors also found reduced brain-derived neurotrophic factor (BDNF) RNA in the hippocampus and oxytocin and vasopressin mRNA in the hypothalamus of antibiotic treated mice, compounds that have previously been associated with cognition (Baj, Carlino, Gardossi, & Tongiorgi, 2013; Gabor, Phan, Clipperton-Allen, Kavaliers, & Choleris, 2012; Mizuno, Yamada, Olariu, Nawa, & Nabeshima, 2000; Skuse et al., 2014; Takayanagi et al., 2005; Tyler, 2002). Lastly, incidence of anxiety-related behaviours have also been associated with antibiotic mediated perturbation of the gut microbiome (Verdú et al. 2006; Bercik et al. 2011; Desbonnet et al. 2015; Tochitani et al. 2016). For example, Bercik et al. (2011) have shown that oral administration of neomycin, bacitracin and primaricin for 7 days to adult mice, reduced anxiety as assessed with the step-down and the light/dark box behavioural tests, and paralleled reductions in hippocampal BDNF.

However, these effects of antibiotics may be confounded by alternative actions of antibiotics – actions not associated with their antimicrobial activity (Forsythe et al., 2016). For example, bacitracin, an antibiotic produced by strains of *Bacillus licheniformis*, is well known to interfere with bacterial plasma membrane permeability and peptidoglycan synthesis (Stone & Strominger, 1971). However, Bacitracin has also been shown to evoke antinociceptive effects and periods of catalepsy when administered intracerebroventricularly or topically to rodents (Herman, Stachura, Laskawiec, Kowalski, & Obuchowicz, 1985; Yilmaz et al., 2015). In addition, the tetracycline derivative minocycline, can directly down-regulate nitric oxide signalling pathways so as to confer neuroprotection and ameliorate depressive and autistic behaviour (e.g., reduced locomotion, social interaction), by regulating free radical damage and serotonin levels in rodents (Cai et al., 2008; Kumar & Sharma, 2016; Saravi, Mousavi, Saravi, & Dehpour, 2016). It is therefore possible that antibiotic mediated effects both locally on gastrointestinal function and on the CNS also result from direct interaction with enteric neuronal circuits (Forsythe et al., 2016).

3. Regulation of Gastrointestinal Motility

Gastrointestinal motility can be defined as the movement of luminal contents inside the gastrointestinal tract (Sarna, 2010). The spatial and temporal patterns of gastrointestinal motility are regulated by the enteric nervous system (Kunze & Furness, 1999). Enteric intrinsic primary afferent neurons (IPANs) readily respond to changes in the chemical or mechanical nature of the intestinal mucosa, and to distension of the musculature. These sensory neurons have large smooth cell bodies in the myenteric and submucosal plexuses and widespread projections into the circumferential axis of the gut (Spiller & Grundy, 2008). Moreover, they are connected to one another through synapses and thus form extensive self-reinforcing assemblies that communicate through slow excitatory post-synaptic potentials (Bertrand, Kunze, Bornstein, Furness, & Smith, 1997; Kunze, Furness, Bertrand, & Bornstein, 1998; Kunze, Furness, & Bornstein, 1993; Kunze & Furness, 1999; Pompolo & Furness, 1988). IPANs also provide outputs to muscle motor neurons and ascending and descending interneurons (Kunze & Furness, 1999). The synaptic relationships IPANs share with most other neurons in the ENS highlights the prominence of IPANs in coordinating unified responses to local stimuli.

Activation of gastrointestinal motility further relies on contraction of the circular smooth muscle layer (Huizinga et al., 2014). While the gastrointestinal tract also contains a longitudinal muscle layer, contractions of this layer result in shortening of the length of the intestine (versus shortening of the diameter) and therefore has a less notable effect on propulsive activity (Sarna, 2010). Hence, research focus has been placed mainly on contractions of the circular muscle layer, which can completely occlude the luminal cavity to propel luminal contents. The intramuscular network of interstitial cells of Cajal, normally generate electrical slow wave potentials that rhythmically depolarize the musculature closer to contractile threshold (Huizinga & Lammers, 2008). Excitatory neurotransmission or inhibition of inhibitory neurotransmission, and distension can all provide the extra surge of depolarization needed to bring the membrane potential to

threshold (Der–Silaphet, Malysz, Hagel, Arsenault, & Huizinga, 1998; Kunze & Furness, 1999). This then facilitates elevation of intracellular calcium levels by opening of voltage-gated calcium channels and through release of calcium from sarcoplasmic reticulum stores, to trigger muscular contraction (Huizinga & Lammers, 2008; Vetterkind & Morgan, 2012). Since all enteric IPANs have projections to the mucosa, they can receive input from a variety of chemical and mechanical stimuli disturbing the mucosal villi and can then communicate those signals to stimulate circular muscle contraction (Spiller & Grundy, 2008). This excitatory response is largely mediated by cholinergic or tachykinin synapses with muscle motor neurons (Bornstein, Costa, & Grider, 2004; Spiller & Grundy, 2008). Essentially, slow excitatory post-synaptic potentials produce long trains of action potentials in motor neurons, resulting in prolonged release and build-up of acetylcholine at the neuromuscular junction, which finally prompts muscular contraction (Sarna, 2010). In addition, mechano-sensitive ion channels on the musculature can also regulate intracellular calcium concentrations and contractility thereby allowing the muscle to respond directly to distension-induced circumferential stretch (Huizinga & Lammers, 2008; Kraichely & Farrugia, 2007; Thorneloe & Nelson, 2005). Contraction of the circular smooth muscle in this manner can lead to activation of mechano-sensitive sites on myenteric IPANs (Costa & Furness, 1982). This results in the downstream activation of ascending excitatory pathways and the sequential inactivation of descending inhibitory reflex pathways to accommodate passage of luminal contents anal to the initial site of contraction (Sarna, 2010; Waterman, Tonini, & Costa, 1994). Inhibition of smooth muscle anally involves the release and interaction of nitric oxide and vasoactive intestinal peptide (Sarna, 2010). The initiation of these reflex pathways does not depend solely on smooth muscle contraction however, as they have been produced when the musculature was relaxed with nicardipine (Smith, Bornstein, & Furness, 1990). This observation further demonstrates the ability of IPANs to respond directly to chemical or mechanical distortion of the mucosa as mentioned earlier (Kunze & Furness, 1999). Whether the circular smooth muscle is activated primarily by IPAN activity or distention, muscular contraction orally and relaxation anally propagates the contractions

and drives luminal contents forward, where another cycle of reflexive responses is then initiated (Kunze & Furness, 1999; Sarna, 2010; Spiller & Grundy, 2008).

The propulsion of intra-luminal content is often characterized by the temporal (e.g., frequency, amplitude) and spatial (i.e., velocity) parameters of propagating contractile clusters (PCCs). These clusters are defined as bands of intrinsic neural excitation that propagate in the same direction (Huizinga & Lammers, 2008; Kunze & Furness, 1999). In relation to in vivo studies, the frequency of PCCs is comparable to the total volume of the bolus propelled per unit of time, and velocity of PCCs can provide reference to the speed of the movement of the bolus (distance per unit time) (Sarna, 2010). PCCs can be identified from both the colon and small intestine segments, however spatiotemporal characteristics vary based on the function of different sections of the intestine. The colon, known for its involvement in the excretion of fecal matter, exhibits rapidly propagating high amplitude PCCs, preventing much absorption of nutrients. More specifically, in the human colon, PCCs have a velocity of 10mm/s, a frequency of 6-10 contractions over a period of 24 hours and mean amplitude of 115 mmHg (Bassotti & Gaburri, 1988; Rao et al., 2009; Sarna, 2010). In rodents, PCCs propagate much more frequently but irregularly over much smaller distances, in the elimination of fecal pellets (Chen et al., 2013; Sarna, 2010). They have a frequency of about 17-45 contractions and 15-25 contractions per hour, in rats and mice respectively (Gourcerol et al., 2009; Li, Johnson, Adams, & Sarna, 2002; Sarna, 2010). Additionally, PCCs are known to be dependent on enteric neural activity because they are abolished when the lumen has been exposed to the neurotoxins, tetrodotoxin or hexamethonium (Chen et al., 2013; Kunze & Furness, 1999). Thus, PCCs portray neuronally-mediated activation and movement of contents from sequential regions of the gut wall (Kunze & Furness, 1999; Sarna, 2010). In addition, as the cluster propagates through a region of the intestine, it can be superimposed by the higher frequency, lower amplitude propagating ripples, which are superficial ring contractions of the circular muscle dependent on electrical slow wave potentials generated by ICCs (Chen et al., 2013; Der–Silaphet et al., 1998; Huizinga & Lammers, 2008; Kunze &

Furness, 1999). These contractions have been shown to be unaffected by TTX and occur in phase with slow waves, substantiating their myogenic origin (Neal, Parry, & Bornstein, 2009). Slow-wave (SW) dependent ripples also propagate in both the anterograde and retrograde directions, suggesting a primary role in absorption of luminal contents (Chen et al., 2013). Changes to features of PCCs and SW- dependent ripples can therefore provide a useful method for investigation of the neuronal and myogenic control of intestinal motor patterns in health and disease (Der–Silaphet et al., 1998; Sarna, 2010). For example, an increase in the frequency and amplitude of PCCs can indicate disruption to the neuronal regulation of gastrointestinal motility and visceral hypersensitivity – likely a consequence of muscle inflammation in patients with IBS and IBD (Sarna, 2010).

3.1. Antibiotic Mediated Changes in Gastrointestinal Motility: Mode of Action?

The importance of the gut microbial community in regulation of gastrointestinal motility is evident from studies of germ-free mice (Caenepeel et al., 1989; Husebye et al., 2001) and from studies employing high-dose antibiotic treatments to alter microbial composition (Abraham & Sellin, 2007; Barbara et al., 2005; Verdú, Bercik, & Collins, 2009). For example, mice fed the broad-spectrum antibiotics ampicillin, neomycin, metronidazole and vancomycin showed a reduction in the richness and diversity of commensal microbiota, which was associated with deregulation of intestinal bile acids and serotonin metabolism and slower colonic transit time (Ge et al., 2017). In another study however, the dysbiotic state induced by a mixture of bacitracin A, neomycin, and amphotericin B, increased basal colonic contractility in mice (Aguilera et al., 2015). Furthermore, a direct effect of the macrolide erythromycin on motility has been demonstrated in animal models, where it has been shown to stimulate gastrointestinal motor activity by acting as motilin receptor agonists on smooth muscle (Catnach and Fairclough, 1992; Itoh et al., 1984). Motilin is a gastrointestinal hormone thought to function in the initiation of propulsive motility (Aguilera et al., 2015). In human subjects, oral administration of erythromycin at therapeutic doses is also known to increase the frequency of contractions in the colon and inhibit contractions in the small intestine

(Lehtola, Jauhonen, Kesäniemi, Wikberg, & Gordin, 1990; Zara, Qin, Pilot, Thompson, & Maskell, 1991). Indeed, outcomes vary depending on the antibiotics and doses administered, initial gut microbial composition, strain of animals used, and length of treatment (Aguilera et al., 2015; Forsythe et al. 2016). However, it is without doubt that treatment with high-dose antibiotics modulates the gut microbiome, leading to functional alterations in gastrointestinal motility.

Interestingly, there is also literature to support the idea that antibiotics can act directly on neurons to exert effects in addition to actions on the microbiome (Figure 1). For instance, acute exposure to high-dose clarithromycin enhanced intrinsic neuronal excitability in rat pyramidal cells from the CA3 region of hippocampus, *ex vivo*, by reducing $GABA_A$ receptor activation (Bichler et al., 2016). In addition, lincosamide antibiotics (closely related to the macrolide class) applied to rabbit colonic epithelium *ex vivo,* have been shown to reduce neuro-epithelial cholinergic neurotransmission by post-junctional inhibition of ion transport (Goldhill et al., 1996). More specifically, while cholinergic secretomotor fibers supplying the rabbit colonic epithelium normally evoke the secretory response to electrical field stimulation, the addition of clindamycin and lincomycin antibiotics attenuated these responses. In the guinea pig small intestine, high dose erythromycin has been shown to inhibit electrically evoked contractions and the ascending excitatory reflex (component of the peristaltic reflex), *in vitro* (Minocha & Galligan, 1991). The authors suggested that the effect of erythromycin in this study is a result of pre-junctional inhibition of the release of substance P and of acetylcholine from nerves in the myenteric plexus, resulting from a reduction in calcium influx through voltage-gated channels in nerve terminals of excitatory motor neurons. This finding was consistent with evidence from a study conducted by Lees and Percy (1981), that showed how increasing extracellular calcium concentrations reversed aminoglycoside antibiotic inhibition of nerve-mediated contractions in the guinea pig small intestine, thereby suggesting that the antibiotic inhibited calcium entry into nerve terminals. Finally, in human airway smooth muscle, macrolide antibiotics including erythromycin, roxithromycin, and clarithromycin, have been shown to depress contractile responses to

electrical field stimulation by inhibiting cholinergic neurotransmission, most likely through reducing release of acetylcholine from nerve terminals (Tamaoki et al., 1995).

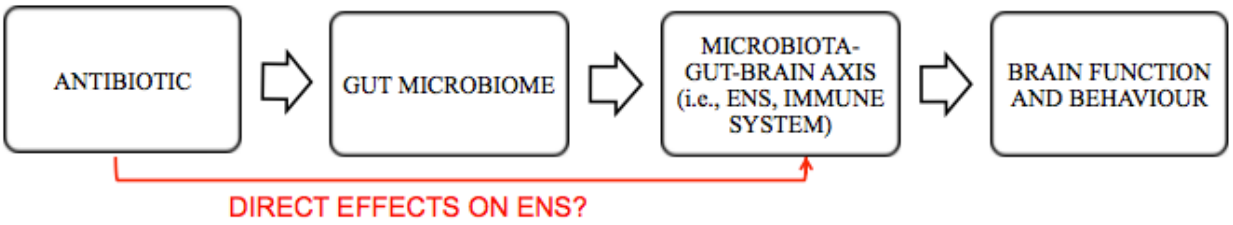

Figure 1: Alternative hypothesis (red) highlighting possibility for direct antibiotic modulation of enteric nervous system in comparison to conventional hypothesis (black).

The use of an organ bath apparatus for ex vivo assessment of gastrointestinal motility is preferred as it allows real time evaluation, troubleshooting and preparing of next steps. The experimental procedure is relatively simple and so several experiments can be completed within one day. Furthermore, as tissue segments are in an isolated system, confounding effects are controlled and a true response of the tissue to the treatment applied can be measured.

4. Antibiotics Can Alter Hard-Wired Gut-Brain Signalling Pathways

If luminal antibiotics can evoke direct responses in the enteric nervous system, it is highly likely that these local effects may modulate brain function and behaviour via vagal EPANs (Delungahawatta et al., 2017). Vagal EPANs can perceive, integrate and transmit chemical and low-intensity mechanical stimuli to the brain (Grundy & Schemann, 2007). Vagal afferents have cell bodies in the nodose ganglion and central terminals are found mainly in the nucleus tractus solitarius (Berthoud & Neuhuber, 2000). From here, projections arise to medullary motor nuclei and other brainstem interneurons, the parabrachial nuclear complex in the dorsolateral pons and various forebrain sites that process sensory input. Alterations in the concentrations of serotonin, norepinephrine, GABA, and glutamate within these brain regions have been shown following electrical

stimulation of vagal afferents, thereby contributing to homeostatic regulation and interoception, and emotional well-being (Grundy & Schemann, 2007; Perez-Burgos et al., 2013). For this reason, vagal nerve stimulation has been FDA-approved for adjunctive treatment of intractable epilepsy and drug-resistant depression (Perez-Burgos et al. 2013).

The density of vagal innervation is highly concentrated in the upper gut and decreases significantly towards the colon (Bercik et al. 2011). More specifically, the gut wall is innervated by vagal afferent nerve terminals at the level of the mucosal lamina propria, the muscularis externa with terminal distribution found in both the muscle layers and myenteric plexus, and in the serosa in close connection with the mesenteric tissue (Perez-Burgos et al., 2014). The most abundant population of vagal nerve terminals is the IGLEs supplying the myenteric plexus. The terminal branching of IGLEs can surround half or all of a myenteric ganglion. Moreover, IGLEs contain proteins and specialized structures likely involved in vesicle-mediated neurotransmitter release (Spiller & Grundy, 2008). Due to such anatomical features, it is possible that IGLEs engage in synaptic signalling with myenteric neurons (Perez-Burgos et al., 2014; Furness et al., 1998; Spiller & Grundy, 2008). Thus, luminal stimuli activating mucosal IPANs may evoke synaptic transmission with extrinsic vagal afferent terminals to carry sensory information to the brain (Perez-Burgos et al., 2014). This theory is in line with the functional implications of neurotransmitter release from IPAN cell bodies and vagal sensitivity to a large variety of mediators including CCK, 5-HT, somatostatin, IL-1b and GLP-1 (Berthoud & Neuhuber, 2000; Perez-Burgos et al., 2014).

In fact, we have previously shown the existence of autonomic synapses between vagal afferents and the somata of cholinergic IPANs in the myenteric plexus. Luminal exposure to *L. rhamnosus,* increased both vagal afferent firing rates (Perez-Burgos et al., 2013) and firing of enteric IPANs (Kunze et al., 2009), a phenomenon found to be dependent on IPAN to vagus calcium-dependent synaptic transmission (Perez-Burgos et al., 2014). In another study, it was found that the effects of *B. longum NCC3001* on inflammation induced anxiety-like behavior and related CNS biochemical changes were dependent on

reduced excitability of enteric neurons in the myenteric plexus and on vagal integrity, highlighting the possibility of synaptic transmission of sensory information from enteric neurons to vagal pathways (Bercik et al., 2011). Nevertheless, vagal afferent responses have been evoked directly by chemical and mechanical stimuli, forgoing any synaptic transmission. For instance, the short-chain fatty acid butyrate has been shown to have a direct effect on vagal afferent discharge in the mucosal lamina propria, unlike longer chain fatty acids which are dependent on enteroendocrine release of CCK to activate vagal afferents (Lal, Kirkup, Brunsden, Thompson, & Grundy, 2001). Taken together, gut-brain communication relies on both intrinsic and extrinsic neural pathways and likely the interactions that may exist between them.

The gastrointestinal wall is attached to the mesenteric organ emerging from the superior mesenteric root (Coffey & O'Leary, 2016). This structure is known as the intestinal hilum, allowing entry of blood vessels, lymphatic vessels and extrinsic nerves to innervate the full span of the intestine. Importantly, the collection of nerves it encompasses includes vagal and spinal EPANs (Kuramoto & Furness, 1989). Thus, *ex vivo* nerve recordings made at this level of dissection can effectively capture activity of all extrinsic afferents (Hillsley & Grundy, 1998) in order to determine effects of luminal stimuli on signal transduction to the brain.

Central Question and Hypothesis

For decades antibiotics have been incorporated into the standards of practice for the treatment of bacterial infections. Harmful effects of antibiotics on several limbs of the gut-brain axis have now been well documented in literature. The underlying mechanisms remain equivocal but are crucial for development of clinical intervention. This thesis addresses the following question: **What is the mechanism by which high-dose antibiotics produce changes in gut-brain signalling to affect pathophysiological outcomes?**

I hypothesize that high-dose antibiotics, as used in prior experimental models that have investigated microbial mediation of gut-brain signalling pathways, can also induce changes in brain function and behaviour by direct interaction with enteric neuronal circuits.

Aims

I will test the hypothesis with the following two aims:

1. To identify whether transient exposure of the gastrointestinal lumen to high-dose antibiotics can acutely modulate gut motility reflexes that depend on the enteric nervous system.

2. To determine whether acute exposure of the gastrointestinal lumen to high-dose antibiotics affects patterns of electrical discharge produced by vagal afferent fibres.

Chapter 2: Materials and Methods

1. Animals

For these experiments, intestinal tissue was collected from 6–8-week-old, 20–30 g male Swiss Webster mice from Charles River Laboratories (Quebec, Canada). About 3–5 mice were housed in one cage, assigned a 12 h light/ dark cycle and provided food and water *ad libitum*. Experiments began 1 week after arrival of the mice to allow adequate time for mice to acclimatize with housing conditions. All procedures were conducted *ex vivo* following cervical dislocation in accordance with the Animal Research Ethics Board of McMaster University (permit 16-08-30).

2. Ex Vivo Gut Motility Recordings

A paired before and after experimental design was employed to assess changes to gut motility parameters between control and treatment with either bacitracin, neomycin or penicillin V antibiotics (see West et al., 2016). First, four cm colon and jejunal segments were excised and oral ends were cannulated to the inflow tubing of an organ bath chamber. The chamber contained 20 mL of 34˚C oxygenated Krebs buffer solution (cycled in at 5 mL min^{-1}) of the following composition (mM): 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). The inflow tubing was connected to two Mariotte bottles which have openings at the top for inlet tubes attached to bags of carbogen gas. These airtight bottles allowed constant inflow of either the Krebs buffer or antibiotic treatment, which can be altered by changing height (pressure) of bottle (McCarthy, 1934). Once the intestinal segment was attached to the inflow tubing, luminal contents were gently flushed by perfusing the lumen with an inflow of room temperature (19–22 °C), carbogen-gassed Krebs buffer at a rate of 0.5 mL/min and perfusion pressure of 5 hPa (Wu et al., 2013). The anal ends were then cannulated to the outflow tubing of the organ bath. Any mesenteric tissue was removed to ensure detection of the true diameter of segment and to limit artifacts generated during analysis. Next, heights of the inflow and

outflow tubing were adjusted to create a negative pressure differential of 1-3 hPa, in order to evoke regular ENS-dependent PCCs (Neal, Parry, and Bornstein, 2009).

Gut motility of entire segment in the organ bath (except for sections fixed to inflow and outflow tubing) was then recorded by a video camera mounted 7 cm above the preparation. Control recordings captured intestinal motility with luminal perfusion with Krebs buffer for 20 min. Next, the perfusate was switched to the Mariotte bottle containing the antibiotic treatment allowing inflow of different concentrations of either bacitracin, neomycin or penicillin V for another 20 min of video recording. These recordings were subsequently converted to spatiotemporal diameter maps (Dmaps) for quantitative analysis using Image J, as described in Wu et al. (2013). Dmaps show the intestinal diameter changes as a hue value that ranges from white (diameter during relaxation) to black (diameter during contraction) per unit time (s). As a unique display of alternating light and dark bands representing movement of PCCs from the oral to anal direction can be generated from each experimental recording, Dmaps have been referred to as 'motility fingerprints' (Wu et al., 2013). PCC velocities were then measured from the slope of the wave front (distance/time), frequencies from intervals between 3-4 successive contractions, and amplitude as the difference in gut diameter between rest and peak contraction (West et al., 2016). Mixing patterns of motility such as SW- dependent contractions can also be assessed with Dmaps. Since these contractions only change luminal diameter superficially, they are represented as relatively small, frequent, antegrade and retrograde dark bands. SW-dependent velocities and frequencies were measured in the same manner as that of PCCs.

2.1. Data Analysis and Statistics

Paired t-tests were performed for before and after measurements, and significance was set at $P<0.05$. N is reported as the number of mice tested. At most, 4 repeated measurements for each gut motility parameter were made from each mouse.

3. **Ex Vivo Mesenteric Nerve Recordings**

Experimental design was adapted based on previous work by Perez-Burgos et al. (2013). A proximal jejunal segment (about 2.5 cm) was excised and placed in a Sylgard-coated recording petri dish filled with 2 mL Krebs buffer of the following composition (in mM): 118 NaCl, 4.8 KCl, 25 $NaHCO3$, 1.0 NaH_2PO_4, 1.2 $MgSO_4$, 11.1 glucose, and 2.5 $CaCl_2$ bubbled with carbogen (95% O_2-5% CO_2). The anal end was then cannulated to plastic tubing and luminal contents flushed using an attached syringe filled with oxygenated Krebs buffer. The entire gut segment and attached mesenteric tissue was then pinned out allowing for isolation of the mesenteric nerve bundle by dissection under a stereomicroscope. The preparation was then moved to an inverted microscope, where the petri dish (serosal compartment) received inflow of 22°C oxygenated Krebs buffer solution (cycled in at 3-5 ml min^{-1}). The oral end of the segment was cannulated to inflow tubing attached to several Mariotte bottles that initially gravity-perfused the lumen at 0.5–1 ml/min with room temperature (22°C) oxygenated Krebs. The isolated nerve bundle was sucked onto a glass micropipette with a wire electrode attached to a patch-clamp electrode holder (CV-7B; Molecular Devices, Sunnyvale, CA). Using Multi-Clamp 700B amplifier and Digidata 1440A signal converter (Molecular Devices), extracellular multi-unit nerve recordings were made. The gut lumen was perfused with Krebs buffer in this manner for 15 min, followed by penicillin V or bacitracin solutions for 30 min. Following the treatment period, the perfusate was switched back to the Krebs solution for 10 min to allow for washout. We used a micropipette to then dispense 5 μM of CCK directly into the serosa to identify vagal fibres (Hillsley & Grundy, 1998; Richards, Hillsley, Eastwood, & Grundy, 1996). After 10 min we dispensed 50 μM of a $5HT_3$ receptor agonist to the serosa to identify small proportion of single vagal afferent fibres that were not sensitive to CCK (Hillsley & Grundy, 1998). To ensure that the effect on multiunit activity was not mediated by muscle contraction evoked by enteric motor neurons, the smooth muscle was paralyzed using the L-type calcium channel blocker nicardipine (Perez-Burgos et al. 2013). 3μM of nicardipine was added to the Krebs buffer perfusing

both the serosa and the lumen, before applying the antibiotic treatment. Finally, as central connections have been severed, signals from vagal efferent fibers from the dorsal motor nucleus are thought to be lost or negligible.

3.1. Data analysis and statistics

Single-units from extracellular multi-unit nerve recordings were identified using the template-based spike recognition tool in DataView. The program identifies single waveforms whose shape, duration and size match previously built templates (Heitler 2007; Perez-Burgos et al. 2013). Any change in excitability of each identifiable single fiber induced by antibiotic treatment was then visualized on a spike frequency histogram. The mean interval time (ms) between successive spikes of the single fiber was measured for control and treatment periods. Firing frequency (Hz) was then calculated by taking the inverse of the interval means. Paired comparisons between control and treatment firing frequencies were then assessed for each individual fiber as constitutive multiunit electrical activity can differ significantly between preparations (Rong et al., 2004). *N* is reported as the number of individual vagal fibers. Differences were considered significant if $p < 0.05$. Errors on bar graphs are SEM. GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) was used for all statistics.

4. Luminal Stimuli

Bacitracin, neomycin and penicillin V antibiotics were purchased in powder formulations from Sigma-Aldrich Canada Co. (Oakville, ON, Canada). The antibiotics were then dissolved with Krebs buffer solution, to achieve a homogenous mixture with pH around 7.02-7.45 before application to the lumen. The amount of antibiotic dissolved in Krebs solution was initially measured as molarity (mol/L) but we have expressed these values as mg/ml for ease of comparison to other studies. Many previous studies however, have tested the effects of oral antibiotics *in situ* and there is little precedence for determining the equivalent *ex vivo* concentrations, as it is difficult to estimate

bioavailability of the antibiotic and luminal volume. In an effort to include the effective *in situ* concentration, we have tested a broad range of concentrations for each antibiotic. Specifically, in the motility experiments, we tested the antibiotics bacitracin at concentrations of 0.43 ($3x10^{-4}$ M), 1.42 (10^{-3} M), 4.27 ($3x10^{-3}$ M) and 14.23 mg/ml (10^{-2} M), neomycin at concentrations of 0.27 ($3x10^{-4}$ M), 0.91 (10^{-3} M), 2.73 ($3x10^{-3}$ M) and 9.09 mg/ml (10^{-2} M), and penicillin V at 1.17 ($3x10^{-3}$ M), 3.88 (10^{-2} M) and 11.65 mg/ml ($3x10^{-2}$ M). For the extracellular nerve recordings, we tested the antibiotics bacitracin and penicillin V diluted with the Krebs and nicardipine buffer before intraluminal application at concentrations of 0.0043 (3×10^{-6} M), 0.43 (3×10^{-4} M), 1.42 (10^{-3} M), 4.27 (3×10^{-3} M), and 14.23 mg/ml (10^{-2} M) bacitracin, and penicillin V at 0.0012 (3×10^{-6} M), 0.39 (10^{-3} M), 1.17 (3×10^{-3} M), and 3.88 (10^{-2} M). Initially, we chose to test the higher doses of bacitracin and neomycin because they had been used in many prior experimental studies that have associated antibiotic- mediated disturbances in the microbiota to neurophysiological and behavioral outcomes (Bercik et al., 2011; Verdú et al., 2006; O'Mahony et al., 2014; Desbonnet et al., 2015; Aguilera et al., 2015; Fröhlich et al., 2016; Tochitani et al., 2016). Finally, we chose to test the effects of pencillin V because it is a widely used broad-spectrum antibiotic, that can be assessed to compare potency and effectiveness.

4.1. Absorption and Metabolism

Penicillin V is also one of the most absorbable antibiotics as about 20-30% of a clinically prescribed oral dose is absorbed from the intestine, mostly from the duodenum (Bunn et al., 1950). Approximately one-fifth of the amount reaching the jejunum and ileum is absorbed, and almost 5% of the amount reaching the colon is absorbed. Bacitracin and neomycin on the other hand, are categorized as non-absorbable antibiotics and are generally thought to be not systemically available (often administered intramuscularly rather than orally for this reason). However, this is not true in every case, as it is clinically well known that aminoglycoside antibiotics (i.e., streptomycin, neomycin) can impair hearing (Brummett & Fox, 1989; Leake, 1988; Leis, Rutka, &

Gold, 2015; Sage, Nazareth, & Noone, 1987). Thus, it is likely that high doses of bacitracin and neomycin exhibit the same distribution patterns as that of penicillin V. If the antibiotics are poorly absorbed from the gastrointestinal tract, local retention and cumulation still remains uncertain as luminal volume is difficult to estimate. Additionally, these antibiotics are not metabolized in the liver, in fact, most antibiotics are not metabolized at all or only minimally, and are predominantly excreted through glomerular filtration in the kidneys (Wright, 1999). While the bioavailability limits the clinical relevance of our findings, the purpose of this study was to demonstrate an effect of low to high dose antibiotics on vagal afferent discharge for comparison to other experimental studies and the interpretation of effects with respect to the microbiome.

4.2. Antibiotic Cocktails

Some experimental studies have also employed 'antibiotic cocktails'– mixtures of multiple antibiotics rather than application of a single antibiotic (Bercik et al., 2011; Verdú et al., 2006; Desbonnet et al., 2015; O'Mahony et al., 2014; Aguilera et al., 2015; Fröhlich et al., 2016; Möhle et al., 2016; Tochitani et al., 2016). These mixtures are commonly used because it increases the antibacterial spectrum and therefore amplifies any systemic effects (Fantin, 1992, Eliopoulos and Eliopoulus, 1988). In our study however, use of a single antibiotic was sufficient to demonstrate rapid neuronal effects and so, no further attempt was made to determine if such effects would be different if antibiotics were instead combined.

Chapter 3: Results

1. Impact of Antibiotics on Ex Vivo Measures of Gut Motility

A complete experiment involved cervical dislocation and extraction of intestinal segments, recording of colonic motility when luminally perfused with control Krebs saline solution (20 min), followed by recording of colonic motility when perfused with antibiotic treatment (20 min). The same procedure was then repeated to test changes to jejunal motility. The total amount of time that each segment was handled *ex vivo* was about 60 mins. The tissue is expected to have maintained its integrity during this time as we have previously shown no rundown of spatiotemporal characteristics during recording periods of up to 2 hours (Wu et al., 2013). Paired comparisons of PCC velocity, frequency, amplitude was made between the Krebs control and antibiotic recordings and between different concentrations of a single antibiotic.

1.1. Antibiotics Did Not Affect Myogenic Regulation of Gastrointestinal Motility

When the serosal compartments of colon and jejunal segments were exposed to 1μM tetrodotoxin, no PPCs could be observed from *ex vivo* motility recordings, indicating their neuronal dependence. This observation is consistent with previous work performed by Wu et al. (2013). Furthermore, the addition of antibiotics (1.42 mg/ml bacitracin, 0.91 mg/ml neomycin and 3.88 mg/ml penicillin V) in the presence of TTX failed to produce any statistically significant changes to baseline SW-dependent contractions, a marker of intestinal muscle or interstitial cell of Cajal driven activity (Figure 2) (Zhu et al., 2013).

1.2. Antibiotics Evoked Changes to Colonic and Jejunal PCC Velocity

Antibiotics Produced Significant Increases in Colonic PCC Velocity

In the colon, all three antibiotics produced dose-dependent changes to PCC velocity (Figure 3, left). The log-dose vs. reponse curves for the effect of bacitracin on

colonic PCC velocity showed the most prounounced change with application of 1.42 mg/ml bacitracin where velocity increased from 0.26±0.06 to 0.35±0.1 mm/s (53% increase, $p<0.001$, n=5) (Figure 3A). At the smallest dose of 0.43 mg/ml bacitracin, PCC velocity decreased from 0.2±0.04 to 0.18±0.02 mm/s ($p=0.04$, n=4). Whereas with doses of 4.27 and 14.23 mg/ml bacitracin, colonic PCC velocity increased from 0.28±0.11 to 0.33±0.13 mm/s ($p=0.03$, n=5) and from 0.28±0.11 to 0.28±0.11 mm/s ($p=0.82$, n=5), respectively. For neomycin, the greatest change was achieved with a dose of 0.91 mg/ml where PCC velocity increased from 0.2±0.04 to 0.24±0.05 mm/s (19% increase, $p<0.02$, n=4) (Figure 3B). At doses of 0.27 and 2.73 mg/ml neomycin, PCC velocity increased from 0.21±0.05 to 0.22±0.12 mm/s ($p=0.57$, n=4) and from 0.21±0.02 to 0.22±0.02 mm/s ($p=0.04$, n=4), correspondingly. At the 9.09 mg/ml of neomycin, colonic PCC velocity decreased from 0.2±0.1 to 0.19±0.07 mm/s, although it was not statistically significant ($p=0.34$, n=4). Lastly, with penicillin V, the dose of 3.88 mg/ml produced the greatest response where the velocity increased from 0.2±0.05 to 0.24±0.06 mm/s (19% increase, $p<0.002$, n=4) (Figure 3C). At both the lowest dose of 1.17 mg/ml and highest dose of 11.65 mg/ml penicillin V, non-significant decreases to PCC velocity from 0.3±0.24 to 0.25±0.09 mm/s ($p=0.21$, n=5) and from 0.25±0.1 to 0.23±0.12 mm/s ($p= 0.55$, n=4) were observed, respectively.

Antibiotics Produced Varying Changes to Jejunal PCC Velocity

Changes to jejunal PCC velocity was specific to the antibiotic tested (Figure 3, right). Bacitracin increased sample velocity at all concentrations less than and greater than 4.27 mg/ml, but changes were not significant (Figure 3A). More specifically, at 4.27 mg/ml bacitracin, PCC velocity was reduced from 0.72±0.58 to 0.57±0.17 mm/s ($p=0.22$, n=5). On the other hand, at doses of 0.43, 1.42 and 14.23 mg/ml bacitracin, velocity increased from 0.2±0.02 to 0.22±0.04 mm/s ($p=0.1$, n=4), from 0.64±0.26 to 0.98±1.66 mm/s ($p=0.38$, n=5) and from 0.33±0.17 to 0.42±0.18 mm/s ($p=0.36$, n=5). With the antibiotic neomycin, another triphasic log-dose vs. response curve was established (Figure 3B). Most notably, PCC velocity was reduced from 0.23±0.05 to 0.19±0.04 mm/s (16%

decrease, p<0.001, n=4) at 0.91 mg/ml. At 0.27 mg/ml and higher doses including 2.73 and 9.09 mg/ml of neomycin, PCC velocity increased from 0.26±0.08 to 0.28±0.06 mm/s (p=0.63, n=4), from 0.2±0.02 to 0.21±0.02 mm/s (p=0.39, n=4), and from 0.23±0.08 to 0.32±0.17 mm/s (p=0.009, n=4). Lastly, penicillin V increased velocity from 0.31±0.16 to 0.34±0.18 mm/s (p=0.1, n=5) at a dose of 1.17 mg/ml and from 0.22±0.06 to 0.25±0.1 mm/s (p=0.22, n=4) at a dose of 3.88 mg/ml (Figure 3C). These changes did not reach statistical significance. At the highest dose of 11.65 mg/ml however, PCC velocity decreased from 0.32±0.06 to 0.28±0.04 mm/s (12% decrease, p=0.04, n=4), producing an overall decreasing linear dose-response trend.

1.3. Antibiotics Evoked Changes to Colonic and Jejunal PCC Frequency

Antibiotics Produced Significant Increases in Colonic PCC Frequency

All intermediary concentrations of bacitracin, neomycin and penicillin V tested increased colonic PCC frequency (Figure 4, left). With doses of 1.42 and 4.27 mg/ml bacitracin, PCC frequency slightly increased from 0.009±0.002 to 0.011±0.003 Hz (p=0.04, n=5), and from 0.012±0.003 to 0.013±0.003 Hz (p=0.06, n=5), respectively (Figure 4A). Although at the lowest dose of 0.43 mg/ml bacitracin, PCC frequency was slightly reduced from 0.011±0.003 to 0.01±0.003 (p=0.013, n=4), and at the highest dose of 14.23 mg/ml bacitracin, frequency remain unchanged at 0.01±0.004 Hz (p=0.89, n=5). A multiphasic log-dose vs. response curve was plotted with intraluminal neomycin perfusion (Figure 4B). Specifically, PCC frequency increased from 0.009±0.002 to 0.01±0.003 Hz (p=0.03, n=4) and similarly from 0.009±0.003 to 0.01±0.002 Hz (p=0.09, n=4) with 0.91 and 2.73 mg/ml neomycin, respectively. However, at 0.27 and 9.09 mg/ml neomycin, colonic PCC frequency insignificantly decreased from 0.01±0.004 to 0.009±0.003 Hz (p= 0.1, n=4) and from 0.008±0.002 to 0.006±0.003 Hz (p=0.16, n=4). Lastly, penicillin V exposure increased frequency of peristaltic contractions from 0.009±0.002 to 0.01±0.002 Hz (p=0.003, n=4) at 3.88 mg/ml (Figure 4C). Yet, at the lowest and highest doses tested, penicillin V failed to produce significant changes. At

1.17 mg/ml, penicillin V reduced PCC frequency from 0.008±0.003 to 0.007±0.003 Hz (p=0.24, n=5) and at 11.65 mg/ml, PCC frequency remained unchanged at 0.01±0.005 Hz (p=0.7, n=4).

Antibiotics Produced Varying Changes to Jejunal PCC Frequency

Bacitracin produced insignificant changes to jejunal PCC frequency at all doses (Figure 4A, right). Specifically, at doses of 0.43, 1.42, 4.27 and 14.23 mg/ml, PCC frequency remained at 0.01±0.002 Hz (p=0.84, n=4), 0.01±0.002 Hz (p= 0.37, n=5), slightly decreased from 0.03±0.03 to 0.02±0.01 Hz (p=0.08, n=5), and remained at 0.02±0.01 Hz (p=0.21, n=5), accordingly. Next, luminal perfusion with neomycin produced a multiphasic log-dose vs. response curve (Figure 4B, left). PCC frequency initially remained unchanged at 0.01±0.005 Hz (0.07, n=4) with 0.27 mg/ml neomycin. At intermediate doses of 0.91 and 2.73 mg/ml, PCC frequency decreased from 0.011±0.003 to 0.009±0.002 Hz (p=0.01, n=4) and then increased from 0.009±0.003 to 0.011±0.002 Hz (p=0.04, n=4), respectively. At the highest dose of 9.09 mg/ml neomycin, PCC frequency increased from 0.01±0.004 to 0.02±0.046 Hz, although not significantly (p=0.2, n=4). Moreover, PCC frequency remained at 0.015±0.01 Hz (p=0.87, n=5) and at 0.01±0.002 Hz (p=0.03, n=4) with acute luminal exposure to 1.17 and 3.88 mg/ml penicillin V (Figure 4C, right). In contrast, at 11.65 mg/ml penicillin V, the frequency decreased from 0.02±0.01 to 0.01±0.003 Hz (p=0.002, n=4).

1.4. Antibiotics Evoked Changes to Colonic and Jejunal PCC Amplitude

Antibiotics Produced Significant Decreases in Colonic PCC Amplitude

Figure 5 (left) shows significant decreases in colonic amplitude with all three antibiotics. Firstly, at the intermediate dose of 4.27 mg/ml bacitracin, amplitude decreased significantly from 0.9±0.54 to 0.53±0.25 mm (p=0.01, n=5) (Figure 5A). However, PCC amplitude remained unchanged at 1.0±0.5 mm (p=0.67, n=4), 0.8±0.4 mm (p=0.7, n=5) and 0.32±0.18 mm (p=0.94, n=5) for doses of 0.43, 1.42 and 14.23 mg/ml

bacitracin, respectively. For neomycin, amplitude decreased from 1.02±0.32 to 0.72±0.36 mm (p=0.02, n=4) at 0.27 mg/ml (Figure 5B). Amplitude increased from 0.91±0.3 to 1.06±0.67 mm (p=0.25, n=4) and from 0.6±0.27 to 0.74±0.37 mm (p=0.24, n=4) with 0.91 and 2.73 mg/ml neomycin, however these changes did not reach statistical significance. At the dose of 9.09 mg/ml neomycin, the amplitude was reduced again from 0.68±0.17 to 0.6±0.46 mm, but not significantly (p=0.47, n=4). Lastly, penicillin V decreased amplitude from 1.06±0.36 to 0.8±0.36 mm (p=0.02, n=4) and from 0.66±0.43 to 0.39±0.28 mm (p=0.01, n=5) at 3.88 and 11.65 mg/ml, respectively (Figure 5C). At the smallest dose of 1.17 mg/ml penicillin V, amplitude insignificantly increased from 0.53±0.35 to 0.7±0.51 mm (p=0.09, n=5).

Antibiotics Produced Varying Changes to Jejunal PCC Amplitude

Jejunal amplitude was relatively unaffected by 0.43 and 4.27 mg/ml bacitracin where amplitude remained at 0.6±0.4 mm (p=0.45, n=4) and decreased insignificantly from 0.48±0.27 to 0.37±0.36 mm (p=0.09, n=5), respectively (Figure 5A, right). At doses of 1.42 and 14.23 mg/ml bacitracin, amplitude was increased from 0.48±0.22 to 0.6±0.18 mm (p=0.05, n=5) and from 0.28±0.16 to 0.44±0.29 mm (p=0.05, n=5), correspondingly. Neomycin did not produce any significant changes to jejunal amplitude at all the doses tested (Figure 5B, right). At doses of 0.27 and 0.91 mg/ml neomycin, amplitude remained the same at 0.42±0.2 mm (p= 0.94, n=4) and at 0.5±0.2 mm (p=0.45, n=4). At a dose of 2.73 mg/ml neomycin, jejunal amplitude increased from 0.57±0.2 to 0.68±0.3 mm, although not significantly (p=0.33, n=4). Lastly, at 9.09 mg/ml neomycin, amplitude was reduced from 0.62±0.24 to 0.47±0.3 mm (p=0.06, n=4). With penicillin V intraluminal perfusion, the amplitude was reduced from 0.57±0.28 to 0.38±0.12 mm (p=0.01, n=4) at a dose of 3.88 mg/ml, and from 0.78±0.21 to 0.65±0.18 mm (p=0.02, n=4) with 11.65 mg/ml (Figure 5C, right). At the lowest dose of 1.17 mg/ml penicillin V however, amplitude remained around 0.51±0.2 mm (p=0.52, n=5).

1.5. No Difference in Baseline Motility Between Segments Tested at Different Concentrations

We compared the baseline motility of segments tested at different concentrations to ensure that changes to gut motility parameters were not confounded by such initial differences. Results from multiple comparison analysis testing in ANOVA have shown that mean values of PCC velocity, frequency and amplitude largely remained the same between colon (Figure 6) and jejunum (Figure 7) segments tested at different concentrations. With the colon segments used, baseline differences were only found between the colon segments tested at the 0.27 and 9.09 mg/ml doses of neomycin for PCC frequency (Figure 6B, center) and between segments tested at 0.43 and 14.23 mg/ml doses of bacitracin for PCC amplitude (Figure 6C, left). Similarly, for jejunal segments used, baseline differences were distinguished between segments used at all concentrations of bacitracin for PCC velocity (Figure 7A, left), between segments tested at 0.43 mg/ml bacitracin, and 4.27 and 14.23 mg/ml for PCC frequency (Figure 7B, left), and between segments tested at 1.17 and 11.65 mg/ml penicillin V for PCC amplitude (Figure 7C, right). However, these baseline differences do not correspond to significant differences in spatiotemporal characteristics with antibiotic treatment. Therefore, the previously stated dose related changes to spatiotemporal characteristics of gut motility accurately reflect effects of the concentration of the antibiotic used and are not influenced by baseline differences between segments used. For example, acute jejunal perfusion with neomycin produced a multiphasic log-dose vs. response curve for PCC frequency, however no significant differences in baseline PCC frequency were found between segments tested at the different concentrations (Figure 8).

2. Impact of Antibiotics on *Ex Vivo* Afferent Responses from the Mesenteric Nerve Bundle

Nerve bundles were isolated from a total of 42 male Swiss Webster mice. At most, 2 preparations were made from one mouse to restrict mouse-specific effects. Multi-unit

activities from all nerve bundles was then recorded for about 1.5 h. Within this time, it is assumed that the preparation has remained intact as it has previously been shown that jejunal tissue can maintain its integrity for up to 4 h *ex vivo*, provided sufficient oxygen and an energy source (Inagaki et al. 2005; Perez-Burgos et al. 2013). From recording each multi-unit nerve bundle, about 15-20 CCK-sensitive single vagal fibers (about 70% of total identifiable single units) were discriminated using computerized waveform analysis. Single-unit firing frequency was assessed from plots of discharge frequency against time that were derived from taking the inverse of the mean interval time between each template-matched spike. In response to all concentrations of bacitracin and penicillin V, single vagal afferent discharge increased for an average period of 17.31 min after a mean latency of 8.03 min.

2.1. Bacitracin Increased Spontaneous Firing Frequency of Afferent Mesenteric Vagal Fibers

Figure 9 shows the percent mean difference to baseline vagal firing frequency as a function of the concentration of bacitracin. Non-linear regression of raw data best fits a sigmoidal dose-response curve with EC50 of 0.55 mg/ml. Initially, with 0.0043 mg/ml bacitracin, mean vagal firing frequency was shown to decrease by 12.42% (from 0.86±0.13 to 0.66±0.09 Hz), although not significantly ($p= 0.37$, $n=33$). Mean vagal firing frequency then increased 23.11% (from 0.54±0.07 to 1.0±0.1 Hz) by 0.43 mg/ml bacitracin ($p= 0.002$, $n= 63$), 55.35% (1.13±0.09to 1.87±0.19 Hz) by 1.42 mg/ml bacitracin ($p<0.0001$, $n= 79$), 83.1% (from 1.16±0.09 to 2.29±0.21 Hz) by 4.27 mg/ml bacitracin ($p=0.005$, $n=79$) and 47.87% (from 0.57±0.07 to 0.93±0.1 Hz) by 14.23 mg/ml bacitracin ($p=0.03$, $n=65$).

2.2. Penicillin V Increased Spontaneous Firing Frequency of Afferent Mesenteric Vagal Fibers at Concentrations >0.0012 mg/ml

The percent mean difference to baseline vagal firing frequency as a function of the concentration of penicillin V was also assessed (Figure 10). Non-linear regression of raw data best fits a second order polynomial model with maximal effectiveness at 3.88 mg/ml

penicillin V. At the lowest dose of 0.0012 mg/ml penicillin V, mean vagal firing frequency did decrease 16.31% (from 1.26 ±0.12 to 1.04 ±0.12 Hz, p=0.001, n=41). At subsequent doses of 0.39, 1.17 and 3.88 mg/ml however, mean vagal firing frequency increased by 58.65% (from 0.66±0.05 to 1.03±0.08 Hz, p<0.0001, n=65), 58.77% (from 0.75±0.05 to 1.09±0.08 Hz, p=0.007, n=77) and 65.46% (from 0.72±0.06 to 2.22±0.1 Hz, p<0.0001, n=62), respectively.

2.3. Mecamylamine Blocked Antibiotic Effects on Vagal Afferent Discharge

Mecamylamine is a non-competitive nicotinic acetylcholine receptor antagonist that will preferentially block nicotinic receptors at autonomic ganglia. As shown in Figure 11, baseline frequency did not change significantly with the addition of 50 μM mecamylamine (before and after mecamylamine: 1.005 ± 0.08 Hz to 0.91± 0.07 Hz; n=86). However, bacitracin and penicillin V both failed to maintain significant increases in vagal afferent discharge in the presence of mecamylamine (Figure 12). Specifically, while 1.42 mg/ml bacitracin initially produced a 55.35% increase in baseline firing (p<0.0001, n=79), the effect was reduced to a 13.61% increase (p=0.68, n=40) with 50 μM mecamylamine. Thus, bacitracin evoked changes in vagal afferent discharge were reduced by 41.74% (p=0.002). Similarly, while 0.39 mg/ml penicillin V produced a 58.65 % increase in baseline firing (p<0.0001, n=65), a 33.5% decrease in vagal firing frequency (p<0.0001, n=37) was observed with addition of mecamylamine. Hence, application of mecamylamine completely abolished penicillin V mediated effects on mesenteric vagal firing frequency (p<0.0001).

2.4. Nicardipine Did Not Affect Baseline Vagal Firing Frequency

As vagal afferents are sensitive to mechanical and chemical stimuli, we continuously perfused the lumen with a constant hydrostatic pressurized Krebs solution to minimize changes in intraluminal pressure (Perez-Burgos, Mao, Bienenstock, & Kunze, 2014). To further reduce transduction of any mechanical stimuli, we added nicardipine to the Krebs solution that perfused both the luminal and serosal compartments. Nicardipine is an L-type calcium channel blocker commonly used to relax the smooth muscle. Since transduction of a mechanical/stretch stimulus can begin in the muscle, nicardipine can

effectively prevent communication to mechanosensitive sensory neurons (Kunze, Clerc, Bertrand, & Furness, 1999; Kunze, Furness, Bertrand, & Bornstein, 1998). However, mucosal exposure to nicardipine has been shown to reduce the release of neurotransmitters, neurohormones and other mediators (e.g., 5-HT) from EC cells, which may interfere with neuronal excitability (Racké & Schwörer, 1993). Therefore, we measured changes to baseline vagal firing before and after nicardipine. As shown in Figure 13, intraluminal perfusion of 3μM nicardipine did not significantly change baseline frequency (frequency remained around 1.04 ± 0.11 Hz, n=27). This also confirmed previous research demonstrating a lack of effect of nicardipine at low concentrations (1-3 μM) on mucosal sensory activation by IPANs and transmission in myenteric ganglia (Iyer et al., 1988; Y. K. Mao et al., 2013b; Tonini & Costa, 1990).

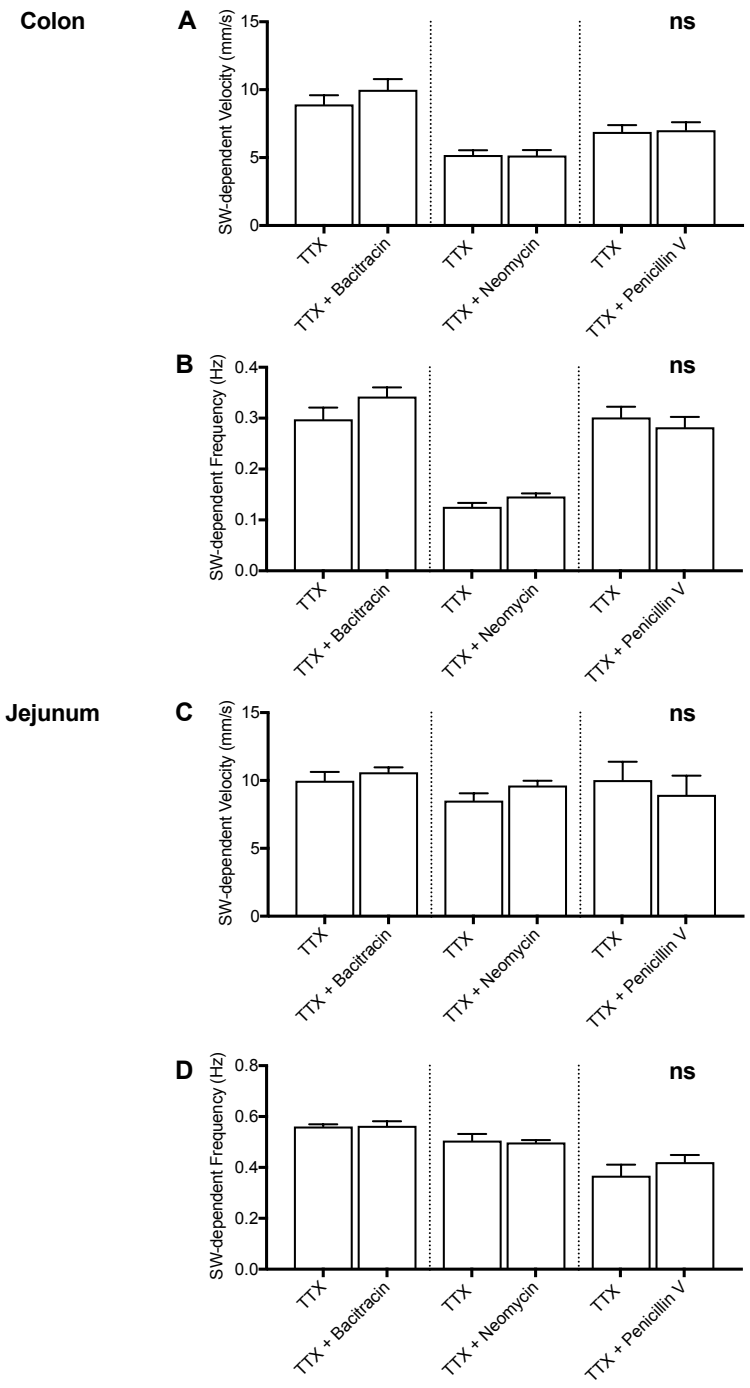

Figure 2: Lack of effect of antibiotics in the presence of TTX on colon and jejunal slow wave-dependent ripples. No statistically significant changes on (A) Colon SW-dependent Velocity (B) Colon SW-dependent Frequency (C) Jejunum SW-dependent Velocity and (D) Jejunum SW-dependent frequency with bacitracin, neomycin, and penicillin V treatment in the presence of 1μM TTX in the colon (n = 4) and jejunum (n = 8).

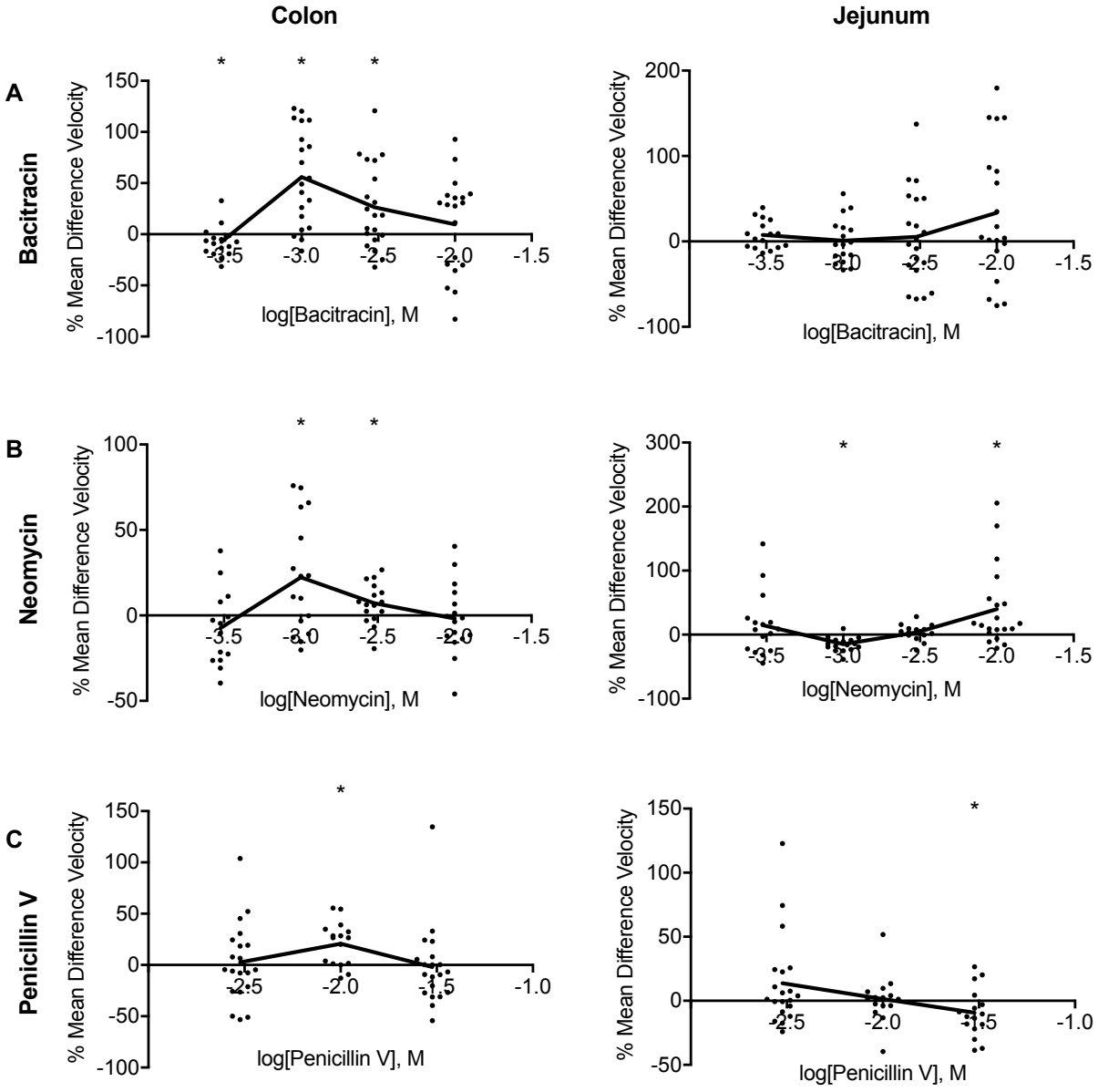

Figure 3: Log-dose vs. response curves for the effect of (A) bacitracin (B) neomycin and (C) penicillin V on PCC velocity (mm/s) of Swiss Webster mouse colon (left) and jejunum (right). Response variable is given as the percent mean difference between control and treatment experiments (* denotes $p < 0.05$). All data points (including repeated measures) are presented. Adjacent means are connected.

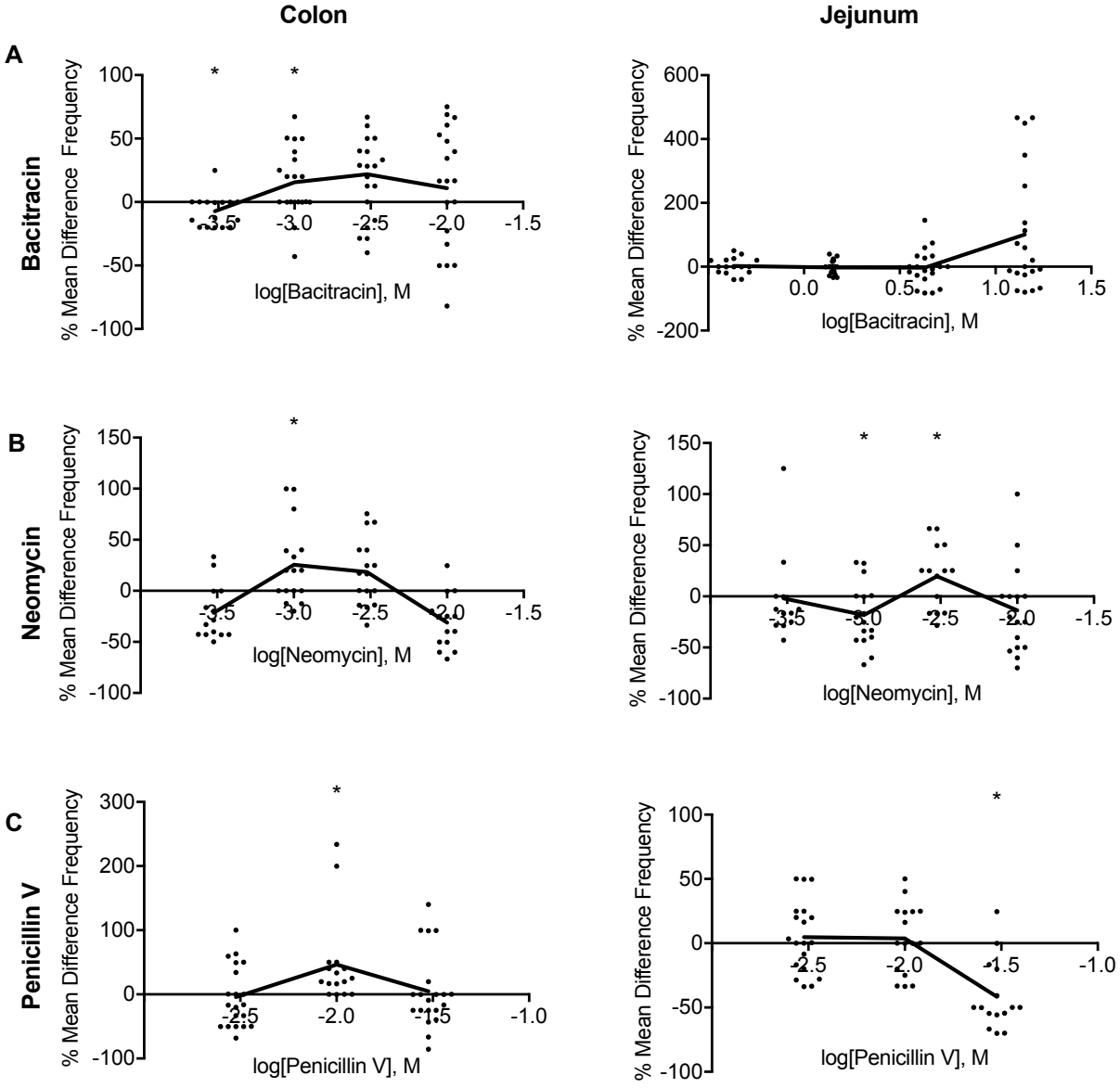

Figure 4: Log-dose vs. response curves for the effect of (A) bacitracin (B) neomycin and (C) penicillin V on PCC frequency (Hz) of Swiss Webster mouse colon (left) and jejunum (right). Response variable is given as the percent mean difference between control and treatment experiments (* denotes $p < 0.05$). All data points (including repeated measures) are presented. Adjacent means are connected.

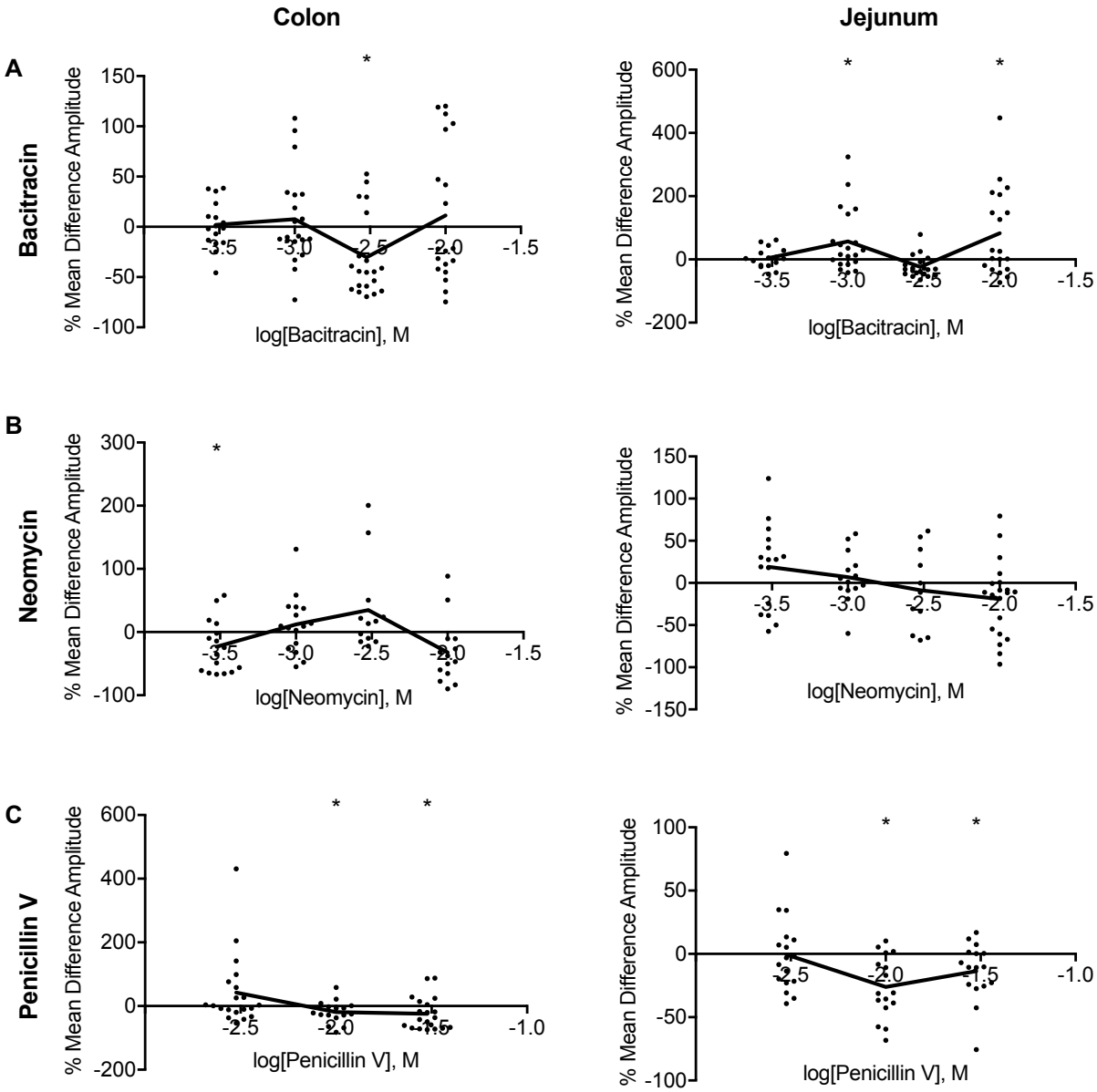

Figure 5: Log-dose vs. response curves for the effect of (A) bacitracin (B) neomycin and (C) penicillin V on amplitude (mm) of Swiss Webster mouse colon (left) and jejunum (right). Response variable is given as the percent mean difference between control and treatment experiments (* denotes $p < 0.05$). All data points (including repeated measures) are presented. Adjacent means are connected.

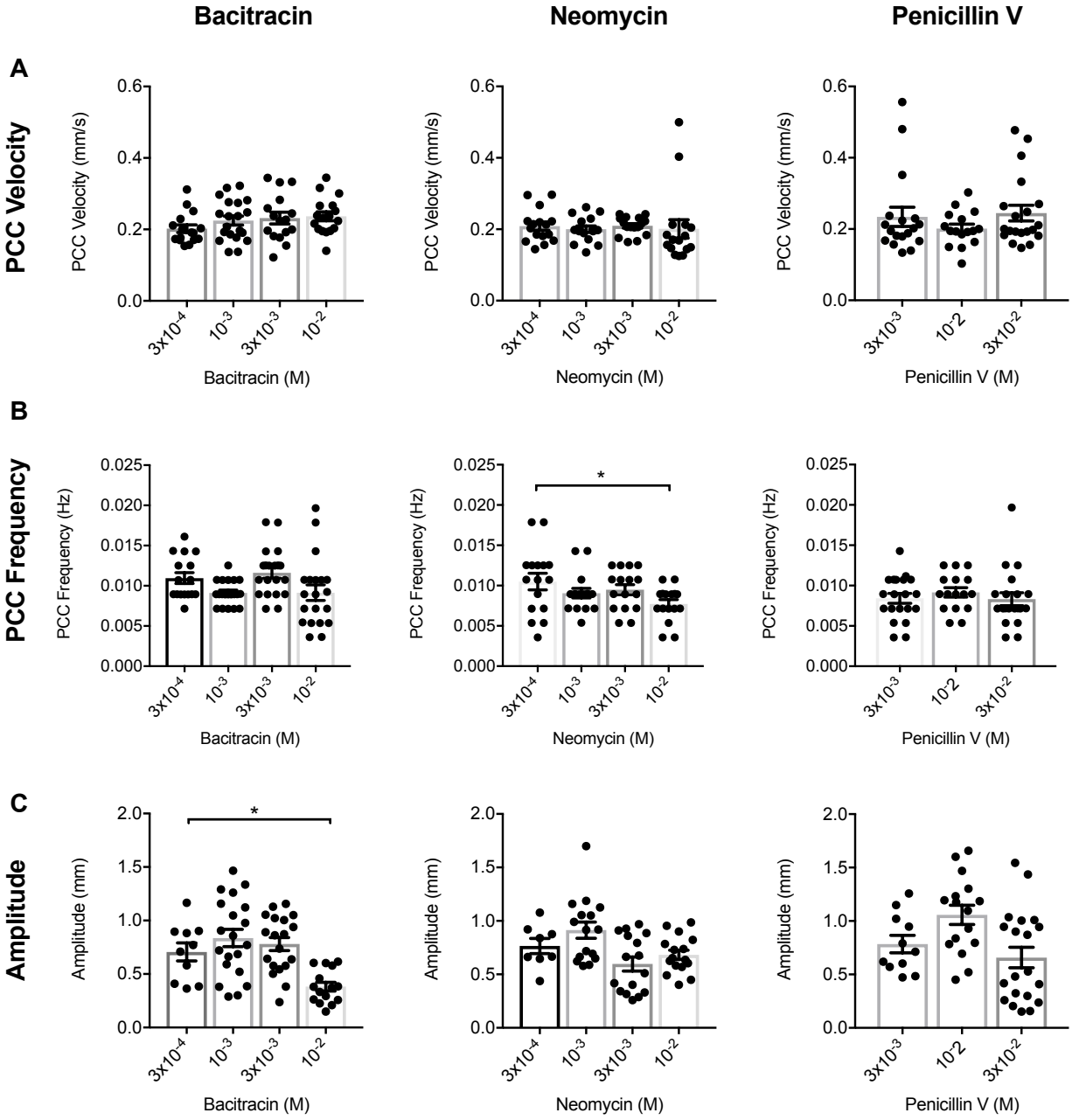

Figure 6: Baseline effect of Bacitracin (left), Neomycin (center) and Penicillin V (right) on colonic (A) PCC velocity (mm/s), (B) PCC frequency (Hz) and (C) Amplitude (mm). * denotes $p < 0.05$. All data points (including repeated measures) are presented.

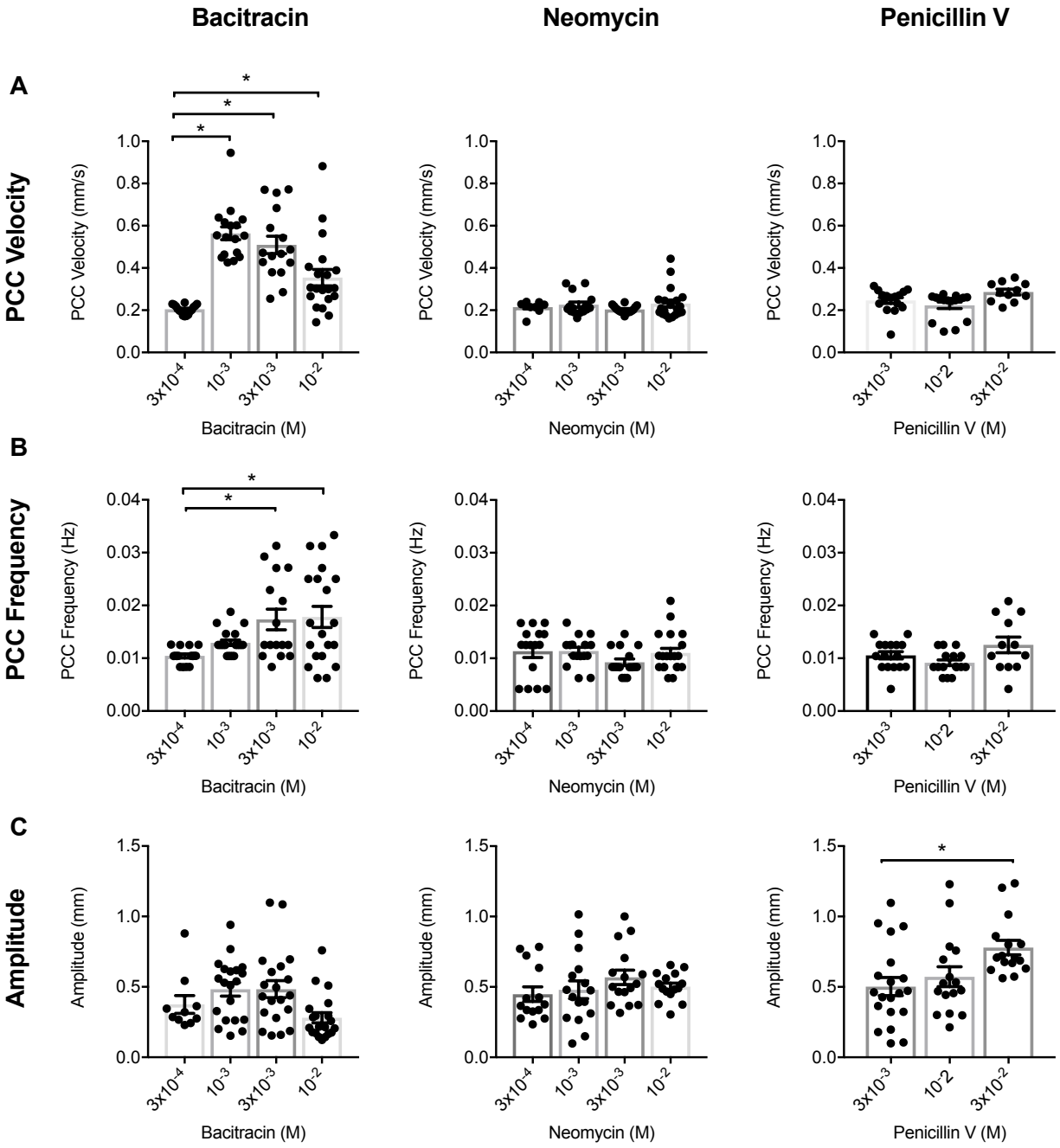

Figure 7: Baseline effect of Bacitracin (left), Neomycin (center) and Penicillin V (right) on jejunal (A) PCC velocity (mm/s), (B) PCC frequency (Hz) and (C) Amplitude (mm). * denotes $p < 0.05$. All data points (including repeated measures) are presented.

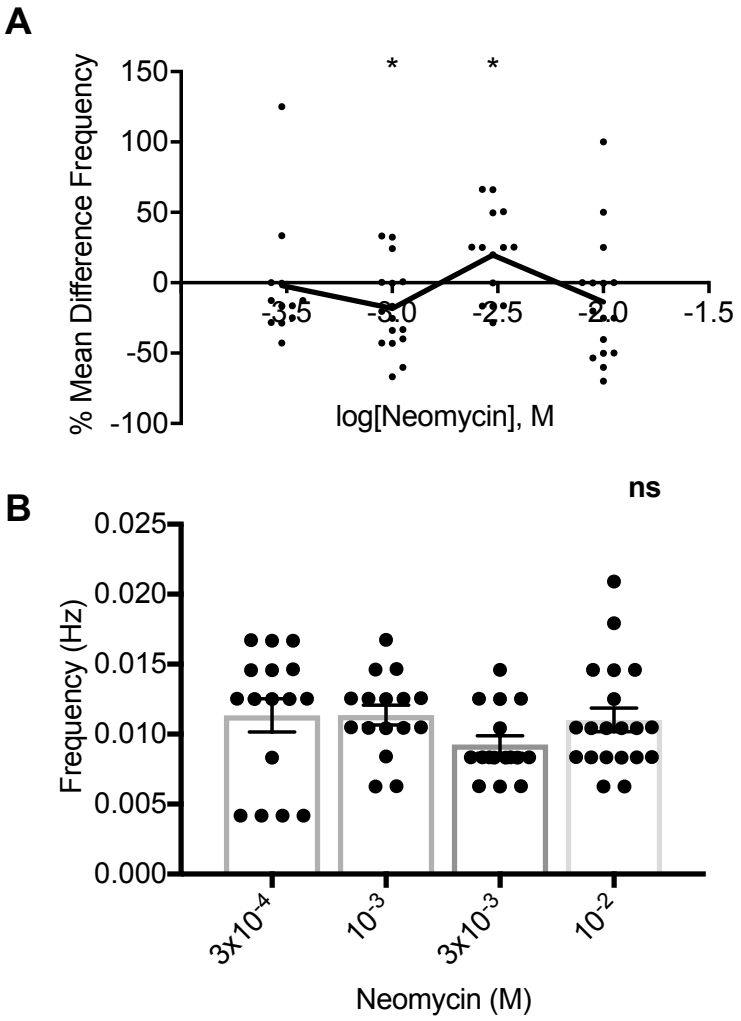

Figure 8: (A) Multiphasic log-dose vs. response curve for the effect of neomycin on PCC frequency (Hz) of Swiss Webster mouse jejunum, despite (B) lack of differences in baseline PCC frequency (Hz) between jejunal segments (n = 4). One-way ANOVA summary details P = 0.2989, F = 1.25, R square = 0.0554. ns, not significant.

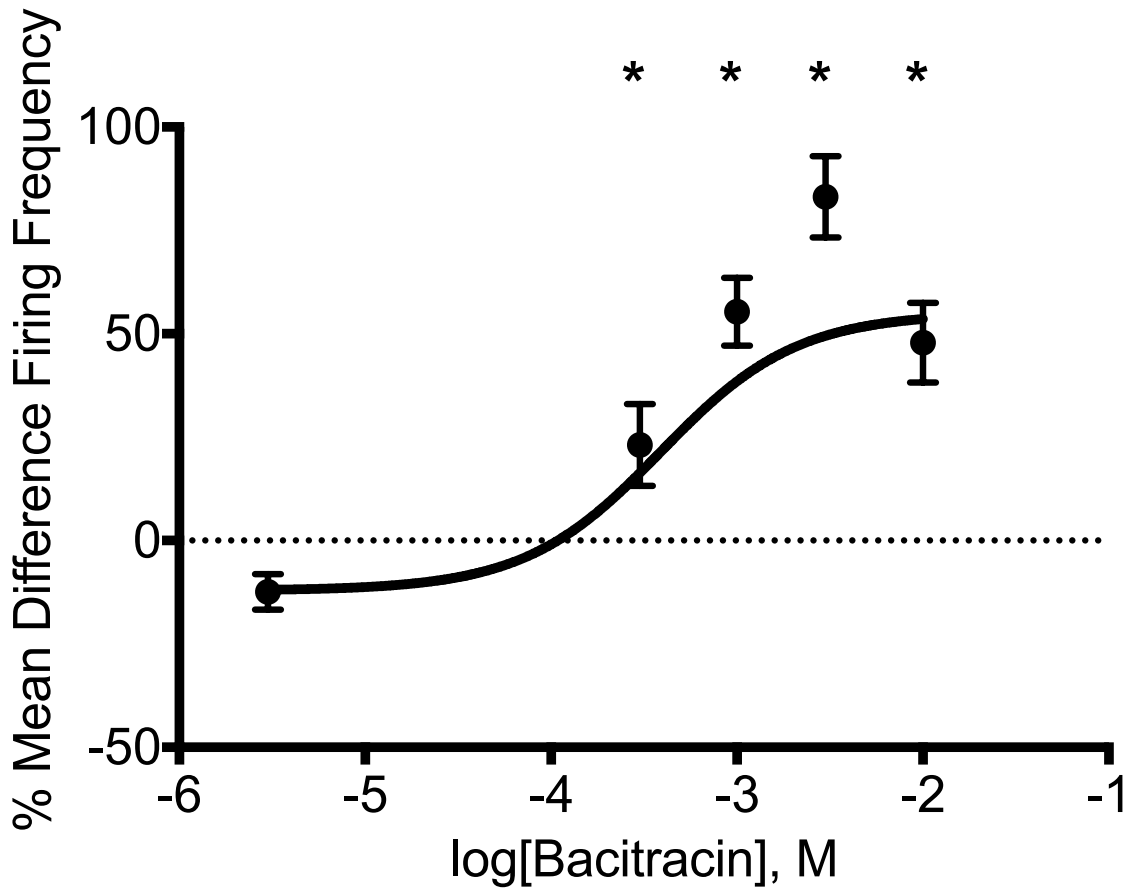

Figure 9: Log-dose vs response curve for the effect of Bacitracin on percent mean difference in baseline vagal firing frequency. Data are expressed as means ± SEM (* denotes $p<0.05$).

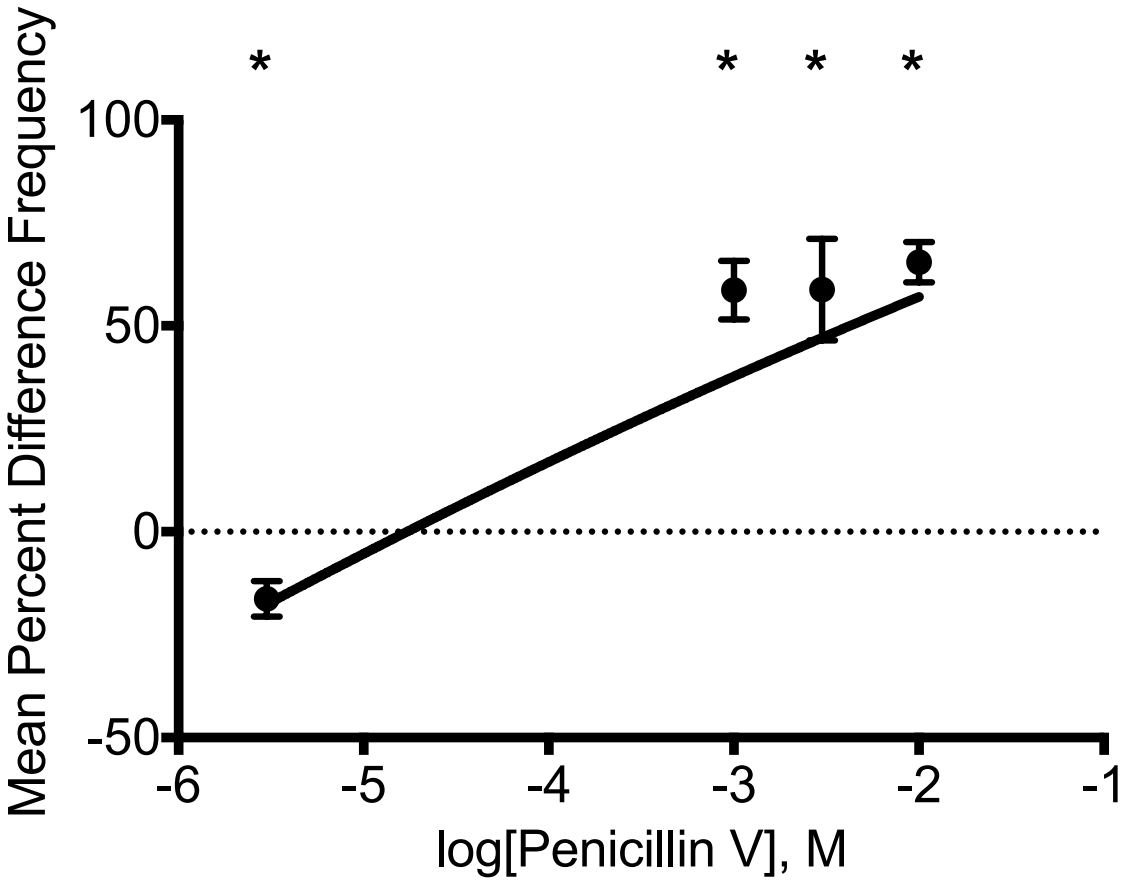

Figure 10: Log-dose vs response curve for the effect of Penicillin V on percent mean difference in baseline vagal firing frequency. Data are expressed as means ± SEM (* denotes $p<0.05$).

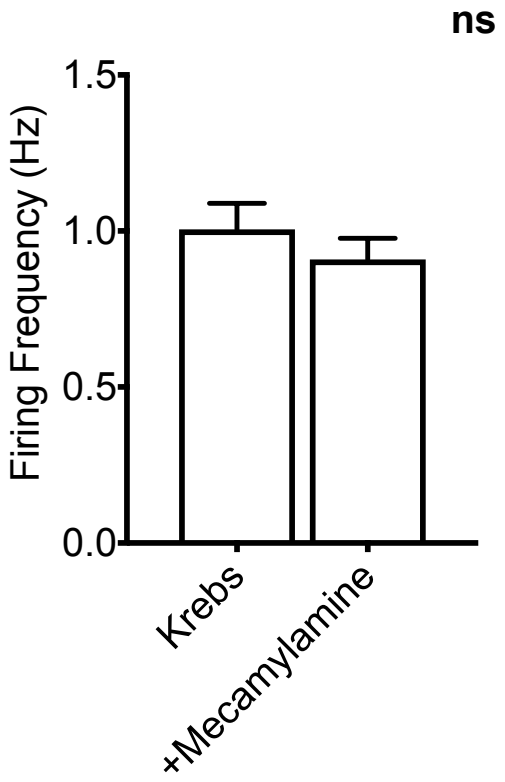

Figure 11: Effect of 50 µM mecamylamine on baseline vagal firing frequency (Hz). Data are expressed as means ± SEM.

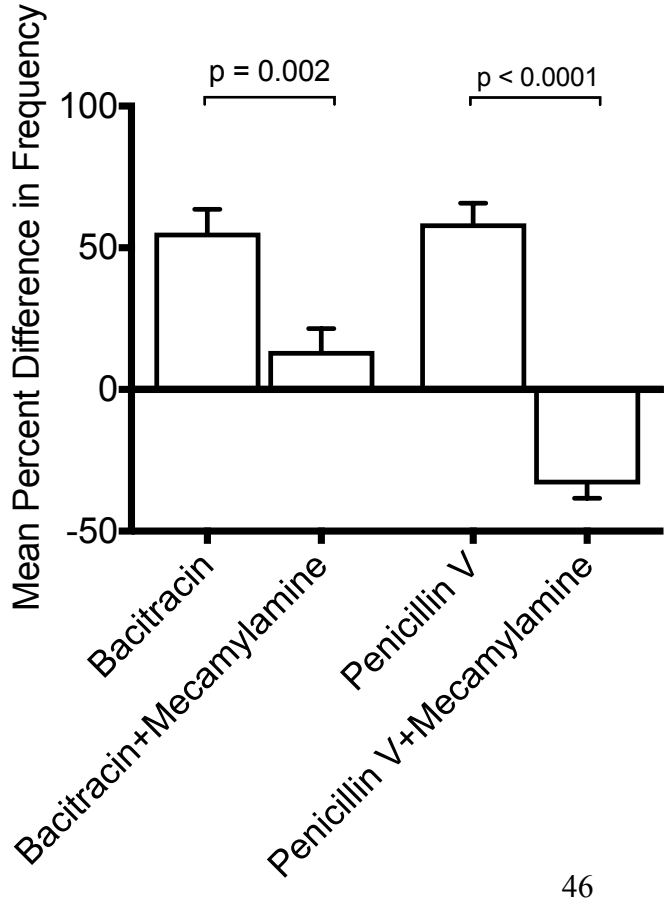

Figure 12: Mecamylamine diminished vagal firing frequency induced by 1.42 mg/ml Bacitracin and 0.39 mg/ml Penicillin V. Data are expressed as means ± SEM.

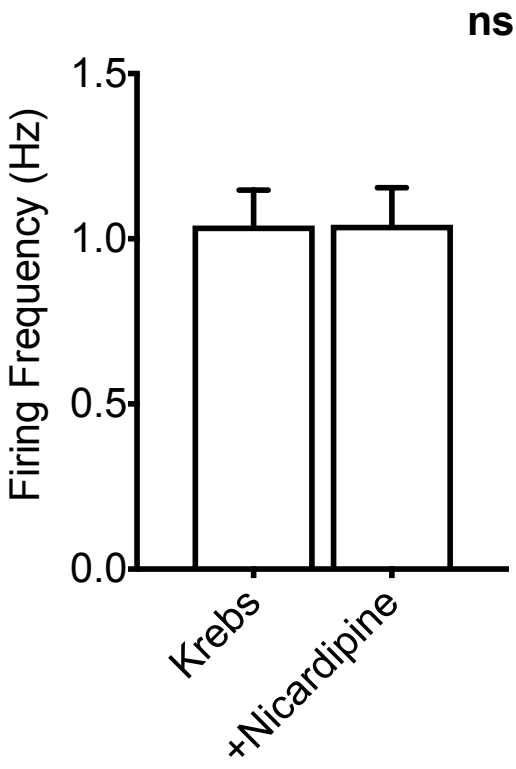

Figure 13: Effect of 3μM nicardipine on baseline vagal firing frequency (Hz). Data are expressed as means ± SEM.

Chapter 4: Discussion

As hypothesized, high-dose antibiotics, previously tested in experimental models that have explored the causative involvement of substantial alterations in resident microbiota in pathogenesis of brain and behavioural disorders, can also affect health by direct interaction with enteric neuronal circuits. From two sets of experiments, we have demonstrated direct effects of high-dose antibiotics on gastrointestinal motility and on gut-brain neuronal signalling pathways.

1. **Direct Effect of Antibiotics on Propagating Contractile Clusters**

Firstly, we have shown that intraluminal perfusion with bacitracin, neomycin and penicillin V antibiotics, can evoke direct dose-dependent changes in ENS-dependent PCCs in both mouse colon and jejunal segments. Specifically, increases in colonic PCC velocity and frequency, and decreases in amplitude were observed following intraluminal perfusion with all three antibiotics. In the jejunum however, antibiotic-specific changes were identified. These changes were dose-dependent but not confounded by differences in baseline motility of segments tested at the different concentrations. Furthermore, responses of the gut microbiota are relatively minimal in these experiments as luminal contents were flushed before the recordings of motility, and because the antibiotics evoked rapid effects (with latencies as short as 5 minutes). Equally quick responses have been observed directly from neurons in the myenteric plexus following application of luminal stimuli to *ex vivo* intestinal preparations (Perez-Burgos et al., 2014). Moreover, in the presence of the neurotoxin TTX, the antibiotics did not produce any changes to SW-dependent propagating ripples suggesting that antibiotics may directly alter neuronal but not myogenic regulation of gastrointestinal motility.

There is evidence in support of direct non-antibacterial actions of antibiotics on neuronal modulation of gastrointestinal function. In one study, effects of lincosamide antibiotics on rabbit colonic mucosal function was investigated (Goldhill et al., 1996).

Gut mucosal epithelial ion transport in particular, is tightly regulated by intrinsic neuronal innervation. Addition of lincosamide antibiotics attenuated epithelial secretory response to electrical field stimulation, resulting from decreased activity of cholinergic secretomotor fibres. This observation is supported by previous findings that have shown how stimulation of submucosal neurons in the rabbit distal colon under similar conditions, increase epithelial chloride secretion – a response that was TTX-sensitive, requiring neuronal activation (Biagi, Wang, & Cooke, 1990). Furthermore, high dose erythromycin has been shown to depress nerve-mediated circular muscle contractions and the ascending excitatory reflex in guinea pig small intestine *in vitro* (Minocha and Galligan, 1991). The result has been attributed to inhibition of acetylcholine and substance P release from nerves of the myenteric plexus as a result of decreased calcium entry. This is consistent with a study conducted by Lees and Percy (1981), that demonstrated how increasing extracellular calcium concentrations can reverse inhibition of nerve-mediated contractions of guinea pig small intestine by aminoglycoside antibiotics.

Similarly, we believe that antibiotics may act directly on the membranes not only of the target gut bacteria but also of the enteric neurons, to produce the changes in gut motility we have observed. Although, the exact mechanism is unclear from our experiments, we predict that the antibiotics affect neuronal membrane integrity in a manner similar to that of the antifungal agent, amphotericin B. In fact, when high-dose amphotericin B, as well as β-lactam (ampicillin) and quinolone (ciprofloxacin) antibiotics, were separately tested on rat intestinal epithelial cells *in vitro*, all three compounds demonstrated similar cytotoxic effects on epithelial ion transport (Jornot et al., 2005; Upreti et al., 2008). Fortunately, the mechanism of action of amphotericin B has been extensively explored and so its biochemical influence on epithelial cell homeostasis is well-defined. In general, this compound is able to form pores on cellular membranes, allowing inflow of Na^+ (Jornot et al., 2005). In an effort to normalize the intracellular Na^+ concentrations, apical Na^+ channel activity is suppressed and the activity of sodium-potassium pumps is enhanced. Continuous or repeated exposure to amphotericin B over a

long period of time, can diminish intracellular Na^+ levels, resulting in an inhibition sodium-potassium pump activity and altered transcriptional activities. Along these lines, amphotericin B and other antibiotics, may impair neuronal membrane integrity to result in an influx of calcium ions from the calcium rich extracellular solution. If this is the case, then it is reasonable to infer that the absence of significant changes in PCC parameters at the low doses of antibiotics tested reflects insufficient effects on membrane excitability. This may be due to regulatory removal excess calcium ions by the mitochondria and endoplasmic reticulum (Upreti et al., 2008). The most significant changes to PCC parameters at intermediate doses of antibiotics may then reflect the effects of sustained increases in intracellular calcium levels on neuronal function. Finally, at the highest doses of antibiotics, the variation in changes to PCC parameters may be a result of antibiotics acting on calcium dependent ion channels or receptors that differ in their association with neuronal excitability and consequently motility. In some cases, at these high doses, clinical manifestations of neurotoxicity have been observed. For instance, the administration of macrolide and β-lactam antibiotics has been shown to supress postsynaptic responses of GABA so as to enhance neuronal excitability and lead to adverse effects on the CNS (i.e., epileptic convulsions, confusion) (Sugimoto et al., 2003; Chow et al., 2005; Bichler et al., 2016).

Although uncommon, the multi-phasic dose-response relationships demonstrated in our experiments have also been shown in studies involving toxicological stressors (Janiak et al., 2017; Kong et al., 2016), pharmaceutical drugs (Gill and Khanna, 1975; Afzal et al., 2016), and biologicals (Ozcan et al., 2011). In fact, antibiotics from many classes, including β-lactams, aminoglycosides, macrolides, fluoroquinolones and tetracyclines, have been shown to evoke hormetic-like responses (Kaplan, 2011). At low sub-inhibitory concentrations, beneficial effects on bacterial growth (Kendig et al., 2010), cellular responses (Davies et al., 2014; Gutierrez et al., 2013; Fajardo and Martínez, 2008) and biofilm formation (Linares et al., 2006; Kaplan, 2011), have been documented. Whereas,

bacterial cell growth inhibition and death have been demonstrated at higher concentrations.

Despite evidence from supporting studies, these preliminary experiments could not directly address the underlying mechanisms of cellular transduction and signalling between antibiotics and enteric neuronal circuits. Although the short latency effects are suggestive of direct neuronal interaction, it is not clear which enteric neurons may be involved. Electrophysiological recordings from enteric IPANs, likely key neuronal targets due to their sheer density in the gastrointestinal tract, following luminal application of the antibiotics should be conducted.

2. **Antibiotics Increase Vagal Afferent Firing Frequency**

Secondly, we have demonstrated that luminal application of high-dose bacitracin and penicillin V can produce short latency (about 8 mins) increases to baseline mesenteric vagal afferent firing frequency. Furthermore, these antibiotic evoked increases in vagal afferent discharge were diminished both partially and completely in the presence of the nicotinic acetylcholine receptor antagonist, mecamylamine. Therefore, it is likely that the observed antibiotic mediated effects on vagal afferent discharge depend on excitatory synaptic connection with intrinsic neurons of the ENS.

The ENS is one of the main divisions of the autonomic nervous system, encompassing several ganglionated plexuses including the submucous plexuses and the myenteric plexus (Spiller & Grundy, 2008). The myenteric plexus alone contains about 60% of all enteric neurons, including cell bodies of enteric IPANs, interneurons, excitatory and inhibitory motor neurons. Enteric IPANs in particular, engage in extensive projection networks and therefore are likely to provide the major excitatory output to all enteric neuronal circuits. Moreover, extrinsic afferent innervation of the gut comes from spinal and vagal afferent neurons. Spinal afferents are activated by low- and high-intensity

mechanical stimuli and carry nociceptive information about the gut. Vagal afferents on the other hand, are sensitive to chemical and low-intensity mechanical stimuli. Interestingly, recent studies have suggested that vagal IGLEs can synapse with neurons of the myenteric plexus (Perez-Burgos et al. 2014; Furness et al. 1998; Spiller and Grundy 2008). Thus, we speculate that the observed increases in vagal afferent discharge may result from antibiotic-mediated activation of mucosal sensory endings of myenteric IPANs and subsequent synaptic signal transduction with vagal IGLEs. Indeed, vagal afferents are sensitive to a variety of mediators that are released from IPAN cell bodies (Furness et al., 2004; Brehmer et al., 2004; Welch, Tamir, Gross et al., 2009), including acetylcholine (Perez-Burgos et al. 2014), substance P (Krowicki & Hornby, 2000) and oxytocin (Charpak, Armstrong, Muhlethaler et al. 1984).

Our results are also comparable to previous work by Perez-Burgos et al. (2014), who have shown that the effect of *L. rhamnosus,* was dependent on IPAN to vagus synaptic transmission. More specifically, authors demonstrated how luminal application of the bacteria increased both firing of enteric IPANs (Kunze et al., 2009) and vagal afferent firing rates (Perez-Burgos et al., 2013). When synaptic transmission was blocked with the Ca^{2+} channel blocker $CdCl_2$, Ca^{2+} channel blocker (N, P and Q-type) ω-conotoxins GVIA and MVIIC or with the nicotinic receptor blocker mecamylamine, the proportion of vagal afferent fibers once excited by *L. rhamnosus* was greatly reduced. In another animal study, gut luminal exposure to the probiotic *B. longum NCC3001* was associated with reduced excitability of enteric neurons in the myenteric plexus and reduced anxiety-like behaviour (Bercik et al., 2011). Such behavioural outcomes were absent however in mice that had been vagotomised. Collectively, these findings provide evidence for the potential of synaptic transmission of sensory information from enteric neurons to vagal pathways.

The vagal primary afferent fibres that were still able to respond to luminal bacitracin in the presence of mecamylamine may represent a portion of fibers with terminals in the mucosa that can be activated directly by chemical and mechanical stimuli, or vagal units

able to engage in sensory synapse through other vagal receptors (Perez-Burgos et al., 2014). First, studies have shown direct vagal stimulation by luminal commensals, forgoing any synaptic transmission. For instance, luminal butyrate has been shown to depend solely on enteroendocrine release of CCK to activate vagal afferents (Lal et al., 2001). It is not clear however, whether antibiotics can activate these vagal EPAN terminals directly (through ion channel opening) or whether there are specific sensory mediators involved in signal transduction. If not directly stimulated, epithelial and ECs release a wide range of sensory mediators including 5-HT, CCK, somatostatin, GABA, ATP, leptin, orexin, or incretin hormones that might play a role in activating these vagal afferents (Perez-Burgos et al. 2014). In addition, gastrointestinal vagal EPANs have several chemoreceptors including the CCK, 5-HT_3, $GABA_B$, κ opioid, NK-1, and purinergic receptors (Blackshaw & Grundy, 1990, 1993; Krowicki & Hornby, 2000; Ozaki, Sengupta, & Gebhart, 2000; Page & Blackshaw, 1999; Page, O'Donnell, & Blackshaw, 2000), that may also facilitate signal transduction. Thus, future studies are needed to test other potential vagal receptors involved in transmitting signals from luminal antibiotic exposure. It may prove useful for instance, to test whether a NK-1 receptor antagonist also diminishes vagal afferent firing as it has been shown that intestinal concentration of substance P increases following antibiotic application (Verdú et al., 2006).

Furthermore, it remains to be elucidated exactly how the increase in the constitutive vagal firing frequency may affect homeostatic regulation of brain neurotransmitters that maintain normal behaviour and brain chemistry. However, increases in vagal afferent activity has been implicated with changes in the concentrations of serotonin, norepinephrine, GABA, and glutamate within brainstem and higher centers that regulate interoception, and emotional well-being (Grundy and Schemann 2007; Perez-Burgos et al. 2013). In an animal study employing high-dose antibiotic cocktails consisting of neomycin, bacitracin, and pimaricin to disturb the microbiome, increases in exploratory behaviour and enhances in BDNF expression in the hippocampus of mice were reported

(Bercik et al., 2011). Similar anxiolytic effects were also seen in another animal study employing a cocktail of neomycin, cefoperazone and ampicillin antibiotics in mice (Farzi, Gorkiewicz, & Holzer, 2012). In clinical studies, administration of rifaximin (550 mg orally, twice a day) for 8 weeks in patients diagnosed with subclinical hepatic encephalopathy, revealed cognitive improvement and increased brain activity in specific fronto-parietal regions (Ahluwalia et al., 2014). These results were validated by another clinical study conducted by Bajaj et al. (2013), who employed the same methods and also observed improved cognitive performance after the rifamaxin treatment. While these studies have focused on the role of the gut microbiome in these alterations to homeostatic regulation of brain neurochemistry, our findings suggest that the ENS and extrinsic vagal afferents in particular, may also play a role. Decoding neuronal spike trains transmitted to the brain is necessary for understanding how the information carried by vagal firing frequency and firing patterns impacts brain function.

Chapter 5: Concluding Remarks

Adverse clinical effects of antibiotics on gut immunity (Aguilera et al., 2015; Verdú et al., 2006), cognitive function (Cryan et al., 2012; Desbonnet et al., 2015; Farzi et al., 2012; Fröhlich et al., 2016; Lurie et al., 2015; Möhle et al., 2016; Rogers et al., 2016), and psychological well-being (Bichler et al., 2017; Reeves, 1992; Weis, Karagülle, Kornhuber, & Bayerlein, 2006) have become increasingly common. Patients who have renal or liver insufficiency, compromised blood brain barrier permeability, high-dose and/or chronic treatment plans or advanced age, are especially at risk of developing such antibiotic-induced clinical manifestations (Grill & Maganti, 2011). The underlying mechanisms however, remain debatable. Many experimental studies have focused on how high-dose antibiotic-mediated reductions in the gut microbiota contribute to such neurophysiological and behavioural consequences (Bercik et al., 2011; Verdú et al., 2006; O'Mahony et al., 2014; Desbonnet et al., 2015; Aguilera et al., 2015; Cryan and Dinan, 2012; Fröhlich et al., 2016; Rogers et al., 2016; Lurie et al., 2015; Möhle et al., 2016; Tochitani et al., 2016). These studies however, have neglected evidence that antibiotics might also act directly on the host nervous system (Bichler et al. 2016; Goldhill et al. 1996; Minocha and Galligan 1991; Tamaoki et al. 1995). Therefore, we aimed to investigate whether high-dose antibiotics, as used in prior experimental models that have investigated the role of the gut microbiome in antibiotic-mediated pathogenesis, can also induce negative health outcomes by direct interaction with enteric neuronal circuits. This work focused on the following two questions:

1. Does acute exposure of the gastrointestinal lumen to antibiotics directly modulate enteric neurons and neuronal reflexes with consequences for gut motility?

2. Does acute exposure of the gastrointestinal lumen to antibiotics alter responses of vagal afferent pathways?

Our findings have shown that acute luminal exposure to high-dose antibiotics can not only evoke direct changes in spatiotemporal characteristics of ENS-dependent PCCs but can also affect responses of vagal afferent fibers from the mesenteric nerve bundle. Taken together, this work suggests that antibiotics directly signal to enteric IPANs, which locally regulate motility reflexes and through sensory synapses can convey those signals to vagal IGLEs, to influence homeostatic regulation of brain functions. Indeed, a comprehensive understanding of antibiotic-associated pathophysiology requires considerable further investigation of the ENS at several levels. Future studies may include close examination of how antibiotic exposure produces direct changes in enteric IPAN excitability, and how these changes contribute to alterations in synaptic transmission with vagal afferents and to changes in neuronally coded messages sent to the brain. If accomplished, these studies can offer novel approaches in the development of antibiotic therapies for mental health and anxiety-related behaviours. Nevertheless, our current data highlight the potential for high-dose antibiotics to directly affect the host nervous system. Therefore, experimental studies attributing the effects of high-dose antibiotics on brain function and behaviour solely to alterations in the gut microbiome, should be carefully interpreted.

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