IMPACT OF INTESTINAL MICROBIOTA ON THE NERVOUS SYSTEM

IMPACT OF COMMENSAL INTESTINAL MICROBIOTA ON NERVOUS SYSTEM DEVELOPMENT AND FUNCTION

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ABSTRACT

Commensal intestinal microbiota number in the realm of 10^{14} organisms per gram of colonic contents. This considerable bacterial load is acquired during birth and in the early postnatal days and has a defining, extensive impact on host physiology. We now have persuasive evidence that the intestinal microbiota influence the development of the nervous system. The following body of work describes alterations in the nervous system of germ free mice – mice bred and maintained with no exposure to bacteria of any kind. Here we examine diverse measures of neural activity, ranging from stress reactivity and stress-associated behaviours, to changes in neurochemistry of brain regions mutually involved in feeding and stress, to electrophysiological measures of sensory cells in the enteric nervous system. We see that in the absence of colonizing microbiota that neural activity is considerably altered both peripherally and centrally. Specifically, germ free mice exhibit a reduction in basal anxiety-like behaviour accompanied by consistent changes in mRNA gene expression of plasticity-related genes in brain tissue, lifelong reduction in circulating plasma leptin, increases in mRNA gene expression of hypothalamic leptin receptors and neuropeptide Y, and decreased excitability in sensory neurons in the myenteric plexus of the enteric nervous system. Furthermore, while it appears that central systems responsible for stress may have an early critical window for bacterial-induced change, it would seem that the peripheral enteric nervous system retains plasticity into adulthood. This novel work provides insight into the microbial-gut-brain axis and suggests potential avenues for therapies aimed at treating the frequently comorbid gastrointestinal and psychiatric illnesses.

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LIST OF ALL ABBREVIATIONS

5-HT1A receptor	Serotonin 1A receptor
α-MSH	Alpha melanocyte-stimulating hormone
ACTH	Adrenocorticotropic hormone
AH	Afterhyperpolarization
AP	Action potential
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CORT	Corticosterone
CRD	Colorectal distension
CRH	Corticotropic releasing hormone
DRG	Dorsal root ganglion
EAE	Experimental autoimmune encephalomyelitis
ENS	Enteric nervous system
EPM	Elevated plus maze
FBD	Functional bowel disorder
FIAF	Fasting-induced adipose factor
GABA	Gamma-aminobutyric acid
GF	Germ free
GI	Gastrointestinal
HPA	Hypothalamic-pituitary-adrenal axis
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IPAN	Intrinsic primary afferent neuron
MMC	Migrating motor complex
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartic acid
NPY	Neuropeptide Y
ObR	Leptin receptor
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
POMC	Proopiomelanocortin
PVN	Paraventricular nucleus
RAG-1	Recombination activating gene-1
RMP	Resting membrane potential
sAHP	Slow afterhyperpolarization
SCFA	Short-chain fatty acid
SD	Sprague Dawley
SHRP	Stress hyporesponsive period
SPF	Specific pathogen free
TLR	Toll-like receptor
Tregs	Regulatory T-cell

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CHAPTER 1. INTRODUCTION

1.1. Intestinal Microbiota

The human intestine is home to a staggering number and variety of commensal microorganisms. Current estimates of this bacterial load are 10^{14} organisms per gram of colonic contents, with roughly 1800 genera represented (Frank and Pace, 2008). These numbers are especially astonishing when we consider that the intestinal lumen houses 150-fold more bacterial genes than human genes (Backhed, 2011). Gut bacteria have evolved alongside their human hosts, and not only perform a variety of functions essential for host health and survival, but also act to shape various aspects of physiology and metabolism (Backhed, 2011). Our close physiological relationship with bacteria is thus not only normal but in fact imperative to optimal health and well-being. We, and indeed all vertebrates, are "germ-free" *in utero* with colonization of the intestinal tract beginning during birth and the immediate postnatal period. At a very basic level, the host-bacterial relationship is mutualistic. The bacteria are provided with a protected environment, rich in energy resources, and the host is provided with microorganisms capable of breaking down otherwise indigestible food components. In addition, these favourable bacteria provide a barrier for pathogenic microorganisms in that they compete for space and resources with potentially harmful invaders. But the extent to which intestinal bacteria benefit the host is in fact much greater.

Initially, much of the research conducted on microbiota involved its necessity for immune

system development, and it is now well established that commensal microflora in the gut are essential for the normal maturation of the mucosal and systemic immune systems. In order to determine this immune dependency, researchers made use of germ-free (GF) mice, animals that are bred and maintained in specialized housing units with no exposure to bacteria of any kind, including commensal intestinal microbiota. Examination of these animals shows that both immune composition and lymphoid structures are vastly different in the absence of gut bacteria. GF mice exhibit an undeveloped mucosal immune system with hypoplastic Peyer's patches (lymphoid structures specific to the mucosa of the small intestine which incubate B and T cells), and far fewer IgA producing plasma cells and $CD4^+$ T cells in the gut mucosa. Outside the mucosal immune system, GF mice are characterized by an undefined spleen and lymph nodes, reduced serum levels of immunoglobulins and fewer circulating plasma lymphocytes (Macpherson and Harris, 2004; Smith et al., 2007). If GF mice are "conventionalized" through introduction of bacteria, these immune deficits are normalized within several weeks (Macpherson and Harris, 2004). Indeed, even the presentation of certain single bacterial species to GF mice have been demonstrated to correct some if not all immune system abnormalities (Umesaki et al., 1995; Talham et al., 1999; Umesaki et al., 1999; Mazmanian et al., 2005).

Aside from their indispensable role in immune development, commensals also play a formative role in postnatal intestinal formation, including strengthening epithelial barrier function (Hooper and Gordon, 2001). GF mice have been demonstrated to have elongated, thinner intestinal villi which may be more vulnerable to infection. If GF mice are conventionalized with normal gut microbiota, the structure of villi alters, shortening

and widening, and epithelial barrier function improves (Hooper et al., 2001; Backhed, 2011). During weaning, dramatic changes typically occur to the developing gut. One of the most obvious of these is the development of a major capillary network in the intestinal villi (Stappenbeck et al., 2002). GF mice do not fully develop intestinal blood vessels, demonstrating impaired angiogenesis. Again, exposing these mice to bacteria, even the single species *Bacteroides thetaiotaomicron*, will stimulate normal capillary development (Hooper, 2004). Intestinal production of a number of carbohydrates and proteins is also diminished in the absence of colonizing bacteria. GF mice do not produce the same types of glycoconjugates in the intestine as normally housed specific-pathogen free (SPF) control animals. Glycoconjugates are produced by all cells and lie on the cell surface providing a protective coating and such functions as cell recognition and signalling. Newborn conventionally housed SPF mice express intestinal epithelial glycans that mainly terminate with sialic acid, but within 2 to 3 weeks shift production to those that terminate with the sugar fucose. This shift in production does not occur in GF mice, but again colonizing GF mice with microbiota at any stage throughout their lifetime normalizes the glycosylation program (Umesaki et al., 1995; Bry et al., 1996). Similar to the fucosylated glycans, Paneth cells (a component of small intestine epithelium) make angiogenin-4, a granule protein, for which production is initiated during early life. Angiogenin-4 is a bactericide with a strong role in host defense, and GF mice never express the high levels observed in conventional animals. This would indicate that the host needs bacterial interaction in order to shape production of their full antimicrobial range (Hooper, 2004). Taken together, these findings indicate that the intestine is

designed to interact with the bacteria it houses, and when these bacteria are absent, as in GF animals, developmental programming is altered.

There is currently keen interest in the role that intestinal microbiota plays in metabolism. Recent work has shown that gut bacteria are an environmental factor that can potentially contribute to the development of obesity. GF mice are protected against obesity, show improved glucose tolerance and sensitivity to insulin (Backhed et al., 2004; Backhed et al., 2007). The mechanisms of this protection are only just beginning to be unravelled, but GF mice are unable to fully harvest energy from their diet, this in spite of regular or even increased food intake (Wostmann, 1981; Backhed et al., 2004; Backhed et al., 2007). When inoculated with a single species of bacteria, *B. thetaiotaomicron,* GF mice demonstrate improved food absorption and processing (Hooper and Gordon, 2001), with specific changes observed in the breakdown and uptake of both glucose and lipids (Hooper et al., 2002). Colonization induces increased production of Na^+/glucose co-transporter in the ileum, which is the main transporter for glucose from the gut lumen into the epithelium. Increased expression of a number of molecules involved in lipid absorption are also observed, namely those involved in the breakdown and transport of triacylglycerols and fatty acids (Hooper et al., 2002). Colonizing GF mice also induces a down-regulation of fasting-induced adipocyte factor (FIAF). FIAF is known to be upregulated in both the liver and the adipocytes in response to fasting, so the fact that FIAF production decreases after monoassociation of GF mice with bacteria is consistent with the other changes in lipid absorption (Hooper et al., 2002). Therefore, while the absence of microflora does not allow for normal metabolic programming, in fact this state

is considerably protective when considering obesity and the potential for development of diabetes. Toll-like receptors (TLRs) are part of the innate immune system and are expressed on intestinal mucosa (Santaolalla et al., 2011). TLR5 knockout mice are hyperphagic and express characteristics that when grouped together are termed "metabolic syndrome" (Vijay-Kumar et al., 2010). Metabolic syndrome is characterized by hyperlipidemia, hypertension, insulin resistance and increased fat storage. TLR5 knockout mice also show alterations in their intestinal microbiota, and a fascinating study has demonstrated that conventionalizing GF mice with feces from TLR5 knockout mice results in a transfer of the metabolic syndrome-like characteristics to the GF mice (Vijay-Kumar et al., 2010). This paper postulates that the gut bacteria to which one is exposed during early life can thus predispose individuals to certain metabolic phenotypes. In keeping with this hypothesis, work that has examined variations in human microbial profiles has shown that 7-year-old children who are overweight have an increased *Staphylococcus aureus* bacterial count during infancy than do 7-year-olds of normal weight. The children in the normal weight category have a greater representation of *Bifidobacterium* in their fecal samples at infancy than do the overweight children (Kalliomaki et al., 2008). Interestingly, clinical studies have demonstrated that adult humans with type II diabetes have a different composition of gut microbiota than healthy volunteers (Furet et al., 2010; Larsen et al., 2010) indicating that an altered microbial profile may play a role in disease progression.

1.2. Microbial-Gut-Brain Axis

Bidirectional communication between the brain and gut has been well established, both pre-clinically and clinically (Mayer, 2000). Research has focused on top-down communication, or how the brain affects gastrointestinal motility, blood flow and secretion, and on the bottom-up effects of intestinal activity on visceral perception and central nervous system (CNS) activity. The gut and brain are known to communicate through the hypothalamic-pituitary-adrenal (HPA) stress axis, but also through ascending and descending neural activity of the autonomic nervous system, the immune system and the endocrine system (Shanahan, 1999; Drossman, 2005; Berthoud, 2008). Communication via these routes is not mutually exclusive, and in fact often involves an interplay between these systems.

There is now strong evidence to suggest that the 10^{14} bacterial organisms that normally reside in the human intestine are also involved in brain development and function, and the concept of the gut-brain axis is now being extended to include the bacterial contents of the gut lumen. This is reflected by a number of recent reviews highlighting the emerging understanding of the role that intestinal microbiota play in the bidirectional crosstalk between the intestine and CNS. Articles describing the "microbial-gut-brain axis" (Rhee et al., 2009; Bercik, 2011; Cryan and O'Mahony, 2011; Fetissov and Dechelotte, 2011) demonstrate this extension of the key players in gut brain communication, and review some of the findings leading to the increased understanding of the bacterial role in CNS function. One of the more dramatic pieces of evidence for commensal bacteria-gut-brain

communication has come from clinical case studies of hepatic encephalopathy. Hepatic encephalopathy is a complication of liver cirrhosis, and symptoms can range from fairly mild cognitive impairment to coma. Pathogenesis is not fully understood, but it is currently believed that accumulated by-products from gut bacteria contribute to the observed brain dysfunction (Riordan and Williams, 2010). Standard treatment for this condition is combined antibiotic therapy and laxatives to target the intestinal microbiota, and recent randomized controlled trials have demonstrated that treatment with antibiotic significantly improve quality of life in these patients as well as reducing number of relapses and number of required hospitalizations (Bass et al., 2010; Sanyal et al., 2011). Indirect evidence for the impact of commensal microbiota on brain function comes from pre-clinical work focusing on the effects of dietary alteration on animal behaviour. Increased hindgut bacterial fermentation of carbohydrates has been shown to be accompanied by increased anxiety and aggression in rats in the absence of neurotransmitter change in the CNS (Hanstock et al., 2003). And in a recent paper examining the effects of dietary change on commensal bacterial populations and learning and memory, Lyte and colleagues showed that rats fed a lean beef diet as opposed to a control pellet-powder diet showed increased bacterial diversity, reduced anxiety-like behaviour and enhanced short-term and long-term memory (Li et al., 2009).

In effort to further understand the role of commensal bacteria in normal CNS development and function, we can also look to studies that introduce bacteria, either pathogenic or probiotic to the intestine, and then measure potential changes to brain neurochemistry and behaviour. Evidence from animal studies using pathogenic bacterial

infection with *Citrobacter rodentium* indicates that information regarding the microbial status of the gut is transmitted to the brain, and results in increased anxiolytic-like behaviour in infected rodents. These findings are especially interesting given that they occur in the absence of overt gut inflammation and systemic inflammatory markers, thereby suggesting alternate route(s) of communication other than via the immune system (Lyte et al., 1998). In fact, it has been demonstrated that these sub-clinical intestinal infections impacting anxiety-like behaviour are accompanied by increased c-fos activity in vagal sensory ganglia, indicating that vagal nerve activity could be transmitting information regarding pathogenic microbial content of the intestinal lumen to the brain (Lyte et al., 2006). Earlier this year, Gareau and colleagues similarly used the *C. rodentium* infection model, and demonstrated memory dysfunction at both 10 and 30 days post infection in mice following exposure to water stress. This is interesting because the memory dysfunction remains even after the bacteria had cleared the intestine and intestinal damage is seemingly repaired (Gareau et al., 2011). Examining other bacterial species, chronic infection of mice with *Helicobacter pylori* also leads to changes in behaviour and brain neurochemistry, with persistent abnormal feeding and alterations in mRNA expression levels in the median eminence and arcuate nucleus of the brain reported (Bercik et al., 2009). In a similar study, infecting mice with the noninvasive parasite *Trichuris muris* leads to increased anxiety-like behaviour and a downregulation of hippocampal brain-derived neurotrophic factor (BDNF) (Bercik et al., 2010). Interestingly in this case, prior vagotomy has no impact on the parasitic effect, in contrast to the previous findings with pathogenic bacterial infection (Lyte et al., 2006) indicating

that mechanisms for communication may differ depending on the mode of gastrointestinal infection. However, while all of these studies are pertinent to the study of gut-brain communication, it is not clear whether non-pathogenic bacteria communicate with the brain via similar mechanisms, so for this we need to look to experiments using probiotic bacteria.

Probiotics are living bacteria that can transiently inhabit the gut and thereby confer health benefits to the host (Sherman et al., 2009). Earlier clinical work with probiotics has demonstrated that giving certain strains of these beneficial bugs can improve the altered gastrointestinal symptoms observed in patients with irritable bowel syndrome (IBS) (Brenner et al., 2009; Moayyedi et al., 2010; O'Mahony et al., 2010). Interestingly the CNS associated symptoms often observed in IBS patients also seem to be ameliorated with probiotic treatment. Abdominal pain is the hallmark symptom of IBS, and feeding both mice and rats *Lactobacillus reuteri* has been shown to increase expression of both opioid and cannabinoid receptors in the intestinal epithelium (Rousseaux et al., 2007). In addition, feeding the rats probiotic reduced their ability to perceive visceral pain due to colorectal distension (Rousseaux et al., 2007). Clinical studies are lending weight to the theory that probiotic treatment can improve the anxiety and related mood symptoms frequently associated with both IBS and chronic fatigue syndrome (Logan and Katzman, 2005; Rao et al., 2009; Silk et al., 2009). A number of recent articles have shown the affects of various probiotic species in rodent behaviour, with the phenotypic outcome differing depending upon the particular bacterial species used. Most recently, Bravo and colleagues have shown that feeding mice *Lactobacillus rhamnosus* alters GABAergic

transmission in the brain, and also reduces anxiety-like and depressive-related behaviours, those most commonly associated with IBS. As with previous work using pathogenic bacteria, vagotomized probiotic fed mice failed to show these changes, further indicating the importance of the vagus in the microbial-gut-brain axis (Bravo et al., 2011). *Bifidobacterium infantis* has been demonstrated to have anti-depressant-like properties when probiotic fed rats were assessed for changes in brain neurochemistry, with treated animals also showing increased plasma levels of the serotonin precursor tryptophan (Desbonnet et al., 2008). In the work by Gareau and colleagues mentioned above with respect to *C. rodentium* induced memory dysfunction in mice, they found that pretreating animals with probiotics *L. rhamnosus and Lactobacillus helveticus* protected infected mice from the stress-induced memory defects (Gareau et al., 2011). In a combined animal and human study designed to assess the affects of the combination therapy *L. helveticus and Bifidobacterium longum,* treated rats demonstrated reduced anxiety in a conditioned defensive burying test and healthy human volunteers showed a decreased serum cortisol level which was accompanied by beneficial psychological effects (Messaoudi et al., 2011). This summary of the effects of probiotics in both pre-clinical and clinical research is not exhaustive as this field has become one of increasingly heightened interest, however it is clear from the highlighted studies that probiotics have the ability to affect change in the CNS, with bacterial species-specific behavioural and neurochemical effects.

Disrupting the intestinal microflora is yet another way of examining the relationship between the microbiota, gut and brain. Work using the antimicrobial drugs neomycin and

bacitracin together with the antifungal primaricin for 7 days induced intestinal dysbiosis in mice and led to changes in anxiety-like and/or exploratory behaviour and hippocampal BDNF expression (Bercik et al., 2011). In another study, neonatal rat pups treated with the antibiotic vancomycin were assessed for behavioural changes in adulthood. While no differences were observed in anxiety or depressive-like behaviours in the treated rats, those with early life dysbiosis showed increased visceral pain responses following colorectal distension (O'Mahony et al., 2010). Lastly, when considering the impact of gut microbiota on CNS development and function, data obtained from GF mice must be considered. GF mice are possibly the most extreme example of disrupted microbiota, in that there are no bacteria present in the intestine of these mice at all. While it has been argued that this is potentially so extreme as to be not useful given that there is no obvious clinical parallel to the GF condition, invaluable data has been obtained from studying these mice as we can observe the results of a complete absence of bacterial input. In addition, studies colonizing GF mice with either a single species of bacteria or with the entire commensal cohort provide valuable information regarding developmental changes in physiology solely related to bacterial exposure. Research conducted in the last decade has shown that adult GF mice show HPA axis hyperreactivity in response to restraint stress, with increased plasma corticosterone and adrenal corticotropic hormone (ACTH) compared to conventionally housed controls (Ikeda et al., 1999; Sudo et al., 2004). This is fascinating because it suggests that intestinal bacteria need to be present for the normal development of stress reactivity. Further alterations observed in brain neurochemistry and behaviour of GF mice will be explored in detail as this specific model was employed

in the microbiota-gut-brain primary research making up this PhD dissertation.

Irrespective of the model used, taken together these studies illustrate the direction of a new and compelling branch of neurogastroenterology research, one which will attempt to elucidate mechanisms of gut brain communication through a focus on intestinal microbiota.

1.3. Development of the Hypothalamic-Pituitary-Adrenal Axis & Stress Reactivity

A quick perusal of the many published reviews on the gut-brain axis, and more recently on the microbial-gut-brain axis shows that the primary area of interest in the brain in this communication revolves around stress reactivity and the behaviours related to anxiety and mood disorders. Stress, experienced during early life or in adulthood, plays a recognized role in the functional bowel disorders (FBD) and in inflammatory bowel disease (IBD) such as Crohn's disease and colitis (Barreau et al., 2007; Reber, 2011). There is a high comorbidity between the stress-related mood disorders and both FBD and IBD (Creed and Guthrie, 1987; Whitehead et al., 2002; Walker et al., 2008) but exactly what role psychological stress plays in susceptibility or symptom onset is not yet completely clear.

The functional link between stress reactivity, anxiety and mood disorders is the HPA axis (Landgraf et al., 1999; Dinan, 2005; Jakovcevski et al., 2011). Patients with depression often show increased plasma cortisol levels, the stress hormone released from the adrenal

glands following HPA axis activation, and increased mRNA expression of corticotrophin releasing hormone (CRH) in the hypothalamus (Merali et al., 2004). Amelioration of depressive symptoms via anti-depressant therapy is accompanied by normalized HPA axis activity (Appelhof et al., 2006; Ising et al., 2007). In addition, 50% of patients suffering from severe depression show abnormal responsivity to a dexamethasone challenge, a test of exogenous synthetic glucocorticoid administration designed to assess negative feedback regulation of the HPA axis (Coryell et al., 2006). A number of animal studies have shown that rodent strains known to exhibit high anxiety also show increased activation of the HPA axis (Landgraf and Wigger, 2002; Jakovcevski et al., 2011). The link between HPA axis dysfunction and anxiety disorders in the clinical population is not as clear cut as with depression, however there is considerable evidence to support a role for abnormalities in CRH expression in anxiety disorders like post-traumatic stress disorder (Arborelius et al., 1999). As discussed in the preceding section, the HPA axis is a main target in the brain for information travelling from the gut microbiota, and probiotics are therefore now being assessed as a potential treatment for depression (Desbonnet et al., 2008; Bravo et al., 2011). Interestingly, the relationship between gut microbiota and the HPA axis seems to be bidirectional. Recent work examining "top-down" control has demonstrated that rat pups exposed to maternal separation, a well established stressor known to permanently alter HPA axis activity, show a different intestinal microbial profile than do control rats (O'Mahony et al., 2008).

The early postnatal period is a vulnerable time for the development of the CNS, and particularly the HPA axis (Hensch, 2004; Knudsen, 2004). The neonatal developing

brain needs protection from a variety of stimuli with potentially aversive long-term effects. One such example involves the plasma stress hormone corticosterone. It has been well-documented that rodents undergo a stress hyporesponsive period (SHRP) in the 2 to 3 weeks shortly after birth, during which it is very difficult to raise plasma corticosterone levels irrespective of many applied stressors (Schmidt et al., 2003). This is evolutionarily advantageous to the organism as high levels of glucocorticoids are damaging to the developing brain, and it is thought that for this reason humans have evolved a similarly adaptive stress hyporesponsive period during early childhood (Lupien et al., 2009). It is not clear exactly how SHRP is maintained, but keeping circulating plasma glucocorticoids at low levels in rodent pups has been linked to aspects of maternal care, particularly feeding (Suchecki et al., 1993; Trottier et al., 1998). Research has demonstrated that newborn rats that experience maternal deprivation do not exhibit the protective SHRP period, show significantly raised plasma corticosterone levels during early life, and go on to demonstrate altered HPA activity in adulthood (Stanton et al., 1987; Levine, 2002). Maternally deprived pups that are supplemented with food demonstrate a normal SHRP period, whereas non-deprived pups that remain with their mothers, but are prevented from feeding, show increases in stress responsivity (Rosenfeld et al., 1993). Researchers have suggested that the specific factor that is transmitted from mother to pup during feeding and acts to confer the SHRP is the protein leptin (Ahima et al., 1998; Trottier et al., 1998; Oates et al., 2000). Intriguingly, early life is also the time of bacterial colonization of the intestinal tract and this coincides and overlaps with the postnatal development of the brain, as well as the SHRP period. As referred to earlier,

there is a strong link between intestinal microbiota and metabolism, including the maintenance of circulating plasma leptin levels. It is therefore possible that one of the mediators of the communication between gut microbiota and the CNS is the plasma protein leptin.

1.4. Enteric Nervous System

The enteric nervous system (ENS) is a dedicated nervous system within the wall of the gastrointestinal tract. While it does make connections with the nervous system extrinsic to the gut, the ENS is capable of operating independently of the spinal cord and brain (Bayliss and Starling, 1899). It is made up of 500 million neurons in humans, (500,000 in mice) clustered in ganglia embedded in the wall of the gastrointestinal tract and together these neurons control gut reflexes, motor patterns, secretion, vasodilation and modulate immune system function. The first descriptions of these ganglionated plexi were in the mid 1800's in separate communications by Meissner, Billroth and Auerbach (Furness, 2006). We now know that ganglia are present in two separate layers of the gut wall, the submucosal or Meissner's plexus located in the submucosal layer, and the myenteric or Auerbach's plexus located between the circular and longitudinal muscle layers. There have been a number of classification systems used to describe the neurons of the ENS, but for the purposes of this thesis we will use that of Hirst and colleagues, which places neurons in one of two groups based on their electrophysiological properties, specifically their ability to fire action potentials (Hirst and Holman, 1978; Bornstein et al., 1994). AH

cells fire a short burst of action potentials which is followed by a prolonged long slow afterhyperpolarization during which the neuron is refractory to further firing for a period of up to 25 seconds. This slow afterhyperpolarization is caused by an influx of calcium into the neuron during the action potential, which then induces calcium-dependent potassium channels to open (Vogalis et al., 2002; Mao et al., 2006). The outward flow of potassium causes the neuronal cell membrane to hyperpolarize, which then reduces the likelihood of the neuron firing additional action potentials as the cell membrane potential is driven lower further from the firing threshold. In addition, due to the influx of positively charged calcium ions into the cell during the action potential AH cells show a calcium hump or "shoulder" during the repolarization phase of the action potential (Schutte et al., 1995). The second category of neurons are classed S neurons, and do not demonstrate the slow afterhyperpolarization period or the calcium hump in the falling phase of the action potential (Hirst and Holman, 1978; Bornstein et al., 1994). Functionally, AH cells are intrinsic primary afferent neurons, responding to luminal chemicals and muscular tension, while S neurons act as motor and interneurons (Smith et al., 1990; Song et al., 1991; Smith et al., 1992; Kunze et al., 1993).

The first ENS neurons to be activated by natural stimuli are the sensory AH cells of the myenteric plexus, or as they are also referred to, the intrinsic primary afferent neurons (IPANs) (Kunze and Furness, 1999). These neurons send processes out to the mucosal layer of the gut wall, terminating close to the epithelial layer. IPANs respond to both mechanical and chemical stimulation of the gut epithelium (Kunze et al., 1995; Kunze et al., 1997). A number of chemical stimuli have been shown to evoke sensory potentials in

these neurons, including acids, short-chain fatty acids and serotonin (Kunze et al., 1995; Bertrand et al., 1997). Most recently probiotics have been shown to increase IPAN excitability but it is not yet known if this is a direct sensory response (Kunze et al., 2009). The IPANs of the myenteric plexus are the first point of contact for the intestinal microbiota residing in the gut lumen and the nervous system and are thus in a favourable position to transmit information from the gut bacteria to the central nervous system as a whole. IPANs synapse on enteric motor neurons controlling gut motility and importantly for a lumen to myenteric plexus to central nervous system signalling pathway there is anatomical evidence of close, synaptic-like connections with vagal nerve endings in the gut (Powley et al., 2008).

Previous work has shown that the motility patterns of GF animals deviate substantially from normal (Husebye et al., 2001). Migrating motor complexes (MMC) are waves of activity that sweep through the intestine at regular intervals during fasting. Fewer MMCs reach the midpoint of the small intestine in GF rats, and the time between these waves in the proximal jejunum is significantly longer in GF *vs* SPF animals (Husebye et al., 1994). Both of these are expressions of abnormal gut motility due to the absence of colonizing microbiota and indicate that neurons of the ENS are responding to changes in the microbial status of the intestinal lumen. Findings from studies investigating the activity of enteric neurons in response to bacteria are now beginning to be published. Applying various *Lactobacillus* species to *ex vivo* segments of guinea pig colon has been shown to result in decreased contraction amplitudes (Massi et al., 2006) and applying *Lactobacillus paracasei* to inflamed gut tissue can reduce hypercontractility in muscle strips (Verdu et

al., 2004). Work in our own laboratories is providing insight on the mechanisms by which bacteria may be acting on enteric neurons to influence motility. Specifically, it has been demonstrated that the probiotic *Lactobacillus rhamnosus* the duration of the post action potential slow afterhyperpolarization plus reducing the excitability of AH cells (IPANs). Because of the similarity of the probiotic effect to that of the intermediate conductance calcium dependent potassium channel blocker TRAM-34 it was suggested that the probiotic or its products target that ion channel (Kunze et al., 2009). In terms of function, this translates to a decrease in motility and it has been previously shown that blockers of the same ion channel cause a decrease in fluid propulsion through the gut (Ferens et al., 2007; Wang et al., 2010a). The reason an increase in enteric sensory neuron excitability produces a decrease in motility, specifically the amplitude of motor complex contractions, was related to the observation that normally resting enteric nervous system activity produces a net inhibitory effect on circular smooth muscle (Wang et al., 2010b).

Clearly when these studies are taken together, we see that the enteric neurons are functionally modulated by the bacterial contents of the intestinal lumen. Given that these same neurons have been shown to form anatomical synapses with vagal nerve endings, it is highly possible that information regarding the intestinal microbiota could be transmitted via these enteric neurons to the extrinsic nervous system. This topic will be explored in more detail in Chapter 5 of this thesis.

1.5 Clinical Relevance

As previously alluded to in this introduction, studying the impact of intestinal microbiota on the development and function of the nervous system has strong clinical implications, the most obvious of these being the application to the frequently comorbid anxiety and mood disorders with IBS and IBD (Creed and Guthrie, 1987; Addolorato et al., 1997; Whitehead et al., 2002; Wood, 2007; Bernstein, 2010). Luminal contents have become a focus of study in the etiology of functional bowel disorders, with a number of studies pointing to variations in the composition of gut microbiota in patients suffering from IBS compared to controls (Malinen et al., 2005; Matto et al., 2005; Kassinen et al., 2007). There is also an established role for the microbiota in IBD, as pathogenesis is linked to the host's inflammatory response against the normally tolerated commensal bacteria (Sartor and Muehlbauer, 2007; Lakatos, 2009). In addition, patients suffering from IBD show a reduced bacterial diversity compared to controls (Manichanh et al., 2006; Ott and Schreiber, 2006; Salzman, 2011). We are only now beginning to understand the role that intestinal microbiota may also play in the pathogenesis of anxiety and depression, with animal studies demonstrating altered bacterial flora in animal models of depression (O'Mahony et al., 2008), GF mice showing hyperreactive HPA activity (Sudo et al., 2004), and probiotics being examined for their potential use in the treatment of depressive symptoms (Desbonnet et al., 2008; Bravo et al., 2011).

One of the main symptoms of IBS is abdominal pain, and probiotics have been shown to alter pain perception in studies using colorectal distension (CRD) in rats. Feeding healthy

rats *L. rhamnosus* attenuated both the cardiac and colonic afferent response to distension compared to vehicle fed control rats, both markers of neurovisceral pain response (Kamiya et al., 2006). In addition CRD has been shown to alter the excitability of dorsal root ganglia (DRG) in healthy animals in the absence of inflammation, after isolation and recovery in culture. Feeding rats *L. rhamnosus* for 9 days previous to distension prevents this increase in DRG excitability post-distension (Ma et al., 2009). The authors hypothesize that the sensory pathway sensitization induced by CRD is ameliorated by the probiotic via activity at the level of the ENS. Given that probiotic has been shown to alter excitability of myenteric neurons (Wang et al., 2010a), the tone of the intestinal muscle would also then be altered, and it has already been established that smooth muscle contraction is necessary for nociceptive afferent signalling in response to CRD (Sarna, 2007). Administering antimicrobials to mice prior to colorectal distension has also been shown alter the gut microbial content and to increase visceral pain perception in a model of functional bowel disorder (Verdu et al., 2006). Feeding the mice *Lactobacillus paracasei* normalized the antibiotic-induced hyperalgesia as well as substance P immunolabelling in gut tissue (Verdu et al., 2006). Again, this study demonstrates that the intestinal microbiota is capable of influencing ENS function, observable via the pain response to mechanical stimuli. Another *Lactobacillus* species, *L. acidophilus* has been shown to upregulate the expression of opioid and cannabinoid receptors in the intestine, providing a mechanism by which the reduction in pain perception after probiotic treatment could be mediated. In the clinical population, meta-analyses are now demonstrating the therapeutic benefits of probiotics on IBS symptoms (McFarland and

Dublin, 2008; Hoveyda et al., 2009), again with the abdominal pain the primary symptom reported.

The clinical relevance of the effects of microbiota on the development of the nervous system has broader implications than may be first considered. Children with autism have been reported to have an increase in both severity and frequency of gastrointestinal problems as compared to healthy volunteers, with a significant correlation found between severity of autism symptoms and severity of gastrointestinal dysfunction (Adams et al., 2011). In addition, it has recently been found that children with autism have significantly lower levels of short chain fatty acids, and an altered microbial profile when compared to children without autism (Adams et al., 2011). Interestingly, a severe form of autism, termed "regressive-onset" is characterized by a later, clear deterioration in previously asymptomatic healthy children. There are wide reports of the use of broad-spectrum antibiotics prior to the appearance of the autistic symptoms in this population. Experimental treatment for regressive-onset autism is a minimally-absorbed oral antibiotic as the proposed pathophysiology involves overproduction of neurotoxin-producing bacteria in the gut following the disruption of the commensal indigenous bacteria (Sandler et al., 2000). Furthermore, there is now evidence of a role for commensal intestinal bacteria in the development of experimental autoimmune encephalitis (EAE), the animal model of multiple sclerosis. Modifying the gut microbiota alters the outcome of this demyelinating disease (Ochoa-Reparaz et al., 2009). Feeding mice antibiotics reduces proinflammatory responses and upregulates regulatory T cells (Tregs) in the mesenteric and cervical lymph nodes. Those Tregs produced in the

cervical lymph nodes show increased IL-10 production after antibiotic treatment, and when adoptively transferred into mice confer protection from EAE (Ochoa-Reparaz et al., 2009; Ochoa-Reparaz et al., 2011). In addition, probiotics have also been demonstrated to alter the course of EAE development in mice. When fed to mice prophylactically, the combination therapy of *L. paracasei and L. plantarum* reduces both EAE symptoms and pro-inflammatory responses (Lavasani et al., 2010). Even more clinically relevant, giving mice with established EAE a treatment of various *L. plantarum* strains reduces the severity of the disease significantly (Lavasani et al., 2010).

Clearly, the study of the intestinal microbiota and its impact on nervous system development function is in its very early stages. Given the wide range of diseases that are already being linked to alterations in the intestinal flora it seems reasonable to presume that this list will only continue to grow. The gut microbiota colonize the largest mucosal surface in the human body and interactions at the host surface have far-reaching significance. Investigating the mechanisms by which the bacteria are communicating with and altering the host nervous system will be paramount to understanding not only pathophysiology of these various disease states, but also how we can take advantage of this relationship to provide alternate therapies.

CHAPTER 2. HYPOTHESIS AND AIMS

Central Hypothesis: That intestinal microbiota play a fundamental role in the development and function of the nervous system. This hypothesis is addressed by the following three studies:

Hypotheses: i) that the absence of intestinal microbiota will result in altered anxiety-like behaviour and brain mRNA expression of genes involved in anxiety and stress-reactivity pathways; ii) that the absence of intestinal microbiota and resulting metabolic changes will alter mRNA gene expression in brain regions critical to both stress reactivity and feeding circuitry; iii) that the absence of intestinal microbiota will result in changes in basal activity of intrinsic primary afferent neurons of the enteric nervous system.

Aims:

1. To determine the impact of the absence of intestinal microbiota on stress-related behaviour and CNS stress circuitry.
2. To determine the impact of the absence of intestinal microbiota on circulating metabolic and stress hormones during prepuberty and adulthood, and on central circuitry related to both.
3. To determine the role of gut microbiota on basal excitability and magnitude of response of intrinsic primary afferent neurons of the enteric nervous system.

CHAPTER 3.

Reduced anxiety-like behaviour and central neurochemical change in germ-free mice

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Abstract

There is increasing interest in the gut-brain axis and the role intestinal microbiota may play in communication between these two systems. Acquisition of intestinal microbiota in the immediate postnatal period has a defining impact on the development and function of the gastrointestinal, immune, neuroendocrine and metabolic systems. For example, the presence of gut microbiota regulates the set point for hypothalamic-pituitary-adrenal (HPA) axis activity. We investigated basal behavior of adult germ free (GF), Swiss Webster female mice in the elevated-plus maze (EPM) and compared this to conventionally reared specific pathogen free (SPF) mice. Additionally, we measured brain mRNA expression of genes implicated in anxiety and stress-reactivity. GF mice, compared to SPF mice, exhibited basal behavior in the EPM that can be interpreted as anxiolytic. Altered GF behaviour was accompanied by a decrease in the NMDA receptor subunit NR2B mRNA expression in the central amygdala, increased brain-derived neurotrophic factor (BDNF) expression and decreased serotonin receptor 1A (5HT1A) expression in the dentate granule layer of the hippocampus. We conclude that the presence or absence of conventional intestinal microbiota influences the development of behavior, and is accompanied by neurochemical changes in the brain.

3.1. Introduction

Within the first few days of birth the infant gut is colonized by commensal intestinal microbiota, an event that heralds the beginning of a mutually beneficial lifelong relationship. In the human lower intestine, numbers of bacteria reach 10^{14} organisms per gram of intestinal contents with roughly 1800 different species accounted for (Frank and Pace, 2008). It is well established that intestinal microbiota are essential to gastrointestinal development and function, regulate host inflammatory responses, and develop and sustain immune homeostasis (Cebra, 1999; Backhed et al., 2005). A rapidly growing body of evidence now also indicates that the microbiome acts as a metabolically active organ within the host, capable of influencing fat storage and metabolism (Hooper et al., 2001; O'Hara and Shanahan, 2006; Velagapudi et al., 2009). Gut-brain communication is important to human gastrointestinal and psychiatric illness, which is highlighted by the increased comorbidity found between anxiety disorders and both inflammatory bowel disease and the functional bowel disorders (Whitehead et al., 2002; Wood, 2007; Walker et al., 2008). But while early postnatal bacterial colonization overlaps with a vulnerable time in the development of the central nervous system (CNS) (Hensch, 2004; Knudsen, 2004), to date there has been little research determining the effects of normal bacterial colonization on the development and function of the brain.

Exact mechanisms by which the brain and gut converse are not fully understood, but bi-directional communication through the autonomic nervous system, immune system and the hypothalamic pituitary adrenal (HPA) axis is well documented (Shanahan, 1999;

Drossman, 2005; Berthoud, 2008). Preclinically, intriguing findings report that subclinical doses of pathogenic bacteria alter both anxiety-like behaviour and vagally mediated neural activation in the brain stems of infected mice in the absence of overt gut inflammation (Lyte et al., 2006). But while these experiments are pertinent to the study of gut-brain communication, it is not clear whether non-pathogenic bacteria similarly communicate with the brain. To this end, Li et al have more recently demonstrated that information regarding dietary induced changes to commensal microbiota may be transmitted to the brain and result in changes to learning and memory in rodents (Li et al., 2009). These findings emphasize the importance of understanding the roles that not only intestinal microbiota may play in the communication between the gut and brain, but also diet and the nutritional status conferred by the microbiota to the host.

Based on work to date we hypothesized that commensal intestinal microbiota normally communicate with the brain and their presence influences CNS development and behaviour. To test this hypothesis, we carried out experiments using germ-free (GF) mice. Previous work using these animals has demonstrated that GF mice exhibited hyperresponsive HPA axis activity following stress as compared to SPF mice (Sudo et al., 2004), however no associated behavioral changes were investigated in this study. To this end we investigated the basal behavioral phenotype in adult GF versus SPF mice. Converging lines of evidence from preclinical and clinical literature suggest that plasticity-related genes, particularly brain derived neurotrophic factor (BDNF) in the hippocampus, influence CNS stress circuitry (Smith et al., 1995; Rasmusson et al., 2002; Roceri et al., 2002; Roceri et al., 2004; Alfonso et al., 2006; Berton et al., 2006;

Martinowich and Lu, 2008) and stress-related behaviours (Itoh et al., 2004; Chan et al., 2006; Chen et al., 2006; Hunnerkopf et al., 2007; Bergami et al., 2008) and we selected to examine hippocampal BDNF mRNA expression in GF and SPF mice. Hippocampal gene expression of the serotonin receptor 5-HT1A was also selected for study, as it is the most commonly linked of the serotonin receptors to emotional behaviour and anxiety (Menard and Treit, 1999; Kalynchuk et al., 2006). Also, we selected the NMDA glutamate receptor subunits NR1, NR2A and NR2B for examination, as NMDA receptors are known to play an important role in synaptic development and plasticity, learning and memory and in the extended amygdala are thought to be involved in central expression of anxiety (Lopez de Armentia and Sah, 2003; Kash et al., 2009).

3.2 Methods

3.2.1. Animals. Female Swiss Webster, eight week old GF and SPF mice were obtained from Taconic Farms Inc., Germantown, NY, USA. GF mice were maintained in regular cages (16.5 cm x 28.1 cm x 12.7 cm) inside a guaranteed germ-free shipper with free access to food and water until the beginning of testing. Upon arrival, SPF mice were transferred to microisolator cages (15.2 cm x 26.7 cm x 12.7 cm) and maintained in standard housing until the beginning of testing. All animals were housed under 12 h light-12 h dark cycle, lights on at 7 AM. Housing room temperature was maintained at 20°C and humidity at 60-70%. With the exception of shipping container type, there were no differences in supplier, shipping or receiving of GF and SPF mice.

3.2.2. Ethics. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

3.2.3. Experimental Design. Behavioral testing was performed 48 h after animals arrived in the facility (n=12 per housing condition). While a longer acclimatization period would be better for behavioural testing, this was the maximum time that was logistically possible to keep the animals in their germ-free state. Prior to behavioural testing, GF mice were transferred into microisolator cages (4 per cage). Locomotor activity was tested first and 3 h later elevated plus maze (EPM) testing was completed. Mice were returned to the housing room between tests. In a separate cohort of animals (n=12), blood and brains were collected 48 h after animals arrived in the facility for corticosterone and *in situ* hybridization analysis. All blood was collected between 10 AM and noon.

3.2.4. Locomotor Activity. Animals were transported to a non-colony room for behavioural testing. Plexiglas activity chambers (40x40x35 cm) were interfaced to a Digiscan 16 monitor and a computer that provided automated recording of locomotor activity using VersaMax software (AccuScan Instruments, Columbus, OH). The activity chambers were covered with ventilated Plexiglas lids. Animals were left undisturbed in activity chambers for 30 min and general locomotor activity for each animal was acquired.

3.2.5. Elevated Plus Maze. Animals were transported singly to a different non-colony room. Each animal was placed in the center of the EPM and performance was videotaped

throughout a 5-min session. Video recordings were analyzed. An entry was scored when all four paws were in an arm of the maze. Data were collected on the number of entries into the open and closed arms of the maze and time spent in different arms (Rodgers and Dalvi, 1997).

3.2.6. Tissue Collection. In a second cohort of animals (n=12 per housing condition), blood and brain tissue was collected 48 h after arrival in the facility, as described above. Blood was collected and processed to collect plasma, and stored at -70°C until use. Brains were rapidly removed following decapitation, frozen in -60^{o}C isopentane, and stored at -70^{o}C until cryostat sectioning.

3.2.7. Corticosterone Analysis. Corticosterone (CORT) was measured in duplicate samples using a standard radioimmunoassay kit from MP Biomedicals.

3.2.8. In situ hybridization. Standard *in situ* hybridization methods were used, details of which have been previously described (Whitfield et al., 1990; Foster et al., 2004). The BDNF riboprobe was generously provided by Drs J. Lauterborn and C. Gall, University of California Irvine, Irvine, CA. The antisense probe produced from the BDNF cDNA template is a 382 bp probe complementary to the coding region of mouse BDNF mRNA (bases 1028-1410, NM_007540). The 5HT1A receptor riboprobe was generously provided by Dr. Pat Levitt, University of Vanderbilt, Nashville, TN. NR1, NR2A and NR2B riboprobes were generated in our laboratory. NR1 primers forward 5'-GTCCTCTGCCATGTGGTTTT-3' and reverse 5'-GGACAGGGACACATTTTGCT-3', NR2A primers, forward 5'-CAGCTGAAGAAGATCCACTCCT-3′ and reverse 5′-

GCAGTGGTTAAGATCCCAAGAC-3', and NR2B primers forward 5'-TAGCTATAGAGGAGCGCCAATC-3' and reverse 5'-CTCGATTTCATCAAACTCCCTC-3' were designed using Primer 3 online software (NR1) (Rozen and Skaletsky, 2000) or obtained from the Allen Brain Atlas (NR2A, NR2B) (Lein et al., 2007). Specificity of primers to mouse NR1, NR2A and NR2B mRNAs was confirmed using BLAST (Altschul et al., 1990). PCR generated cDNA (521 bp NR1, 253 bp NR2A, 694 bp NR2B) was inserted in the pGEM T-easy expression vector (Promega, Mississauga, ON, Canada). Antisense and sense probes were transcribed from linearized plasmids with α-35S-UTP (specific activity >1,000 Ci/mmol; Perkin Elmer, Boston, MA, USA) using appropriate RNA polymerases. Hybridization of sense probes did not reveal any signal.

3.2.9. Data Analysis. All data was analyzed using GraphPad Prism (LaJolla, CA, USA). Statistical significance was evaluated using two-way ANOVA with Bonferroni post-tests for locomotor activity (time and housing as factors) and EPM (housing and location as factors). Two-tailed unpaired t-tests were used for comparison of BDNF and 5-HT1A receptor mRNA signal in different hippocampal subregions in GF and SPF mice, and for amygdala subnuclei expression of NR1, NR2A and NR2B mRNAs. Statistical criteria for significant differences were set at $p<0.05$. Data are presented as mean +/- SEM.

3.3. Results

3.3.1. Gut Microbiota

GF status was confirmed by microbiological evaluation and showed no growth for anaerobic, aerobic, and mycotic bacteria (Taconic, Germantown, NY).

3.3.2. Locomotor Activity

Total distance traveled in the activity chambers did not differ between SPF and GF mice (SPF = 9,005 cm $\pm$ 106, GF = 10,654 cm $\pm$ 1455, t=1.24, df=22, p=0.113). Figure 1 shows the distance traveled in the open field at 5 min intervals over the 30 m test period. No difference was observed in these values between GF and SPF mice ($F_{1,105}$=0.86, p=0.36).

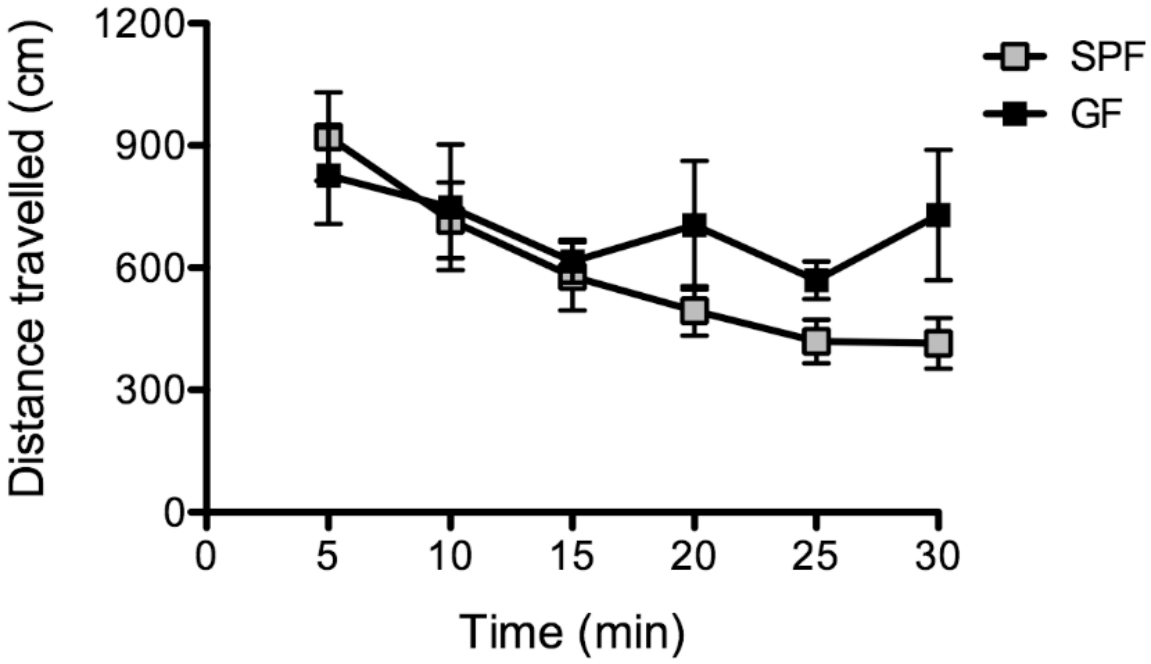

Figure 1. Locomotor activity was measured in SPF and GF mice in the open field. Total distance traveled is shown at 5 m intervals for the 30 m testing period. No differences were observed between activity in SPF and GF mice. Values are means +/- S.E.M.

3.3.3. EPM behaviour

GF mice showed increased open arm exploration in the EPM compared to SPF mice (Fig. 2A; $F_{1,44}$=22.8, p<0.0001). Posthoc analysis showed a significant increase in time spent in the open arms by GF mice compared to SPF mice (Bonferroni, p<0.01) and a significant decrease in time spent in the closed arm by GF mice compared to SPF mice (Bonferroni, p<0.01). This was accompanied by an increased number of open arm entries (Fig. 2B; $F_{1,44}$=8.23, p=0.0063; Bonferroni p<0.001), however, there was no significant difference between GF and SPF mice in number of entries into the closed arms (Bonferroni, p>0.05). We conducted a minute-by-minute analysis of EPM open arm entries which revealed that GF mice continued to explore the open arms with the same frequency as the test progressed while the SPF mice showed less open arm entries per minute as the test continued (Fig. 2C). These behavioural data have been presented previously in poster form at the Neurogastroenterology & Motility meeting in 2008 (Neufeld et al., 2008).

3.3.4. Plasma corticosterone

Plasma corticosterone levels were determined in mice 48 h after arrival in the facility. GF mice showed significantly higher corticosterone levels compared to SPF mice (Fig. 3; t=2.48, df=22, p=0.021). It is possible that elevated corticosterone in GF mice in this experiment reflects an increased stress response related to the short acclimatization period (48 h) prior to tissue collection as previous work did not detect differences in basal corticosterone in Balb/C germ-free mice (Sudo et al., 2004).

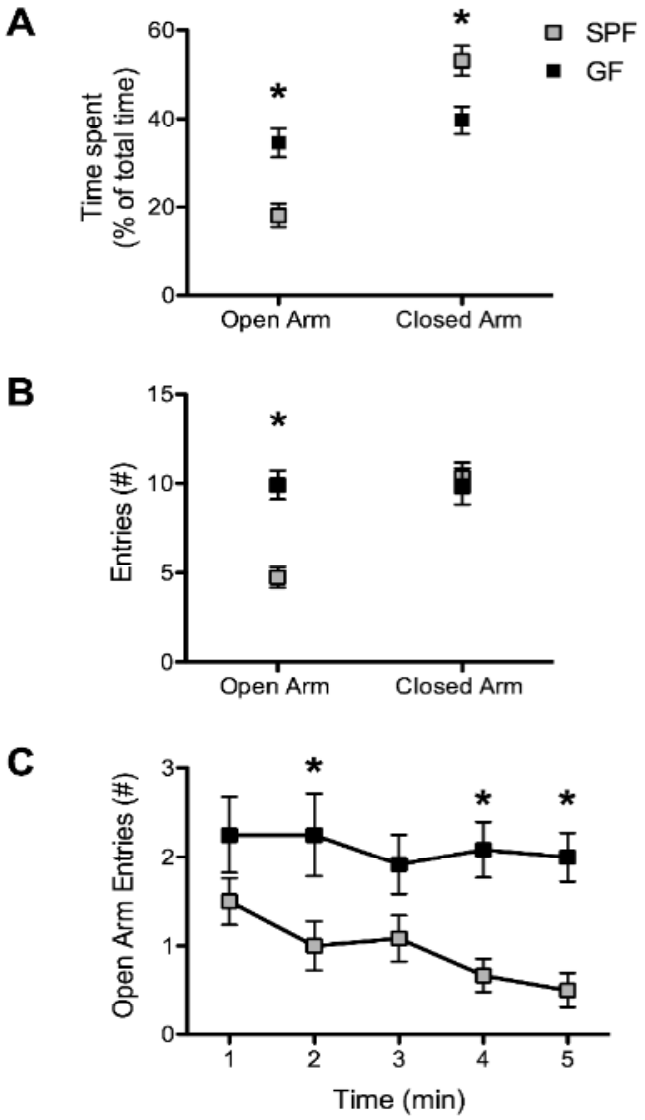

Figure 2. Testing in the elevated plus maze revealed an anxiolytic-like behaviour in GF mice compared to SPF mice. A. GF mice spent significantly more time in the open arms of the EPM and significantly less time in the closed arms than SPF mice. B. GF mice showed increased open arm entries but no differences in closed arm entries (activity) compared to SPF mice. C. Minute by minute analysis of EPM behaviour showed that GF mice continued to enter the open arms for the duration of the test. Values are means +/- S.E.M., *$p<0.05$.

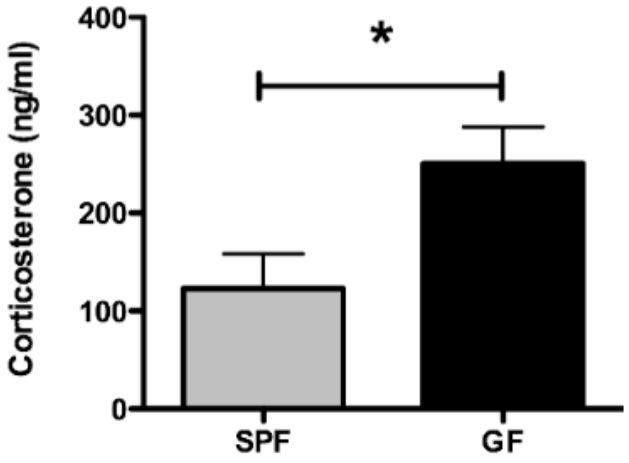

Figure 3. Plasma corticosterone was measured in GF and SPF mice 48 h after arrival in the animal facility. Significantly higher levels of plasma corticosterone were detected in GF mice compared to SPF mice. Values are means +/- S.E.M., *$p<0.05$.

3.3.5. Altered gene expression

Housing (GF vs SPF) had a significant impact on BDNF mRNA expression in the hippocampus. Representative film images are provided for BDNF mRNA signal observed in SPF (Fig. 4a) and GF (Fig. 4b) mice. Analysis revealed a specific significant up-regulation of BDNF mRNA in the dentate gyrus of the hippocampus (Fig. 4c, $t=2.97$, $df=20$, $p=0.0076$). There were no significant differences in BDNF expression in other subregions of the hippocampus ($p>0.05$). Housing (GF vs SPF) also had a significant impact on 5HT1A receptor expression in the hippocampus. Representative film images are provided for 5HT1A receptor mRNA expression observed in SPF (Fig. 5a) and GF (Fig. 5b) mice. Analysis revealed a specific significant down-regulation of 5HT1A receptor mRNA in the dentate gyrus of the hippocampus (Fig. 5c, $t=3.18$, $df=20$, $p=0.0047$). There were no significant differences in 5HT1A receptor expression in the CA1 subregion of the hippocampus ($p>0.05$). Housing (GF vs SPF) had a significant impact on NR2B mRNA expression in the amygdala. Representative film images of NMDA subunit mRNA expression in SPF mice are provided (Fig.6). As indicated by the outline in Fig. 6a and 6d, mRNA for the CeA was measured at Bregma -1.06 mm and for the LA and BLA at Bregma -1.94 mm (Paxinos and Franklin, 2001). Densitometric analysis revealed a significant down-regulation of NR2B mRNA in the CeA region of the amygdala (Fig. 7c, $t=2.82$, $df=22$, $p=0.0099$). There were no significant differences in NR2B mRNA expression in other subregions of the amygdala ($p>0.05$). There were no significant differences between GF and SPF mice in NR1 or NR2A mRNA gene expression in any of the subnuclei of the amygdala (Fig. 7a,7b;$p>0.05$).

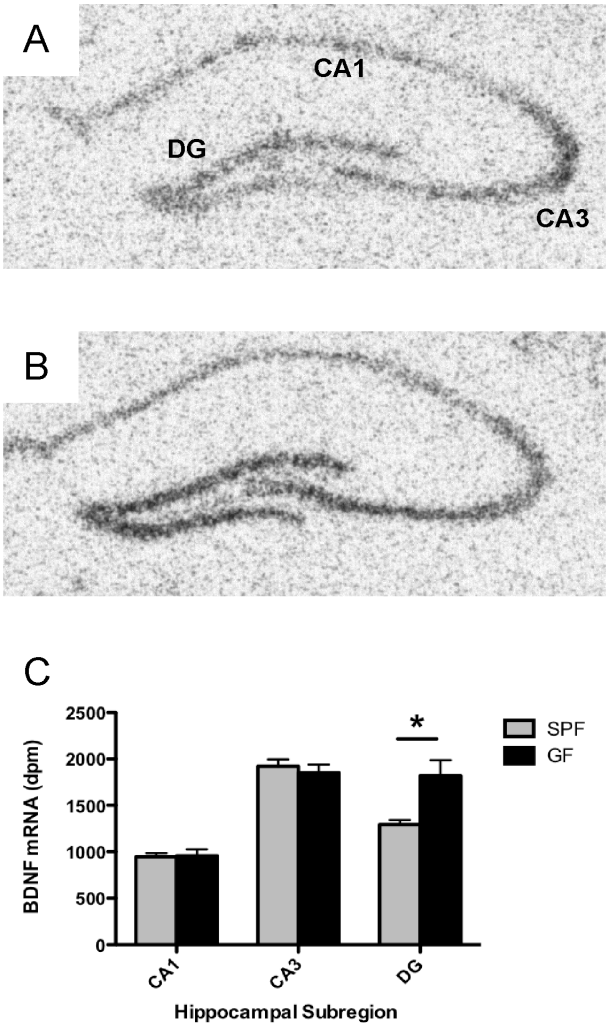

Figure 4. BDNF mRNA gene expression in subregions of the hippocampus of GF and SPF mice. A. Representative film image of basal expression of BDNF mRNA in SPF mouse hippocampus. B. Representative film image of basal expression of BDNF mRNA in GF mouse hippocampus. Qualitatively, increased expression in the dentate granule (DG) can be seen in the GF mouse compared to the SPF mouse. C. Quantitative densitometry revealed that GF mice show a significant increase in BDNF mRNA in the dentate granule region of the hippocampus compared to SPF mice. CA1 and CA3 subregions did not show a difference in BDNF mRNA expression between GF and SPF mice. Values are means +/- S.E.M., * $p<0.05$

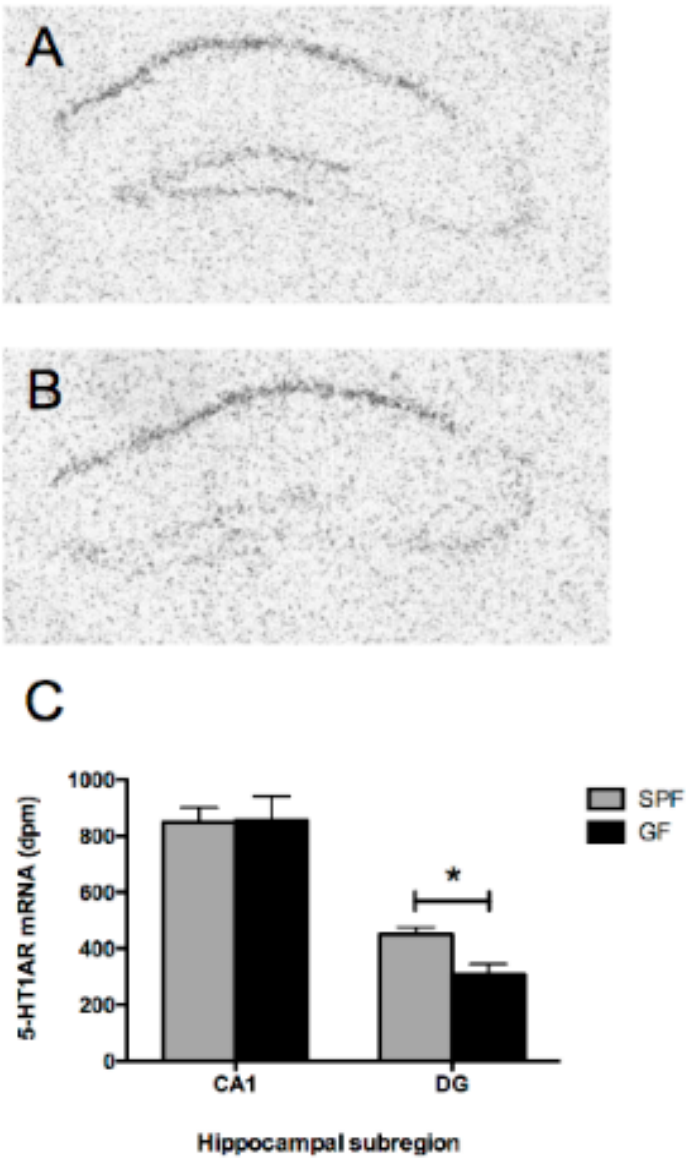

Figure 5. A. Representative film images of 5HT1A mRNA expression in hippocampus of SPF and (B) GF mice. C, Densitometric analysis of 5HT1A mRNA showed a significant decrease in 5-HT1AR mRNA in the dentate granule layer of GF mice compared to SPF mice. Values are means +/- S.E.M., * $p<0.05$

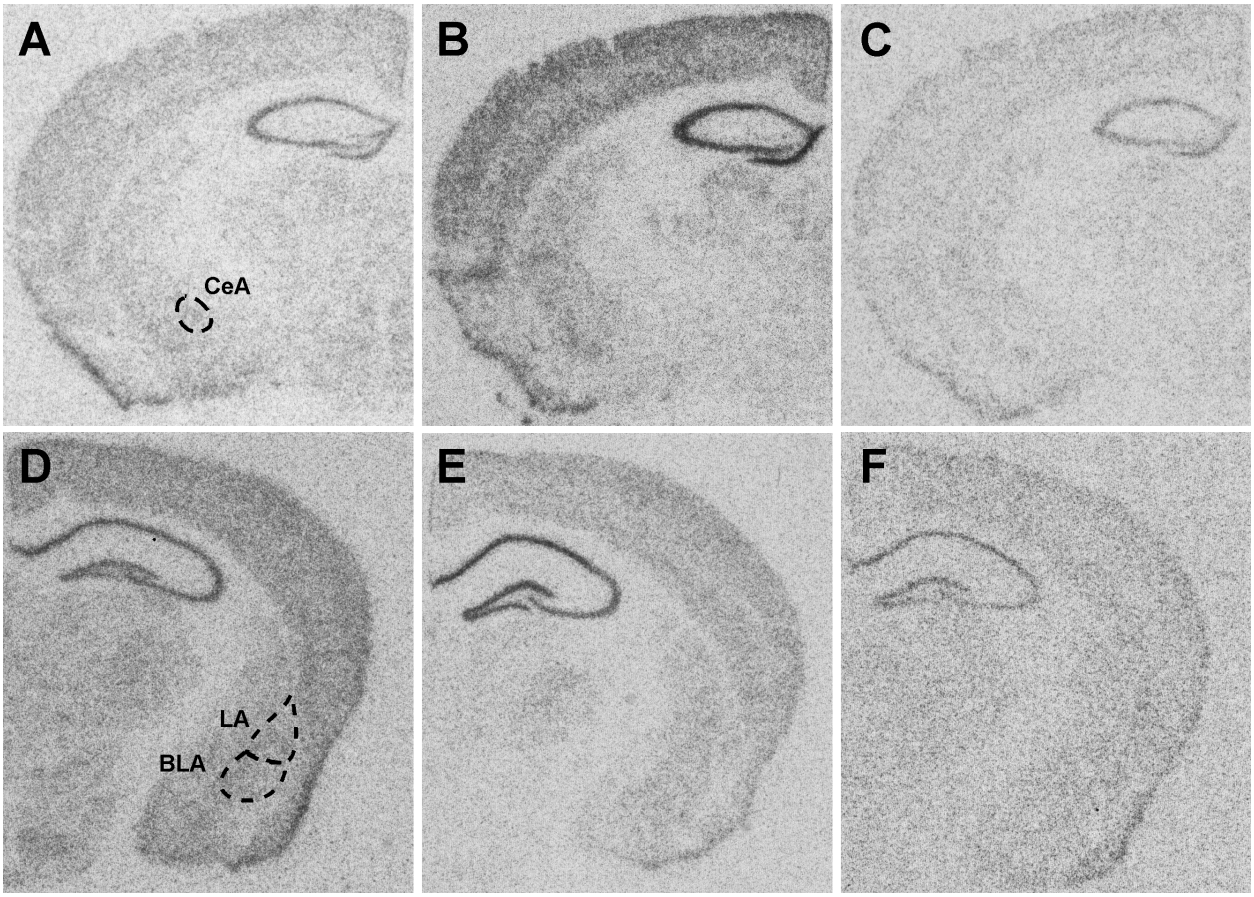

Figure 6. Representative film images of NMDA subunit mRNA expression at the level of the central amygdala (Bregma -1.06 mm), A. NR1 B. NR2A C. NR2B and at the level of the lateral and basolateral amygdala (Bregma -1.94 mm) D. NR1 E. NR2A F. NR2B. Areas of interest are indicated in panel A - central amygdala (CeA) and in panel D - lateral amygdala (LA) and basolateral amygdala (BLA).

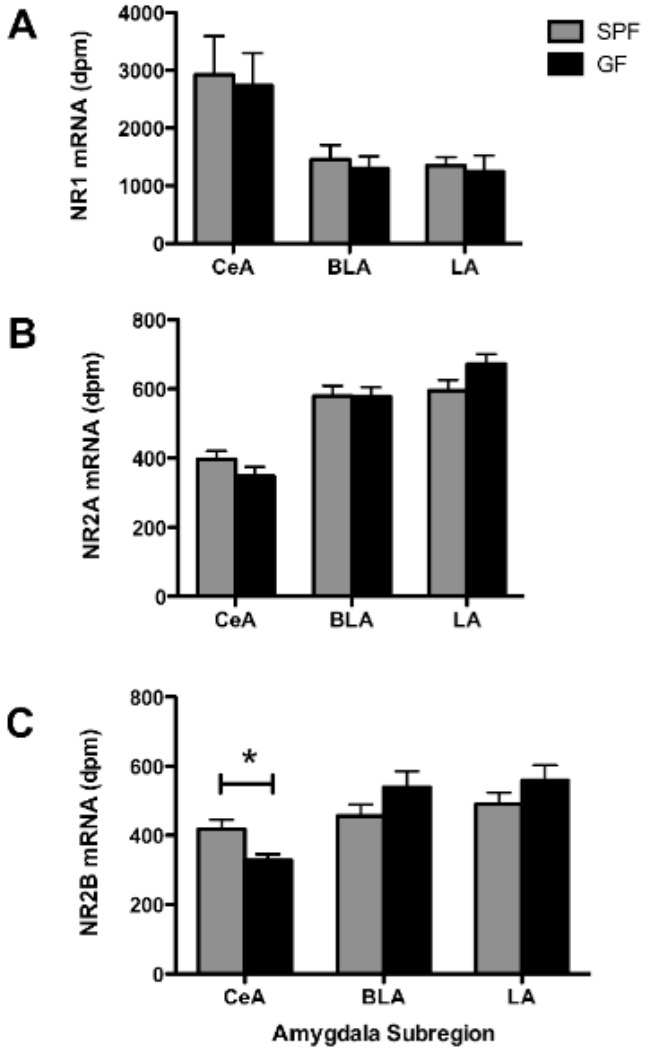

Figure 7. Densitometric analysis of NR1 mRNA (A) and NR2A mRNA (B) did not reveal differences in expression between SPF and GF mice. C. Analysis revealed a significant decrease in NR2B mRNA in the CeA in GF mice compared to SPF mice. Values are means +/- S.E.M., * $p<0.05$

3.4. Discussion

As illustrated by several recent comprehensive reviews, the topic of intestinal microbiota and its impact on the nervous system (Collins and Bercik, 2009; Rhee et al., 2009) has broad applications for both gastrointestinal and psychiatric illness (Neufeld and Foster, 2009; Forsythe et al., 2010), perhaps especially for those like the functional bowel disorders that show co-morbidity across both systems. Clearly then, the study of the impact of gut microbiota on behavior and mRNA gene expression in brain tissue is of high clinical relevance. Our data suggest that gut bacteria influence the development of behavior in mice. In this study we used the EPM, an ethologically and pharmacologically validated tool for the assessment of rodent anxiety-like behavior (Pellow and File, 1986; Carobrez and Bertoglio, 2005), to examine basal behavior of GF and SPF mice. The basal behavioral phenotype observed in EPM activity in adult GF as opposed to SPF mice is interpreted as anxiolytic. Additionally, we report central changes in gene expression in plasticity-related genes that have been established to play a role in emotional behaviour in mice. Specifically we observed a significant basal decrease in NMDA receptor NR2B mRNA expression in the central amygdala, an increase in hippocampal BDNF mRNA expression and a decrease in 5-HT1A receptor mRNA expression in the dentate gyrus of GF compared to SPF mice.

Our behavioural data show no differences in locomotor activity in GF and SPF mice, but increased open arm exploration in GF mice in the EPM compared to SPF mice. These data must be considered within the context of limitations to our experimental design. 1)

The order and timing of behavioural testing in rodents is known to impact the outcome and in particular, testing anxiety-like behaviour in the EPM is influenced by prior testing (Bouwknecht et al., 2004; Cryan and Holmes, 2005). The design of our behavioural testing was not ideal as both behavioural tests occurred on the same day, and therefore we can not be certain that the exposure to the open field did not affect the EPM behavioural data. 2) Our GF mice were exposed to SPF conditions in the 3 h period prior to EPM testing and while GF status was confirmed prior to shipping, we did not assess the microbiota status at time of behavioural testing. 3) Estrous cycle can influence stress-related behaviours (Gangitano et al., 2009; Walf et al., 2009) and differences in estrous may have contributed variation to our data. 4) In a second group of mice, our corticosterone analysis showed higher levels in GF mice 48 h after arrival in the facility. As Sudo et al. have previously shown an exaggerated corticosterone response to stress, it is possible that the increased levels of plasma corticosterone reflect a stress response in GF mice to experimental conditions. Alternatively, our work was completed in female Swiss Webster mice compared to male Balb/C GF mice in previous work so both strain and sex could contribute to the differences observed (Cryan and Holmes, 2005). Considering these limitations is important, however, one would expect that these factors would increase anxiety-like behaviour in mice and we did not observe this. The open arm exploration time and the open and closed arm entry data observed in our SPF are comparable to published data for these measures in Swiss Webster mice that have had longer habituation periods (Rodgers et al., 2002; Adamec et al., 2006). In addition, based on the increased corticosterone level, one would expect to see increased anxiety-like

behaviour in GF mice and we saw a robust anxiolytic behavioural phenotype on several outcome measures in the EPM. These observations are in line with previous findings in male Swiss Webster mice showing that anxiety-like behaviours in the EPM are not related to corticosterone levels (Rodgers et al., 1999). Our molecular data provide initial insights into the neurobiological pathways underlying this behavioural phenotype.

Our finding of a downregulation in gene expression of the glutamate NMDA receptor subunit NR2B in the central amygdala of GF mice is intriguing. NMDA receptors are heteromeric complexes, and are made up of both NR1 and NR2 subunits. Previously, Sudo et al. used PCR and showed downregulation in NR2A mRNA in the cortex and the hippocampus of GF mice compared to SPF mice, however, we did not detect differences in subregions of the hippocampus by *in situ* hybridization. We extended our analysis to the NR2B subtype and to include the amygdala. The NR2B subtype is the critical receptor in amygdala synaptic plasticity and development, as well as in learning and memory (Cull-Candy et al., 2001). Additionally, NMDA receptor antagonists are known to block anxiety in both mice and rats (Jessa et al., 1995; Kotlinska and Biala, 1999). In a previous study examining ethanol-withdrawal induced anxiety in rats, administration of NMDA receptor antagonists resulted in anxiolytic-like behaviour as measured in the EPM, with treated animals showing increases in both time spent in the open arm of the EPM, and open arm entries (Gatch et al., 1999). This behavioural phenotype is comparable to that of our GF animals, and was accompanied by the basal decrease in NMDA receptor expression. Antagonists specific to NR2B block the acquisition of amygdala-dependent fear learning (Rodrigues et al., 2001) thereby further illustrating the

role that this NMDA receptor subtype plays in the expression of anxiety, fear and CNS plasticity. It is possible that the downregulation of NR2B mRNA in our GF mice is contributing the anxiolytic-like phenotype that we observe.

Serotonin receptors are distributed throughout the CNS and receive input from neurons of the dorsal and ventral raphe. Several 5HT receptors have been implicated in anxiety-like behaviours (Kaiyala et al., 2003; Lesch et al., 2003; Dwivedi et al., 2005), however, the 5HT1A receptor has received more attention in both clinical and preclinical work. 5HT1A is both a presynaptic autoreceptor and a postsynaptic receptor. Pharmacological interventions in rodents show that activation of both receptors can reduce firing of serotonergic neurons (Sprouse and Aghajanian, 1988). Reduced firing would result in reduced 5HT synthesis, reduced 5HT release in projection areas, and reduced 5HT turnover (Kennett et al., 1987; Bohmaker et al., 1993). A role for the 5HT1A receptor in anxiety-like behaviour is well supported by pharmacological studies showing an anxiogenic effect by 5HT1A agonists (Blanchard et al., 2003; Li et al., 2004; David et al., 2007), and by genetic studies showing increased anxiety-like behaviours in 5HT1A-deficient mice (Parks et al., 1998; Ramboz et al., 1998; Holmes et al., 2003). Hence, it is reasonable to suggest that changes in serotonergic signalling in GF mice may contribute to the altered anxiety-related phenotype observed. It is of interest to continue this work and consider how serotonergic signalling in the hippocampus and the hypothalamus might be altered in GF mice.

Up-regulation of BDNF mRNA in the dentate region of the hippocampus in the GF mice is also consistent with literature identifying a role for this molecule in anxiety-like behaviours (Ren-Patterson et al., 2005; Martinowich and Lu, 2008). Recent work has demonstrated that impaired BDNF signalling in the dentate gyrus of adult mice results in a marked increase in anxiety-like behavior (Chen et al., 2006; Bergami et al., 2008). Restraint stress is known to decrease expression of hippocampal BDNF (Alfonso et al., 2006), activate the HPA axis (Butterweck et al., 2001; Reyes et al., 2003), and result in increased anxiety-like behavior in rodents (Bhatnagar et al., 2004). While these reports support our suggestion that higher BDNF levels are related to the observed reduction in anxiety-like behaviours in GF mice, the whole story is certainly more complex. Importantly, Sudo et al. observed decreased levels of BDNF protein in the hippocampus of GF mice. These differences may relate to differences at the level of mRNA and protein as a modification at the level of the transcript does not necessarily translate to a similar change at the level of the protein expression and protein levels were not examined in the current study. Alterations in housing conditions such as psychosocial enrichment can also positively impact hippocampal BDNF expression levels and suppress stress-related behaviours (Zhu et al., 2006).

The role of intestinal microflora in shaping a fully functional immune system is established (Cebra, 1999; Hooper et al., 2001; Macpherson et al., 2001; Macpherson and Harris, 2004). Evidence of behavioural alterations in conjunction with adaptive immune deficits have been demonstrated by Cushman et al. who showed that deletion of the recombinase activating gene (RAG-1) in mice which results in absent antibody

synthesizing capability, caused increased exploration in the open field and decreased open arm avoidance in the EPM (Cushman et al., 2003). However, it should be noted that RAG-1 is also expressed in the CNS (Chun et al., 1991) where its function has yet to be elucidated. It is possible that the known immaturity of the adaptive immune system of GF mice contributes to the behavioural phenotype that we observed.

The sensory arm of the autonomic nervous system may be a route whereby gut microbiota affect brain function and several reports make this link. Oral ingestion of a bifidobacterium by conventional Sprague Dawley (SD) rats has been shown to result in changes to serotonin metabolism in the brain stem (Desbonnet et al., 2008). Consumption of *Lactobacillus reuteri* (LR) by conventional SD rats inhibited the perception of pain consequent to visceral distension (Kamiya et al., 2006) and administration of *Lactobacillus paracasei* likewise normalized antibiotic-induced visceral hypersensitivity in mice during colorectal distension (Verdu et al., 2006). Rousseaux et al. have reported that ingestion of yet another commensal, *Lactobacillus acidophilus*, by both conventional mice and rats promoted the expression of opioid and cannibinoid receptors by gut epithelial cells. Here too, ingestion resulted in decreased sensitivity to visceral distension (Rousseaux et al., 2007). Taken together, these reports suggest that commensal bacteria can affect nerve function and pathways even in conventional animals. Recent work in our laboratories has shown that oral treatment of SD rats with LR consistently inhibits calcium dependent potassium channels in a specific subset of enteric neurons in the myenteric plexus (Kunze et al., 2009), inhibits intestinal contractility (Ma et al., 2009), and hyperexcitability in dorsal root ganglion neurons (Wang et al., 2009). These

observations thus afford a direct link between luminal commensals and the enteric nervous system.

To our knowledge, ours is the first work to demonstrate an altered behavioral phenotype associated with the absence of intestinal microbiota. Further work exploring these associations may lead to novel insights into the complex roles of commensal bacteria in the development and function of the CNS. Unravelling these pathways may eventually lead to new therapeutic approaches in dealing, for example, with the known significant comorbidity between irritable bowel syndrome and psychiatric illness (Whitehead et al., 2002).

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CHAPTER LINK

In the previous chapter we demonstrated that adult germ free (GF) mice display a basal anti anxiety-like phenotype as measured in the elevated plus maze as compared to conventionally housed control mice. In addition, we reported central changes in gene expression in plasticity-related genes that have been established to play a role in emotional behaviour in mice. Specifically we observed a significant basal decrease in NMDA receptor NR2B mRNA expression in the central amygdala, an increase in hippocampal BDNF mRNA expression and a decrease in 5-HT1A receptor mRNA expression in the dentate gyrus of GF mice. Given that it has previously been reported that conventionalizing GF mice with commensal intestinal microbiota at 6 weeks of age but not later ameliorated HPA hyperreactivity to restraint stress (Sudo et al., 2004), we decided to examine if a similar critical window existed in our animals. To this end, the following chapter represents an addendum that we published to the original paper, showing our results in GF mice after adult conventionalization with commensal intestinal microbiota.

CHAPTER 4.

Effects of intestinal microbiota on anxiety-like behaviour

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Abstract

The acquisition of intestinal microbiota in the immediate postnatal period has a defining impact on the development and function of many immune and metabolic systems integral to health and well-being. Recent research has shown that the presence of gut microbiota regulates the set point for hypothalamic-pituitary-adrenal (HPA) axis activity (Sudo et al., 2004). Accordingly, we sought to investigate if there were other changes of brain function such as behavioural alterations in germ free (GF) mice, and if so, to compare these to behaviour of mice with normal gut microbiota. Our recent paper showed reduced anxiety-like behaviour in the elevated-plus maze (EPM) in adult GF mice when compared to conventionally reared specific pathogen free (SPF) mice (Neufeld et al., 2011). Here, we present data collected when we next colonized the adult GF mice with SPF faeces thereby introducing normal gut microbiota, and then reassessed anxiety-like behaviour. Interestingly, the anxiolytic behavioural phenotype observed in GF mice persisted after colonization with SPF intestinal microbiota. These data show that gut-brain interactions are important to CNS development of stress systems and that a critical window may exist after which reconstitution of microbiota and the immune system does not normalize the behavioural phenotype.

4.1 Addendum

It is well established that the gut and brain communicate in a bidirectional manner through the autonomic nervous system, immune system and the HPA axis (Shanahan, 1999; Drossman, 2005; Berthoud, 2008). Gut-brain communication is important to human gastrointestinal and psychiatric illness, which is highlighted by the increased comorbidity found between mood and anxiety disorders and both inflammatory bowel disease and the functional bowel disorders (Creed and Guthrie, 1987; Wood, 2007; Walker et al., 2008). Indeed the focus of Rome III, a diagnostic instrument designed to aid clinicians in the diagnosis of functional bowel disorders such as irritable bowel syndrome (IBS), is on gut, brain and spinal cord interactions and their involvement in the generation of symptoms of pain and intestinal dysfunction (Grundy et al., 2006). Luminal contents have also become a focus of study in the etiology of functional bowel disorders, with a number of studies pointing to variations in the composition of gut microbiota in patients suffering from IBS compared to controls (Matto et al., 2005). A recent report found that compared to specific-pathogen free (SPF) mice, adult germ free mice showed an exaggerated stress response, as evidenced by increased plasma corticosterone (CORT) and adrenocorticotrophic hormone (ACTH) levels in response to restraint stress (Sudo et al., 2004). Clearly the study of the impact of gut microbiota on the development of HPA dysfunction and potentially anxiety-like behaviour has important clinical applications in the study of both gastrointestinal and psychiatric health and disease, as they are essentially involved in the communication between the gut and the brain.

We propose that the intestinal microbiota housed within the gastrointestinal tract may act as a mediator in the communication existing between the gut and the brain. In our recent study (Neufeld et al., 2011), we examined behaviour in germ free (GF) mice and provided evidence that gut microbiota are important to the development of stress circuitry and related behaviours. We demonstrated that GF mice show reduced anxiety-like behaviour in the elevated plus maze (EPM) in comparison to specific pathogen free (SPF) mice, a phenotype that was accompanied by changes in plasticity-related genes in the hippocampus and amygdala (Neufeld et al., 2011). Our results provide support for the idea that normal healthy gut bacteria may influence the development of the CNS and thereby its function, as reflected by both the behavioural and molecular changes seen in GF mice. Our findings are consistent with another recent report linking gut microbiota with behaviour and neurochemistry (Heijtz et al., 2011). In both the original work by Sudo et al., and the recent work by Heijtz et al., the authors addressed the issue of critical windows of development. Sudo et al. demonstrated reconstitution of germ free mice with SPF flora at 6 wk (young adult) but not at 14 wk (adult) reversed the HPA axis response to restraint stress (Sudo et al., 2004). Heijtz et al. showed that reconstitution of germ free mice early in life reversed the locomotor and anxiety-like behaviours in the EPM, however, increased exploratory behaviour in the light/dark box persisted in these mice (Heijtz et al., 2011). In a subset of GF mice used in our original study, we colonized the GF mice with SPF faeces thereby introducing normal gut microbiota and reassessed anxiety-like behaviour. Conventionalization of GF mice (CONV-GF) occurred by daily mixing the GF mouse's home bedding with SPF mouse bedding and stool. Studies have

shown that this intervention will reconstitute the full complement of microbiota to a GF animal (Cebra, 1999; Macpherson and Harris, 2004). As shown in Fig. 1, CONV-GF mice showed a persistent anxiolytic behavioural phenotype, as shown by increased time spent in the open arms of the EPM and increased open arm entries (t-test, $p<0.05$). Given that we observed no change in the altered behavioural phenotype post-conventionalization in the adult GF mouse, it is likely that neural pathways are altered early in development and that there is a critical period postnatally in which HPA axis dysfunction and behavioural traits become relatively hard-wired into adulthood.

Evidence of behavioural alterations in conjunction with adaptive immune deficits have been demonstrated by Cushman et al. who showed that deletion of the recombinase activating gene (RAG-1) in mice which results in absent antibody synthesizing capability, caused increased exploration in the open field and decreased open arm avoidance in the EPM (Chun et al., 1991; Cushman et al., 2003). However, it should be noted that RAG-1 is also expressed in the CNS (Chun et al., 1991) where its function has yet to be elucidated. It is possible that the known immaturity of the adaptive immune system of GF mice somehow contributes to the behavioural phenotype we observed. The sensory arm of the autonomic nervous system may be an alternate route whereby gut microbiota may affect brain function. Several reports make this link. Oral ingestion of a bifidobacterium by conventional Sprague Dawley (SD) rats resulted in evidence of changes in serotonin metabolism in the brain stem (Desbonnet et al., 2008). Consumption of *Lactobacillus*

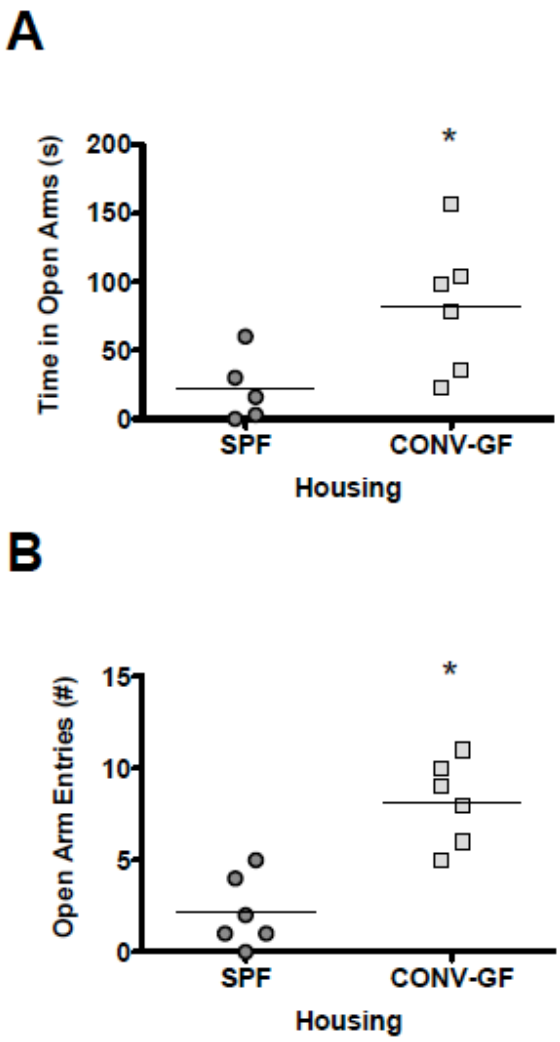

Figure 1. EPM testing of SPF and conventionalized GF mice (CONVGF) showed increased open arm (A) and increased open arm entries (B) in CONV-GF mice compared to SPF mice. * $p<0.05$

reuteri (LR) by conventional SD rats inhibited the perception of pain consequent to visceral distension suggesting that commensal bacteria can affect nerve function and pathways in conventional animals (Kunze et al., 2006). Recent work in our laboratories has shown that oral treatment of SD rats with LR consistently activated calcium dependent potassium channels in a specific subset of enteric neurons in the colonic myenteric plexus (Kunze et al., 2009), thus affording a direct link between luminal commensals and the enteric nervous system. In related studies using minimal doses of two different potentially pathogenic bacteria, *Citrobacter rodentium* and *Campylobacter jejuni*, Lyte and colleagues observed vagally mediated activation in the brain stem of mice in the absence of any evidence of inflammation in the gut, again emphasizing the effects of intestinal microbes upon the gut-brain communication pathway (Goehler et al., 2005; Lyte et al., 2006).

We consider that the behavioural and molecular alterations that we have observed in GF mice may have occurred due to the absence of microbial direct or indirect neural communication. Further work exploring these mechanistic possibilities may lead to novel insights into the complex roles of commensal bacteria in the development and function of the CNS. Unraveling these pathways may eventually lead to new therapeutic approaches in dealing with both gastrointestinal and psychiatric illness.

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CHAPTER LINK

The previous two chapters present data that demonstrates that adult germ free (GF) show altered anxiety-like behaviour with associated changes to brain neurochemistry. Furthermore, these changes are not responsive to adult conventionalization with commensal intestinal microbiota, indicating that it is likely that neural pathways are altered early in development in GF mice and that there is a critical period postnatally in which HPA axis dysfunction and related behavioural traits become relatively hard-wired into adulthood. In the next chapter, I present data examining the interplay between metabolic systems and stress reactivity in GF mice over the lifetime. Previous work has shown that GF mice have altered metabolic activity given their absence of intestinal microbiota, and literature suggests that the metabolic hormone leptin may be involved in early life programming of stress reactivity. Given that our work has demonstrated that early life is likely critical in determining HPA responsivity in GF mice, we look at the interaction of these systems in our model during both prepuberty and adulthood.

CHAPTER 5.

Alterations in stress reactivity and leptin feeding circuitry in germ free mice

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Abstract

It is now well established that the acquisition of intestinal microbiota in early postnatal life has a defining impact on the development of many physiological systems integral to health and well-being. It has been demonstrated that immune, inflammatory and metabolic systems all undergo fundamental postnatal shaping in response to commensal intestinal bacteria. Recent work is now focusing on the extent to which the central nervous system (CNS) may also be influenced. Previous work in our laboratory has shown that mice with no gut microbiota, that is germ free (GF) mice, exhibit reduced anxiety-like behaviour in the elevated plus maze in comparison to specific pathogen-free (SPF) mice. This phenotype is accompanied by changes in plasticity-related genes in the hippocampus and amygdala. Interestingly, the reduced anxiety-like behavioural phenotype observed in adult GF mice persists after colonization with SPF intestinal microbiota suggesting that gut-brain interactions are important in the early development of stress systems. In the absence of gut bacteria, GF mice exhibit altered metabolic functioning, including decreased weight gain. Work by others suggests that circulating plasma leptin is necessary for the normal postnatal development of CNS stress circuitry. In the current study, we investigated the interplay of leptin, corticosterone, and central circuits for stress-reactivity and feeding in the presence or absence of gut microbiota. Our results showed that GF mice have significantly lower plasma leptin levels than SPF mice. *In situ* hybridization showed increased levels of neuropeptide Y (NPY) and leptin receptor (ObR) mRNA in the arcuate nucleus of adult GF mice, suggesting that leptin insufficiency in GF mice leads to central changes in feeding circuitry. With respect to

stress reactivity, adult female GF mice showed higher basal corticosterone compared to SPF controls and exaggerated hypothalamic-pituitary-adrenal (HPA) reactivity to acute stress compared to SPF female mice. In 4 week-old female GF mice, unstressed and stressed levels of corticosterone were extremely high (600 ng/ml) in comparison to unstressed (75 ng/ml) or acute stressed (350 mg/ml) 4 week-old SPF mice. This dysregulation observed during prepuberty somewhat normalize in adult GF mice in spite of the maintenance of low leptin levels, suggesting alternate mediators in the regulation of the HPA axis during puberty. These data demonstrate that the presence of commensal bacteria is important in the development and function of hypothalamic circuitry related to both stress and feeding. Understanding the related direct and indirect signaling pathways may eventually lead to new therapeutic approaches in dealing with both gastrointestinal and psychiatric illness.

5.1 Introduction

In the human lower intestine, numbers of bacteria reach 10^{14} organisms per gram intestinal contents with roughly 1800 species accounted for thus far (Frank and Pace, 2008). It has been well established that these healthy gut bacteria play a major role in normal immunological development, regulating host inflammatory responses and in host metabolism (Hooper and Gordon, 2001; Backhed et al., 2005). Current research now indicates that the impact of intestinal bacteria also extends to the central nervous system (CNS) (Sudo et al., 2004; Gareau et al., 2011; Heijtz et al., 2011; Neufeld et al., 2011). This is perhaps not too surprising given that bacterial colonization occurs during the early postnatal period, and this coincides and overlaps with a vulnerable time for the developing CNS (Hensch, 2004; Knudsen, 2004). Earlier work in adult germ-free (GF) mice has shown that these animals, which are bred and maintained with no exposure to bacteria of any kind, demonstrate stress hyperresponsivity after exposure to an acute stressor, displaying significant increases in both plasma corticosterone and adrenocorticotropic hormone (ACTH) (Ikeda et al., 1999; Sudo et al., 2004). Work published by our laboratory has shown that GF mice also demonstrate reduced anxiety-like behaviour in rodent behavioural tests, accompanied by consistent changes in brain neurochemistry (Neufeld et al., 2011). However to date, the potential mechanisms by which gut microbiota may influence the development of the brain is unknown.

Early observations of GF mice revealed that they required more calories to maintain body weight and had a very lean physique (Wostmann, 1981; Wostmann et al., 1983). Mechanisms for this phenotype were not understood until studies demonstrated that gut microbiota contribute to carbohydrate and fat absorption (Sonnenburg et al., 2005), and through complex interactions with host physiology, regulate fat storage (Backhed et al., 2007; Backhed, 2011). Reconstituting GF mice with intestinal microbiota (referred to as conventionalization) has been shown to increase body fat by 60% within 14 days in spite of reduced food intake (Backhed et al., 2004). Similarly, circulating plasma leptin levels are significantly lower in GF mice than conventional controls, and conventionalization with bacteria will raise these concentrations within 14 days. Interestingly, there is a significant body of work indicating that circulating plasma leptin may influence the development of stress reactivity during early life (Rosenfeld et al., 1993; Suchecki et al., 1993; Ahima et al., 1998; Trottier et al., 1998; Oates et al., 2000). While both the stress hyperreactivity and the low leptin levels have been previously reported in adult GF mice, no attempt has been made to explore how these two phenotypes interact in the GF model over the lifetime.

Leptin acts in the brain via high-affinity receptor binding in the arcuate nucleus (Tartaglia et al., 1995). Receptor binding results in the down-regulation of production and release of neuropeptide Y (NPY) (Stephens et al., 1995; Hakansson et al., 1996), neurons of which terminate at the paraventricular nucleus (PVN) of the hypothalamus. The PVN is the first way station of the HPA, and corticotropin-releasing hormone (CRH) producing neurons originating here terminate at the median eminence to stimulate the release of

ACTH from the anterior pituitary. ACTH is released from the pituitary systemically to act on the adrenal gland where it causes the ultimate secretion of glucocorticoid (corticosterone in rodents, cortisol in humans). NPY has been previously demonstrated to be a potent stimulator of corticosterone release (Zarjevski et al., 1993), therefore downregulation of this peptide via leptin receptor binding results in the ultimate *decrease* of plasma corticosterone. In addition it has been shown that leptin perfused directly into rat hypothalami results in a down-regulation of CRH (Heiman et al., 1997; Huang et al., 1998). The evidence thus demonstrates that leptin can act to dampen HPA reactivity via decreased NPY expression. Leptin receptors are also found on proopiomelanocortin (POMC) producing neurons in the arcuate nucleus (Cowley, 2003; Schwartz et al., 2003; Zigman and Elmquist, 2003). Leptin binding on these neurons acts to up-regulate the production of POMC, an anorectic peptide known to inhibit feeding (Williams et al., 2009). POMC is a precursor for the melanocortin family and gives rise to α-melanocyte stimulating hormone (α-MSH) and ACTH (Pritchard et al., 2002). Given the established functional and anatomical relationships between these feeding peptides and stress circuitry, we selected the leptin receptor (ObR), NPY and POMC as targets for our molecular study of GF brain tissue.

In the current paper we examined whether CNS circuitry related to both feeding and stress was altered in adult GF mice due to the absence of microbiota. In addition we assessed both plasma leptin and corticosterone under stressed and unstressed conditions in the prepubescent period as there is currently no information on either of these levels in early life in GF mice. In Experiment 1 we took brains from adult GF and SPF mice and

compared basal mRNA expression of ObR, NPY and POMC via *in situ* hybridization. We also tested blood plasma for circulating leptin and correlated this to the ObR expression. In Experiment 2 we went on to examine stress reactivity and plasma leptin levels in stressed and unstressed mice both in early life and adulthood in order to test our hypothesis that both circulating leptin and corticosterone are altered in GF mice throughout the lifetime. This research is based on the hypothesis that the microbial contents of the intestinal lumen affect the development and function of the HPA axis via the circulating metabolic hormone leptin. This hypothesis is predicated on the findings that leptin affects HPA axis reactivity through brain receptor binding, and that in the absence of intestinal microbiota, as found in GF animals, circulating plasma leptin levels are altered and the HPA axis is hyperreactive to stress.

5.2 Methods

5.2.1. Animals. For Experiment 1, female Swiss Webster, eight week old GF and SPF mice were obtained from Taconic Farms Inc., Germantown, NY, USA. GF mice were maintained in regular cages (16.5 cm x 28.1cm x 12.7 cm) inside a guaranteed germ-free shipper with free access to food and water until the beginning of testing. Upon arrival, SPF mice were transferred to microisolator cages (15.2 cm x 26.7 cm x 12.7 cm) and maintained in standard housing until the beginning of testing. All animals were housed under 12 h light-12 h dark cycle, lights on at 7 AM. Housing room temperature was maintained at 20°C and humidity at 60-70%. With the exception of shipping container

type, there were no differences in supplier, shipping or receiving of GF and SPF mice. For Experiment 2, female, Swiss Webster, four week old and ten week old GF and SPF mice were obtained from the Animal Research Facility at McMaster University. GF mice were bred and raised in the gnotobiotic facility, while SPF mice were conventionally housed. As with Experiment 1, all mice were maintained in cages with free access to food and water until the beginning of testing and were housed under a 12 h light-12 h dark schedule, with lights on at 7 AM.

5.2.2. Ethics. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

5.2.3. Experimental Design. For Experiment 1, all experiments were carried out 48 h after arrival in the facility, when blood and brain were collected for leptin and *in situ* hybridization analysis. All blood was collected between 10 AM and noon. For Experiment 2, mice were received from McMaster's gnotobiotic unit or conventional housing units at 9 AM. Animals were moved to the experimental room, separately caged, then left for 1 h to acclimatize to surroundings. Mice were randomly assigned to acute restraint stress or unstressed groups. Stressed groups received 1 h restraint stress in their home cage, while unstressed mice were left undisturbed. Following the stress procedure, animals were killed and blood collected for corticosterone and leptin analysis.

5.2.4. Tissue Collection. Blood was collected and processed to collect plasma, and stored at -70°C until use in leptin and corticosterone analysis. Brains were rapidly removed

following decapitation, frozen in -60^{o}C isopentane, and stored at -70^{o}C until cryostat sectioning.

5.2.5. Leptin Analysis. Leptin was measured in duplicate samples using an immunoassay kit from Millipore, the Milliplex Map Kit for mouse gut hormone.

5.2.6. Riboprobes & In situ hybridization. The POMC riboprobe (923 bp) was kindly provided by Dr James Douglass of the Vollum Institute, Portland OR (Hatfield et al., 1989). The ObR and NPY probes were generated in our laboratory. ObR primers forward 5'-GCCTCGGCTTTGAAGGGGGC-3' and reverse 5'-TGGCTCTGGGTACCGGCACA-3' and NPY primers forward 5'-TGGACTGACCCTCGCTCTAT-3' and reverse 5'-GATGAGGGTGGAAACTTGGA-3' were designed using Primer 3 software (Rozen and Skaletsky, 2000). Specificity of primers to mouse was confirmed using BLAST (Altschul et al., 1990). Polymerase chain reaction generated complementary DNA (439 bp ObR, 314 bp NPY) was inserted in the p-GEM T-easy expression vector (Promega, Missisauga, ON, Canada). Antisense and sense probes were transcribed from linearized plasmids with an α-35S-UTP (specific activity >1,000 C/mmol; Perkin Elmer, Boston, MA, USA) using appropriate RNA polymerases. Hybridization of sense probes did not reveal any signal. Standard *in situ* hybridization methods were used, details of which have been previously described (Whitfield et al., 1990; Foster et al., 2004).

5.2.7. Restraint Stress. Animals in the stressed group were subjected to 1 h restraint stress. Animals were held in 7 x 7" wire mesh restrainers, which had been folded and

clipped on all sides to render them immobile throughout the stress period. Restraint stress was carried out inside the home cage.

5.2.8. Corticosterone Analysis. Corticosterone (CORT) was measured in duplicate samples using a standard radioimmunoassay kit from MP Biomedicals.

5.2.9. Data Analysis. All data were analyzed using GraphPad Prism (LaJolla, CA, USA). Statistical significance was evaluated using two-tailed unpaired t-tests for comparison of ObR, NPY and POMC mRNA signal in the arcuate nucleus of GF and SPF mice, and for Experiment 1 leptin analysis. Two-way ANOVA with Bonferroni post-tests were used for corticosterone and leptin analyses in Experiment 2 (housing and stress status as factors). Statistical criteria for significant differences were set at $p<0.05$. Data are presented as mean +/- SEM.

5.3. Results

5.3.1. Gut Microbiota

GF status was confirmed by microbiological evaluation and showed no growth for anaerobic, aerobic, and mycotic bacteria (Taconic, Germantown, NY and Gnotobiotic Unit, McMaster University).

5.3.2. Altered gene expression – Experiment 1

Housing (GF *vs* SPF) had a significant impact on ObR mRNA expression in the arcuate nucleus. Representative film images are provided for ObR mRNA signal observed in SPF mice (Fig. 1A). Analysis revealed a significant up-regulation of ObR mRNA in the arcuate nucleus in GF mice (Fig. 1D, t=3.07, df=17, p=0.007). Housing also had a significant impact on NPY expression in the arcuate nucleus. Representative images are provided for NPY expression observed in SPF mice (Fig. 1B). Analysis again revealed a significant up-regulation of NPY in GF mice (Fig. 1D, t=2.62, df=17, p=0.018). There was no significant difference in mRNA expression levels of POMC in GF *vs* SPF mice (Fig 1C & 1D, t=0.095, df=17, p=0.93).

5.3.3. Plasma Leptin – Experiment 1

Plasma leptin levels were determined in mice 48 h after arrival in the facility. GF mice showed significantly lower plasma leptin levels compared to SPF mice (Fig. 2A; t=2.55, df=16, p=0.021). Across all mice (GF and SPF) leptin receptor mRNA levels in the arcuate nucleus of the hypothalamus were significantly correlated with plasma leptin levels such that higher leptin levels were associated with lower hypothalamic expression of ObR mRNA (Fig. 2B; r=-0.5482, p=0.034).

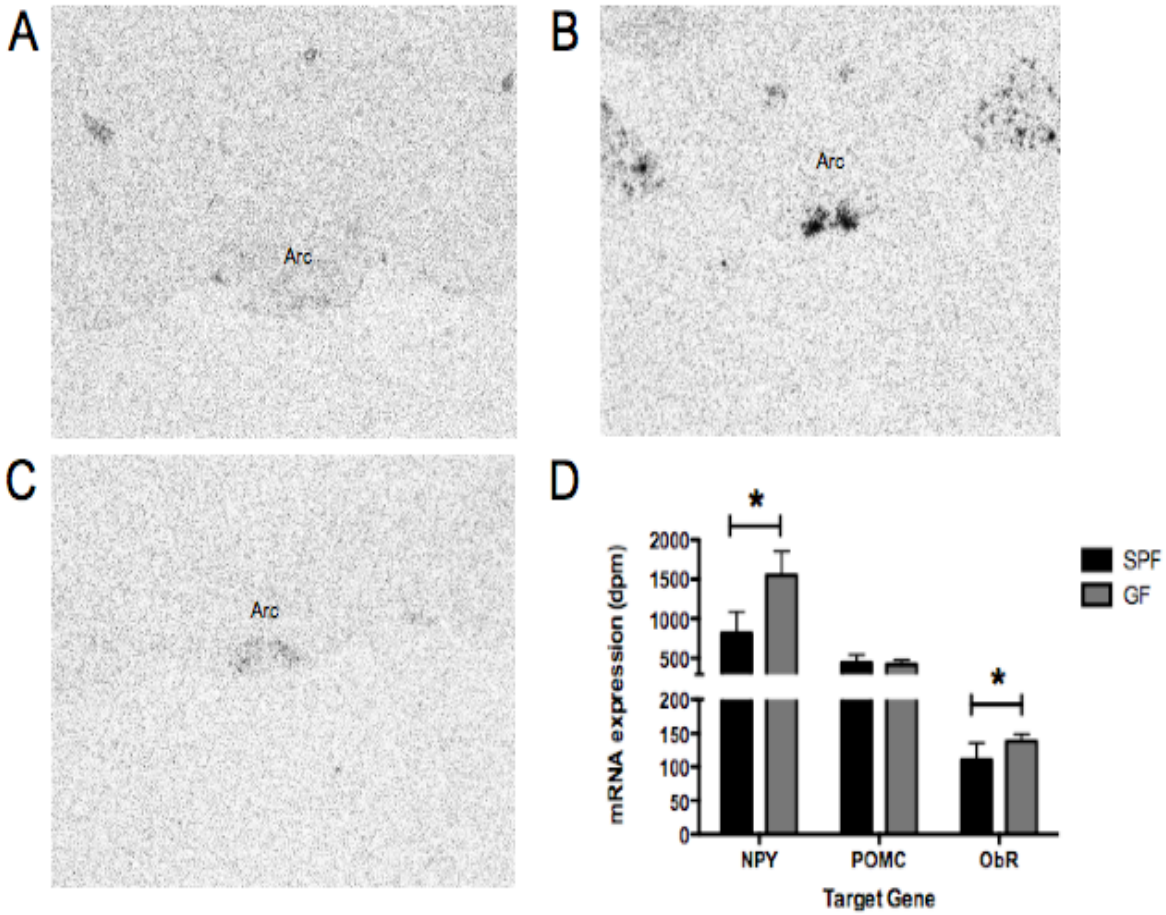

Figure 1. (A) Representative film image of ObR mRNA expression in arcuate nucleus (Arc). (B) Representative film image of NPY mRNA expression in Arc. (C) Representative film image of POMC mRNA expression in Arc. Images A-C are all taken from Bregma -1.82 of SPF mouse. (D) Densitometric analysis revealed a significant increase in both NPY and ObR in GF mice as compared to SPF mice. No difference was found in POMC in GF vs SPF animals. Values are means +/- S.E.M., *$p<0.05$

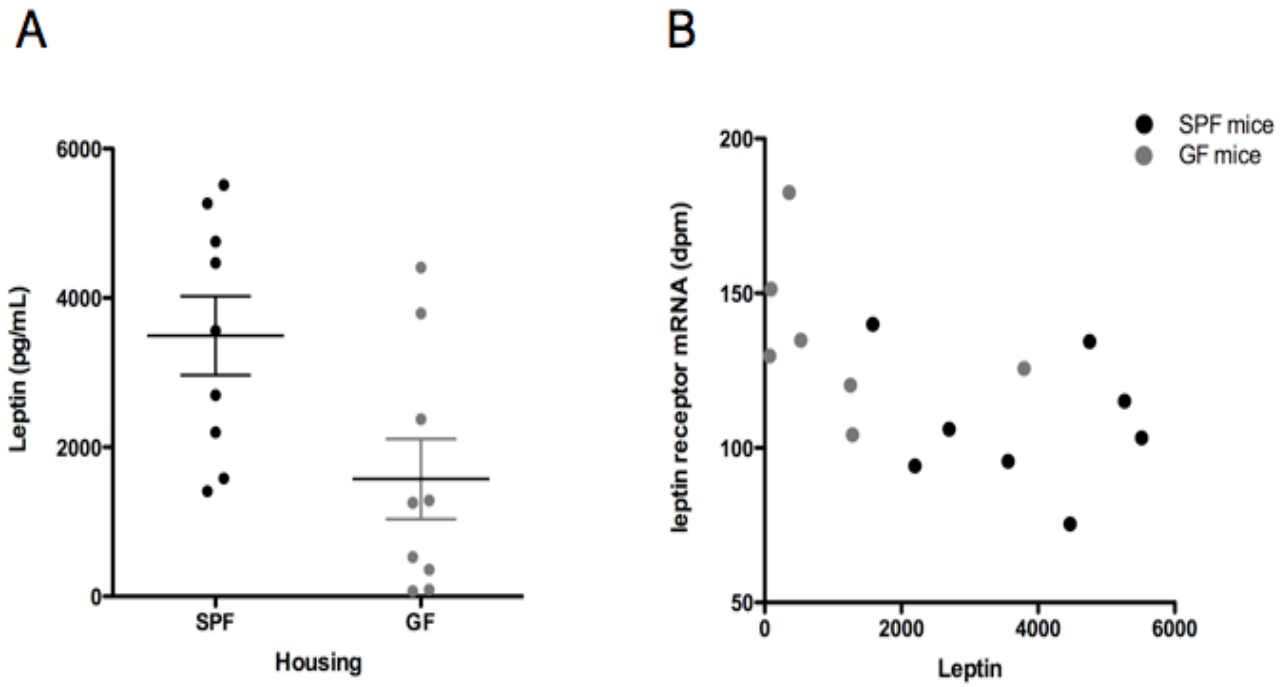

Figure 2. (A) Plasma leptin was measured in GF *vs* SPF mice. GF mice were found to have significantly lower plasma leptin levels than SPF mice. Values are means +/- S.E.M., *$p<0.05$. (B) A significant negative correlation was found between leptin receptor mRNA expression in the Arc and plasma leptin levels for both GF and SPF mice.

5.3.4. Plasma Corticosterone – Experiment 2

Plasma corticosterone levels were determined in 4 week and 10 week old female mice from both stressed and unstressed groups. At 4 weeks of age, there was a significant main effect for housing (Fig. 3A; $F_{1,31}$=85.2, p<0.0001), stress status ($F_{1,31}$=13.2, p=0.001) and a significant interaction ($F_{1,31}$=8.11, p=0.008). Posthoc analysis showed a significant increase in corticosterone levels in GF RS *vs* SPF RS mice (Bonferroni, p<0.001) and in GF unstressed *vs* SPF unstressed mice (Bonferroni, p<0.001). In SPF mice RS resulted in significantly higher corticosterone levels compared to unstressed mice (Bonferroni, p<0.001). At 10 weeks of age there was a significant main effect for housing (Fig. 3B; $F_{1,28}$=13.9, p=0.0009) and stress status ($F_{1,28}$=167.2, p<0.0001). Posthoc analysis showed an exaggerated corticosterone response in RS GF mice when compared to RS SPF mice (Bonferroni, p<0.05). Unstressed GF mice also demonstrate elevated levels of basal corticosterone compared to SPF mice (Bonferroni, p<0.05). The corticosterone response to RS was also significantly increased compared to unstressed within both housing groups (Bonferroni, p<0.001).

5.3.5. Plasma Leptin – Experiment 2

Plasma leptin levels were determined in 4 week old and 10 week old female mice under either stressed or unstressed conditions. At 4 weeks of age there was a significant main effect for housing (Fig. 4A; $F_{1,29}$=11.8, p=0.002). Posthoc analysis showed a significant decrease in leptin levels in GF *vs* SPF mice irrespective of stress status (Bonferroni, p<0.05). At 10 weeks of age there was a significant main effect for housing (Fig. 4B;

$F_{1,28}$=16.32, p=0.0004). Again, posthoc analysis showed a significant decrease in leptin levels in GF *vs* SPF mice irrespective of stress status(Bonferroni,p<0.001).

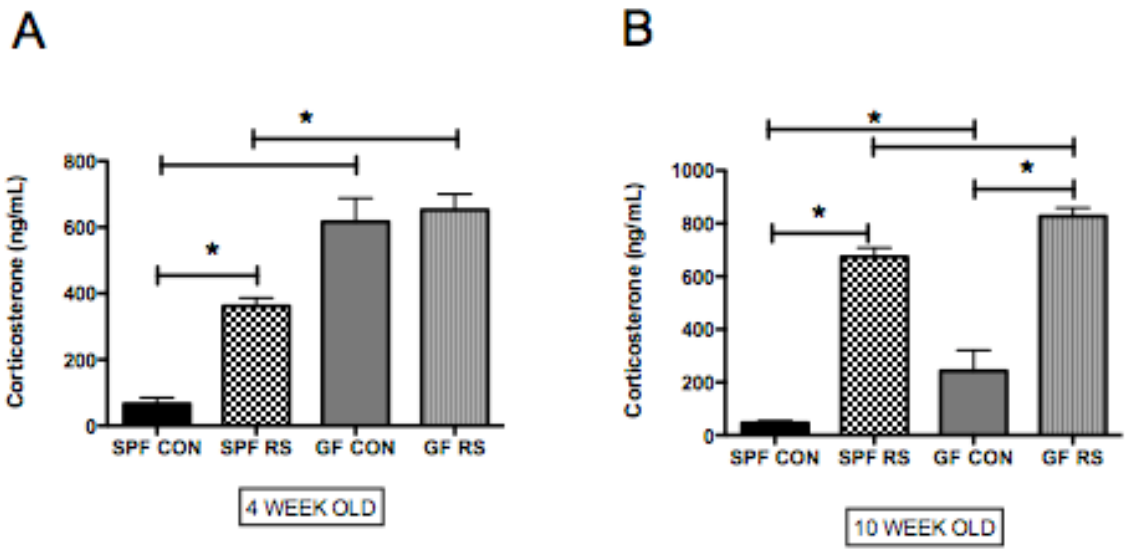

Figure 3. (A) Corticosterone in 4 week old GF and SPF mice. GF mice have significantly higher corticostone in both stressed (RS) and unstressed conditions (CON) compared to both SPF RS and SPF CON. (B) Corticosterone in 10 week old GF and SPF mice. Both GF and SPF groups showed significant increased corticosterone in restraint stress (RS) vs non-restraint stress (CON) groups. In addition GF RS had significantly increased corticosterone compared to SPF CON. Values are means +/- S.E.M., *$p<0.05$.

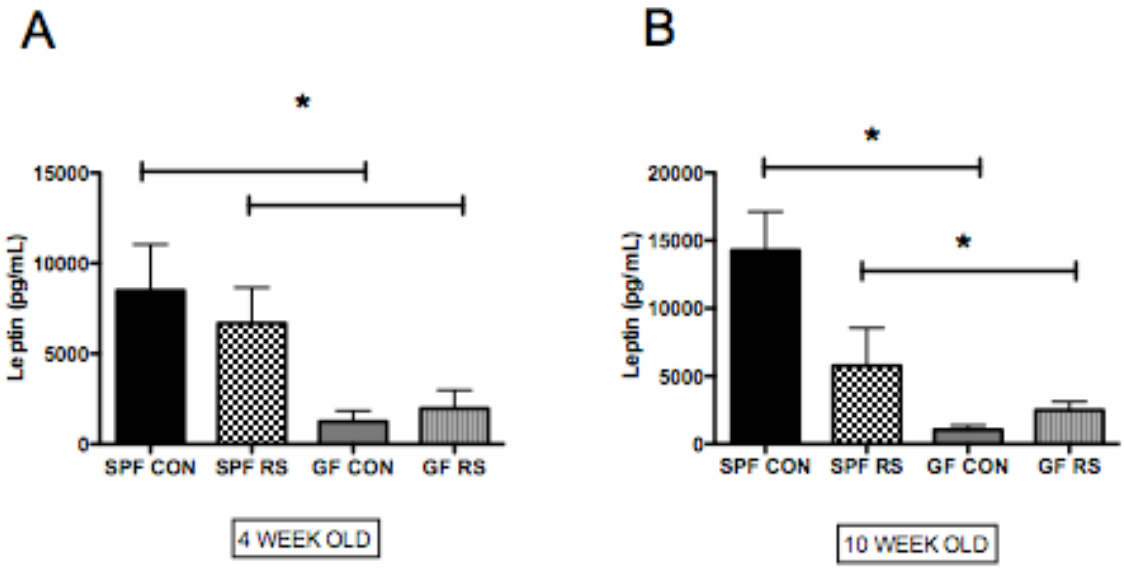

Figure 4. (A) Plasma leptin in 4 week old GF and SPF mice. GF mice have significantly lower plasma leptin irrespective of stress status compared to SPF mice. (B) Plasma leptin in 10 week old GF and SPF mice. Again, GF mice show significantly lower plasma leptin irrespective of stress status compared to SPF mice. Values are means +/- S.E.M., *$p<0.05$.

5.4. Discussion

The current findings demonstrate that GF mice have lifelong low plasma leptin levels, and changes to hypothalamic circuitry important to both feeding and stress. In addition, female GF mice exhibit completely dysregulated stress reactivity in the prepubescent period, as reflected by extremely high plasma corticosterone levels, both in the presence and absence of an acute stressor. While the basic trend of glucocorticoid response to stress normalizes in adulthood (i.e. increased plasma corticosterone after exposure to a stressor compared to baseline), GF mice continue to demonstrate altered stress reactivity with both a significantly higher basal plasma corticosterone level and a hyperreactive stress response to acute restraint stress as compared to control animals. Here we propose that leptin insufficiency in GF mice in early life contributes to an inability to maintain low levels of circulating glucocorticoids during development and results in changes to hypothalamic circuitry related to both feeding and stress, which then contributes to lifelong stress hyperresponsivity.

It has previously been reported that adult GF mice have low plasma leptin levels (Backhed et al., 2004; Backhed et al., 2007). Here, we demonstrate that this shortage of available leptin is actually present in mice as young as 4 weeks of age. The reasons for low plasma leptin in GF mice have already been identified. GF mice have very lean body types and leptin is released from adipocytes, however lack of bioavailability is not the sole reason for the paucity observed in these animals. One role of the commensal microbiota is to aid in the breakdown of food, including the digestion of carbohydrates

into short-chain fatty acids (SCFAs). As GF mice have no bacteria to aid in carbohydrate digestion, they produce very few SCFAs (Backhed, 2011). SCFAs act on G protein-coupled receptors in adipocytes to stimulate the release of plasma leptin (Xiong et al., 2004; Zaibi et al., 2010). Therefore the lack of plasma leptin observable in GF mice is due to not only less adipose tissue, but also to a relative absence of stimulating SFCAs. Colonizing GF mice with intestinal microbiota has been shown to not only increase body fat, but also to increase plasma leptin levels (Backhed et al., 2004; Backhed et al., 2007).

Both the ObR and NPY mRNA expression are significantly increased in the arcuate nucleus of GF mice compared to SPF controls. ObR expression is inversely correlated to circulating plasma leptin levels, and thus our finding of increased expression in brain tissue in GF mice is consistent with this. Interestingly, NPY is also upregulated in GF tissue. NPY is an orexigenic peptide and is highly expressed in the arcuate nucleus (Shi et al., 2010). Previous work has shown that chronic leptin treatment in leptin knockout mice results in a downregulation of hypothalamic NPY expression. In addition, leptin perfused directly onto isolated hypothalamic tissue suppresses NYP release (Stephens et al., 1995). Conversely, decreased leptin receptor binding results in a upregulation of NPY expression and thus increased food intake (Stephens et al., 1995; Hakansson et al., 1996). This is consistent with what is known about GF mice as in spite of their lower body weights these mice do demonstrate increased feeding (Wostmann, 1981; Wostmann et al., 1983; Backhed et al., 2004). NPY neurons terminate at the PVN of the hypothalamus where they are a potent stimulator of corticosterone release (Zarjevski et al., 1993). Experiments have also demonstrated that NPY is sensitive to stress hormones,

as repeated stress stimulates the release of NPY (Bonne et al., 2004). It is therefore reasonable to suggest that higher NPY expression in the arcuate nucleus may drive the increased basal expression of corticosterone observed in adult GF mice. This is the first work to show that the absence of colonizing intestinal microbiota results in changes to brain circuitry related to both metabolism and stress and it points to leptin as a potential mediator in the communication that is known to exist between the gut, brain and microbiota.

Our results support a link between leptin and stress reactivity. There is an extensive body of work arguing a role for plasma leptin in the maintenance of the stress hyporesponsive (SHRP) period during development (Ahima et al., 1998; Trottier et al., 1998; Oates et al., 2000). SHRP is a period in the early life of the rodent whereby corticosterone levels are kept to a minimum in order to protect the developing brain from damaging high levels of glucocorticoids (Bohn, 1980; Levine, 2002). During SHRP stressors that would normally elicit an HPA response generally do not (Sapolsky and Meaney, 1986). However, maternal separation without feeding does activate the HPA axis during SHRP and leads to long-term dysregulation of HPA reactivity in adulthood (Stanton 1987, Suchecki 1993). Maternal separation *with* supplemented feeding does not activate the HPA during SHRP and leptin has been the proposed transmittable factor in feeding that confers SHRP to the infant (Suchecki 1993). Given that we demonstrate here that GF mice show lifelong leptin insufficiency and dysregulated HPA activity at 4 weeks of age, it is interesting to consider that the absence of leptin during the normal SHRP period in GF mice may underlie this dysregulation. Furthermore, given that the corticosterone levels are so high

in the young GF mice, we propose that early life programming of stress reactivity is thus altered in these animals and that similar to the case of maternal separation, this rewiring of the CNS stress circuitry leads to maladaptive stress hyperresponsivity that is observable into adulthood.

The extreme dysregulation of the stress axis observed in GF prepubescent mice was a surprising finding especially given that this level of dysfunction is not maintained into adulthood. While adult female GF mice do show a significant increase in basal corticosterone and HPA hyperresponsivity to stress compared to conventionally housed controls, we do observe a normalization in the stress response in our adult animals when compared to the 4 week old mice. Given that the plasma leptin levels remain low in these GF animals throughout the lifetime, a biological factor other than leptin must play a role in the ultimate maturation of the HPA axis during adolescence. Neuroendocrine sex hormones may play a role in this adolescent programming. The literature suggests that there are two critical time periods when estrogen can act at the level of the HPA axis to contribute to stress programming, with the first of these time periods occurs prenatally, the second during puberty (Evuarherhe et al., 2009). Estrogen receptors are expressed at the level of the PVN in the hypothalamus (Viau and Meaney, 2004), and estrogen has been shown to have an inhibitory effect on HPA activity prior to puberty, and an excitatory effect post-puberty (Evuarherhe et al., 2009). This change in responsivity to estrogen over puberty indicates that there is a HPA maturation occurring during the adolescent period, and that this maturation involves changes with respect to sex steroids. These findings have contributed to the hypothesis that there is in fact a second critical

window for HPA development after early life, one that exists during puberty (Romeo et al., 2006; Romeo and McEwen, 2006; McCormick and Mathews, 2007). It would seem that at least for the GF mice, that this second critical window may be a period during which the extreme HPA dysregulation we observe is somewhat ameliorated.

In summary, this work demonstrates that brain circuitry related to both metabolism and stress reactivity is altered in the absence of commensal intestinal microbiota. We propose that insufficient plasma leptin levels in GF mice contributes to the dysregulated stress reactivity in early life, and alters CNS stress circuitry leading to a maladaptive stress hyperresponsivity in adulthood. In addition, the partial normalization of the stress system during adolescence identifies a second critical window for HPA programming that is independent of both microbiota and leptin. This work is interesting in that it links microbiota to development of both the feeding and the stress systems and suggests that metabolic dysfunction may be an intriguing target for studying the connection between the brain and gut.

5.5 References

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CHAPTER LINK

In the preceding chapters I have presented data demonstrating changes to the central nervous system development and function in germ free (GF) mice as compared to controls. In the following chapter I begin to tease apart potential mechanisms by which commensal intestinal microbiota may be communicating with the brain. I present data from electrophysiological recordings of neurons in the enteric nervous system in GF mice, specific-pathogen free (SPF) controls, and conventionalized GF mice. Given that neurons of the enteric nervous system represent the first way station at which intestinal bacteria and the nervous system may interact, we examined basic electrophysiological measures in these cells.

CHAPTER 6.

The microbiome is necessary for normal gut intrinsic primary afferent neuron excitability

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Abstract

Recently there has been profound interest in the role of intestinal microbiota in gut-brain communication. Germ free (GF) mice are bred and maintained without exposure to bacteria of any kind, and thus provide a valuable research tool to investigate whether the presence of a gut microbiome affects the development or maintenance of the normal functional state of enteric nervous system (ENS) neurons. Here we examine if GF mice differ from their conventionally-reared specific pathogen-free (SPF) counterparts in terms of intestinal intrinsic primary afferent neuron (IPAN) excitability. We chose IPANs as the initial targets for investigation because previous work has shown that ingested commensal bacteria directly act on IPANs rather than inter- or motorneurons. Segments of jejunum from 8 week old GF and SPF mice were individually placed in a silastic-lined recording dish filled with carbogenated Krebs. The segment was opened, pinned flat, and mucosa, submucosa and circular muscle were dissected away to expose the myenteric plexus. Intracellular recordings in current clamp mode were obtained from these IPANs by impaling cells with sharp microelectrodes. Action potential (AP) shapes, firing thresholds, the number of APs fired at 2x threshold as well as passive membrane characteristics were measured. In GF mice the excitability was decreased in afterhyperpolarization (AH) neurons as measured by the number of APs generated at 2x threshold. In addition the post AP slow afterhyperpolarization (sAHP) was extended in GF compared to SPF animals, again indicating decreased excitability in the GF AH neurons. Passive membrane characteristics were also altered in GF mice as reflected by

the membrane hyperpolarization and a decrease in input resistance. The absence of bacteria thus lowered the resting membrane potential for the AH cells and extended the post AP sAHP duration. Both of these measures indicate the potential for decreased IPAN firing in response to sensory stimuli, which could underlie the abnormal gut motility previously reported in GF mice. This is the first work to show that commensal intestinal microbiota are necessary for normal excitability of gut sensory neurons.

6.1. Introduction

Currently, there is profound interest in the role of commensal intestinal microbiota in gut-brain communication. This is understandable given that we now know that intestinal microbiota number 10^{14} organisms per gram of colonic contents (Frank and Pace, 2008). Until recently little was known about the potential role of these microbes in the development and function of the nervous system. The little that had been published demonstrated that germ-free (GF) mice, or mice that had been bred and maintained with no exposure to bacteria of any kind, showed stress hyperreactivity compared to conventionally housed control animals (Ikeda et al., 1999; Sudo et al., 2004). Specifically, GF mice showed a significant increase in both plasma corticosterone and adrenocorticotropic hormone (ACTH) following acute restraint stress as compared to specific pathogen-free (SPF) mice (Ikeda et al., 1999; Sudo et al., 2004). In the last year a number of publications, including one from our own laboratory and reproduced in Chapter 3 of this thesis, have demonstrated that GF mice also have altered stress-related behaviour and associated changes in brain neurochemistry (Gareau et al., 2011; Heijtz et al., 2011; Neufeld et al., 2011). Specifically, our group has shown that GF mice demonstrate basal anti anxiety-like behaviour with an up-regulation of hippocampal brain-derived neurotrophic factor (BDNF) (Neufeld et al., 2011). However, the physiological mechanisms by which the brain is informed of changes to the gut bacterial load are unknown.

The enteric nervous system (ENS) allows for the independent functioning of the intestine from the central nervous system (CNS) (Bayliss and Starling, 1899). Cell bodies of neurons of the myenteric plexus are located between circular and longitudinal muscle layers in the gut wall (Furness, 2006). Sensory neurons (IPANs) located here send long processes into gut mucosa, terminating near the epithelial layer (Kunze et al., 1995; Kunze et al., 1997; Kunze and Furness, 1999). Given that these nerve endings are in such close proximity to the intestinal lumen, and that myenteric neurons have been demonstrated to form anatomical synapses with vagal fibres (Powley et al., 2008), it is reasonable to hypothesize that information regarding the bacterial status of the gut could be transmitted via the enteric neurons through vagal afferents to the brain.

Neurons in the ENS can be divided into two sub-types, based on both anatomical and functional categories (Furness, 2006). Afterhyperpolarization (AH) cells, or Dogiel Type II cells, have long multipolar processes and are chemo- and mechanosensory intrinsic primary afferent neurons (Kunze et al., 1995; Bertrand et al., 1997). S cells, or Dogiel Type I cells, have a single axon and short dendrites and are motor or interneurons (Smith et al., 1990; Song et al., 1991; Smith et al., 1992; Kunze et al., 1993). These cell types can also be identified based on their electrophysiological properties which correlate with the morphotype (Hirst and Holman, 1978; Bornstein et al., 1994). AH cells have a broader action potential, with a calcium hump on the falling region of the action potential (Schutte et al., 1995). In addition, AH cells were so named by David Hirst (Hirst and Holman, 1978) because they have a prolonged slow afterhyperpolarizing potential (sAHP) following an action potential. The sAHP makes the neuron refractory to further

firing for most of the duration of the sAHP. This refractory event is negligible or entirely absent in S cells. Previous work in our laboratory has demonstrated that feeding rats probiotic *Lactobacillus rhamnosus* for a period of one week prior to recording increases excitability in AH cells of the myenteric plexus by reducing the sAHP duration (Kunze et al., 2009). Patch clamp recordings showed that feeding the rats this probiotic mimicked the effect of applying IK_{Ca} channel blocker (TRAM-34) suggesting that the reduced sAHP was caused by a reduction in IK_{Ca} opening (Wang et al., 2010).

Given our findings that feeding probiotic commensal bacteria to normal mice reduced sAHP, we hypothesized that the total absence of bacteria (as in GF mice) might result in decreased excitability associated with abnormally long sAHP in IPANs. We also hypothesized that conventionalizing adult GF mice with normal commensal intestinal microbiota would normalize any differences in excitability and sAHP between GF and SPF groups. To test our hypotheses we carried out intracellular sharp recording experiments on IPANs within the myenteric plexus in GF and SPF male and female, adult Swiss Webster mice. In addition, we conventionalized adult GF mice over a period of one month, and then carried out recordings on this group of animals. We compared electrophysiological behaviour across the 3 treatment groups to determine if there was an overall treatment effect. These hypotheses we tested statistically using analysis of variance and selected post hoc multiple comparisons tests.

6.2 Methods

6.2.1. Animals. Male and female, Swiss Webster, eight week old, SPF or GF mice were obtained from the Animal Research Facility at McMaster University. GF mice were bred and raised in the gnotobiotic facility, while SPF mice were conventionally housed. Conventionalized germ free (CONV-GF) mice were obtained as 8 week old GF adults from the gnotobiotic facility and then housed for 4 weeks in conventional housing. Conventionalization was accomplished by transferring dirty bedding from SPF cages into GF cages on alternate days as described by Harris & Macpherson (Macpherson and Harris, 2004). All mice were maintained in cages with free access to food and water until the beginning of testing. All animals were housed under a 12 h light-12 h dark schedule, with lights on at 7 AM. Housing room temperature was maintained at 20^{o} C and humidity at 60-70%.

6.2.2. Ethics. All experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Research Ethics Board, McMaster University, Hamilton, Ontario, Canada.

6.2.3. Preparation. Mice were received from the Animal Facility at McMaster University daily at 9 AM. Animals were transported to the laboratory and immediately killed by cervical decapitation. A 2 cm segment of jejunum was removed, and tissue placed in a 2 mL recording dish lined with silastic and filled with carbogenated Krebs buffer of the following composition (in mM): NaCl 118.1, KCl 4.8, $NaHCO_3$ 25, NaH_2PO_4 1.0, $MgSO_4$ 1.2, glucose 11.1, and $CaCl_2$ 2.5, gassed with carbogen (O_2 95%,

CO_2 5%). Nicardipine (2-3 uM) and scopolamine (1 μM) were routinely added to the saline to minimize spontaneous muscle contraction. The segment was opened along a line parallel to the mesenteric attachment and pinned flat, under moderate tension, mucosa uppermost. The myenteric plexus was exposed by dissecting away the mucosa, submucosa, and circular muscle. The recording dish was then mounted on an inverted Nikon T 2000 microscope and the tissue continuously superfused (4 mL/min) with carbogenated Krebs that had been warmed to 35-37^{o}C.

6.2.4. Electrophysiology. Intracellular voltage signals measured in current-clamp mode using an Axon Instruments Multiclamp 700A computer-controlled amplifier (Molecular Devices, Sunnyvale, California) and a Digidata 1322A (Molecular Devices) A/D digitizer. Current clamp commands were delivered to the amplifier using Clampex 8 (Axon Instruments) software. Thin-walled boroscilicate glass pipettes were pulled on a Flaming-Brown P97 (Sutter Instruments, http://www.sutter.com) electrode puller to produce sharp electrodes with resistances of 100-120 MΩ. Electrodes were then filled with 1 M KCl and 0.5% Neurobiotin.

6.2.5. Physiological parameters measured. After impalement AH neurons (IPANs) were identified electrophysiologically by the presence of a hump on the repolarization phase of the action potential (AP) and by the presence of a sAHP of ≥ 2 s duration following the AP (Fig. 1). The hump could also be detected as an inflection in the 1st order time derivative of the AP (Fig. 1D & E). A single AP for measuring the hump was evoked by injecting a short (<1 ms duration) depolarizing current pulse which just evoked the spike

on the falling phase of the electrotonic potential (Mao et al., 2006). This data was Bessel prefiltered at 5 and acquired at 20 kHz. For other experiments, data was prefiltered at 2 and acquired at 10 kHz. When neurons were identified as being AH cells, 500 ms duration depolarizing current pulses of increasing intensity were injected until threshold for AP firing was reached (Fig. 1A & B). Then, having determined threshold current intensity another 500 ms depolarizing current pulse of exactly 2 times threshold was injected (Fig. 1C). Finally, the sAHP was evoked by delivering 3 suprathreshold 50 ms duration depolarizing current pulses separated by 100 ms intervals.

6.2.6. Data Analysis. For each neuron off-line analyses included measurements of excitability, that is AP threshold, and the number of APs fired at 2x threshold (Fig. 1A-C). The passive membrane properties of resting membrane potential (RMP) and input resistance were measured using Clampfit software (Molecular Devices). The following AP properties were measured (Fig. 1D-G): spike amplitude (AP amp.), AP width at half amplitude (1/2 width), maximum rate of depolarization (max dV/dt), the amplitude of the fast afterhyperpolarization (fAHP) that continues the AP downstroke, area under the curve (AUC), and duration and amplitude of the sAHP (Fig. 1F & G). It is not always possible to measure the fAHP duration as fAHP frequently merges with a much longer sAHP that follows it for AH cells. Data were stored on a computer and analyzed off-line. Descriptive statistics are given as mean ± SD and number of neurons (*n*) in the sample. Errors on bar graphs are SEM where *, ** and *** and ns denote significance at the P = 0.05, 0.01, 0.001 or >0.05 (not significant) level. One-way ANOVA was used to compare the measured parameters between the different treatments described above.

When a statistically significant ($P \leq 0.05$) treatment was identified, post hoc tests were used to test for hypothesized differences. GraphPad Prism version 5 (GraphPad Software, San Diego, CA) was used for all descriptive statistics, including one-way ANOVA, and Bonferroni or Dunnett's multiple comparison tests.

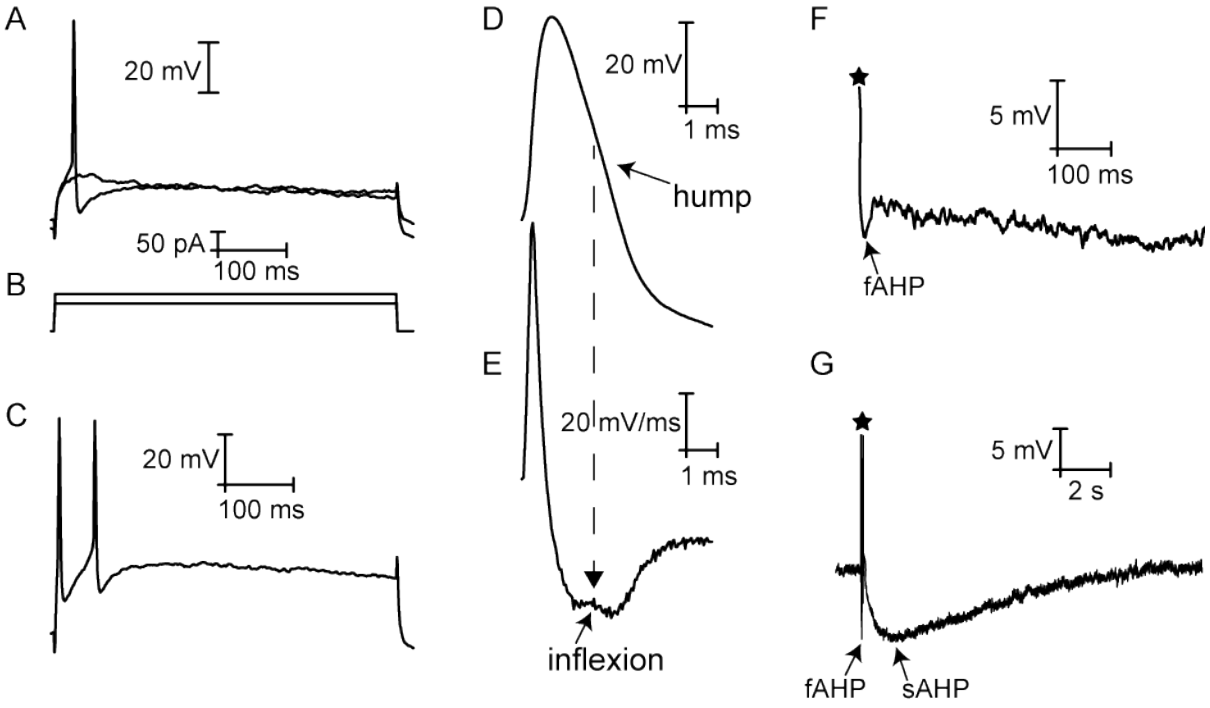

Figure 1. Traces of intracellular recordings were made from a representative AH cell. A) Electrotonic voltage deflection and suprathreshold responds with action potential to B) subthreshold and suprathreshold 500 ms square wave positive current pulses injected into the cell via the recording electrode. C) 2 APs were discharged when depolarizing current at 2 x threshold intensity was injected. D) Single AP showing hump on repolarizing phase (downstroke), E) 1st order time derivative of AP reveals the hump as an inflection. F) Fast afterhyperpolarization shown as continuation of AP downstroke. AP truncated at ★. G) Slow afterhyperpolarization (sAHP) lasts for seconds and peaks with a delay after AP and fAHP

6.3 Results

6.3.1. Excitability

The threshold currents required to evoke an AP using control (SPF) animals were 175 ± 82 pA (16), for GF (9) animals was 289 ± 271 (19), and for CONV-GF animals was 188 ± 135 pA (Fig. 2A); there was no statistical difference in population means (ANOVA, $P = 0.2$). The number of APs evoked at twice threshold current intensity differed between treatments (Kruskal Wallis test, $P = 0.01$), those from SPF animals were 3.6 ± 2.2, GF animals were 1.9 ± 0.6 and CONV-GF were 3.7 ± 2.8 (Fig. 2B). Post hoc Dunnett's multiple comparison test showed that SPF differed from GF ($P < 0.01$, rank sum difference = 15) and GF differed from CONV-GF ($P < 0.05$, rank sum difference = -12).

6.3.2. Passive membrane properties.

The resting membrane potentials (RMP) were -56 ± 3 (15), -66 ± 7 (9) and -59 ± 7 mV for SPF, GF and CONV-GF respectively (Fig 3A), giving a statistical difference between treatments (ANOVA, P = 0.001). A post hoc Bonferroni's multiple comparison test indicated that SPF differed from GF (P < 0.001) and CONV-GF differed from GF ($P < 0.5$). IPAN input resistances (Rin) for SPF, GF and CONV-GF mice were 206 ± 62 (18), 146 ± 45 (8) and 212 ± 64 (16) MΩ (Fig. 3B) with a significant difference in treatments (ANOVA, $P = 0.04$). SPF differed from CONV-GF mice ($P < 0.05$) and CONV-GF differed from GF mice ($P < 0.05$) (Bonferroni's multiple comparison test).

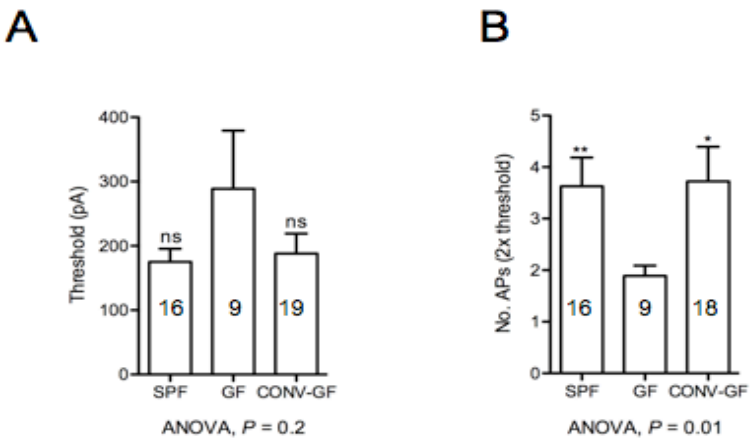

Figure 2. AH cell excitability: summary statistics. A) Sample differences for AP thresholds between treatment groups did not reach statistical significance. B) Number of APs evoked by intracellular stimulus current at 2x threshold intensity differed between treatment groups. Post hoc analysis with Dunnett's multiple comparison test revealed that number of APs discharged for GF differed from those of SPF mice, and those from CONV-GF differed from GF mice. For this and subsequent figures post hoc multiple comparison tests were always between GF *vs* SPF and CONV-GF *vs* GF treatment groups, as these were hypothesized to be different. Numbers inside bars denote n number

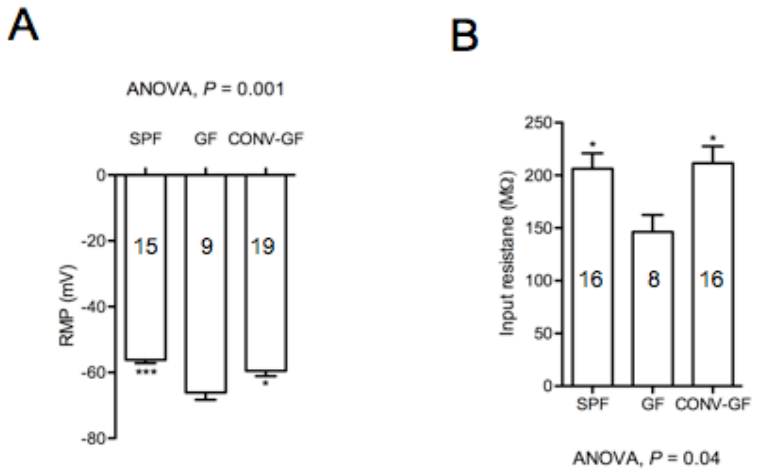

Figure 3. Passive membrane characteristics: summary statistics. A) Resting membrane potential (RMP) and B) input resistance differed between treatment groups, with statistically significant differences between GF and SPF and CONV-GF and GF groups. Selected post hoc tests, with Bonferroni's correction, were between GF *vs* SPF and CONV-GF *vs* GF. Numbers inside bars represents n number.

6.3.3. Action potential parameters.

AP amplitudes were 81 ± 9 (14), 89 ± 22 (8) and 76 ± 12 (18) mV for SPF, GF and CONV-GF mice respectively (Fig. 4A). There was no statistical difference between treatments (ANOVA, P = 0.1). The maximal rates of AP depolarization (dV/dt) were also not affected by treatments (81 ± 10, 89 ± 22 and 76 ± 12 mV.ms) for SPF, GF and CONV-GF treatment groups (ANOVA, $P = 0.1$) (Fig. 4B). The AP widths at half amplitude (1/2 width) were 2.2 ± 0.5 (13), 2.6 ± 0.7 (8) and 2.4 ± 0.4 (17) (ANOVA, $P = 0.2$) (Fig. 4C), and the amplitudes for the fast afterhyperpolarization (fAHP) were -5.5 ± 6.2 (12), -3.6 ± 4.2 (8) and -4.9 ± 7 (18) (Fig. 4D) (ANOVA, $P = 0.8$), all for SPF, GF, CONV-GF treatments respectively.

The area under the curve (AUC) of the slow sAHP was altered by the treatments. SPF, GF and CONV-GF values were -26,874 ± 16,969 mV.ms (16), -58,637 ± 67,143 (9) and -24,539 ± 17,288 (19) mV.ms (Fig. 4E) (ANOVA, $P = 0.04$). Post hoc comparisons between SPF versus GF and CONV-GF *vs* GF revealed that the former sample difference had $P > 0.05$ while the latter had $P < 0.05$ (Bonferroni's multiple comparison test). Durations of the sAHP were SPF: 3.0 ± 1.5 (16), GF: 6.2 ± 3.2 (9) and CONV-GF: 2.8 ± 1.6 (19) (Fig. 4F) (ANOVA, $P = 0.0003$). The difference between SPF *vs* GF and CONV-GF *vs* GF samples were statistically discernable with $P < 0.001$ (Bonferroni's multiple comparison test). However, the treatments had no effect on sAHP amplitudes (Fig. 4G), sample values were SPF: -6.3 ± 2.4 (16), GF: -9.7 ± 6.1 (9) and CGF: -7.3 ± 2.6 (19) (ANOVA = 0.08).

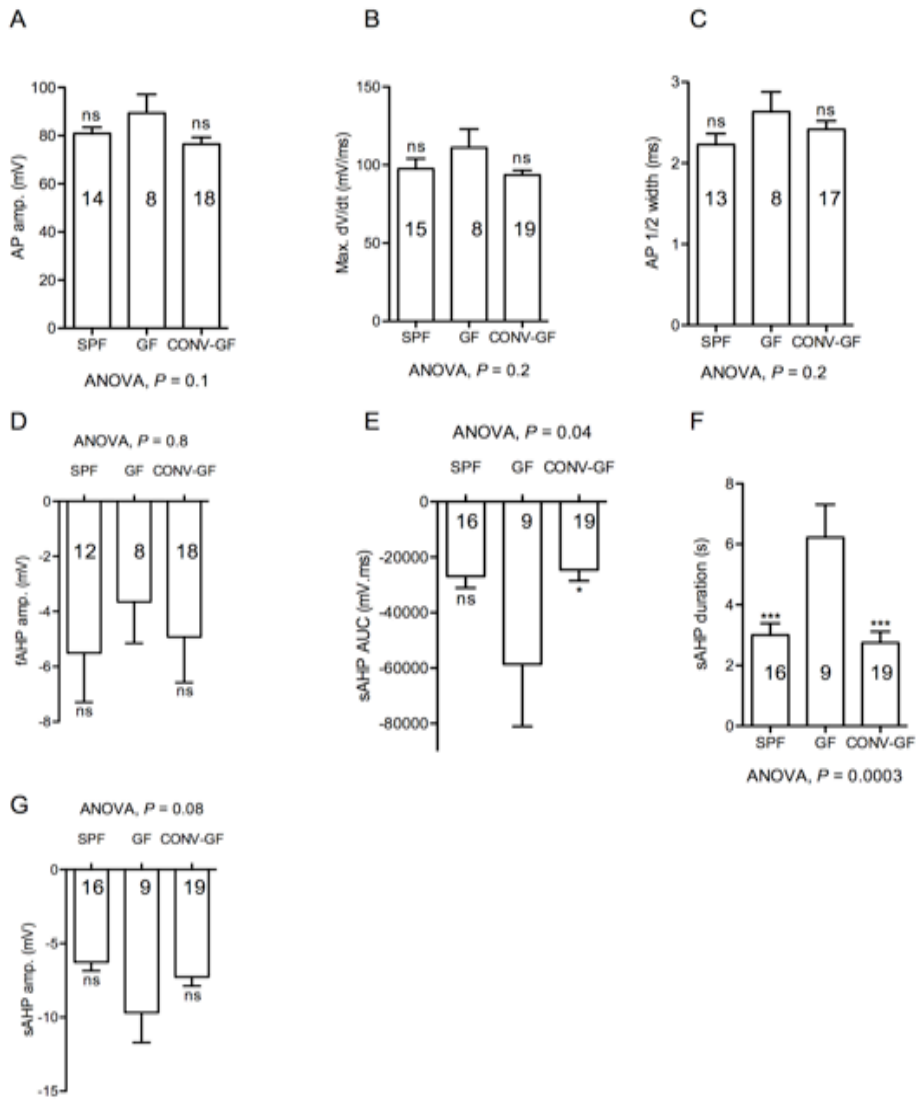

Figure 4. Action potential properties: summary statistics. A-D) AP amplitude (AP amp), maximal rate of depolarization (Max dV/dt), width at 1/2 amplitude (1/2 width) and the amplitude of the fast afterhyperpolarization (fAHP amp) were not significantly altered by the treatment conditions. E) Treatments affected the area under the curve of the slow afterhyperpolarization (sAHP AUC) with post hoc analysis showing a statistically significant difference between CONV-GF *vs* GF treatments, but for the sample size used

only a non-significant trend was evident when SPF was compared with GF treatment. F) sAHP duration was significantly affected by the treatments, with SPF *vs* GF and CONV-GF *vs* GF treatments producing highly significant differences in the durations. G) sAHP amplitude (sAHP amp) was not statistically affected by the treatments.

6.4 Discussion

This is the first work to demonstrate that in the absence of colonizing commensal intestinal microbiota the activity of neurons in the myenteric plexus of the mouse intestine is altered. Specifically, in GF mice we observe a lowered excitability of sensory AH cells in the mouse myenteric plexus. This effect was associated with both a lower resting membrane potential and decreased input resistance suggesting that the background (constitutive) resting conductance was altered in GF mice. With respect to action potential characteristics the duration of the sAHP was significantly increased. The functional net effect of these changes would be to *decrease* the likelihood of cell firing in response to exposure to normally adequate sensory stimuli. Exposing adult GF mice to intestinal microbiota for a period of 4 weeks restored the AH cells of the myenteric plexus to normal levels of excitability and appeared to reverse the effects on passive membrane characteristics and sAHP duration that were associated with the germ free state. These findings are intriguing because they directly demonstrate altered electrophysiological properties of ENS neurons due to the absence of commensal intestinal microbiota.

The ENS provides sensory innervation of the gut mucosa, and nerve fiber endings of the myenteric plexus terminate in close proximity to epithelial cells lining the lumen (Kunze et al., 1995; Furness, 2006). Given that these neuronal endings are thus adjacent to gut luminal contents, i.e. trillions of commensal bacteria, it is reasonable to hypothesize that bacteria may signal to the central nervous system as a whole via these enteric neurons. It

is intriguing that differences were specifically found in the myenteric sensory AH neurons, as opposed to S neurons, which are primarily motor and interneurons and which generally do not have processes projecting to the luminal epithelium. AH/Dogiel Type II neurons are thought to be crucial to influencing ENS processing (Kunze and Furness, 1999; Bertrand and Thomas, 2004) and indeed previous work in our own laboratory has demonstrated changes to these particular neurons after the feeding of live probiotic *L. rhamnosus* to rats (Kunze et al., 2009). Rats fed the probiotic as opposed to vehicle control for 9 days demonstrated increased neuronal excitability via a number of electrophysiological measures including; lower threshold for eliciting action potentials, increased number of elicited action potentials during a suprathreshold depolarizing pulse, and decreased duration of sAHP in AH neurons. A follow up study demonstrated that *L. rhamnosus* likely worked to increase excitability of AH neurons by blocking the potassium-dependent calcium channel IK_{Ca} (Wang et al., 2010). These measures are all consistent with the hypothesis that probiotic *L. rhamnosus* works on myenteric neurons to increase excitability in healthy animals, which is in contrast to the current work demonstrating that AH neurons show *less* excitability in the absence of colonizing microbiota. Interesting too is the observation that similar to the present findings, probiotic appeared to have no effect on electrical activity of S cells. While patch clamp techniques were not carried out in the current work, and thus direct measures of ion channel activity were therefore not possible, the fact that GF mice differ in terms of the duration of the sAHP in AH neurons when compared to SPF mice, and that normal duration is apparently restored after conventionalizing GF mice indicates that the IK_{Ca}

channel activity could also be altered in the absence of gut bacteria as in the presence of probiotic, albeit with opposite effect.

Early work in GF mice has demonstrated altered gut motility in the absence of intestinal microbiota (Husebye et al., 2001). Specifically, when motility in the GF gut was examined, researchers found that fewer migrating motor complexes (MMC) reached the midpoint of the small intestine, and the duration between MMCs was increased in GF mice as compared to controls (Husebye et al., 1994). Both of these findings are indicative of decreased motility in the GF gut and suggest that the enteric neurons are responding to the bacterial status of the intestinal lumen. Our findings of reduced excitability in AH neurons of the myenteric plexus in GF mice are consistent with these reports of decreased motility.

Our findings of altered excitability in the IPANs of the myenteric plexus are particularly interesting given that cell bodies from ganglia in the myenteric plexus have been demonstrated to form anatomical synapses with vagal nerve fibres (Powley et al., 2008; Phillips et al., 2010). Given this, it is reasonable to hypothesize that information regarding the status of the intestine, and indeed the luminal bacterial contents, could be transmitted to the CNS via the myenteric neurons and the vagus to the brain. Previous work in our laboratory has demonstrated that GF mice show changes in CNS function and brain circuitry, and information travelling from the ENS via the vagus to the brain is therefore a potential pathway by which the brain activity could be modulated by intestinal bacteria. Previous work in using pathogenic bacterial infection has demonstrated the

necessity of an intact vagal pathway for CNS response (Lyte et al., 2006) and work published this year demonstrated that antidepressant and anti anxiety-like effects of probiotic *L. rhamnosus* on mice were abolished after vagotomy (Bravo et al., 2011). These studies not only emphasize the importance of the vagus in gut-brain communication, but are now beginning to highlight the importance of neural communication in transmitting information regarding intestinal bacterial load.

The current study is the first examination of the electrophysiological properties of myenteric neurons in the absence of colonizing commensal intestinal microbiota. Our novel finding of reduced excitability of AH sensory neurons in GF mice is a window into how intestinal bacteria can induce change in the enteric nervous system and provides a preliminary look into how the nervous system as a whole may be initially responding to alterations in intestinal bacterial status.

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CHAPTER 7. DISCUSSION

7.1. Summary of Findings

The present thesis examines the impact of commensal intestinal microbiota on the development and function of the nervous system. As the extent to which the intestinal bacteria can influence host physiology is both extremely broad and considerably unknown, we investigated outcome measures as diverse as animal behaviour, HPA axis stress reactivity, brain neurochemistry and electrophysiological properties of the ENS. In order to begin to determine the effects of intestinal microbiota on each of these outcome measures, we used GF animals throughout our investigations, examining in turn the influence that a complete absence of gut bacteria has on the nervous system's various properties. In the publication that comprises Chapter 3 of this thesis we show that GF mice demonstrate a reduced anxiety-like behaviour in the EPM, which is accompanied by associated changes in brain neurochemistry. Specifically we see an increase in expression of hippocampal BDNF mRNA, a decrease in NMDA receptor subunit expression in the amygdala and a decreased serotonin receptor 1A expression in the hippocampus. All of these neurochemical changes are consistent with the basal behavioural phenotype that we observe in GF mice, that is one of decreased anxiety-like behaviour, and suggests that the absence of intestinal microbiota acts to modify both CNS development and function. Chapter 4 of the thesis is an addendum to the published paper that makes up the previous chapter. In this publication we show that the reduced anxiety-

like phenotype we observe in GF animals as compared to conventionally housed controls is maintained after adult conventionalization with commensal microbiota demonstrating that for at least anxiety-like behaviour the adult brain is not responsive to change in the intestinal commensal microbial status. Chapter 5 of the thesis investigates the role of microbiota on circulating plasma leptin and HPA hyperresponsivity in both prepubescent and adult female GF mice. In this chapter, exposure to 1 hour acute restraint stress results in a significant increase in plasma corticosterone in adult female GF mice compared to similarly stressed SPF control mice. In addition, HPA reactivity is completely dysregulated in GF mice prepuberty, with significantly increased levels of corticosterone exhibited irrespective of stress status. Plasma leptin is demonstrated to be significantly lower throughout the lifetime in GF mice and we see that this reduction in leptin is associated with changes to brain mRNA expression of genes key to both feeding and stress circuitry. We hypothesize that the lower plasma leptin levels observed in GF mice play a role in early life stress programming, which contributes to the altered stress reactivity we observe in these mice both prepuberty and in adulthood. Finally, in Chapter 6 of the thesis we demonstrate changes in electrophysiological measures of AH neurons in the myenteric plexus of GF mice *versus* conventionally housed SPF control mice. We see that these primary sensory neurons are less excitable in the absence of colonizing microflora, and that this is reversible after adult conventionalization. This is fascinating given that these primary neurons of the ENS have been previously demonstrated to form anatomical synapses with vagal nerve fibers in the gut, thus potentially providing a route

whereby information regarding the bacterial load of the gut could travel to the extrinsic nervous system, including the brain.

7.2. Germ Free as a Model of Early Life Environmental Challenge

Early life is a vulnerable time for the developing organism, and it has been well established that environmental challenges or adverse events experienced during this time can have a significant impact on the development and ultimate function of the CNS (Shanks et al., 1995; Shanks et al., 2000; Nilsson et al., 2002; Boisse et al., 2004; Walker et al., 2004; Bilbo et al., 2005a; Bilbo et al., 2005b; Ellis et al., 2005; Spencer et al., 2005; Bilbo et al., 2006; Spencer et al., 2006). Early life stressors in many forms can alter the trajectory of CNS development and many lead to long-term alterations in stress reactivity with associated behavioural change. A significant amount of the literature focusing on early life adverse events involves immune challenge (Shanks et al., 2000; Bilbo et al., 2005a; Bilbo et al., 2005b; Spencer et al., 2007). For example, exposing neonatal rats to endotoxin is known to modify the HPA stress axis, stress related behaviour and learning in adult animals (Shanks et al., 1995; Shanks et al., 2000; Walker et al., 2004). However, early life events that impact the development of the brain are certainly not limited to those that are immune in nature. Changes to adult HPA activity have been demonstrated in rodents that have been subjected to maternal separation, altered maternal care, (i.e. high or low licking and grooming), or the amount of handling received (Weaver et al., 2006). Indeed a recent publication has shown that the stress hyperreactivity and cognitive

deficits observed in rats exposed to early life bacterial infection can be reversed by increased handling of the animals in the early post-natal days (Bilbo et al., 2007). Based on the existing literature related to early life challenge in combination with my results in the GF mouse, *I propose that the absence of colonizing gut microflora in the early postnatal days acts as an environmental challenge* to the GF mice and is thereby an animal model of early life stress. This challenge then alters the HPA axis in such a way as to become hyperresponsive to stress later on in adulthood but is also manifest in basal alterations in anxiety-like behaviour. In Chapters 3, 4 and 5 of this thesis, I present data demonstrating that GF mice show a basal reduction in anxiety-like behaviour as measured in the EPM compared to conventionally housed SPF mice. I also show that female adult GF mice show HPA hyperreactivity following acute restraint stress compared to adult female SPF mice, and that prepuberty female GF mice have totally dysregulated HPA activity with exceedingly high plasma corticosterone levels irrespective of stress status. These findings are in keeping with previously published work demonstrating stress hyperreactivity in adult male GF mice following acute restraint stress (Ikeda et al., 1999; Sudo et al., 2004). The end result for the adult GF mice in terms of stress reactivity and associated behavioural abnormalities is thus consistent with changes observed in other models of early life challenge.

I propose that one of the ways that the absence of colonizing microbiota in the GF mice is acting as an early life environmental stressor is related to the altered metabolic pathways observed in this animal model. Because there are no gut bacteria in GF animals, there is also a significantly lower level of plasma leptin (Backhed et al., 2007; Zaibi et al., 2010).

Leptin is a protein hormone released from the adipocytes and is known to play a key role in energy balance and metabolism (Houseknecht and Portocarrero, 1998). The paucity of plasma leptin observed in GF mice is not only due to their lean body types and resultant lack of adipocytes, but is also directly due to the absence of microbiota itself (Hooper et al., 2002; Backhed et al., 2004). One role of commensal microbiota is to aid in the breakdown of otherwise indigestible food sources, specifically polysaccharides. Microbes break down polysaccharides into short-chain fatty acids (SCFAs), which GF mice are known to produce little to none of (Backhed, 2011). SCFAs act on G protein-coupled receptors in adipocytes to stimulate the release of plasma leptin (Xiong et al., 2004; Zaibi et al., 2010). Therefore the lack of plasma leptin observable in GF mice is due to not only less adipose tissue, but also to a relative absence of stimulating SFCAs. Colonizing GF mice with intestinal microbiota has been shown to not only increase body fat, but also to increase plasma leptin levels (Backhed et al., 2007). Interestingly, one critical function of plasma leptin during early life is thought to be the maintenance of the stress hyporesponsive period (SHRP). As mentioned previously, given that we know that early life stressors can be damaging to the developing brain, organisms have evolved certain responses in order to protect the CNS from harm during early life. High levels of circulating glucocorticoids are known to be damaging to the developing brain, and for this reason, both rodents and humans are thought to undergo SHRP (Bohn, 1980; Levine, 2002). During SHRP, plasma glucocorticoids are maintained at very low levels and stressors that would otherwise initiate the normal HPA neuroendocrine cascade resulting in increased plasma corticosterone (in rodents) or cortisol (in humans), are not able to

induce a stress response (Sapolsky and Meaney, 1986). The exact mechanisms by which organisms are able to maintain SHRP are unknown, but extensive research in rodents has strikingly demonstrated that plasma leptin may play a key role (Ahima et al., 1998; Trottier et al., 1998; Oates et al., 2000). One stressor that *is* known to raise corticosterone levels in rodents during SHRP is maternal deprivation (Stanton et al., 1987). Of all the maternal behaviours, feeding seems to be the critical factor in maintaining SHRP, as maternally deprived pups that continue to be fed demonstrate normal SHRP, whereas pups that remain with their mothers but are not permitted to feed do not exhibit SHRP (Suchecki et al., 1993). It has been hypothesized that leptin is the transmittable factor during feeding that results in normal development of the HPA axis (and thus normal SHRP) (Ahima et al., 1998; Trottier et al., 1998; Oates et al., 2000). I hypothesize that due to the absence of colonizing microbiota and the lifelong low plasma leptin levels observed in GF mice, and the data we have collected in prepubescent stressed and unstressed GF mice which shows complete HPA axis dysfunction, that GF animals do not undergo the protective SHRP. This was not tested directly in my thesis, but future experiments examining stress reactivity during the mouse SHRP period (2-3 weeks of age) in GF mice would provide a clearer understanding of the interconnected relationship between intestinal microbiota, leptin and stress reactivity in very early life. As shown in Chapter 5 of this thesis our data on stress reactivity during prepuberty shows drastic differences between mice raised with normal gut microbiota *versus* those without. Conventionally housed prepuberty mice show normal stress responsivity, albeit with slighter lower corticosterone levels than is observed in adult control mice. This is not too

surprising given that SPF control animals are just coming out of the SHRP period at this early puberty time point, and thus glucocorticoid levels have yet to reach their peak. GF mice however show exceedingly high glucocorticoid levels in early puberty, irrespective of stress status. These abnormal levels demonstrate a complete dysregulation of stress responsivity in adolescence, and given that we know that high circulating glucocorticoid levels are damaging to the young brain, are likely contributing to the alterations in stress responsivity we observe in adult GF mice, i.e. increased stress reactivity and altered anxiety-like behaviour. In this manner I propose that the lack of intestinal microbiota is acting as an early life environmental stressor via the absence of normal leptin signaling to the brain, and thus results in the observed abnormal development of stress reactivity and associated behaviours we see in the GF mice.

7.3. Critical Window for Bacterial Induced Change in the CNS *vs* PNS

If you reconstitute commensal intestinal microbiota in GF mice early enough in life, the brain is still sufficiently plastic for normal HPA activity to be restored. Sudo and colleagues demonstrated that giving commensal microbiota to GF mice prior to 6 weeks of age but not later normalized HPA activity in response to acute restraint stress (Sudo et al., 2004). In our behavioural work, reconstituting GF mice with SPF microbiota at 8 weeks of age was not sufficient to normalize the basal abnormalities in anxiety-like behaviour. Our finding of decreased anxiety-like behaviour in GF mice as measured in the EPM was recently replicated by another research group who also found that

conventionalizing GF mice from birth normalized this behaviour (Heijtz et al., 2011). Taken together, these data indicate that there is likely a critical window during early life whereby the introduction of commensal gut bacteria can normalize both stress reactivity and associated stress-related behaviours.

Intriguingly, our experiments examining the ENS of GF animals would suggest that the while the concept of a critical window whereby microbiota can induce permanent change in the nervous system seems to be true for the CNS, it is not so in the periphery. In our electrophysiology experiments we showed that the intrinsic primary afferent sensory neurons of the intestine in GF animals were less excitable overall than those of conventionally reared animals. When we conventionalized adult GF mice with normal commensal microbiota we saw a normalization of excitability, indicating that the intestinal bacteria induced change in the activity of the neurons of the myenteric plexus. Thus, the neurons in the intestine seem to retain their plasticity into adulthood, unlike those of the CNS, and are capable of responding to changes in the commensal bacterial load of the intestinal lumen. The same has been reported for pain sensitivity in the gut after treatment with probiotics. As discussed previously, feeding adult rats probiotic *L. reuteri* both upregulated cannibinoid and opioid receptors in intestinal epithelium and decreased visceral sensitivity to colorectal distension (Rousseaux et al., 2007). In another study, treating rats with antibiotic both altered the intestinal microbial profile and increased visceral sensitivity to CRD. Treating these rats with probiotics was able to ameliorate the antibiotic-induced changes (Verdu et al., 2006). By the same token, feeding rats *Lactobacillus rhamnosus* has also been shown to alter the typical

neurovisceral pain response to colorectal distension (Kamiya et al., 2006). All of these studies were carried out in adult rats, well past the 6 week age that seems to be the upper limit for inducing long term change in the CNS stress circuitry via microbiota. It should be noted that studies using probiotics may need to be interpreted slightly differently than studies such as ours which colonize a previously GF intestine with commensal intestinal microbiota. Probiotics are known to transiently alter the microbial profile of the intestine, and administration needs to be constant in order to retain an altered profile from baseline.

Our ENS data is particularly interesting not only because it is another illustration of the impact of gut bacteria on nervous system activity, and one in which there does not appear to be a critical window for change, but also because it provides another potential mechanism by which the gut could talk to the brain regarding intestinal bacteria. We see that the AH sensory neurons of the myenteric plexus are different in GF mice compared to controls, showing less excitability overall. The potential mechanism by which this information is translated to the brain comes via the findings that these neurons form anatomical synapses with the vagus nerve (Powley et al., 2008). It is possible that the change in activity we observe in the myenteric sensory neurons is being transmitted to the brain via the vagus nerve, and thus information regarding the intestinal bacterial load. Studies discussed earlier in the introduction of this thesis have demonstrated the importance of an intact vagal connection between the gut and brain in transmitting information regarding pathogenic bacteria to the CNS (Lyte et al., 2006).

One question that remains then is what does all of this mean in terms of clinical applications? Extensive alteration to microbial flora in early life is an environmental challenge that may be difficult to rectify after a certain developmental window. This is thus most critical when we consider the period of human infancy. The potential long-term effects of neonatal antibiotic treatment (Brunser et al., 2006), of vaginal versus caesarean births (Biasucci et al., 2010), of formula feeding versus breast milk (more complex microbiota in formula fed infants thus conferring a greater ability to ferment complex carbohydrates) (Edwards and Parrett, 2002), and of probiotic supplemented formulas (Braegger et al., 2011) would all need to be considered in light of this information. If colonization patterns are significantly altered in early human life, what pathways are being changed in the brain, especially those where stress reactivity and anxiety are housed? And if the peripheral nervous system is remaining plastic into adulthood in terms of responsivity to bacterial input, how can we exploit this characteristic to indirectly induce change at the level of the CNS? This is likely one of the more exciting avenues for research in the microbial-gut-brain axis, one in which there is evidently much promise for intervention and therapeutics.

7.4. Future Directions

We are currently working on developing a method whereby we can conduct electrophysiological recordings directly from single units within the gut mesenteric nerve bundle *in vivo,* with vagal *versus* spinal signals identified by vagotomy and responses to

cholecystokinin. The ability to record directly from this nerve has important implications in terms of understanding what information is sent from the intestine to the brain regarding the bacterial status (germfree, normal and probiotic) of the intestinal lumen. I intend to pursue this work in my postdoctoral studies, continuing to work with the GF animal model, but focusing too on the effects of monoassociation with a single bacterial species to examine the impact of individual probiotic/commensal species and strains on gut to brain signalling. I can couple this work with the molecular techniques I have learned throughout my graduate studies which will enable me to examine concurrent changes occurring at the level of the periphery and the CNS. This work will help to elucidate pathways by which bacteria may be used to confer benefits to the host CNS function.

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APPENDIX

Effects of gut microbiota on the brain: implications for psychiatry

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It may be surprising to learn that the human gastrointestinal tract is home to 10^{14} bacterial organisms (Savage, 1977). In fact, there are more bacteria in your gut than there are somatic cells in your body. These resident bacteria are referred to as commensal microbiota and their arrival during the first few postnatal days sets up a symbiotic relationship that is necessary and crucial to normal physiology. This life-long relationship is essential to host pathogen defense and plays an important role in nutrient uptake and metabolism (Nicholson et al., 2005). Immunologists have been aware of this system and its importance to the development of the mucosal and systemic immune systems for a long period of time (Cebra, 1999; Backhed et al., 2005). What is new and noteworthy is emerging evidence that gut microbiota influence behaviour and CNS function (Sudo et al., 2004; Neufeld et al., 2011). This commentary provides a brief overview of research related to gut-brain communication in a context that allows neuroscientists and psychiatrists to take note and consider the role of microbiota in their research related to CNS function and behaviour.

Colonization of the gastrointestinal tract, predominantly the colon, begins at birth, continues over the early developmental years, and remains throughout life. The early profile of microbiota is influenced by genetics and postnatal environmental exposure. Several bacterial phylotypes are distributed in the human gastrointestinal (GI) tract, and while each person's microbial profile is distinct, relative abundance and distribution along the GI tract of these bacterial phylotypes is similar between healthy individuals (Ley et al., 2006a; Ley et al., 2006b; Peterson et al., 2008). Several physiological functions are served by commensal flora. Gut microbiota facilitate nutrient uptake and metabolism,

providing us with otherwise inaccessible nutrients and vitamins (Guarner and Malagelada, 2003; Puupponen-Pimia et al., 2005; Martin et al., 2008). Colonization and the presence of microbiota is important to the development, function, and maintenance of a healthy gastrointestinal tract (Chu et al., 2004; Pull et al., 2005; Turnbaugh et al., 2007). Interestingly, gut microbiota are also essential and necessary for the proper development of the mucosal and systemic immune systems (Cebra, 1999; Macpherson and Harris, 2004; Backhed et al., 2005), a relationship we believe to be central when considering the impact that microbiota may have on the development and function of the brain.

Gastrointestinal research has for many years highlighted the importance of the "gut-brain axis", especially in relation to the functional bowel disorders like irritable bowel syndrome, but much of this work has been focused on "top-down" control, or the examination of the impact that the brain can have on general gut function (Aziz and Thompson, 1998; Wood, 2007). New work involving intestinal microbiota, the resident bacteria present in the healthy gastrointestinal tract, is indicating that events occurring in the gut also have an impact on the development and function of the CNS. Using the "top-down" approach, recent work has demonstrated that early life stress in a rodent, known to lead to altered stress-reactivity later in life, in parallel leads to an altered profile of gut microbiota (O'Mahony et al., 2008). Gut microbiota are also known to influence energy balance and in turn, emerging evidence demonstrates the importance of gut microbiota to the pathophysiology of obesity (Turnbaugh et al., 2006). Energy balance and food intake are centrally-mediated processes, however, the direct link between gut microbiota and

central feeding circuits has not yet been made. This is an example of the less-studied "bottom-up" control, which we believe will have a significant impact on both the study and treatment of diseases that have traditionally been viewed as being solely housed within the CNS.

Almost 50 years ago, Gustafsson (Gustafsson et al., 1957; Gustafsson and Laurell, 1958; Gustafsson, 1959) developed germ-free (GF) animals as a scientific tool (Haavik et al., 1997). These mice have no commensal intestinal microflora and as such exhibit an undeveloped immune system (Boman, 2000; Macpherson and Harris, 2004; Macpherson and Uhr, 2004; Tlaskalova-Hogenova et al., 2005). GF mice have proved to be a useful tool for investigations into differences between adaptive and innate immunity. We propose that a vital pathway of communication from the gut to the brain is through the immune system and therefore suggest that experimentation in GF mice related to stress-reactivity and related behaviours will provide answers to how intestinal microbiota influence CNS function.

A recent report found that compared to specific-pathogen free (SPF) mice, adult GF mice show an exaggerated stress response. GF mice showed no difference in basal stress hormones but showed increased plasma corticosterone (CORT) and adrenocorticotropic hormone (ACTH) levels in response to restraint stress (Sudo et al., 2004). An additional interesting finding in this report was that colonization of the gut microbiota, and the resultant constitution of the immune system, at 6 weeks of age (adolescence) resulted in normalization of the stress axis, however when mice were colonized in early adulthood (8

weeks of age or later), the altered stress response was persistent throughout adulthood. Our group extended this work and examined the behavioural phenotype of the germ-free mouse in early adulthood and observed a significant basal increase in open arm exploration in germ-free mice compared to SPF controls on the elevated plus maze (unpublished data). Retesting of the same mice following colonization with SPF microbiota showed that this altered anxiety-related phenotype was persistent. Therefore in the unstressed state, GF mice show less anxiety-like behaviour than SPF comparisons. This observation was surprising since Sudo et al. (Sudo et al., 2004) demonstrated an exaggerated HPA activation in response to restraint stress, however, it should be noted that the basal levels of stress hormones (CORT and ACTH), in their GF mice, were not elevated. While preliminary in nature, these data are provocative and suggest the microbiota influence CNS development of stress-reactivity and anxiety-like behaviour. Additional work using germ-free mice will provide an avenue to tease out the underlying mechanisms by which gut microbiota communicate with the CNS and influence behaviour.

Clinically, psychiatric illness does not stand alone. It is well recognized that many gastrointestinal disorders demonstrate a high co-morbidity with psychiatric illness. This is particularly true for the functional bowel disorders, such as irritable bowel syndrome (IBS) and functional dyspepsia. Mood disorders are the most common of these psychiatric illnesses, with studies demonstrating that more than 50% of patients with IBS also meet criteria for mood disorder (Whitehead et al., 2002). Indeed, antidepressants are one of the most common pharmaceutical interventions for IBS. While most clinical and

preclinical studies have focused on top-down treatment options for primarily intestinal disorders, emerging work from germ-free mice suggests the possibility of treatment options for psychiatric diseases potentially being targeted to systems outside of the CNS. New directions in preclinical work considering the importance of microbiota in combination with clinical work examining the impact of antibiotic and probiotics on CNS development and function will inform us of the importance of bottom-up control to brain function. The results of work in this emerging area may provide novel targets for intervention in psychiatric illnesses.

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