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GUT BACTERIA, THE VAGUS NERVE AND THE BRAIN

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SUMMARY

There is now strong evidence from animal studies that gut microorganisms can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain and behaviour. The vagus can differentiate between commensal and potentially pathogenic bacteria even in the absence of overt inflammation and vagal afferent signals can induce both anxiogenic and anxiolytic effects depending on the nature of the stimulus. In addition to direct afferent signalling to the brain circuitry associated with stress and anxiety there are well described anti-inflammatory efferent responses that may contribute to behavioural effects of vagal stimulation in certain circumstances. Advances in our understanding of the microbiome-gut-brain axis will come from studies of how distinct microbial stimuli activate the vagus and the nature of the signals transmitted to the brain that lead to differential neurochemical changes that subsequently drive distinct behavioural changes.

THE VAGUS NERVE

The vagus (10^{th} cranial nerve) innervates the pharynx, larynx and visceral organs. It contains more afferent than efferent nerve fibres and projects from the medulla oblongata in the brain stem to the colon. Indeed, the vagus nerve is the main afferent pathway from the abdominal cavity to the brain. Information from the heart, lungs, pancreas, liver, stomach and intestines are delivered tonically to the brain via sensory fibres in the vagus nerve (*Browning* and *Mendelowitz*, 2003). Sensory vagal inputs arrive in the nucleus of the solitary tract (NTS), and are thence transmitted to widespread areas of the CNS, including the cerebral cortex and medulla oblongata. Neurones of the rostral ventrolateral medulla oblongata (RVLM) provide one of two major sources of afferent inputs to the locus coeruleus (*Aston-Jones* et al., 1986), which in turn projects to areas of the cortex that are associated with stress-related behaviour and affective disorders. The locus coeruleus is also considered a major site for integrating stress-responses (*Aston-Jones* et al., 1996). Following repeated activations, a feed-forward system between noradrenergic locus coeruleus neurones and areas of the forebrain that produce corticotropin-releasing factor (CRF) can lead to altered behavioural responses (*Ziegler* et al., 1999). Chronic activation of this system induces changes in neuronal activity that underlies anxiety, panic disorders and depression (*Arborelius* et al., 1999). The concept of interoception and experimental data suggesting that changes in visceral sensation can affect the perception and interpretation of external inputs (*Crucian* et al., 2000) has

lead to the suggestion that altered sensory vagal inputs can influence our attitude to the outside world and that pathological changes in sensory vagal inputs may increase the risk of affective behavioural disorders. It has been proposed that chronic sensory vagal inputs could act as 'natural' breaks for augmentation of stress-related behavioural responses via tonic modulation of the neuronal activity in the locus coeruleus and in turn the forebrain (*Zagon*, 2001).

THE VAGUS AND SICKNESS BEHAVIOUR

Some of the earliest indication of the role of the vagus in modulating behaviour came from studies of animals exposed to endotoxin. Sickness behaviour is a term used to describe the drastic changes in behaviour that occur in physically ill patients and animals, and is a motivational state responsible for re-organizing perceptions and actions to enable ill individuals to cope better with infection (*Dantzer* et al., 2000). The associated behaviours include lethargy, depression, anxiety, and loss of appetite, sleepiness, hyperalgesia, and reduction in grooming. These behavioural changes are mediated by pro-inflammatory cytokines particularly IL-1β and TNF (*Dantzer* et al., 2000).

The role of vagal afferents in the induction of sickness behaviour following intraperitoneal administration of the cytokine inducer lipopolysaccharide (LPS) or IL-1 β has been assessed in laboratory animals that have been submitted to subdiaphragmic vagotomy (*Luheshi* et al., 2000; *Konsman* et al., 2000). In these experiments carried out in rats and mice, sickness behaviour was measured by decreased social exploration and by depressed operant responding for a food reward in mice that had been trained to repeatedly poke their noses into a hole for getting a food pellet. After recovery from surgery, a dose of LPS or IL-1 β that induced consistent sickness behaviour in sham-operated animals was no longer able to decrease social exploration in rats and mice (*Laye* et al., 1995; *Bluthe* et al., 1994). In the same manner, vagotomy blocked the depressing effects of LPS on food-motivated behaviour in mice (*Bret-Dibat* et al., 1995).

VAGAL STIMULATION AS AN ANTI-DEPRESSANT

While vagal activation by cytokines has been associated with sickness and depressive type behaviour it is also emerging that stimulating the vagus can lead to a reduction in anxiety and depression associated behaviours. In one study, rats were administered 30 minutes per day of continuous vagus nerve stimulation for 4 days, and were then subjected to the forced swim test, a well validated assessment of antidepressant activity. Vagus nerve stimulation significantly reduced immobility time compared to unstimulated controls, reflective of antidepressant effects (*Krahl* et al., 2004). Interestingly, vagal nerve stimulation-induced decreases in immobility were associated with increased swimming behaviour, which has been linked to a predominantly serotonergic mechanism of action *Cryan* et al., 2005). In a subsequent controlled trial, rats received desipramine or vagal nerve stimulation for 2 h at three time points over a 24 hour period, prior to undergoing the

forced swim test and both treatments resulted in reduced immobility compared to saline control (*Cunningham* et al., 2008). However, chronic vagal nerve stimulation for 1 month failed to show any behavioural alterations in rats subjected to the forced swim test or the elevated plus maze test, in contrast to treatment with another classical antidepressant, imipramine (*Biggio* et al., 2009). Vagal stimulation is an FDA accepted alternative treatment for intractable depression and has also been used successfully in the treatment of refractory epilepsy, demonstrating clear behavioural effects of modulating vagal afferent signals (*Walsh* and *Kling*, 2004). While this treatment is controversial, largely due to a lack of positive sham treatment controlled clinical trials, there have been reports that vagal nerve stimulation is beneficial in at least some patients with depression and may be particularly effective with chronic treatment (*Rizvi* et al., 2011; *Martin* and *Martin-Sanchez*, 2012).

VAGAL INFLAMMATORY REFLEX

The vagus innervates tissues known to participate in immune functions and/or contain important immune elements, such as thymus, lung, liver, and gastrointestinal tract. Furthermore, trunks or branches of the vagus are often associated with lymph nodes that drain regions in which immune activation occurs. It has recently been identified that the vagus plays a critical role in a neural circuit that controls the inflammatory response in a reflex-like manner. The vagus nerve senses inflammation sending afferent signals to the brain that then activates an efferent response, releasing mediators including acetylcholine that, through an interaction with immune cells, attenuates inflammation.

Tracey and colleagues first highlighted the anti-inflammatory role of the vagus demonstrating that direct electrical stimulation of the peripheral vagus nerve in vivo during lethal endotoxaemia in rats prevented the development of shock through the inhibition of TNF synthesis (*Borovikova* et al., 2000). The vagus nerve also plays a counter-inflammatory role in the experimental colitis (*Ghia* et al., 2006). Macrophages have been identified as the major source of TNF during endotoxaemia, and are suggested to be the main target of the anti-inflammatory function of the vagus nerve in a murine model of inflammatory bowel disease (*Ghia* et al., 2006).

In addition to suppressive effects on macrophages the vagus nerve also acts to regulate T cell function. *O'Mahony* et al. (2009) demonstrated that transfer of $CD4^+$ T cells from vagotomised donors into non-vagotomised with DSS induced colitis reduced the number of splenic $Foxp3^+$ regulatory T cells in recipient animals, and was associated with aggravated disease symptoms mimicking the effects of vagotomy on colitis. Subdiaphragmatic vagotomy leads to a dramatic increase in T cell proliferation and production of inflammatory cytokines when compared to cells from sham-operated animals (*Karimi* et al., 2010). The effect of vagotomy is not limited to the spleen as lymphocytes isolated from the mesenteric lymph nodes also demonstrated a significant increase in inflammatory cytokine production. Overall this data suggests that $CD4^+$ T cell are also under tonic inhibitory control from the vagus. This anti-inflammatory efferent response may, in certain circumstances play a

role in mediating the anti-depressive effects of vagal nerve stimulation.

Immune system dysfunction has also been linked to depression (*Connor* and *Leonard*, 1998; *Miller* et al., 2009; *Li* et al., 2011). Approximately one-third of people with depression, without co-morbid disease, have higher levels of inflammatory markers compared with the normal, non-depressed population. Furthermore, inflammatory illnesses are associated with greater rates of major depression while patients treated with cytokines for various illnesses are at increased risk of developing major depressive illness. Conversely, successful treatment with an antidepressant decreases levels of pro-inflammatory cytokines such as IL-6 and TNF (*Capuron* and *Dantzer*, 2003; *O'Brien* et al., 2007; *Hernandez* et al., 2008). While it is as yet unclear whether neurostimulation therapies for depression affect immune function, there is evidence in vagal nerve stimulation treated epilepsy patients that pro-inflammatory cytokine levels were reduced with successful treatment (*De Heerdt* et al., 2009; *Majoie* et al., 2011).

VAGUS AND THE GUT

Vagal primary afferents innervate the muscular and mucosal layers of the gut. They are 30,000 to 80,000 vagal afferent nerves (and as many spinal afferents) that supply the intestine with the ratio of afferent to efferent fibres in peripheral nerve bundles being 9:1 (*Mei*, 1983; *Blackshaw* et al., 2007). Vagal afferents innervate all of gut with the coeliac branch supplying the intestine from proximal duodenum to the distal part of the descending colon (*Wang* and *Powley*, 2007). Vagal innervation is densest proximally but still substantial for the colon. Histological and electrophysiological data reveal that visceral afferent chemosensitive endings are free endings (*Mei*, 1983) that express a large mixture of chemical and mechanosensitive receptors (*Blackshaw* et al., 2007).

Chemosensitive receptors are the targets of gut hormones and regulatory peptides such as ghrelin, CCK, GLP-1 and PYY(3–36) that activate vagal afferent neurons, whose terminals lie in the mucosa that can powerfully influence the control of food intake and regulation of energy balance (*Blackshaw* et al., 2007). Labelling experiments have identified vagal afferent fibres in the lamina propria of duodenal and jejunal villi and crypts of Lieberkühn, but they do not cross the basal membrane to innervate the epithelial layer (*Wang* and *Powley*, 2007). Thus, vagal afferents are not in a position to sense luminal nutrients directly, but are in close anatomical apposition to the basal membrane of entero-endocrine cells (*Li*, 2007).

Intraganglionic laminar vagal afferent endings (IGLEs) are located in the connective tissue capsule of myenteric plexus ganglia, between the longitudinal (outer) and circular (inner) muscle layers. These fibres respond to muscle tension generated by both passive stretch and active contraction of the muscle layers (*Berthoud* et al., 2001). This type of vagal afferent ending is found in large numbers throughout the oesophagus and gastrointestinal tract and is thought to be important for generating vagal afferent tone for balanced interoceptive awareness and emotional well-being.

Thus, given the key role of the vagus in communicating visceral signals to brain and particularly to neural circuitry associated with mood and anxiety it is perhaps not surprising that many investigations of communication between gut bacteria and the CNS have examined the role of the vagus. There is now strong evidence from animal studies that gut microorganisims can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain and subsequently, behaviour.

Such evidence came early from the study of animals infected with pathogens. Subdiaphragmatic vagotomy attenuated c-fos expression in the PVN of rats inoculated with *Salmonella typhimurium* (*Wang* et al., 2002). Although *S. typhimurium* infection was accompanied by intestinal inflammation subsequent studies have indicated that microorganisms in the gastrointestinal tract can directly activate neural pathways even in the absence of an identified immune response (*Goehler* et al., 2005). The anxiogenic effect of orally administered subclinical doses of *Campylobacter jejuni*, in mice was associated with a significant increase in c-Fos expression in neurons bilaterally in the vagal ganglia and activated visceral sensory nuclei in the brainstem. The areas of brainstem activation, the NTS and lateral parabrachial nucleus, participate in neural information processing that ultimately leads to autonomic neuroendocrine and behavioural responses (*Goehler* et al., 2005).

Non-pathogenic bacteria also activate vagal signalling from gut to brain. *Tanida* et al. (2005) demonstrated that intraduodenal injection of the bacterial strain *Lactobacillus johnsonii* La1 reduced renal sympathetic nerve activity and blood pressure while enhancing gastric vagal nerve activity. All of these effects could be abolished by pre-treatment with a histaminergic H3-receptor antagonist. Similarly the effects were absent in animals that had bilateral lesions of the hypothalamic suprachiasmatic nucleus, a major regulator of circadian rhythm. These findings suggest that the influence of the bacteria on autonomic neurotransmission and subsequently blood pressure is mediated centrally, likely through histaminergic nerves and the suprachiasmatic nucleus (*Tanida* et al., 2005).

Consequently, infradiaphragmatic denervation of vagal nerve fibres surrounding the oesophagus eliminated the ability of *L. johnsonii* La1 to reduce renal sympathetic nerve activity and blood pressure indicating that at least some of the effects of this bacterium on autonomic nerve responses were elicited by interaction with afferent vagal nerve fibres (*Tanida* et al., 2005).

Recently it was demonstrated that oral administration of a *L. rhamnosus* strain (JB1) could alter the normal behaviour of adult balb/c mice (*Bravo* et al., 2011). Chronic treatment with the bacteria reduced anxiety-like behaviour as assessed in an elevated plus maze and decreased the time spent immobile in a forced swim test. In addition, stress-induced plasma corticosterone levels were lower in treated mice a similar effect to subchronic or chronic treatment with antidepressants that can prevent forced swim stress-induced increases in plasma corticosterone in both mice and rats. Overall, changes induced with *L. rhamnousus* were indicative of reduced anxiety, and decreased depression-like behaviour Assessment of neural correlates to behavioural changes determined that mice receiving *L. rhamno-*

sus had alterations in central GABA receptor subunit mRNA expression. *L. rhamnosus* administration decreased expression of GABA type B (GABAB) subunit 1 isoform b (GABAB1b) mRNA in the amygdala and hippocampus, while increasing expression in cortical areas. Expression of GABAAα2 receptor mRNA was reduced in the amygdala and cortical areas, whereas levels were increased in the hippocampus (*Bravo* et al., 2011). It is difficult to attribute a causal relationship between behavioural effects observed and neural correlates. However, reduced expression of GABAB1b mRNA, in the amygdala, hippocampus, and locus ceruleus is consistent with the antidepressant-like effect of GABAB receptor antagonists (*Cryan* and *Slattery*, 2010) and with studies of GABAB1b-deficient animals, indicating an important role for this subunit in the development of cognitive processes, including those relevant to fear (*Jacobson* et al., 2007a, 23007b). It is also interesting to note that in a recent study of transcriptomes from the mucosa of the proximal small intestines of healthy human subjects following treatment with different lactobacillus species, there was a strong correspondence between *in vivo* transcriptional networks altered after consumption of one of the strains, *Lactobacillus casei*, and the response of human cells to the anxiolytic GABA A receptor modulator, Tracazolate (*van Baarlen* et al., 2011).

Subdiaphragmatic vagotomy blocked the anxiolytic and antidepressant effects of chronic *L. rhamnosus* ingestion in normal adult Balb/c mice while also preventing the associated alterations in GABAAα2 mRNA expression in the amygdala (*Bravo* et al., 2011). Similarly, the ability of *B. longum* to attenuate DSS colitis induced anxiety was abolished by vagotomy (*Bercik* et al., 2011a).

CONCLUSION AND FUTURE DIRECTIONS

Overall, studies indicate that vagal pathways mediate signals that can induce both anxiogenic and anxiolytic effects depending on the nature of the stimulus and, interestingly, the vagus appears to differentiate between non-pathogenic and potentially pathogenic bacteria even in the absence of overt inflammation.

It is therefore clear that the involvement of the vagus in microbiota-gut-brain communication is not straightforward or simply dependent on "activation". Assuming that an increase in c-fos expression always uniquely reflects an increase in neuronal firing rates, a dubious assumption, existing anatomical data cannot answer the question why in some cases vagal activation causes depression and in others, for example, electrical stimulation of the vagus, eases depression. What is currently lacking is relevant data on the electrophysiology of the system. In spite of our obvious lack of understanding of how probiotics encode vagal neural discharge, very little work has been published on a probiotic neural code. *Tanida* et al. (2005) showed that injecting *L. johnsonii* into rat duodenum increased gastric vagal multiunit firing rate by about 10% within 15 minutes, and this slowly grew to a 90% increase over the baseline 1 h after the injection was delivered. Clearly, much more work of this sort needs to be done and should compared with vagal responses to anxiogenic and anxiolytic peripheral stimuli.

Electrophysiology may also be utilized to determine the nature of the peripheral signal acting to stimulate the

vagus nerve in the gut following exposure to specific bacteria. Single chemosensitive vagal afferent units supplying the gut are normally silent or have a low resting discharge of 0-3 Hz (*Blackshaw* and *Grundy*, 1993). They respond to most luminal molecules by increasing their firing rate. Response latencies vary according to the chemical nature of the stimulus. The short chain fatty acid butyrate had a response onset latency of 2-3 ms (*Lal* et al., 2001), the long chain fatty acid sodium oleate had a latency of 15 ms (*Lal* et al., 2001), amino acids evoked responses within about 9 ms (*Mei*, 1983) the response to casein acid hydrolysate has a latency and of 19 ms (*Eastwood* et al., 1998), and glucose takes 20 ms (*Hardcastle* et al., 1978). *Salmonella typhimurium* lipopolysaccharide evoked an increase in the mesenteric nerve discharge with 30 min (*Liu* et al., 2009) while LPS from a commensal *E. coli* had no effect (*Liu* et al., 2009). Mesenteric vagal afferents are mainly unmyelinated fibres with conductions speeds of 0.7 m/s (*Cervero* and *Sharkey*, 1988), so the conduction from mucosa to the mesenteric recording electrode (less than 10 mm distance) would be $\leq$ 7 ms. Thus most of the response times could be attributed to mucosal endocrine cells sensing and transduction, transmission to adjacent vagal endings and generation of a suprathreshold neurite receptor potential.

Certainly, important advances in our understanding of the microbiome-gut-brain axis will come from studies of how distinct microbial stimuli activate the vagus and the nature of the signals transmitted to the brain that lead to differential changes in the neurochemistry of the brain and behaviour. However, while it appears that the vagus in critical to mediating gut-brain communication by specific bacteria in some model systems, it is by no means the only potential signalling method. Indeed, largely due to technical difficulties, few studies have investigated the role of spinal afferents in mediating bacteria induced changes in behaviour and brain chemistry. It is certainly possible that the observed changes in brain chemistry behaviour induced by gut bacteria require parallel input from both the vagal and spinal afferents. Furthermore, behavioural changes induced through disruption of the microbiota by antibiotic treatment have been demonstrated to be independent of vagal signalling (*Bercik* et al., 2011b) with some additional evidence that neither sympathetic afferents nor immune modulation is required. This clearly suggests that the bacteria in the gut can communicate to the brain through multiple pathways. A potential means of communication, that has been somewhat neglected in existing studies, involves direct hormonal signalling to the brain. Nevertheless understanding the induction and transmission of anxiolytic signals in the vagus nerve may have important implications for the development of microbial-based therapeutic strategies for mood disorders.

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