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MICROBIAL ENTERIC NEUROPHYSIOLOGY

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SUMMARY

The myenteric plexus contains a type of neuron that innervates the mucosa, and projects to all other classes of myenteric neurons. This neuron has a specific multipolar morphotype and possesses a characteristic action potential whose firing frequency is moderated a potassium current dependent post-spike slow after-hyperpolarisation. The neuron, termed AH cell, is functionally an intrinsic intestinal primary afferent neuron (IPAN) with chemosensory and mechano-sensory responses to adequate stimuli. With about 80% of sensory neuropeptide containing nerve fibres in the mucosa belonging to IPANs they are ideally positioned to respond to signals from commensal or probiotic gut bacteria. Application of *Lactobacillus rhamnosus* JB-1 or *Bacteroides fragilis* to the mucosal epithelium evokes sensory action potentials in the IPANs within a few seconds of administering the bacteria. In the case of *B. fragilis*, a capsular polysaccharide A is necessary and sufficient to carry the signal from microbe to neuron. This sensory activation leads to a longer-term increase in the intrinsic excitability of the IPAN sensory neuron network caused at least in part by a decrease in a calcium dependent potassium conductance. We have identified a molecular and neuronal target of commensal signalling. The neuron's rapid sensory responses may activate the neuronal circuits that mediate some of the motility and central nervous system effects of ingesting beneficial microorganisms.

INTRODUCTION

It may be time to consider the idea of probiotic or microbial neurophysiology. This notion derives from observations that microbes, especially symbiotic, commensal or probiotic bacteria, can change how the host nervous system functions. The concept has parallels to other constructs such as microbial endocrinology (*Lyte*, 2010, 2011; *Roshchina*, 2010) or microbial immunology (*Artis*, 2008; *O'Hara* and *Shanahan*, 2006) that seek to elucidate how the host systems interact with the microflora. It is tacitly recognised that within the whole animal, or even for individual organs, there must be reciprocal interplay between these systems. A simple example of this is if behavioural stress (nervous system) and increased stress hormones (endocrine system) co-exist to alter the constituents of the microbiome (see *Lyte*, 2011). Within the enteric nervous system, neuronal behaviour may be changed by luminal microbes, mast cell products or inflammation or paracrine

hormones (*Nurgali* et al., 2011; *Furness* and *Poole*, 2011; *Buhner* and *Schemann*, 2012; *Sundler* et al., 1989; *Ahlman* and *Nilsson*, 2001).

The gut microbiome forms a complex ecosystem with its associated "metabolome" producing myriads of metabolites including chemical messengers and foodstuffs. The totality of this microbial organ has profound effects on the host during development and adult life. Such effects include education of the immune system and the normal development of the gut-brain axis. Much of this knowledge has been deduced from work with germ-free animals or alterations of the microbiome using antibiotics (*Collins* and *Bercik*, 2009; *Bienenstock* and *Collins*, 2010; *Heijtz* et al., 2011). Such major experimental perturbations cannot unaided explain the remarkable observation that ingesting about 1 billion cells of a single probiotic strain (that may or may not be a commensal) can have a therapeutic effect (*Moayyedi* et al., 2010) on an adult who at the same time carries 100 trillion or more gut commensal organisms.

The FDA defines beneficial bacteria that are intended to prevent or help treat disease as drugs (*Hoffman*, 2008). Homeostatic organ responses to repeated dosing with probiotics may mask the underlying physiological mechanisms responsible for the direct neuronal action of active probiotic derived ligand(s) (*Dykstra* et al., 2011). In addition, pharmacological tolerance (*Koch* and *Höllt*, 2008), sensitisation and priming (*Kostrzewa*, 1995) are potential additional confounding factors. To clarify the mechanisms of action of beneficial bacteria, quantitative measurements of their acute actions on individual neurons and other target cells should be made. In the same way, the mechanisms of action of opium, antidepressant or anxiolytic plants, or other biologics could not have been deduced solely from their long-term chronic actions.

DO COMMENSALS, PROBIOTICS ACT ON THE NERVOUS SYSTEM?

Even in the absence of overt inflammation, probiotics may influence behaviour or alter brain chemistry. Some of the symptoms of chronic fatigue syndrome have been reported to be decreased by ingestion of a Shirota strain of *Lactobacillus casei* (*Rao* et al., 2009). Ingestion of a *Lactobacillus rhamnosus* strain (JB-1) had antidepressive and anti-anxiety effects (*Bravo* et al., 2011). On the other hand, *Citrobacter rodentium*, when given at a dose that produces no inflammation or increase in plasma inflammatory cytokines, induced anxiety in mice (*Lyte* et al., 2006). A formulation of *Lactobacillus helveticus* plus *Bifidobacterium longum* reduced anxiety in a rat model and decreased anxiety scores in humans (*Messaoudi* et al., 2011).

Probiotics can modulate physiological parameters controlled by the autonomic nervous system. Intra-duodenal injection of *Lactobacillus johnsonii* (La1) has been shown to reduce systemic and renal blood pressure in rats (*Yamano* et al., 2006; *Tanida* et al., 2005) within 15 min of injection. Feeding milk fermented with *Lactobacillus helveticus* appeared to decrease ambulatory blood pressure in hypertensive patients (*Jauhiainen* et al., 2005; *Aihara* et al., 2005). There is also evidence that certain probiotic strains moderate gut migrating motor complexes (MMCs) (see below).

It is possible that the behavioural and autonomic effects ascribed to pro-

biotic ingestion could be caused by alterations in the immune status of peripheral tissue or the release of circulating hormones (*Bansal* et al., 2010; *Bercik* et al., 2012). Another explanation, not mutually exclusive, is that primary afferent neurites are activated within the gut wall and these then transmit synaptically to the higher order autonomic neurons whose processing modulate peripheral autonomic reflexes or limbic system function centrally. To resolve this question, it will be important to determine if neurons in the intestine are in fact direct or early targets of probiotic application. Early perturbations of primary afferent neuron firing by commensals could also underlie later longer-term effects on immune, endocrine systems (*Logan* and *Katzman*, 2005; *Lyte*, 2011).

SPINAL PRIMARY AFFERENTS

Spinal primary afferent neurons have their soma in the dorsal root ganglia that lie on either side of the spinal cord. Gut spinal primary afferents have sensory terminals that ramify throughout the width of the gastrointestinal wall where they are activated by mechanical or chemical stimuli, generally of a nociceptive nature (*Blackshaw* et al., 2007).

Some probiotic strains attenuate afferent pain signals. Feeding a non-absorbable antibiotic to decrease colon *Lactobacillus* species has been reported to increase thresholds of pseudo-affective pain responses to colorectal distension (CRD) (*Verdu* et al., 2006), suggesting a possible role of these species in visceral pain transmission or transduction. In a more strain-specific study, *Kamiya* et al. (2006) showed that 9 day feeding of JB-1 to rats decreased pain responses to CRD, and that this was paralleled by a reduction of CRD evoked increases in spinal dorsal root single unit firing. It is important to realise that the anti-nociceptive action of JB-1 occurred in the absence of experimentally induced or a detectable peripheral inflammation. Therefore, even if the bacterium exerted anti-inflammatory actions on the host, this may not explain its pain-suppressing ability. Similarly, feeding *L. acidophilus* (NCFM) reduced pseudo-affective responses to CRD in rats, and this was accompanied by an increase in opioid and cannabinoid receptor expression in mucosal epithelial cells (*Rousseaux* et al., 2007). It is not clear how such receptors in epithelial cells could alter firing in nociceptive neurons.

Probiotic bacteria may be able to block activity-dependent sensitisation of pain pathways. Afferent pain pathways can be sensitised by repeated painful stimuli (*Woolf* and *Salter*, 2000). Such sensitisation represents a form of cellular memory of pain, and is implicated in maintaining pathological pain states (*Woolf* and *Salter*, 2000). CRD induces greater than normal substance P expression in rat DRG neurons that persists at least 24 h after the distensions (*Lu* et al., 2005). Functionally, the substance P overexpression was accompanied by hyper-excitability in the pseudo-affective response to CRD (*Ma* et al., 2009). It is noteworthy that this type of sensitisation occurred in the absence of histological or chemical markers for inflammation. Prior feeding of JB-1 was able to block this nociceptive sensitisation (*Ma* et al., 2009).

INTESTINAL PRIMARY AFFERENT NEURONS

The putative intestinal primary afferent neuron (IPAN) is a plausible target for beneficial microbes. IPANs are part of the enteric nervous system, which, long regarded as a part of the parasympathetic division of the autonomic nervous system, is now considered to be a third independent division (*Gershon*, 1999). It has been acknowledged that the gut can perform its motor and secretory roles in the absence of connections with nervous systems extrinsic to the gut. This has led to the proposition (*Furness* et al., 1998) that there are neurons (IPANs), with somata within the wall of the intestine, whose activation by chemo- or mechano-sensory stimuli lead to propulsive reflexes. Patterned on-going activity in networks of IPANs contributes to motor or secreto-motor programs that produce propulsive MMCs, and possibly stationary motor complexes that result in mixing (*Gwynne* and *Bornstein*, 2007; *Bornstein* et al., 2002, 2004). Yet, whether IPANs truly exist, or what their identity might be, and if the apparent autonomy of the gut results from axon reflexes of extrinsic primary afferent fibres embedded in the gut wall is still under discussion.

IPANs were first anticipated because of the morphology and physiology of a specific type of myenteric neuron. Such neurons have a large flattened oval soma and multiple long neurites that innervate adjacent myenteric ganglia and the epithelial cell layer of the mucosa (Dogiel Type II morphology). This morphotype led *Dogiel* (1899) to propose that the neurons were sensory in function. David Hirst (*Hirst* et al., 1972; *Hirst* and *Spence*, 1973; *Hirst* et al., 1974) found that the action potentials of Dogiel type II neurons were broad with a calcium hump on their repolarising phase. Significantly, because of its effects on firing patterns, the action potential was followed by a slow inhibitory afterhyperpolarisation (sAHP) lasting several seconds (*Hirst* et al., 1985, 1974). Such action potential characteristics are very similar to those of unmyelinated spinal DRG neurons (*Hay* and *Kunze*, 1994; *Schild* et al., 1994). These putative IPANs, termed "AH cells" by Hirst, did not appear to receive the classical fast (inotropic) synaptic input that characterises ganglionic inter- and motor- or relay-neurons (*Hirst* et al., 1974). The absence of synaptic input from other neurons suggested that the neurons are by default sensory. Hirst argued that if AH cells were not activated by other neurons they must be the first or primary afferent neuron whose activation arises from sensory stimuli (*Hirst* et al., 1974). Interneurons or motorneurons would necessarily receive synaptic input from sensory or other interneurons. Later, *Kirchgessner* et al. (1992) blocked nicotinic transmission in the submucosal plexus and then mechanically stimulated the mucosa. They reported enhanced c-fos expression in some neurons, even under nicotinic receptor blockade. Lack of nicotinic synaptic input has been taken as proof that some submucosal neurons are primary afferent by *Furness* (2006a). Against this, there remained the possibility of non-nicotinic synaptic transmission in the submucosal plexus.

SENSORY RESPONSES TO CHEMICAL LUMINAL AND MECHANICAL STIMULI

The “classic schema” has been that luminal chemicals and bacterial products are detected by the immune system which then signals via the release of mediators to the enteric nervous system (*Cooke*, 1994). Experimental data, however, suggest that myenteric AH cells can respond directly to luminal chemicals with varying degrees of involvement enteroendocrine cells (EECs) playing the role of "taste cells" (*Bertrand*, 2009). Orthodromic action potentials can be recorded from guinea pig myenteric AH cells in response to applying brief puffs of HCl to the mucosal epithelium (*Kunze* et al., 1995). "Orthodromic" means travelling in the "right" or normal direction; for a peripheral sensory neuron action potential this is from the receptive field towards the soma and, in the case an AH cell, from the mucosal epithelium to the myenteric plexus. AH cells were shown to be IPANs when the response to HCl persisted even if all synaptic transmission was blocked by removing extracellular calcium and raising extracellular magnesium tenfold (*Kunze* et al., 1995). Since the response could not have come from other neurons, it either must have been generated by sensory transduction occurring in the neuronal endings near the lumen or in closely associated mucosal entero-endocrine "taste" cells (*Bertrand*, 2003, 2009). Sensory responses have also been recorded in myenteric AH cells in response to epithelial applications of short chain fatty acids for mouse small intestine (*Mao* et al., 2006) and rat colon (*Kunze* et al., 2009).

AH cells are additionally mechano-sensitive neurons responding to tension. Generator and action potentials have been recorded from myenteric AH cells when their processes within the ganglia are distorted. In contrast, compression of the soma inhibits firing (*Kunze* et al., 2000). Such responses were recorded during total synaptic blockade establishing their primary afferent nature. Active muscle contraction was required for these responses to occur, and passive stretch with paralysed muscle could not evoke them (*Kunze* et al., 1998). Thus, tensions sensitive mechano-sensitive IPANs are also Dogiel type II AH cells (*Kunze* et al., 1999, 2000). Sub-modalities and multimodal properties have been ascribed to IPANs (*Mayer*, 2011); so far, experimental evidence for this is lacking.

Mechano-sensory responses have also been recorded from significant proportions of myenteric S cells (so named because they receive prominent fast synaptic input) (*Schemann* and *Mazzuoli*, 2010; *Mazzuoli* and *Schemann*, 2009; *Spencer* and *Smith*, 2004), which have been previously been assumed to be inter-or motor-neurons within the enteric circuits (*Nurgali* et al., 2004; *Furness* et al., 1998, 2004; *Kunze* et al., 1999). These responses occurred even when the muscle was paralysed with nicardipine, suggesting that these S cells are stretch rather than tension receptors. Since MMCs require active muscle contraction for their initiation (*Lüderitz*, 1891) it is not easy to know what might be the functional role of these mechano-sensory S cells.

The promiscuous proliferation in the literature of putative intrinsic gut primary afferent neurons has not gone without criticism (see: *Wood*, 2008). It is argued that intramural vagal or spinal axon reflexes are sufficient to account for the functional independence of the gut (*Christofi* and *Wood*, 1993; *Wood*, 2008). Peristaltic reflexes that persist in

ex vivo gut segments are proposed to be mediated by the severed stumps of extrinsic sensory fibres that remain in the organ wall. Also, the idea that the enteric nervous system contains its own primary afferent neurons has been rejected on principles of evolutionary parsimony (*Christofi* and *Wood*, 1993; *Wood*, 2008). It is suggested that extrinsic vagal and spinal primary afferents would suffice for all the sensory innervation of the gut. However, the idea that in biology evolution designs the simplest system for a particular function (ontological parsimony) has been refuted many times (for a discussion of the general issue and a distinction between ontological and methodological parsimony see for example: *Crisci*, 1982). Occam's razor (in the form of ontological parsimony) cannot reasonably be used as an argument against the existence of IPANs. Finally, *Wood* (2008) has argued that since chemosensitive AH cells appear to require sensory transduction from specialised entero-endocrine cells, they cannot be primary afferent neurons. If this argument is granted, then the lingual nerve, for example, which appears to require type 4 taste cells for some of its responses would not be primary afferent.

RESPONSES TO LUMINAL PROBIOTIC OR COMMENSAL BACTERIA

A key function of chemosensory IPANs may be to monitor and respond to the microbiome and its metabolome. There are 500 million enteric neurons in humans and 500,000 in mice. Dogiel Type II/AH cells make up 15 to 20% of the total neurons (*Kunze* et al., 1999; *Furness* et al., 1998). An important teleological question arises; why would the gut have so many chemosensory neurons all of which innervate the mucosa (*Song* et al., 1994)? The total numbers of cells contributing to the microbiome is not known with certainty, but the concentrations of dominant commensal genera can reach 8 log cfu/ml in the small, and 11 log cfu/ml in the large intestine (*Tappenden* and *Deutsch*, 2007; *Reuter*, 2001). By far the richest innervation (compared to spinal or vagal afferents) for mucosal epithelial layer cells derives from the myenteric plexus, which provides in excess of 90% of sensory neuropeptide containing fibres to the mucosal layer (*Ekblad* et al., 1987; *Keast* et al., 1984). Each enteric AH neuron innervates 80-120 villi (*Kunze* et al., 1999) and there are about 500,000 neuropeptide (calcitonin gene related peptide, CGRP) containing AH cells in the mouse (*Furness*, 2006b). Therefore, IPANs are ideally positioned to sample signals from the microbiome and ingested beneficial bacteria; possibly having evolved just so, to monitor this large source of metabolites and foreign DNA.

There is experimental evidence that probiotics influence myenteric IPANs. Using a modified Trendelenburg *ex vivo* gut segment preparation to record intraluminal pressure, MMCs were reduced 50% in amplitude 9-16 min after 7.7 log cfu/ml (*Wang* et al, 2010a) were introduced into the lumen. Nine day ingestion 9 log cfu JB-1 similarly reduced MMC amplitudes (*Wang* et al., 2010b). Since the MMC were blocked by the neuron sodium channel blocker tetrodotoxin (TTX) (*Wang* et al., 2010a, 2010b), these results suggest that the probiotic acted on enteric neurons. Slow wave related contractions persisted when all neural activity was blocked with tetrodotoxin (*Wang* et al.,

2010a, 2010b) were not altered by JB-1. Kamm and colleagues fed 7.3 log cfu/g of *Saccharomyces boulardii* per day to pigs for 9 days, after which jejunal myenteric neurons were assayed histochemically for chemical markers known to correlate with major functional subpopulations within the enteric nervous system (ENS) (*Kamm* et al., 2004). The authors reported decreased expression of the vitamin D dependent calcium binding protein, calbindin, in myenteric Dogiel Type II neurons (*Kamm* et al., 2004; *Jungbauer* et al., 2006). They also assayed for choline acetyltransferase, substance P, calcitonin gene-related peptide, vasoactive intestinal polypeptide, nitric oxide synthase and the calcium binding protein calretinin. These chemicals identify AH cell/Dogiel Type II neurons or each of the major classes of inhibitory and excitatory motor neurons or interneurons (S cells). For none of these chemicals (except for calbindin) was there a change in the amount expressed in the ENS, demonstrating what appears to be a high functional selectivity in perturbation of the ENS by the fungus. (*Kamm* et al., 2004; *Jungbauer* et al., 2006). Nine day feeding of 9 log cfu per day of JB-1 increased the intrinsic excitability (decreased firing thresholds and increased number of action potentials fired during a standard stimulus current pulse) of rat colon myenteric IPANs. S cell (inter- and motor-neuron) excitability was unaffected (*Kunze* et al., 2009). Since myenteric IPAN\Dogiel Type II cells, but not S cells, innervate the mucosa (*Furness* et al., 1998; *Bertrand*, 2004; *Song* et al., 1994) it is likely that for each ingestion, IPANs are the first type of neuron to be activated (*Furness* et al., 1999; *Kunze* et al., 1995). Then, the increase in IPAN firing is transmitted to S cells by metabotropic synaptic transmission (*Kunze* et al., 1993). Such daily activation of IPANs would cause them to evoke transient slow postsynaptic potentials and firing in their target S cells (*Kunze* et al., 1993). IPANs (*Clerc* et al., 1999) but not S cells (*Alex* et al., 2002) have a form of activity dependent long-lasting memory that is entrained by their repeated, frequency dependent, excitation. This may be why only IPANs were found to have heightened intrinsic excitability after 9 day feeding with JB-1. That is, inter- or motor-neurons may have been activated during and after presentation of the bacteria to the mucosal epithelial surface but this effect would have waned when the intestine was excised for subsequently electrophysiological analysis. IPANs have the ability to induce long-term potentiation of their excitability many hours beyond the duration of their sensory stimulation (*Clerc* et al., 1999). How long such potentiation could ultimately last, and what are the optimal intervals between probiotic ingestion periods, are questions that require further research and may well be specific for individual probiotic strains and host species and gut regionalisation.

IPAN ION CHANNEL TARGETS FOR PROBIOTIC ACTION

IPANs excitability and discharge properties are determined by a complex interaction of membrane ion channel currents. In general, and with the exception of the pig, myenteric AH cells have electrophysiological properties that are well conserved from mouse to man (*Mao* et al., 2006). The action potential upstroke, which is generated by transient and persisting Na^+ (*Zholos* et

al., 2002; *Rugiero* et al., 2002, 2003), and N (and R) -type Ca^{2+} currents (*Rugiero* et al., 2002; *Bian* et al., 2004), is followed by a fast (fAHP) and then a slow (sAHP) after-hyperpolarisation. The fAHP is generated by a mixture of voltage sensitive K^+ currents including the delayed rectifier, an A current (*Starodub* and *Wood*, 2000) and a Ca^{2+} dependent large conductance K^+ conductance (*Kunze* et al., 2000; *Vogalis* et al., 2002). The sAHP is produced by the generation an intermediate conductance Ca^{2+} dependent K^+ (IK_{Ca}) current and is opposed by a coincident hyperpolarisation activated cationic current (I_h) (*Mao* et al., 2006). The duration and frequency of action potential firing are determined by the sAHP and fAHP, I_h and the inactivation characteristics of the Na^+ currents. Action potential firing thresholds depend on the activation characteristics of Na^+ currents and on the total plasmalemma leak conductance. The resting membrane potential is determined by various background or leak conductances, which include I_h , IK_{Ca} current, Na+ window currents and at least one type of tandem pore K^+ channel current (*Matsuyama* et al., 2008).

The IK_{Ca} current underlying sAHP in IPANs is at present the most plausible target for JB-1 although other bacteria might act on any other or combination of other IPAN ion channels. The reduction in MMC amplitude by JB-1 (see above) was reproduced within 5-15 min (see for example Figure 4 in *Wang* et al, 2010a and Figure 3 in *Wang* et al, 2010b) of adding the specific intermediate conductance Ca^{2+} dependent K^+ (IK_{Ca}) channel blocker TRAM-34 to the Krebs buffer superfusing segments of rat colon or mouse small intestine (*Wang* et al., 2010b). In the absence of overt inflammation, only IPANs (AH cells) but not inter- or motor-neurons (S cells) express functional IK_{Ca} channels. We deduce that the probiotic altered motility by altering IPAN function, probably by decreasing the IK_{Ca} dependent inhibitory slow after-hyperpolarisation current (*Wang* et al., 2010a, 2010b). It is important to note that a similar effect on MMCs was produced for rat colon after log 9 cfu JB-1 was fed to the animals daily for 9 days (*Wang* et al., 2010b), suggesting IK_{Ca} may be a probiotic target for both acute and repeated probiotic applications and for more than one host species.

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