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MICROBIOTA, STRESS AND THE BRAIN

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SUMMARY

Bacterial colonisation of the intestine has a major role in the post-natal development and maturation of the immune and endocrine systems. These processes are key factors underpinning central nervous system (CNS) signalling. Regulation of the microbiota-gut-brain axis is essential for maintaining homeostasis, including that of the CNS. Moreover, there is now expanding evidence for the view that commensal organisms within the gut play a role in early programming and later responsivity of the stress system. Research has focused on how the microbiota communicates with the central nervous system (CNS) and thereby influences brain function. The routes of this communication are not fully elucidated but include neural, humoral, immune and metabolic pathways. This view is underpinned by studies in germ-free animals and in animals exposed to pathogenic bacterial infections, probiotic agents or antibiotic agents which indicate a role for the gut microbiota in the regulation of mood, cognition, pain and obesity. Thus the concept of a microbiota-gut brain axis is emerging which suggests that modulation of the gut microflora may be a tractable strategy for developing novel therapeutics for complex stress-related CNS disorders where there is a huge unmet medical need.

INTRODUCTION

The fields of microbiology and neuroscience in modern medicine have largely developed in distinct trajectories, with the exception of studies focused on the direct impact of infectious agents on brain function, which include early investigations of syphilis to more recent studies of neuroAIDS. In addition to the direct effect of bacteria on CNS function, the field of psychoneuroimmunology has emerged to provide a framework as to how pathogenic bacterial agents can alter brain function. Thus, the role of the CNS in mediating the behavioural responses to infections ranging from "sickness behaviour" to septic encephalopathy has been advanced significantly. More recently, a new concept has emerged which indicates that commensal bacteria can also affect brain function in both health and disease. In this review we discuss the evidence to date and delineate how harnessing such pathways may provide for a novel approach to treat a variety of disorders of the brain-gut axis.

It is increasingly recognized that the brain-gut axis provides a bi-directional homeostatic route of communication which, if dysfunctional, can have important pathophysiological consequences (*Mayer*, 2011). This axis is

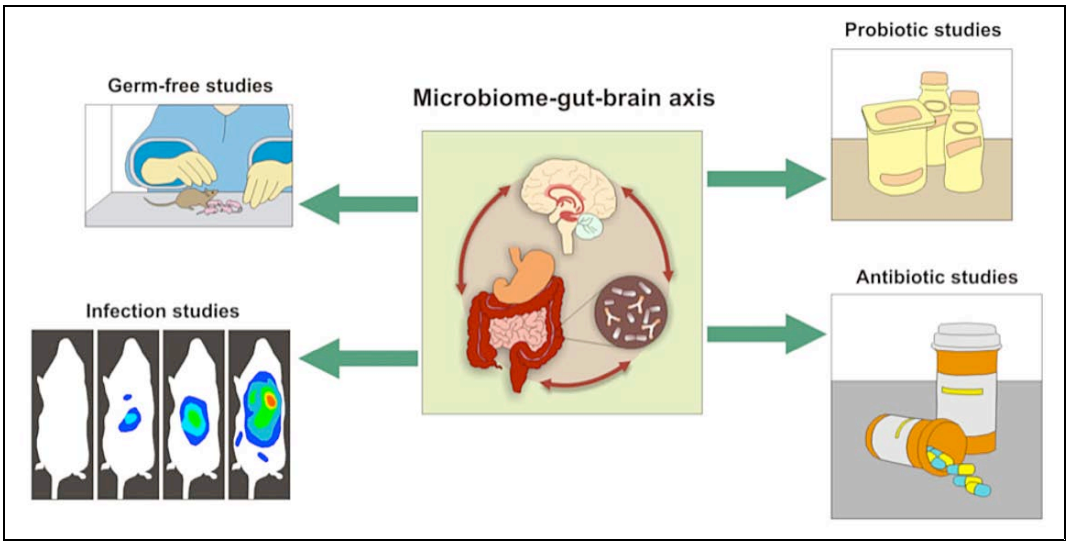

Figure 1: Strategies used to investigate the role of the microbiome gut brain axis in health and disease

regulated at neural, hormonal and immunological levels and alterations in brain-gut interactions are associated with gut inflammation, chronic abdominal pain syndromes and eating disorders (*Mayer*, 2011). Indeed, modulation of brain-gut axis function is associated with specific alterations in the stress-response and overall behaviour (*Rhee* et al., 2009). The high comorbidity between stress-related psychiatric symptoms such as anxiety with gastrointestinal disorders including irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) (*Reber*, 2012) is further evidence of the importance of this axis. Thus modulation of the brain-gut axis is being seen as an attractive target for the development of novel treatments for a wide variety of disorders ranging from obesity, mood and anxiety disorders to GI disorders such as IBS (*Mayer*, 2011). In addition, increasing evidence also suggests that the enteric microbiome can greatly influence all aspects of physiology (*Clemente* et al., 2012; *Sekirov* et al., 2010) not least of which is its ability to impact on gut-brain communication.

THE MICROBIOTA

The human gastrointestinal tract (GIT) is inhabited with 10^{13}-10^{14} microorganisms, which is >10 times that of the number of human cells in our bodies and 150 times as many genes as our genome (*Gill* et al., 2006; *Qin* et al., 2010), and thus often referred to as the "forgotten organ" (*O'Hara* et al., 2006). Our appreciation of the relationship between the microbiome and host is changing rapidly and it now can be viewed as being mutualistic (with both partners experiencing increased fitness) (*Backhed* et al., 2005), playing a crucial role in the development and functionality of the innate and adaptive immune responses (*Olszak* et al., 2012; *Round* et al., 2010) regulating gut mo-

tility, intestinal barrier homeostasis, nutrient absorption and fat distribution (*Backhed* et al., 2004; *Bercik* et al., 2012). Over the past 5 years significant advances have been made in the technology for assessing microbiota composition at the genetic level (*Fraher* et al., 2012; *Qin* et al., 2010) that is also having an immense impact on increasing our understanding of host-microbe interactions.

THE MICROBIOTA-GUT BRAIN AXIS AND BEHAVIOUR

Taken together, it is thus perhaps not surprising that there is a growing body of literature focused on assessing the impact of enteric microbiota on brain and behaviour and the concept of the microbiome-gut-brain axis is emerging. The general building blocks of this axis include the CNS, the neuroendocrine and neuro-immune systems, the sympathetic and parasympathetic arms of the autonomic nervous system (ANS) and the enteric nervous system (ENS) along with the intestinal microbiota, which is being increasingly viewed as the cornerstone. These components converge to form a complex reflex network with afferents that project to integrative CNS structures and efferents to smooth muscle (*Mayer*, 2011). Crucially, this axis functions bi-directionally (*Mayer*, 2011). Approaches used to parse the role of gut microbiota on brain function (see Figure 1) include using germ-free animals, assessing the impact of probiotic agents, antibiotic-induced dysbiosis and pathogenic infections (*Cryan* and *O'Mahony*, 2011). In addition, accumulating studies have also shown that manipulations known to impact brain function (e.g. stress) also impacts microbiota composition (*Dinan* et al., 2012).

GERM-FREE ANIMALS

The use of germ-free (GF) animals enables the direct assessment of the role of the microbiota on all aspects of physiology. In these animals surgical delivery replaces the normal birthing process, thus eliminating the opportunity for post-natal colonization of the GIT and allows for direct comparison with their conventionally colonized counterparts. In a landmark study, Sudo and colleagues (*Sudo* et al., 2004) provided direct evidence that intestinal microbiota plays a key role in the development of the hypothalamic-pituitary-adrenal (HPA) axis. In GF mice a mild restraint stress induced an exaggerated release of corticosterone and adrenocorticotrophin hormone compared to the specific pathogen free controls. The stress response in the GF mice was partially reversed by colonization with faecal matter from control animals and fully reversed by mono-association with *Bifidobacterium infantis* in a time dependant manner (*Sudo* et al., 2004). These data clearly demonstrated that the microbial content of the GIT is critical to the development of an appropriate stress response later in life and also that there is a critical window in early life where colonization must occur to ensure normal development of the HPA axis. From a neuronal point of view, a decrease in brain derived neurotrophic factor (BDNF), a key neurotrophin involved in neuronal growth and survival, and decreased expression of the N-methyl-D-asparaginezuur (NMDA)

receptor subunit 2a (NR2a) in the cortex and hippocampus of GF animals compared to controls was also observed. However, it took a further seven years for these findings to be followed up at a behavioural level. Three independent groups have now shown that GF animals (from different strains and genders) have reduced anxiety in the elevated plus maze or light dark box (*Clarke* et al., 2012a; *Heijtz* et al., 2011; *Neufeld* et al., 2010), but see (*Gareau* et al., 2011) The mentioned tests are widely used to assess anxiety related behaviour (*Cryan* and *Sweeney*, 2011). These findings are somewhat puzzling as it is opposite to what one would have been predicted based on the exaggerated HPA axis. Interestingly, Neufeld and colleagues (*Neufeld* et al., 2010) also reported changes in BDNF, NR_{2B} and $5\text{-}HT_{1A}$ receptor mRNA expression in GF mice. However, it is worth noting that the direction of such changes is not in agreement with data reported by *Sudo* et al. (2004) From a cognitive point of view GF mice displayed deficits in simple non-spatial and working memory tasks (novel object recognition and spontaneous alternation in the T-maze). Future studies should focus on enhancing the repertoire of behavioural cognitive assays employed. However, maintaining animals GF and conducting complex behavioural studies is not a trivial logistical hurdle.

More recently, we have shown that GF animals have a significant elevation in the hippocampal concentration of 5-HT and 5-HIAA, its main metabolite, compared with conventionally colonised control animals (*Clarke* et al., 2012a). Concentrations of tryptophan, the precursor of serotonin, are also increased in the plasma of GF animals, suggesting a humoral route through which the microbiota can influence CNS serotonergic neurotransmission. Interestingly, colonisation of the germ-free animals post-weaning is insufficient to reverse the CNS neurochemical consequences in adulthood of an absent microbiota in early-life despite the peripheral availability of tryptophan being restored to baseline values. Gender may play a role in such effects. Indeed, we have recently shown that many of the neurochemical but not endocrine or immune effects of growing up in a germ-free environment are only evident in male animals (*Clarke* et al., 2012a). Further behavioural studies, including the use of other species such as germ-free rats will greatly expand our knowledge of the role of microbiota in stress-related disorders.

PROBIOTICS

Probiotics are live organisms that, when ingested in adequate quantities, exert a health benefit on the host (*Gareau* et al., 2010; *Quigley*, 2008). Probiotics are reported to have a wide variety of effects in both human and animal studies (*Gareau* et al., 2010; Quigley, 2008). Increasing evidence points to beneficial effects of various probiotics in the treatment of the gastrointestinal symptoms of disorders such as IBS (*Clarke* et al., 2012b). Moreover, there is some clinical evidence to support the role of probiotic intervention in reducing the anxiety and stress response as well as improving mood in IBS patients and those with chronic fatigue (*Logan* et al., 2005; et al., 2009). Recently, a study assessing the effect of a combination of *Lactobacillus helveticus* and *B. longum* on both human subjects and rats

demonstrated the ability of this probiotic cocktail to reduce anxiety in animals and had beneficial psychological effects with a decrease in serum cortisol in patients (*Messaoudi* et al., 2011). While the mechanism of action is not known it has been postulated that probiotic-induced effects on pro-inflammatory cytokines, oxidative stress and in nutritional status may contribute to such effects (*Cryan* and *O'Mahony*, 2011; *Logan* et al., 2005).

Recently we have shown that a bacterium of the *Lactobacillus* species *L. rhamnosus* (JB1*),* decreases anxiety and despair-like behaviour as well as reducing the stress-induced increase of corticosterone in mice (*Bravo* et al., 2011). Moreover, this potential probiotic alters the mRNA expression of both $GABA_A$ and $GABA_B$ receptors in the CNS. Alterations in these receptors are associated with anxious and depressive-like behaviours in animal models. Interestingly, these effects are vagus-dependent as vagotomy prevented the anxiolytic and antidepressant effects of this bacterium as well as the effects on the central GABA receptors. This suggests that parasympathetic innervation is necessary for *L. rhamnosus* to participate in the microbiota-brain interaction. Whilst there have been studies showing that potential probiotics can reverse the effects of colitis, infection or stress these data are the first to our knowledge to show beneficial effects in animal assays used to assess anxiolytic or antidepressant activity (*Cryan* and *Sweeney*, 2011).

Indeed, previous studies have shown that the probiotic *B. longum* NCC3001 but not *L. rhamnosus* NCC4007 reversed colitis-induced anxiety and alterations in hippocampal BDNF without impacting gut inflammation or circulating cytokines (*Bercik* et al., 2010; *Bercik* et al., 2011a). The anxiolytic effect of *B. longum* NCC3001 was absent in mice with vagotomy, suggesting a neural mechanism, which was confirmed by *ex vivo* electrophysiological studies, in enteric neurons (*Bercik* et al., 2011b). We have also shown that probiotic agents such as *B. infantis* can modulate antidepressant-like behaviour in a maternal separation model (*Desbonnet* et al., 2010) and modulate peripheral pro-inflammatory cytokine and tryptophan concentrations both of which have been implicated in depression. Finally, exciting studies are emerging showing that brain fatty acid concentrations (including stearic acid, arachidonic acid, and DHA) can be elevated in mice whose diets were supplemented with *B. breve* NCIMB 702258 (*Wall* et al., 2012). Interestingly, this effect was bacterial strain dependent as it was not induced by another B. *breve* strain DPC 6330. Taken together, certain probiotic strains can modulate various aspects of the microbiome-gut-brain axis some of which are vagus dependent. However, it is clear that caution needs to be exercised when generalising such effects from one bacterial strain to another and efforts need to be directed at identifying the mechanism of how each strain induces their effects.

PAIN

Some of the most convincing data on the microbiome-gut-brain axis emerges from the pain field. Visceral pain is a pronounced and, at times, dominant feature of a variety of gastrointestinal disorders, including IBS. Recurrent, episodic but often unpredictable painful events can exert a disabling impact on daily life and result in impairment of several domains of quality of life. Vis-

ceral pain perception is regulated by complex mechanisms. These include peripheral sensitization of sensory nerves whereas the central processing of visceral nociception is mediated at both cortical and subcortical levels by pathways also involved in the processing of psychological stress. Specifically, the prefrontal cortex has been shown to play an integral role in these processes, with imaging studies in humans (*Mayer* et al., 2008; *Mertz* et al., 2000) and animal (*Gibney* et al., 2010; *O'Mahony* et al., 2010; *Wang* et al., 2008) showing activation of the anterior cingulate, pre-limbic and infra-limbic cortices in response to painful and stressful stimuli. Growing evidence suggests that both central and peripheral mechanisms could be affected by intestinal microbiota. In animal studies, probiotics (mostly *Lactobacilli and Bifidobacteria*) have been shown to alleviate visceral pain in stress models (*Ait-Belgnaoui* et al., 2006; *Gareau* et al., 2007; *Johnson* et al., 2011; *McKernan* et al., 2010; *Rousseaux* et al., 2007; *Verdu* et al., 2006). This has also been also the case clinically with many different probiotics showing beneficial effects in abdominal pain (*Bercik* et al., 2012; *Clarke* et al., 2012b). The mechanisms of action of such effects remain unclear currently and as in animal studies may involve a combination of effects on neural, immune and endocrine effects. We have recently demonstrated that *B. infantis* 35624 is effective at increasing the pain threshold in rats and reducing the number of pain behaviours following CRD in both a normo-sensitive and a hypersensitive rat strain (*McKernan* et al., 2010). Recently, it has been shown that *Lactobacillus acidophilus* reduces visceral hypersensitivity in rats by inducing cannabinoid (CB2) and opioid receptor (MOR1) expression in the colonic epithelium (*Rousseaux* et al., 2007). Furthermore, in a number of recent studies, *Lactobacilli* have been shown to affect the excitability of enteric neurons and nerves innervating the gut, which in turn has been shown to have effects on colonic motility (*Kunze* et al., 2009; *Ma* et al., 2009; *Wang* et al., 2010).

MICROBIOTA AND STRESS

It is long known that stress and the HPA axis can influence the composition of gut microbiome (*Tannock* et al., 1974). However, the functional consequences of such changes are now only being unravelled (*Dinan* et al., 2012). Maternal separation, an early life stressor which can result in long-term HPA axis changes (*O'Mahony* et al., 2011), has been shown to cause a significant decrease in faecal lactobacilli on day 3 post separation, which returns to baseline by day seven as assessed by enumeration of total and Gram-negative aerobic and facultative anaerobic bacterial species (*Bailey* et al., 1999). However, we have more recently shown that early life stress can also have long-term effects on the microbiome. Analysis of the 16S rRNA diversity in adult rats exposed to maternal separation for three hours per day from post natal days 2-12 revealed a significantly altered faecal microbiome when compared to the non-separated control animals (*O'Mahony* et al., 2009). A study using deep sequencing methods demonstrated that the community structure of microbiota from mice exposed to chronic restraint stressor was significantly different to that in non-stressed control mice (*Bailey* et al., 2011). More

recently chronic psychosocial stress decreased the relative abundance of *Bacteroides*, while increasing the relative abundance of bacteria in the genus *Clostridium* in the caecum. The stressor also increased circulating levels of IL-6 and MCP-1, which were significantly correlated with stressor-induced changes to three bacterial genera (i.e., *Coprococcus, Pseudobutyrivibrio,* and *Dorea*). These data show that exposure to repeated stress affects gut bacterial populations in a cytokine dependent manner (*Bailey* et al., 2011).

CONCLUSIONS AND PERSPECTIVE

A growing body of experimental data and clinical observations support the existence of the microbiome-gut-brain axis and suggest that it is poised to control canonical aspects of brain and behaviour in health and disease. Future research should focus on deconvoluting the relative contributions of immune, neural, and metabolic pathways to this axis. A better understanding of these relationships will inform our understanding of a host of GI and extra-GI disorders including neuropsychiatric diseases such as depression and anxiety. Much work is needed to tease apart the various constructs at play in this complex communication network. It is not clear how the various microbial strains can differentially affect CNS functioning but metabolite production, polysaccharide actions, structural effects and direct and indirect activation of the immune system are likely factors at play. Indeed, the bacterial metabolism of dietary fibre to short-chain fatty acids is a significant energy source for humans and these metabolites are of importance for gut motility, have a trophic effect on epithelial cells, impact on immune system development and modulate entero-endocrine hormone secretion (*Grenham* et al., 2011). Certain microorganisms including *Lactobacilli* are able to convert nitrate to nitric oxide, a potent regulator of both the immune and nervous systems whilst others can produce neuroactive amino acids such as GABA (*Forsythe* et al., 2010). Elucidating the mechanisms underlying such effects will be crucially important for the development of any microbiota-based therapeutic strategies for CNS diseases.

It is clear that the clinical translation of any animal data especially those in complex areas of anxiety, depression and cognition is now warranted and that this should be done with the same rigour as pharmaceutical drug development. Overall, research to data clearly shows that behaviours, physiology, and neurochemistry can be affected in many ways by modulation of gut microbiota. Whether this translates to microbial-based CNS therapeutics remains a tempting possibility and one that is worthy of much further investigation.

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