UNDERSTANDING THE EFFECT OF POSTNATAL EXPOSURE TO ANTIBIOTICS ON THE MICROBIOTA-GUT-BRAIN AXIS

By

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TITLE: Understanding the Effect of Postnatal Exposure to Antibiotics on the Microbiota-Gut-Brain Axis

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Lay Abstract

Oral antibiotics are commonly prescribed for infants and children. These drugs, although useful, can change the normal state of the body, whether temporarily or permanently. Antibiotics kill both harmful and beneficial bacteria that live in the gut. These bacteria, collectively called the microbiota, make up 100 trillion cells in the gut and work with the metabolic and immune systems to maintain a healthy state. Disruption to the microbiota by antibiotics at a critical developmental period may have consequences on health and disease. Some beneficial bacteria, called probiotics, are being studied to help reverse the damage antibiotics present. In this study, the effects of a real-life dose of an antibiotic, PenecillinV, and a probiotic, JB-1, are examined in a mouse model. We found that the antibiotic was able to change short- and long-term measures of behavior, brain chemistry, immunology, and intestinal endocrinology. Interestingly, most of these changes were reversed by simultaneous probiotic feeding. These results suggest that the body is vulnerable to change mediated by the microbiota at the postnatal stage of life.

Abstract

Introduction. A 100 trillion bacteria reside in the human gastro-intestinal tract. These microbes are involved in the gut-brain axis. Disruption to their pattern of growth can alter gut-brain communication, and thus health and disease outcomes. Probiotics are being explored for their therapeutic use relative to early-life insults that alter the microbiota, such as antibiotics and stress. In our study, we use a clinically relevant dose of PenicillinV, a beta-lactam antibiotic, and *Lactobacillus rhamnosus* JB-1, a probiotic, to examine their postnatal effects on long-term immune, nervous, and enteroendocrine status.

Methods. BALB/c male and female mice were orally feed, from postnatal day (PND) 14 to 21, twice a day for seven days with either PBS and antibiotics, antibiotics and probiotic, or PBS for both feeding times. For the first experiment, all mice were subjected to behavior tests that measured their anxiety-like and social behavior at PND 70. Fecal samples were collected for microbial diversity analysis 24 hours and 3 weeks after treatment cessation. For the second experiment, mice were sacrificed at PND 22. After decapitation, tissue and serum was collected for analysis of immune, neurochemical, and enteroendocrine markers for both experiments.

Results. Antibiotic altered microbial diversity in the short and long term. Male mice exposed to antibiotics showed a decrease in social behavior associated with immune changes. JB-1 attenuated the effects on behavior and immunology. Sex-dependent antibiotic-induced differences in tight junction expression in the brain and ileum were prevented by concurrent JB-1 treatment.

Conclusion. Our findings support the principle that early-life perturbations to the gut microbiota can affect other physiological systems in a transient and lasting way. The results also confirm the psychoactive and immune-modulatory function of JB-1 in attenuating immune and behavioral defects induced by antibiotic treatment. The clinical relevance of the antibiotic treatment further shows that the postnatal period is a critical window for microbial, immune, and brain development and is vulnerable to environmental challenges.

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Table of Contents

List of Figures

Abbreviations

AB: antibiotics

APC: antigen presenting cell

AVPR1a: vasopressin receptor-1a

AVPR1b: vasopressin receptor-1b

BBB: blood-brain barrier

BDNF: brain-derived neurotrophic factor

CLDN-5: claudin-5

CNS: central nervous system

CNTL: control

DC: dendritic cells

DSS: dextran sodium sulfate

ENS: enteric nervous system

GF: germ free

GI: gastrointestinal

GLP-1: glucagon-like peptide-1

IBD: inflammatory bowel disease

IBS: inflammatory bowel syndrome

IL: interleukin

JB-1: *Lactobacillus rhamnosus* JB-1

LDP: low-dose penicillin

LPS: lipopolysaccharides

MLN: mesenteric lymph nodes

OCLD: occludin

OTR: oxytocin receptor

PenV: PenicillinV

PND: postnatal day

POMC: pro-opiomelanocortin

PYY: peptide yy

Th: helper T

TJ: tight junction

T_{reg}: regulatory T

ZO-1: zona occludin-1

Introduction

Oral antibiotics (ABs), specifically penicillin, are the most widely used form of pediatric medication (Clavenna and Bonati, 2011). AB administration in childhood is linked to non-communicable diseases in later life, including inflammatory bowel disease (IBD) (Hviid et al., 2011), asthma (Russell et al., 2013), obesity (Trasande et al., 2013) and diabetes (Hu et al., 2016; Livanos et al., 2016). Furthermore, exposure to AB, specifically in early life, seems to play a role in dysregulating the microbiota-gut-brain axis, which may have long-term neurocognitive consequences (Farzi et al., 2012; Slykerman et al., 2016; Fröhlich et al., 2016). AB reduce microbial diversity (Panda et al., 2014; Yassour et al., 2016). Hence, changes correlated to early-life administration of AB may be mediated by microbial dysbiosis occurring at a critical time point. However, there is a gap in understanding how AB influence immune, metabolic, and behavioral pathologies through the changes they exert on the gut microbiota.

Gut microbial maturation and neurodevelopment share critical growth periods vulnerable to damage (Borre et al., 2014). Early-life events that perturbate microbial colonization can mold mental health in adulthood (Heijtz et al., 2011; de Theije et al., 2014). Three windows are critical for growth and development: prenatal, postnatal, and adolescence. The focus of this project is on the postnatal period. This period is implicated more in growth, as most organs and tissue structures develop prenatally in mammals. However, the central nervous system (CNS) is an exception because a considerable amount of morphological maturation occurs postnatally (Wise and Jones 1976; Herschkowitz et al.,

1997; Barker and Ullian, 2010). A major transition event that occurs at infancy in humans in synaptic refinement, which is proposed to set a foundation of strengthened neuronal circuits for adulthood (Petanjek et al., 2011). Postnatal environment insults may shape this process to determine long-term psychiatric disorders (Borre et al., 2014). Perinatal AB exposure and maternal separation are used as early-life models of adversities shaping disease and disorders of gut-brain communication (O'Mahony 2014; Forsythe et al., 2016). To understand the microbiota-brain-axis better, both models will be explored here. However, the focus of this study is postnatal exposure to clinically relevant AB.

The development of a healthy gut microbial pattern in early life is important for childhood health. This "normal" pattern of microbial development in the gut is influenced by early-life environmental factors. For example, babies born via Caesarian section, formula-fed, and exposed to AB have different microbial composition to those born vaginally, breast-fed, and not exposed to AB (Tamburini et al., 2016). Altering the intestinal microbiome via stress or AB exposure is a form of challenge that seems to have long-lasting consequences.

It was believed for a long time that a fetus is first colonized by microbial organisms at birth, but recent studies have challenged this notion. Bacteria reside in the umbilical cord, amniotic fluid, and meconium (Steel et al., 2005; Jiménez et al., 2008). Bacteria found in meconium appear to resemble maternal oral microbiota (Gosalbes et al., 2013). However, how bacteria transfer from mother to fetus remains unclear. This argument of initial colonization is highly controversial and remains to be settled. In the first month, the infant gut is dominated by aerobic bacteria, *Enterobacteriaceae*, before yielding to anaerobes,

mainly *Bifidobacterium*, *Bacteroides*, and *Clostridium*, within the first six months (Bokulich et al., 2016). By age two, the fetal microbiota profile begins to resemble an adult state. In humans, *Bacteroidetes* and *Firmicutes* phyla are dominant, and there is a low abundance of *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Eckburg et al., 2005). In contrast, genus and species diversity is great. Regardless of this variation, the microbiota carries out the same function in healthy individuals resulting in the term "core microbiome" and divergence from this core is commonly referred to as "dysbiosis" (Qin et al., 2010). However, because there is no normal microbial species composition, use of the word "dysbiosis" may be somewhat misleading. A common feature of microbial community health is high species diversity.

Microbiota-gut-brain axis: An emerging system

There are 100 trillion bacterial residents of the human gastrointestinal (GI) tract (Bäckhed et al., 2005; Frank and Pace, 2008). It has been recently realized that these microbes interact with both the gut and the brain, two organs that have known connections. Humans share a mutualistic relationship with their gut microbes. The human gut provides a safe habitat for the microbes and, in return, they carry out and promote metabolic and immune functions, such as processing indigestible polysaccharides, producing essential nutrients, and providing protection against parasites (Chow et al., 2010). In addition, microbes in the gut produce neuroactive elements, such as neurotransmitters, oligosaccharides, and short chain fatty acids (SCFA), that signal to the nervous system. These bacteria also play a role in the regulation of GI tract epithelium, mucosal

immunology, enteric nervous system (ENS), and vagal nerves, which use molecules such as cytokines, hormones, and neurotransmitters to communicate. This, in turn, influences the CNS and specific neuroendocrine responses, such as the hypothalamic pituitary adrenal axis (HPA) and the ENS. The HPA is the main endocrine stress system in humans. It is also a major pathway for communication between the brain and intestinal immunity (de Weid et al., 1993). Products of those various systematic pathways shape gut microbe diversity, although the way in which this happens is still unclear. The proposed mechanistic interaction of the microbe-gut-brain axis forms a bidirectional and complex network between the nervous, immune, and endocrine systems (Forsythe et al., 2016).

Immunology. The immune system depends on microbial colonization for its maturation (Sansonetti and Di Santo, 2007). Perinatally, gut microbes interact with immune and epithelial cells of the intestinal mucosa to establish a tightly controlled homeostatic immune state in the GI tract. This occurs through a complex microbial sampling process via M cells and dendritic cells (DCs). M cells transfer antigens to DCs, where they are processed by toll-like receptors (TLR). DCs then migrate to mesenteric lymph nodes (MLN) to initiate an adaptive immune response and direct specific IgA production in the lamina propria, preventing luminal bacterial over-growth (Neura et al., 2001; Akira and Hemmi, 2003; Fagarasan and Honjo, 2003; Macpherson and Uhr, 2004). This immune response is mediated by local cytokines suppressing T cell differentiation, including but not limited to T helper (Th) cells (Th1, Th2, and Th17), potentially by activating CD25+ regulatory T (T_{reg}) cells (Chen et al., 2003). The initiation of this process in early life seems to play a role in gut homeostasis mediated by resident bacteria and their interaction with

intestinal epithelial cells, influencing barrier operations (Rakoff-Nahoum et al., 2004; Fukata et al., 2005). Commensal gut bacteria are pivotal for the GI immune fine-tuning to allow controlled commensal bacteria growth and to protect against bacterial pathogen attacks.

Germ free (GF) rodent models have been employed as a proof of concept method to study the function of the microbiota-gut-brain axis in human physiology. GF mice show a compromised immune system with smaller Peyer's patches, reduced IgA secretion, downregulated TLR expression, fewer villi, and decreased intra-epithelial lymphocytes (Abrams et al., 1962; Wostmann et al., 1970; Weinstein and Cebra, 1991). GF mice have a smaller number of CD3+ T cells in the lamina propria, which is associated with epithelial barrier permeability (Williams et al., 2006). An immature or weakened immune system due to the absence of colonizing bacteria highlights the dependence of immune development on the microbiota. It has also become clear that early life is critical for healthy immune development. Supporting this, colonization of GF at weaning has been shown to prevent chemically induced colitis, while colonization in adulthood does not (Olzac et al., 2012). Thus, disrupting gut microbe colonization in the developmental period has immunological consequences, and this idea will be explored further below.

Commensal microbes utilize the matured immune system to communication with the rest of the host's systems. Epithelial cells are not immune by nature; however, they are the largest number of cells lining the intestinal lumen and are in direct contact with microbes that exert immuno-modulatory effects through manipulating intestinal permeability (Furness et al., 2013). The "leaky gut" phenomenon explains a main feature

of the gut-brain axis. The proposed mechanism is that the gut barrier integrity is comprised by psychological or physical stress causing the translocation of gram negative bacteria across the mucosal lining, where they can interact with immune cells and the ENS (Gareau et al., 2008). The transfer of bacteria from the lumen to circulation activates the immune response, which leads to the production of pro-inflammatory cytokines interleukin 6 (IL-6) and IFNg.

Gut microbiota have the power to alter production of metabolites that act as precursors to gut-brain signaling molecules. Bacterial lipopolysaccharides (LPS), peptidoglycans, and SCFA are such molecules. LPS can travel to the brain via blood circulation and bind to TLR, inducing an inflammatory process and influencing brain function (Arroyo et al., 2011). SCFA are able to control macrophage anti-inflammatory behavior and promote T_{reg} cells (Furusawa et al., 2012). Bacterial components and metabolites also communicate with the ENS and the enteroendocrine cells (EEC) to modulate brain signaling (Aidy et al., 2015; Farzi et al., 2018).

Entero- and neuro-endocrinology. SCFAs, such as acetate, propionate, and butyrate, are products of the fermentation of complex carbohydrates, that can act as signaling molecules from the GI tract to the CNS. N-butyrate, specifically, functions to regulate energy homeostasis and promotes the production of glucagon-like peptide-1 (GLP-1), a hormone involved in insulin regulation (Burcelin et al., 2007). More than 20 hormones are produced by 12 distinct EEC types comprises the enteroendocrine system (Furness et al., 2013). These hormones regulate metabolic homeostasis in the gut by controlling gut motility and energy production or consumption. ECCs in the gut receive information from

microbial metabolites and in turn communicate with neighboring epithelial cells, arteries, and neurons (Furness et al., 2013). In GF mice, the expression of intestinal PYY and GLP-1 is downregulated, along with other changes, influencing feeding behavior (Duca et al., 2012). The enteroendocrine system interacts with the neuroendocrine system, the gut's own nervous system and the so-called "second brain" (Forsythe and Kunze, 2013). Expression of neuropeptides is altered in GF mice: in the hypothalamus, orexigenic neuropeptides are increased and anorexigenic neuropeptides are decreased (Schéle et al., 2013). The neuroendocrine system affects processes beyond the gut, possibly shaping brain and behavior.

Brain and behavior. One of the first papers using GF mice in behavior research (Sudo et al., 2004), demonstrated an exaggerated stress reaction of the HPA to acute restraint stress in adult male GF mice compared to specific-pathogen-free control mice. The authors found reduced mRNA gene expression of both cortical and hippocampal brain-derived neurotrophic factor (BDNF), a gene involved in learning and memory, among many other functions. GF mice colonized prior to 6 weeks of age showed normalized HPA reactivity, that later colonized mice did not attenuate. This study identified adolescence as a critical developmental window. A later paper reported adult female GF mice exhibit anxiolytic-like behavior compared to specific-pathogen-free mice accompanied by increased expression of BDNF and decreased serotonin 1A receptor expression in the hippocampus (McVey Neufeld et al., 2011). Heijtz et al. (2011) confirmed these behavioral finding in GF male mice. In addition, they found a higher turnover of monoamine neurotransmitter in the striatum, possibly contributing to the observed behavioral

differences. GF male mice also exhibit reduced social behavior with reduced sociability and preference for social novelty in the three chamber sociability test (Desbonnet et al., 2014). This deficit in sociability was reversed in GF mice colonized shortly after weaning. However, preference for social novelty was not altered by post-weaning microbial colonization. This showed that the postnatal period is another critical developmental window. These studies done in GF rodent models have shown that endogenous gut microbes are vital to mammalian physiology and healthy brain development. Further, they show that the microbiota-gut-brain axis employs nervous, endocrine, and immune networks for communication and function. Yet the mechanisms by which this axis works in the human body are not fully understood.

Antibiotics: An interrupter of healthy development

In 2010, it has been estimated that 70 billion doses of AB were consumed worldwide (Blaser, 2016). In the USA, 79% of children are exposed to AB before 24 months of age (Ajslev et al., 2011). Given that the penicillin family is the most widely used for of pediatric oral AB, it is used in many early-life models of AB exposure (Clavenna et al., 2011). PenicillinV (PenV) belongs to the beta-lactam class of AB. Beta-lactam AB interfere with cell wall synthesis of bacteria, causing it to lyse. More specifically they inhibit the cross-linkage of peptidoglycans by transpeptidases, called penicillin binding proteins (Strominger and Tipper, 1965). Beta-lactams are clinically used for throat, ear, respiratory tract, urinary tract, and skin infections (Laurence et al., 2006).

Chromic immune diseases. It is recognized that AB exposure in early life is

associated with an increased risk of chronc immune pathologies such as IBD and inflammatory bowel syndrome (IBS). IBD is a disease characterized by chronic inflammation of the gut and is divided into two forms: ulcerative colitis and Crohn's disease (Podolsky, 2002). IBS is an GI functional disorder often associated with infection or stress; it is characterized by increased visceral hypersensitivity (Talley, 1998). The high comorbidity of IBD and IBS with stress-related disorders such as anxiety and depression is further evidence of the vital role of the microbiota-gut-brain axis in health and disease (Bonaz and Bernstein, 2012; Moloney et al., 2014). In the peripartum period, AB administration in an IL-10 deficient colitis mouse model causes higher susceptibility to dextran sodium sulfate (DSS)-induced colitis and a skewing of the immune system in the offspring (Miyoshi et al., 2017). AB-treated offspring show a persistent decrease in alpha diversity until 8 weeks after treatment cessation. In an experimental T cell transfer model for IBD, mice treated with an AB cocktail prenatally to weaning were found to have faster onset CD4+ T cells shown when transferred to Rag1-deficient mice (Scheer et al., 2017). CD4+ T cells play a role in T cell differentiation, which is involved in IBD development (Sellon et al., 1998). In a study of maternal separation in rats, increased corticosterone, an LPS-induced immune response, and visceral hypersensitivity were associated with an altered microbial profile (O'Mahony et al., 2009). Meta-analyses of maternal and early childhood use of AB in humans show that AB use is associated with immunological dysfunction and diseases. IBS patients have an over-active HPA axis and an exaggerated pro-inflammatory cytokine presence (Dinan et al., 2006). An epidemiological study reported that AB administration in early life is correlated with risk of IBD in adulthood,

and that risk increased with the number of courses of AB taken (Haviid et al., 2011). This association between high AB exposure in early life and emerging diseases may be mediated by the disturbance of gut microbiota during a pivotal developmental period for the immune system (Forsythe and Bienenstock, 2010).

Behavior and neurochemical abnormalities. In mouse models, exposure to AB results in abnormalities in brain neurochemistry with associated changes in behavior. AB exposure in adolescence is linked to reduced anxiety-like behavior associated with reduced oxytocin and vasopressin mRNA expression in the brain (Desbonnet et al., 2015). Moreover, AB administration is correlated with downregulation of receptors implicated in neurogenesis, such as BDNF receptors in the amygdala and hippocampus, which has been linked to increased exploration, decreased apprehension, and increased aggression (Bercik et al., 2011). Given that AB disrupts brain function, Fröhlich et al. (2016) hypothesized that AB-induced microbial dysbiosis mediates these metabolic and neurochemical changes (Farzi et al., 2012). Indeed, they found diminished BDNF expression in the prefrontal cortex, hippocampus, and hypothalamus in AB-treated mice. In addition, some tight junction (TJ) protein expression was decreased in the hippocampus and others were increased in the amygdala. Levels of SCFA in the colonic lumen were dramatically reduced by AB. These molecular changes were associated with reduced novel object recognition memory. Adult mice treated with AB showed decreased hippocampal neurogenesis and memory retention (Möhle et al., 2016). However, recolonization coupled with probiotic or exercise reversed this effect in the brain, which was associated with an increase in $Ly6C^{hi}$ monocytes. Adoptive transfer of monocytes also attenuated the effect of the AB.

Understanding how AB induce these changes in the CNS will contribute to the knowledge of mechanisms involved in psychiatric disorders and, thus, to the further development of therapies.

Clinical relevance. Cox et al. (2015) were among the first to use a clinically relevant or low dose of penicillin (LDP) in early life to assess long-term effects on microbial diversity, metabolism, and immunity. Mice either received LDP for 28 weeks from birth or from weaning (postnatal day [PND] 28) . Although phylogenetic diversity was increased in early life (week 4), specific taxa (*Lactobacillus*, *Candidatus Arthomitus*, *Allobaculum*) prominent in early life were significantly reduced relative to control mice. LDP also reduced non-fasting serum peptide YY (PYY) at late adulthood (week 30) associated with a percentage increase in body fat, a characteristic typically present in obesity. CD4+IL17- percentage, a Th17 cell marker, was decreased in distal intestinal lamina propria after 8 weeks of LDP, suggesting that the intestinal immune response was dampened by the treatment. In non-obese diabetic mice, early-exposure pulsed AB increased the incidence of type 1 diabetes (Livanos et al., 2016). In the same study, mice treated with a therapeutic dose of AB daily, prenatally to week 12, had reduced Th17 and T_{reg} in the intestinal lamina propria. The phylogenic diversity of gut microbiota in the AB-treated mice was lowered at all time points from week 3 to 13.

Leclercq et al. (2017) studied the early effects of perinatal clinically relevant AB treatment in mice. Dams were treated with the same LDP from an earlier period of time: from embryonic day 14 to PND 21, so pups were colonized by an altered maternal microbiota and received AB through breast milk. An additional group was treated with

concurrent AB and a psychoactive probiotic *Lactobacillus rhamnosus* JB-1 (JB-1). AB-treated male mice showed a decrease in anxiety-like and aggressive behavior in adulthood. The aggressive behavior was associated with an increase of vasopressin receptor 1b (Avpr1b) expression and inflammatory cytokines in the frontal cortex. The AB-induced decrease in sociability and preference for social novelty was attenuated by JB-1. While the probiotic treatment did not attenuate most of the changes to microbial diversity caused by the AB, there was a dramatic change in the increase in Proteobacteria in AB and AB+JB-1 groups relative to control at PND 21. There was also a decrease in Bacteroidetes and increase in Firmicutes at PND 21. These changes and the general decrease in alpha diversity caused by AB remained to PND 42, 3 weeks after treatment. However, the therapeutic mechanism of JB-1, relative to clinically relevant early AB exposure, remains unknown and was explored in my thesis project. Using a low or clinically relevant dose of AB, as an alternative to GF models, allows the microbiota-gut-brain axis to be understood at a translational research level.

The GF state is an artificial one. It does not resemble the state of the human gut at any point in life. It is simply a useful method to study the role of microbes relative to other physiological systems (Luczynski et al., 2018). The GF model allows the manipulation of intestinal microbe colonization, whether it is the time of colonization or the species being colonized. This gives a basic understanding of the microbiota-gut-brain axis and thus provides a foundation to build future knowledge in this field. Since GF animals lack microbes, the model is limiting in the study of the microbiota and early life interventions. AB and probiotic treatments offer a more suitable, clinically relevant research model.

Probiotics: A potential defense against environmental perturbations

Microbes play a role in modulating behavior through the CNS, immune system, and neuroendocrine system. This is a reason to study the potential therapeutic benefits of specific beneficial microbes (probiotics). Probiotics are live microorganisms that when administered in adequate amounts may confer a health benefit for the host (World Health Organization, 2002). Many probiotic strains are under study for their beneficial effects on inflammatory and psychiatric diseases. In mice models, probiotics show immune-modulatory and neuroprotective effects. For example, feeding rats *Bifidobacterium infantis* for 14 consecutive days resulted in reduced inflammatory markers in the gut and altered markers of depression in the brain and blood circulation, suggesting the bacteria have an anti-depressive property (Desbonnet et al., 2008). A probiotic formulation of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 lowered anxiety-like behavior in rats after 14 days of feeding (Messaoudi et al., 2011). The same formulation used in a double-blind, randomized control trial reduced psychological stress in healthy volunteers after 30 days of administration. In a maternal separation model, *Bifidobacterium seudocatenulatum* CECT 7765 fed in early life, during chronic stress, prevented elevated corticosterone levels and intestinal inflammatory markers (Moya-Pérez et al, 2017). Anxiety-like behavior in adulthood associated with maternal separation were attenuated and expression of certain neurotransmitters (adrenaline, noradrenaline, serotonin, and dopamine) in the hypothalamus was normalized with the probiotic treatment. Also, *B. pseudocatenulatum* CECT 7765 was protective against AB-induced dysbiosis. The same probiotic ameliorated

the exaggerated stress response and anhedonia behavior in high-fat diet fed obese mice, which was associated with a reduced expression of obesity induced intestinal and hippocampal TLR2 (Agusti et al., 2017).

As metabolic and immune diseases are intertwined, GI inflammatory diseases and stress-related psychiatric disorders are correlated, highlighting the gut-brain cross-talk (Holzer at al., 2017). Studies indicate that intestinal inflammation and anxiety or depressive-like behavior are directly proportional, and this is exemplified in IBS models. Exposure to infection-causing pathogens increases anxiety-like behavior, measured with different tests, given absence of a peripheral or systematic immune response (Lyte et al., 1998l; Lyte et al., 2006; Goehler et al., 2008). These studies suggest that with a stable immune status, the presence of a pathogenetic bacteria in the GI can have anxiogenic effects (Foster and McVey Neufeld, 2013). In addition, studies using probiotics show that intervention attenuates anxiety-like behavior and beneficially alters inflammatory markers. *Bifidobacterium longum* ameliorates anxiety-like behavior present in a DSS-induced colitis mouse model (Bercik et al., 2011). However, the anxiolytic effect of the microbe was absent in anxious mice vagotomised before the last DSS treatment cycle. Ait-Belgnaoui et al. (2012) demonstrate that prevention of the leaky gut syndrome by *Lactobacillus farciminis* ameliorates the HPA response to acute stress. In a randomized controlled, double-blind study, a mixture of probiotics (mainly made up of *Lactobacillus* and *Bifidobacterium* species) administered to children with IBS for 6 weeks led to successful relief of symptoms (Guandalini et al., 2010).

The dampening effects of specific bacteria on the inflammatory and stress responses in animal models and clinical trials of infection and chronic stress have made bacteria great potential therapies (Braegger et al., 2011; Foster et al., 2017). JB-1 is a probiotic species that has been under study in the Brain-Body Institute in relation to the microbiota-gut-brain axis. This bacterial strain was studied in my master's project to further understand its ability to attenuate the persistent effects of early-life clinically relevant doses of AB exposure. Administration of JB-1 changes the expression of $GABA_{B1b}$ receptor in the brain, which is associated with reduced stress-induced corticosterone, anxiety-like and depressive behaviors (Bravo et al., 2011). GABA is the main CNS inhibitory neurotransmitter, highly implicated in anxiety and depressive behavior (Cryan and Kaupmann, 2005). JB-1 also attenuates behavioral deficits of chronically defeated mice (Bharwani et al., 2016). After 28 days of treatment, JB-1 prevented the stress-induced activation of DCs and increased the population of IL10+ T_{reg} cells in the spleen. JB-1's immunoregulatory properties have been established in a number of studies showing that it prevents IL-8 stimulation by TNFα in a human colonic epithelial cell line and protects against allergic airway response mice by inducing T_{reg} cells (Karimi et al., 2009; Karimi et al., 2012). These findings suggest that JB-1 has consistent immunomodulatory and neuro-active effects.

Rationale

GF and high-dose AB studies in rodents have helped in understanding how microbiota, or lack thereof, affects overall health and disease. Using a clinically relevant dose of penicillin aims to imitate a clinical setting, and thus provide a better understanding of the realistic long-term harms of AB use in early life. The effects of postnatal AB

administration on the microbe-gut-brain axis have yet to be fully explored. I aim to provide a holistic view and specific mechanisms by which early-life AB administration leads to abnormalities in adulthood through microbial-mediated changes.

Many different probiotic strains are being studied to understand GI diseases. The idea that probiotics can be used as therapy for psychiatric disorders, which are highly associated with immune disease, is relatively new. Exploring how JB-1 can prevent lasting brain, behavioral, and immune abnormalities caused by early-life AB exposure may provide new therapies for those affected.

Objectives

The project has three main objectives:

1. Identify the postnatal critical window in which AB exposure has the most robust effects on immune, nervous, and enteroendocrine systems;
2. Explore mechanisms through which these effects occur; and
3. Investigate the potential therapeutic effects of probiotic treatment on lasting effects mediated by AB-induced dysbiosis of gut microbes

Hypotheses

In my thesis, I aim to understand how AB influence the microbiota-gut-brain axis and explore possible therapeutic approaches involving the probiotic bacterium JB-1. Thus, I hypothesize the following:

1. Disruption of the microbiota-gut-brain axis through postnatal exposure to clinically relevant doses of penicillin leads to long-term changes in immune, nervous, and enteroendocrine profiles;
2. AB-induced changes may be attenuated by concurrent JB-1 treatment

Methods

Animals

The model organism being used is the BALB/c mouse. BALB/c mice have been characterized as anxious (Michalikova et al., 2009), and thus represent a susceptible population: a portion of the population prone to anxiety disorders. This makes the mouse model more suitable because the targeted population is one that is in most need for treatment.

BALB/c male and female mice were acquired from Charles River and delivered to St. Joseph's Healthcare Hamilton. After two weeks of habituation in the animal facility, breeding pairs, each in a cage, were housed together for 10 days. The sire was removed 16 days after pairing. Litters were randomly assigned to AB, AB+JB-1, or control group. At weaning (PND 21), males and females were separated and assigned to mixed litter cages of the same experimental group.

In the first experiment, animals were sacrificed at week 10-11 after behavioral tests, 7-8 weeks after treatment cessation. In the second experiment, animals were sacrificed at week 3 (at weaning), around 36 hours after treatment cessation. All sacrifices were done by decapitation using a guillotine. The total sample size was achieved with two cohorts for each experiment.

Treatment

BALB/c pups were orally feed twice a day for 7 days, with a 4-hour difference between treatment times (10 am and 3 pm), from PND 14 until weaning (PND 21). Each cohort was comprised of three treatment groups: AB, AB+JB-1 and control. The AB group was fed PBS in the morning and AB in the afternoon. The AB+JB-1 group was fed JB-1 in the morning and penicillinV in the afternoon. The control group was fed PBS in the morning and the afternoon. The volume of treatment per feed was adjusted depending on litter weight gain, which was measured every other day. The volume fed did not exceed 60-80 µl per day, complying with pup oral feeding guidelines (Turner et al., 2011). The clinically relevant AB dosage was calculated using the following ratio: 33 mg/kg of pup mass. JB-1 concentration was fixed with 10^9 CFU per feed.

Behavioral Tests

At adulthood (week 6-7), mice were subjected to a total of four behavioral tests for males and three for females, with a 4- to 6-day rest between each test. The behavioral tests included open field, elevated plus maze, three chamber sociability test and social avoidance following acute social defeat to assess behavior related to anxiety, sociability, and aggression. MotorMonitor (Thomas Scientific) or EthoVision XT (Noldus) programs were used for video analysis of all behavioral tests.

The open field test measures for locomotion and anxiety-related behaviors. The experimental mouse is put in the middle of a rectangular arena and is left to move freely

for 30 minutes. The time and distance travelled in the center and parameters of the field are recorded by MotorMonitor.

The elevated plus maze is an apparatus used to test for anxiety-like behavior. There are four arms to the maze, two are closed and two are open. The number of entries into the open arms is scored by MotorMonitor.

The three chamber sociability test measures sociability and preference for social novelty. In the first round, the experimental mouse is put in the middle of three chambers to habituate for 5 minutes, with no access to the side chambers. In the second round (sociability test), a stranger mouse is put in one of the side chambers under a wire cup, then the subject mouse is put back in the middle and is left to move freely for 10 minutes. After this sociability test, the experimental mouse is temporarily removed from the apparatus. In the third round (social novelty test), a new stranger mouse is put under a wire cup in the side chamber left empty from the previous test, then the subject mouse is put back in the middle chamber and is left to move freely for 10 minutes. The number of entries and time spent in each chamber interaction and non-interaction zones is recorded by EthoVision XT.

Flow Cytometry Analysis

After decapitation, spleens and MLN were collected and kept in 2 ml of cold PBS. Spleens were harvested and dispersed using a cell strainer in cold, sterile PBS. Cell suspensions were centrifuged at 1500 rpm for 10 min at 4 °C, then resuspended in red blood cell lysis buffer for 2-3 minutes. The resulting solution was centrifuged and the remaining palette was washed with 6 ml of complete RPMI medium. Viable cell numbers were

assessed by dilution of 90 ml Trypan Blue to 10 ml of cell solution. MLN were harvested in cold, sterile PBS then transferred into 1 ml of 5% FBS-RPMI medium on ice and dispersed through a cell strainer. The single-cell suspension was then added to 10 ml of 5% FBS-RPMI and centrifuged at 1500 RPMI for 10 minutes at 4 ℃. Cells were then resuspended in 2 ml of 5% FBS-RPMI. Viable cell numbers were assessed by dilution of 90 ml Trypan Blue to 10 ml of cell solution. Splenocytes and MLC cells were stained for markers of DC maturation and activation (CD11c-PerCP-Cy5, MHCII-FITC, CD80-PE, CD86-APC), T_{reg} cells (CD4-FITC, CD3-APC-Cy7, CD25-PE-Cy7, IL10-PE), Th1 (CD4-PerCP-Cy5.5, CD3-APC-Cy7, IFNg-APC), Th2 (CD4-PerCP-Cy5.5, CD3-APC-Cy7, IL4-AlexaFluor488), and Th17 (CD4-PerCP-Cy5.5, CD3-APC-Cy7, PE-Cy7-IL17, PE-RORgt). All samples were run on the BD FACSCelesta (BD Bioscience). Data were analyzed using FlowJo software (Tree Star).

Serum Cytokine Assays

At decapitation, trunk blood was collected from neck blood vessels. Blood was allowed to clot at 8 °C for 3 hours. Serum was extracted by centrifuging the samples at 10000 rpm at 4 ℃ for 15 minutes. The serum was immediately transferred to a new tube and stored in -80℃ until transfer. Before being sent, samples were diluted by a two-fold dilution with PBS, according to instructions provided by Eve Technologies. Cytokines were analyzed by using the Moue Cytokine Array Pro-inflammatory Focused 10-plex Discovery Assay (Eve Technologies).

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction Analysis

After decapitation, brains 1 cm of the distal ileum was collected and placed in RNA*later* (Thermo Fisher) solution. The tissue was incubated overnight at 4 ℃ and then transferred to -20 ℃ for storage. The frontal cortex, hypothalamus, and hippocampus were micro-dissected and returned to -20 ℃ for storage in RNA*later* solution for further processing. Following tissue homogenization with a Bio-Gen PRO200 Homogenizer (PRO Scientific), RNA extraction was carried out using RNeasy Mini Kit (Qiagen) for intestinal tissue and RNeasy Lipid Tissue Mini Kit (Qiagen) for brain parts. RNA quality was assessed using a NanoDrop™ One/One^{C} Microvolume US-Vis Spectrophotometer (Thermo Fisher). cDNA synthesis was done using SuperScript IV VILO Master Mix (Invitrogen) for brain and intestinal tissue. Diluted cDNA was used for PCR reactions using PowerUp SYBR™ Green Real-Time PCR Master Mix (Thermo Fisher) to determine gene expression. The qPCR reactions were performed in the fast mode (UDG activation 50 ℃, 2 minutes; Dual-Lock DNA polymerase 95 ℃, 2 minutes; denaturation: 95 ℃, 1 second; annealing/extension 60 ℃, 30 seconds; number of cycles: 40-50) by using QuanStudio3 machine (Applied Biosystem). Data were normalized to the endogenous control GAPDH and the relative quantification was analyzed using the ddCt method. Primer sequences used for PCR analysis are:

Target	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
AVPR1a	GGGATACCAATTTCGTTTGG	GGGATACCAATTTCGTTTGG
AVPR1b	TCTACTCTCCGTCTTAGCCTTAACCT	CTCCATCCACCTGCTCCAA
BDNF	CTG ACA CTT TTG AGC ACG TCA TC	CACCCGGGAAGTGTACAAGTC
CLDN-5	TCAGCTTCCCGGTCAAGTACT C	CCGCCCTTAGACATAGTTCTTCTT
GAPDH	TGGCCTCCAAGGAGTAAGAAA C	GGGATAGGGCCTCTCTTG
GLP-1	GGCACATTCACCAGCGACTAC	CAATGGCG ACTTCTTCTGGG
OCLD	TGAACAGCCCCCCAATGT	TCAACTCTTTCCGCATAG TCAGAT
OTR	ATGGCCTGCCCCAGTCTCGC	CCGGGCTGCAGCAGATGCCT
POMC	TGCTTCAGACCTCCATAGATGTGT	GGATGCAAGCCAGCAGGTT
PYY	CCTACCCTGCCAAACCAG	GGACATCTCTTTTTCCATACCG
ZO-1	GTGGATAGATCATTCAGTGAGAAACGT	TGGGCGCCC TGGAA

16S rRNA Gene Sequence Analysis

Fecal pellets were collected at week 3 (after treatment) and week 6 (before behavioral tests. Fecal pellets were stored in a –80 °C freezer until shipping. Fecal samples were sent to Bar-Ilan University for purification and 16s rRNA gene sequence analysis of microbial diversity. Below is the protocol that was used.

DNA was extracted using the PowerSoil HTP DNA Isolation Kit (MoBio) according to the manufacturer's instructions with a bead beater (BioSpec) set on high for 2 minutes. Following DNA extraction, the V4 variable region of the bacterial 16S rRNA gene was amplified by PCR using the 515F and 806R (each well received a separate 515F barcoded primer). 515F (barcode) 5'-AATGATACGGCGACCACCGAGATCTACACGCTAGCC

TTCGTCGCTATGGTAATTGTG TGYCAGCMGCCGCGGTAA-3' and 806R 5'-CAAGCAGAAGACGGCATACGAGATAGTCAGTCAGCCGGACTACHVGGGTWTCTAA-3'. PCR reactions were carried out with the Primestar taq polymerase (Takara) for 30 cycles of denaturation (95 °C), annealing (55 °C) and extension (72 °C), and a final elongation at 72 °C. Products were purified using AMPure magnetic beads (Beckman Coulter) and quantified using Pico-green dsDNA quantitation kit (Invitrogen). Samples were then pooled at equal concentrations (50 ng ml^{-1}), loaded on 2% E-Gel (Thermo Fisher) and purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Purified products were sequenced using the Illumina MiSeq platform. Data analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline version 1.8.0. Paired–end sequences were joined using fastq-join, demultiplexed and quality filtered with an average quality threshold of 25. Chimeric sequences were identified using USEARCH and removed, and reads were clustered into operational taxonomic units (OTUs) using the open reference UCLUST method against the GreenGenes 08/13 database74, with a cutoff of 97% sequence identity. Core OTUs were 30 calculated by filtering for OTUs presents in at least 50% of subjects in the same treatment group. Analyses were performed on the core OTUs using a rarefied table of 10,200 sequences per sample. In addition, alpha diversity was estimated using Faith's phylogenetic diversity and Pielou's evenness index. Beta diversity was calculated using weighted and unweighted UniFrac75.

Statistical Analysis

For all behavioral and tissue analyses, results across groups were analyzed by a Kruskal-Wallis one-way analysis of variance and a post-hoc test (Tukey's multiple comparisons test). Results between groups were analyzed using Student's t-tests. All data are represented as mean +/- standard error of mean. A p value of less than 0.05 was considered to be significant. Statistical significance is denoted by * ($p < 0.05$), ** ($p < 0.01$) ***, and ($p < 0.001$). Grubb's test, with a significance value of alpha = 0.05, was used to exclude outliers.

Results

AB-induced sex-dependent changes in social but not anxiety-like behavior (Figure 1)

Treatment did not affect locomotion in the open field test. There were no statistically significant differences in the center distance travelled between all three groups in male ($p = 0.142$) and female ($p = 0.491$) mice. In addition, treatment did not alter anxiety-like behaviors tested in the EPM. There were no statistically significant differences in the entries into open arm scores between all three groups in males ($p = 0.829$) and female ($p = 0.079$) mice.

AB decreased social behavior in adult male mice. During the first portion of the 3SCT, male mice treated with AB exhibited less social behavior. AB-treated male mice spent less time in the mouse chamber interaction zone compared to the control group ($p = 0.045$).

JB-1 attenuated the effect of AB on social behavior (Figure 1)

The reduction in social behavior observed in male mice exposed to AB was not evident in AB+JB-1 mice, with social interaction times comparable to controls ($p = 0.087$). AB+JB-1 treatment did not affect sociability in females ($p = 0.669$).

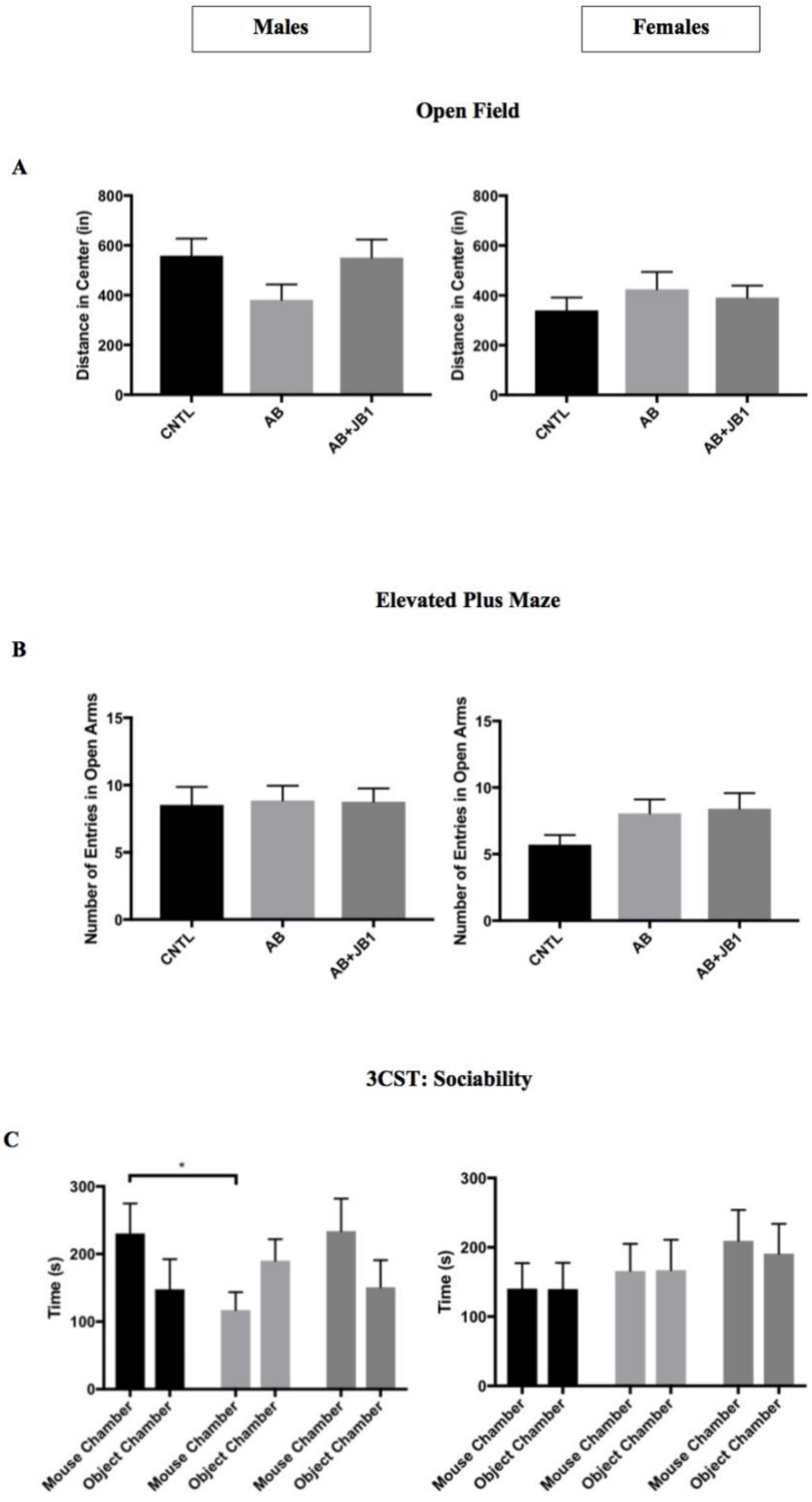

Males
Females
Open Field
A
Distance in Center (in)
800
600
400
200
0
CNTL
AB
AB+JB1
Elevated Plus Maze
B
Number of Entries in Open Arms
15
10
5
0
3CST: Sociability
C
Time (s)
300
200
100
*
Mouse Chamber
Object Chamber

Figure 1. Behavior tests for locomotion, anxiety, and sociability.

A. **Males**: n = 15 CNTL, 15 AB, 14 AB+JB-1. **Females**: n = 21 CNTL, 20 AB, 17 AB+JB-1. **B**. **Males**: n = 15 CNTL, 15 AB, 14 AB+JB-1. **Females**: n = 21 CNTL, 20 AB, 17 AB+JB-1. **C**. **Males**: n = 15 CNTL, 14 AB, 14 AB+JB-1. **Females**: n = 19 CNTL, 17 AB, 16 AB+JB-1.

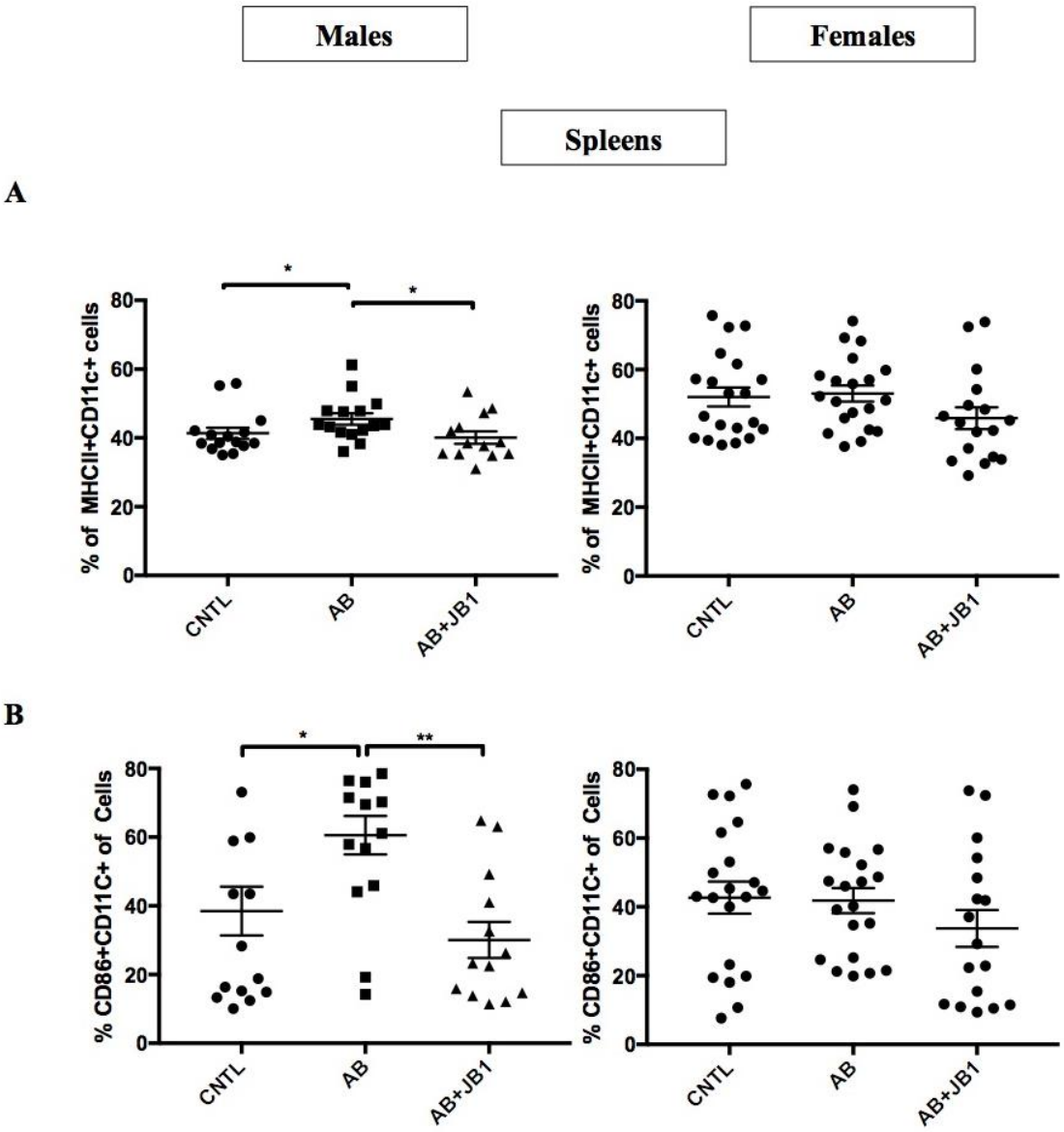
AB-induced changes in spleen and MLN immune cell populations

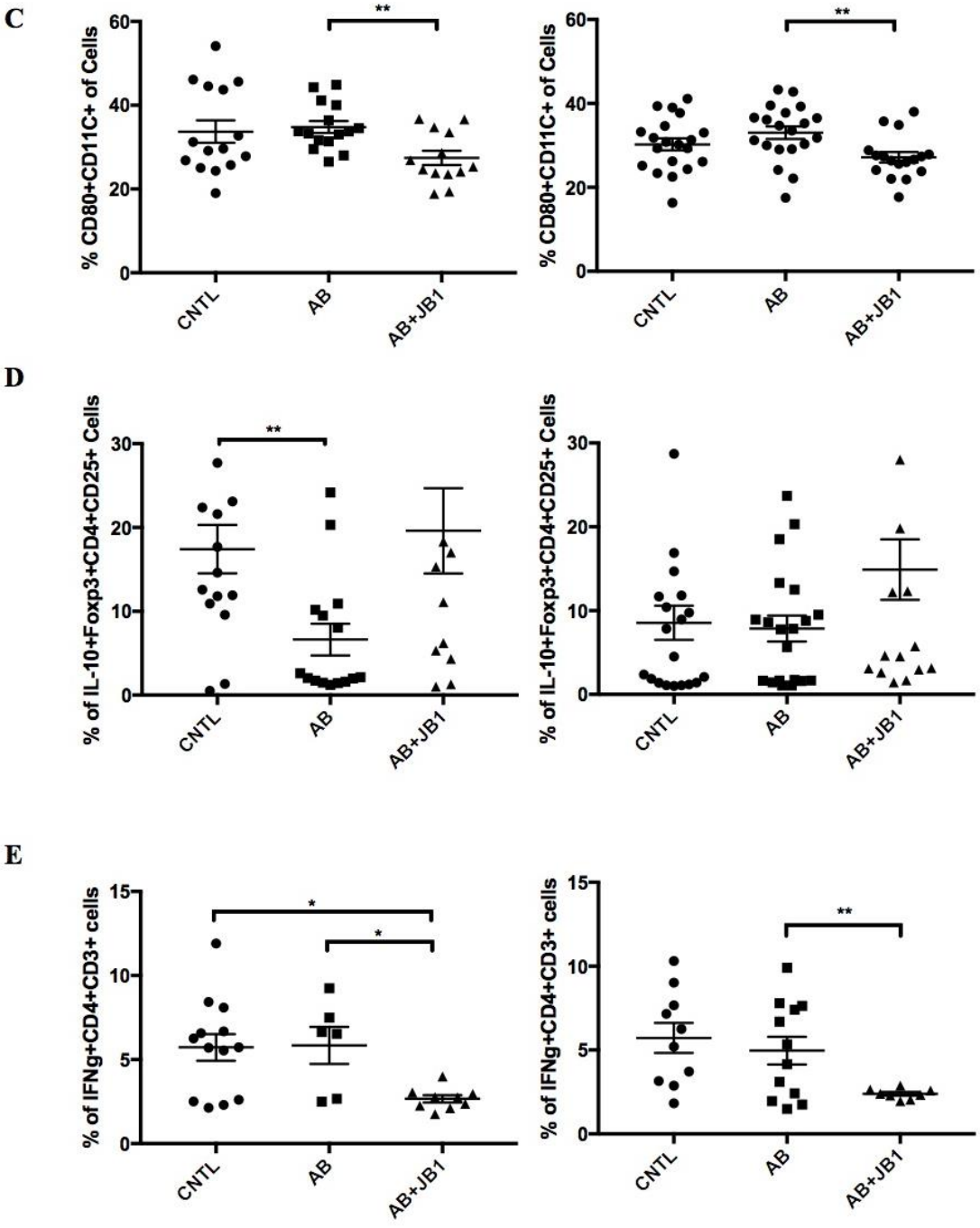
(Figure 2)

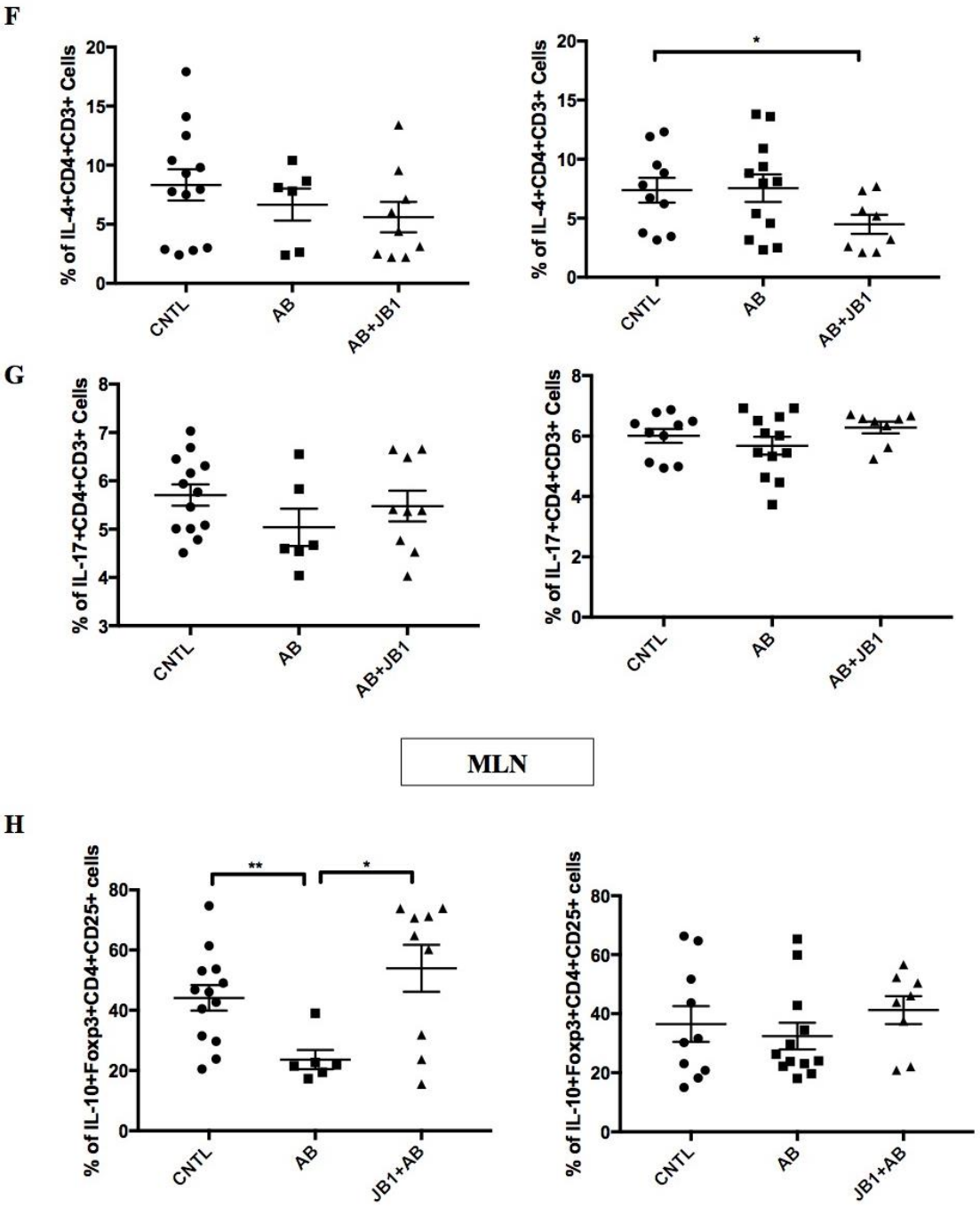
There was a statistically significant difference in percentages of DC (MHCII+CD11c+) in spleens between the three groups of male mice (p = 0.027). AB treatment early in life significantly increased the percentage of DC compared to the control group in adult males (p = 0.024). CD86+ activated DC followed the same pattern of MHCII+CD11c+ cell percentages in adult males (p = 0.007) with an increase in AB-treated mice (p = 0.029). There were no differences in CD86+ DC across the three adult female groups (p = 0.307). The population of DCs did not differ across the three male and female groups at weaning. However, both markers for activated DC were increased with AB in weaning females (CD80+ T: p = 0.011; CD86+ t: p = 0.021). AB treatment decreased T_{reg} (IL-10+ Foxp3+CD4+CD25+) cell populations in the spleen and MLN (p = 0.024, AB vs. CNTL: p = 0.006) of adult male mice. At weaning, there were no statistically significant differences in T_{reg} cell population in males (p = 0.268) or females (p = 0.242). Splenic Th17 cells (IL17A+ CD4+CD3+) were decreased in the AB group (p = 0.020) and further decreased in the AB+JB-1 group (p = 0.008) in male mice at weaning but not in adulthood.

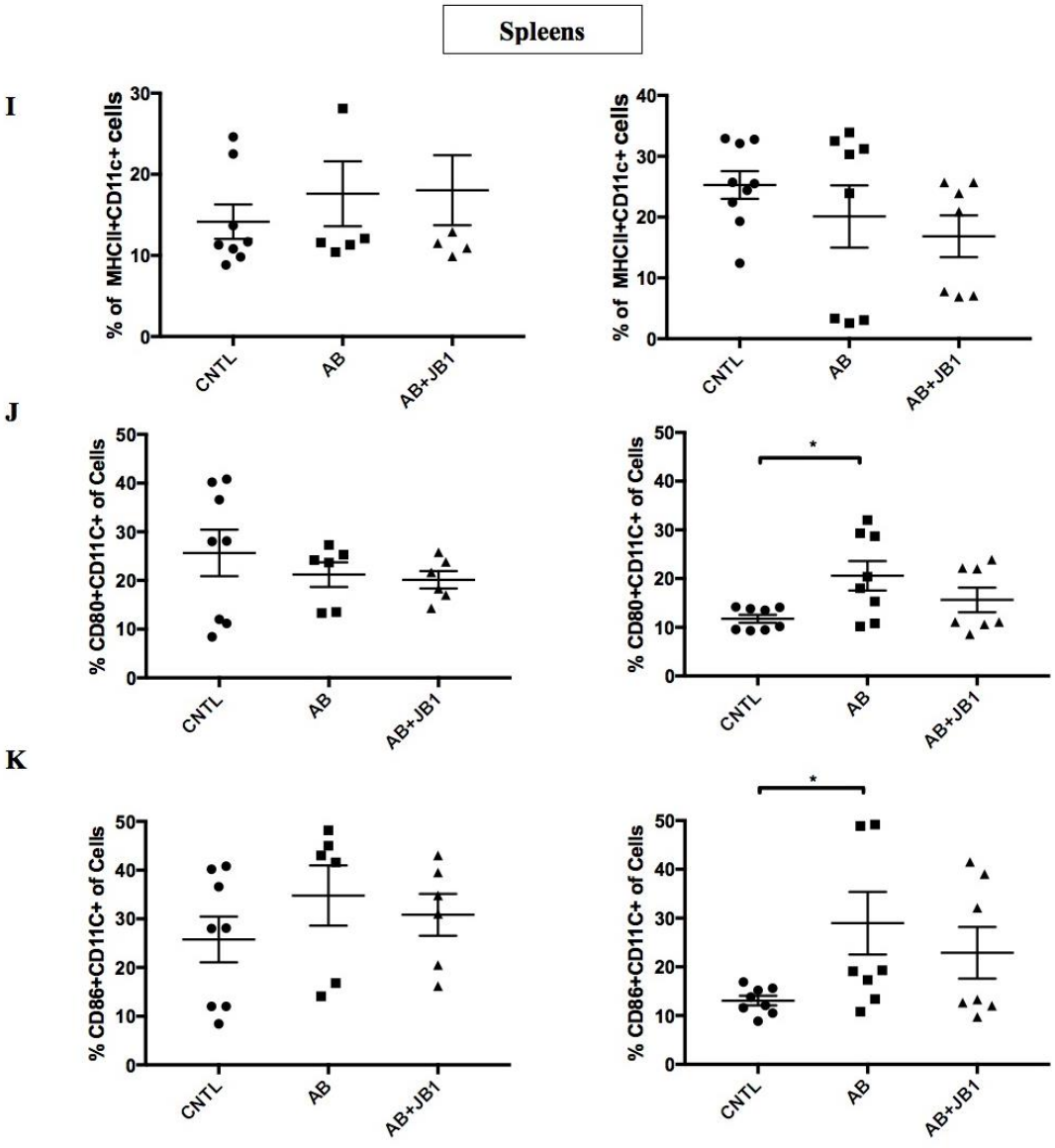
JB-1 attenuated the effect of AB on spleen and MLN immune cell populations (Figure 2)

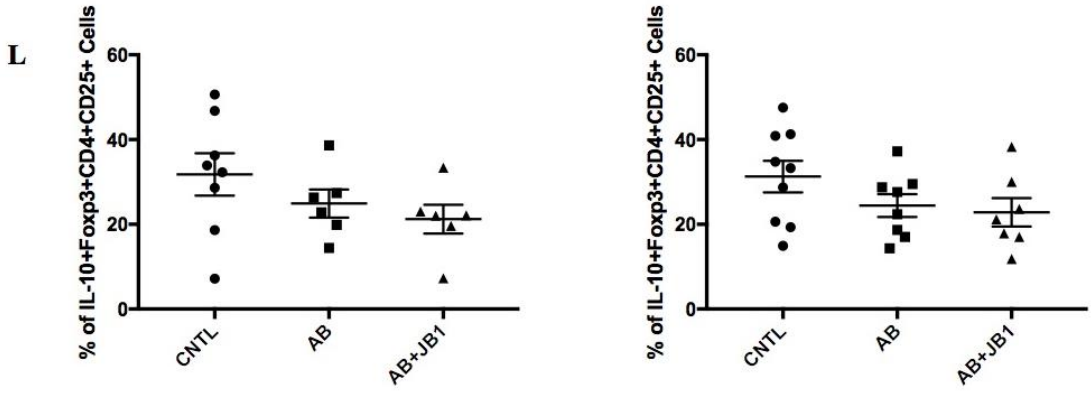
JB-1+AB treatment significantly decreased the population percentage ($p = 0.022$), resembling the control group DC population ($p = 0.517$). JB-1+AB treatment significantly increased the percentage of DCs compared to the AB-treated group in adult female mice ($p = 0.042$). JB-1 attenuated the AB-induced increase in activated CD86+ DCs ($p = 0.001$) compared to controls ($p = 0.547$). For CD80+, another type of activated DC, there was a significant difference across male groups ($p = 0.029$), with a significant decrease in AB+JB-1-treated mice compared to AB alone ($p = 0.010$). There were no differences in the CD80+ DC population across adult female groups ($p = 0.013$). However, as in males, AB+JB-1 significantly decreased the percentage of activated CD80+ DC compared to AB-treated group ($p = 0.003$) in these adult females. JB-1 attenuated the decrease of splenic and MLN T_{reg} cells (AB+JB-1 vs. CNTL: $p = 0.9278$) in adult male mice. Th1 cells (IFNg+CD4+CD3+) in the spleen were decrease with simultaneous AB+JB-1 treatment in males ($p = 0.017$) and females ($p = 0.001$) at adulthood, and only in males at weaning ($p = 0.005$). At weaning, the splenic Th2 cell (IL-4+CD4+CD3+) population was decreased in the AB+JB-1 group relative to the control group in females ($p = 0.0221$). No changes in this cell population were observed in males at either time points.

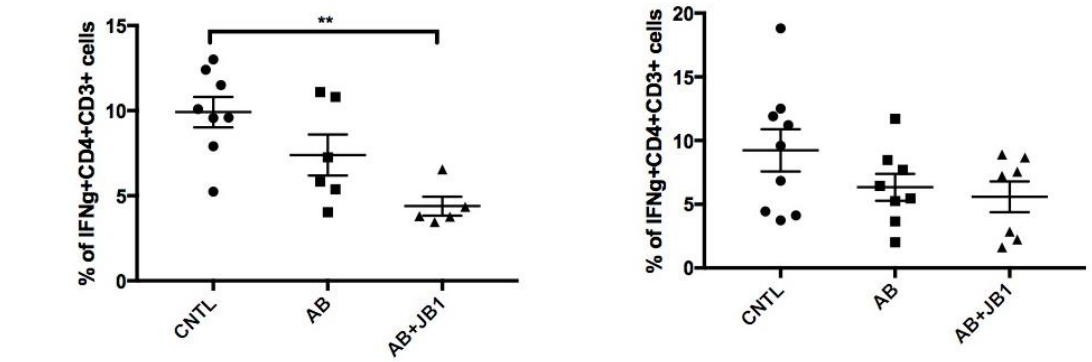

Males
Females
Spleens
A
% of MHCII+CD11c+ cells
0
20
40
60
80
CNTL
AB
AB+JB1
*
*
B
% CD86+CD11C+ of Cells
0
20
40
60
80
CNTL
AB
AB+JB1
*
**

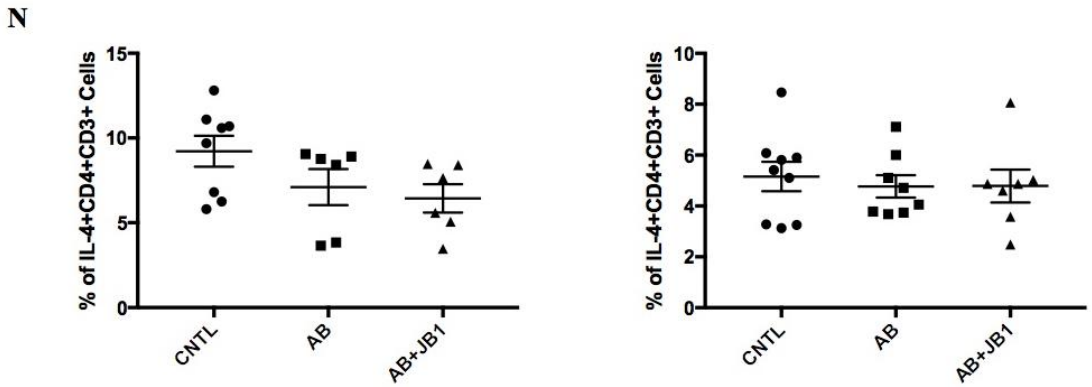

C
% CD80+CD11C+ of Cells
60
40
20
0
**
CNTL
AB
AB+JB1
% CD80+CD11C+ of Cells
60
40
20
0
**
CNTL
AB
AB+JB1
D
% of IL-10+Foxp3+CD4+CD25+ Cells
30
20
10
0
**
CNTL
AB
AB+JB1
% of IL-10+Foxp3+CD4+CD25+ Cells
30
20
10
0
CNTL
AB
AB+JB1
E
% of IFNg+CD4+CD3+ cells
15
10
5
0
*
*
CNTL
AB
AB+JB1
% of IFNg+CD4+CD3+ cells
15
10
5
0
**
CNTL
AB
AB+JB1


F
% of IL-4+CD4+CD3+ Cells
CNTL
AB
AB+JB1
*
G
% of IL-17+CD4+CD3+ Cells
MLN
H
% of IL-10+Foxp3+CD4+CD25+ cells
**
*
JB1+AB


Spleens
I
% of MHCII+CD11c+ cells
CNTL
AB
AB+JB1
J
% CD80+CD11C+ of Cells
*
K
% CD86+CD11C+ of Cells
*


L
% of IL-10+Foxp3+CD4+CD25+ Cells
60
40
20
0
CNTL
AB
AB+JB1
% of IL-10+Foxp3+CD4+CD25+ Cells
60
40
20
0
CNTL
AB
AB+JB1


M
% of IFNg+CD4+CD3+ cells
**
15
10
5
0
CNTL
AB
AB+JB1
% of IFNg+CD4+CD3+ cells
20
15
10
5
0
CNTL
AB
AB+JB1


N
% of IL-4+CD4+CD3+ Cells
15
10
5
0
CNTL
AB
AB+JB1
% of IL-4+CD4+CD3+ Cells
10
8
6
4
2
0
CNTL
AB
AB+JB1

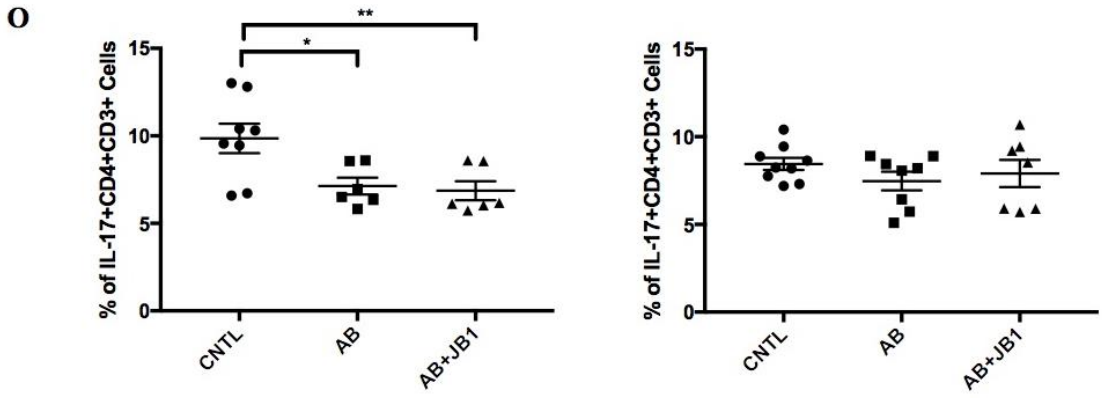
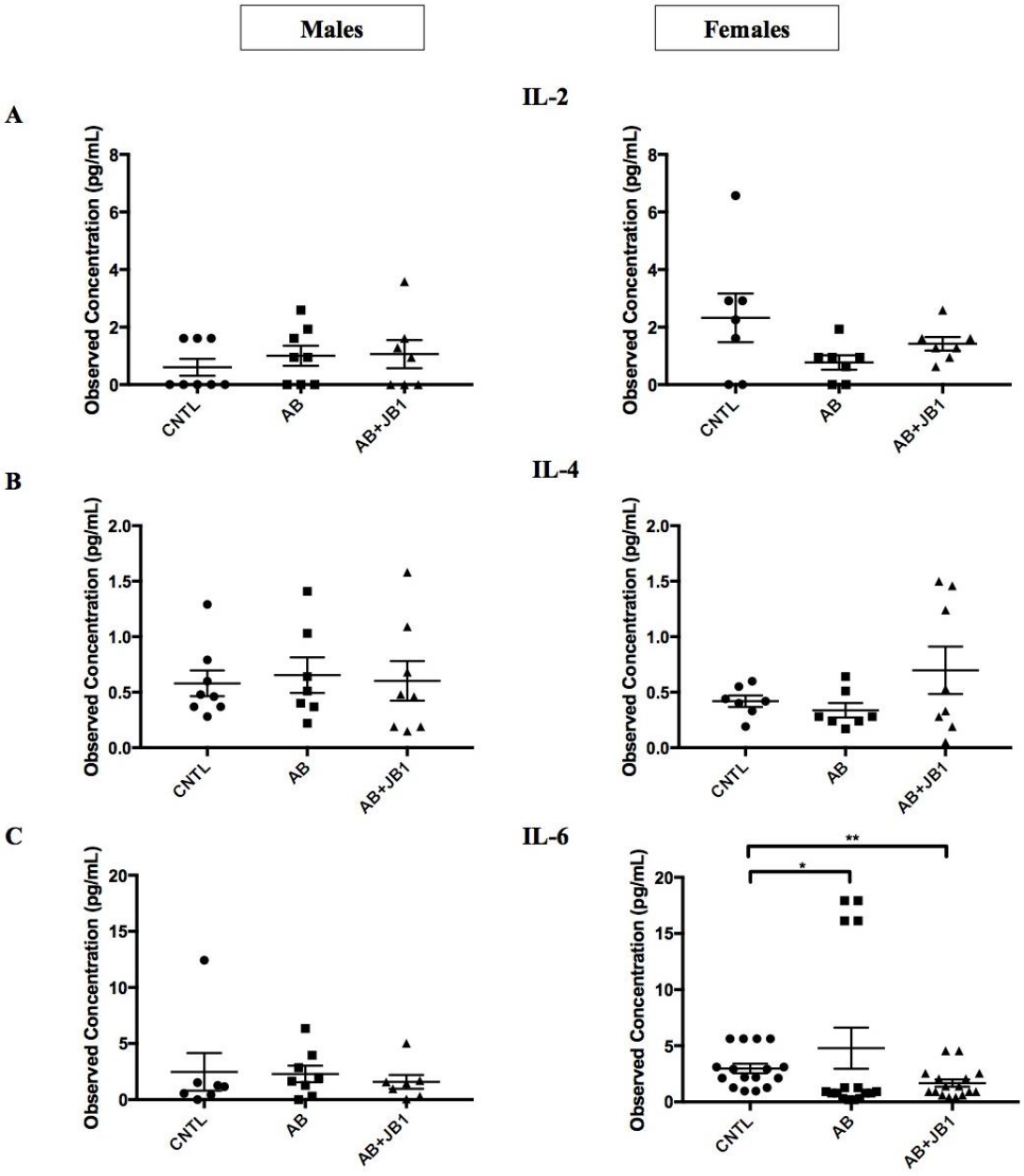


Figure 2. Flow cytometry analysis of spleen and MLN immune cell populations.

A-H are graphs from Experiment 1 (PND 70). Spleen **Males**: n = 15 CNTL, 15 AB, 14 AB+JB-1. **Females**: n = 20 CNTL, 20 AB, 17 AB+JB-1. MLN **Males**: n = 13 CNTL, 6 AB, 9 AB+JB-1. **Females**: n = 10 CNTL, 12 AB, 8 AB+JB-1. **I-O** are graphs from Experiment 2 (PND 21). **Males**: n = 8 CNTL, 6 AB, 6 AB+JB-1. **Females**: n = 9 CNTL, 8 AB, 7 AB+JB-1.

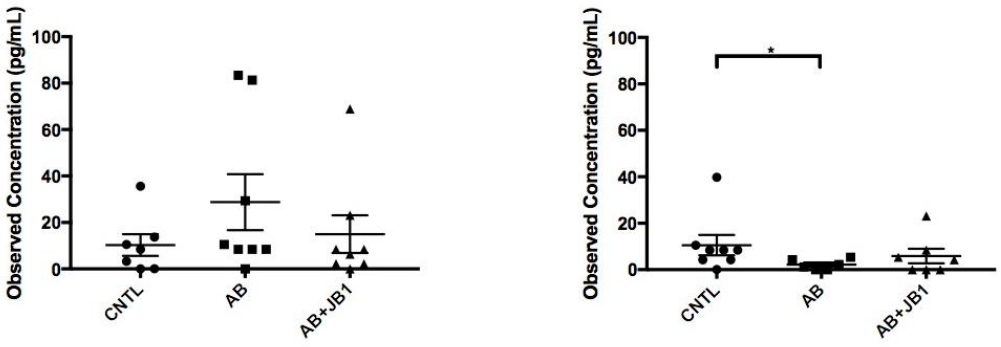
AB effects pro-inflammatory serum cytokine levels (Figure 3)

AB induced an increase in serum cytokines at weaning and a female-specific decrease in adulthood. At weaning, there was an increase in IL-4 in males ($p = 0.013$) and IL-2 in females ($p = 0.017$) treated with AB compared to AB+JB-1 mice. Regardless of the change in IL-4, all groups show very low cytokine levels. In adulthood, changes in serum cytokine levels were only present in females: with a AB-induced decrease in IL-12 ($p = 0.027$) and TNFα ($p = 0.046$) compared to controls, and MCP1 ($p= 0.004$) and TNFα ($p = 0.031$) compared to AB+JB-1 mice. The reduced levels of IL-12 ($p = 0.931$) and TNFα ($p = 0.979$) relative to controls were prevented by concurrent JB-1 feeding. IL-6 was increased with AB ($p = 0.028$), which was attenuated with AB+JB-1 ($p = 0.006$) treatment.


Males
Females
A
IL-2
B
IL-4
C
IL-6
Observed Concentration (pg/mL)
CNTL
AB
AB+JB1
*
**

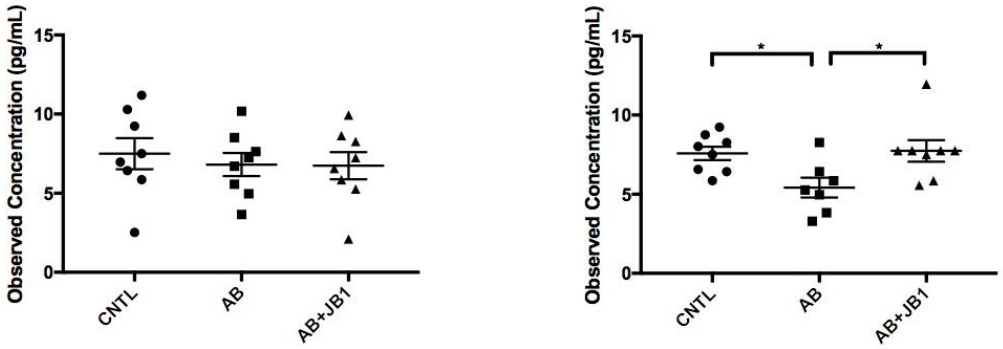
IL-12

D



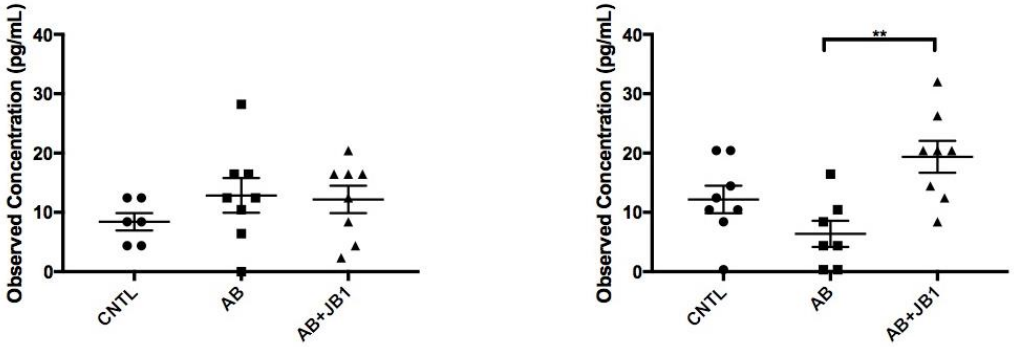
TNFα

E

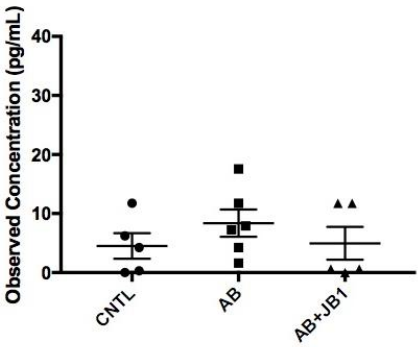


MCP1

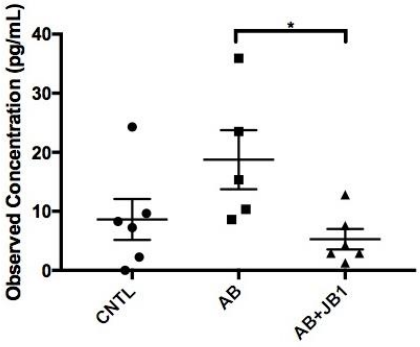
F



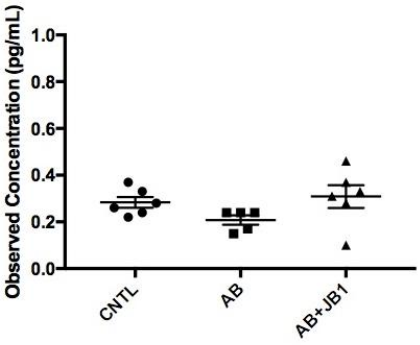
G



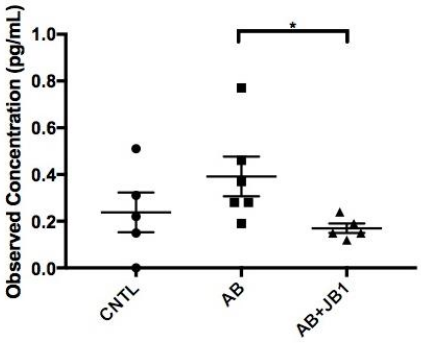
IL-2



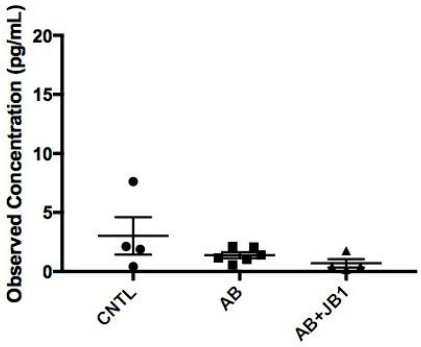
H



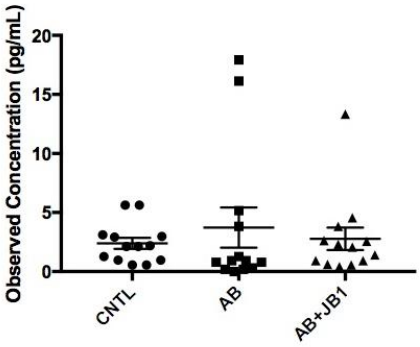
IL-4



I



IL-6



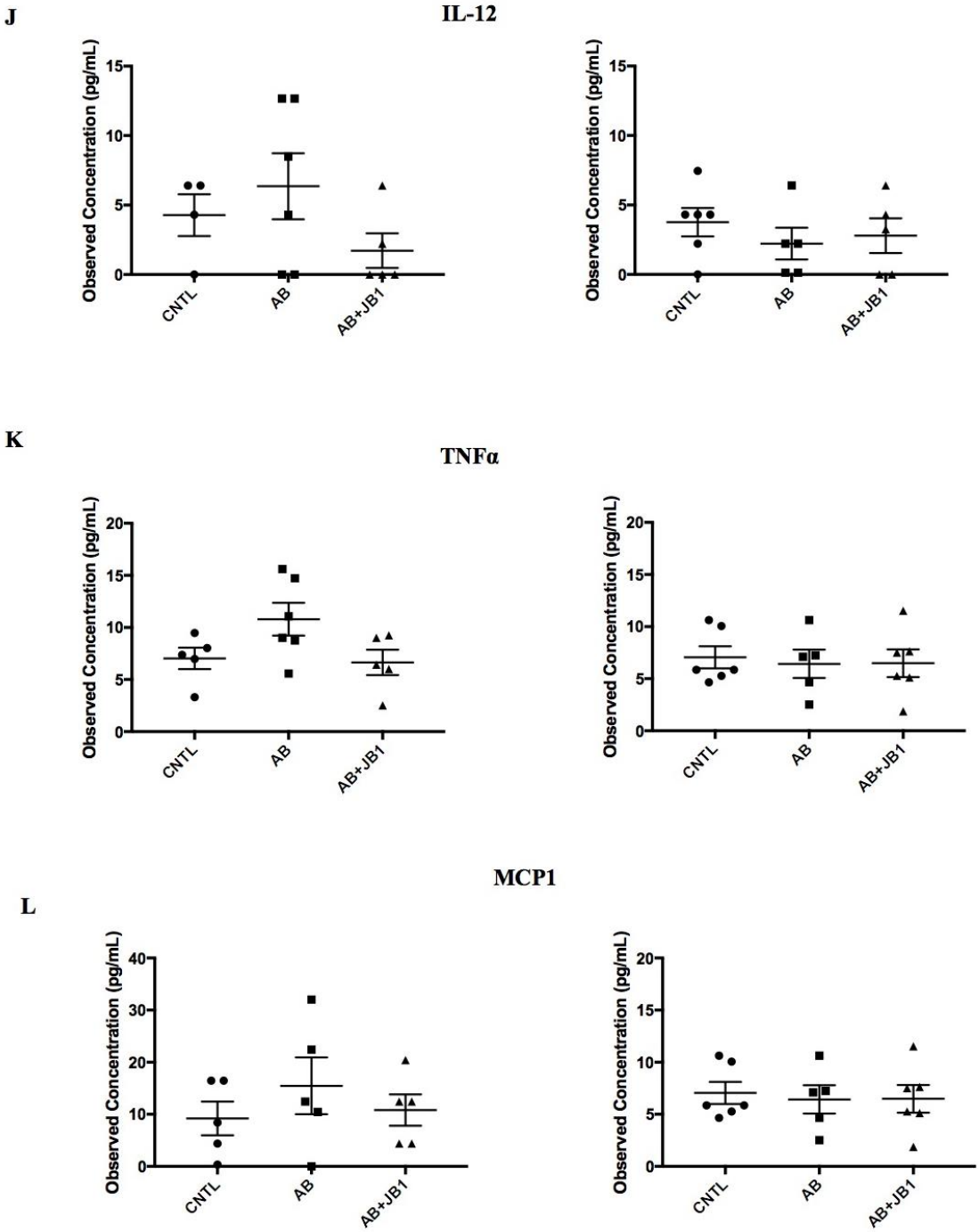


Figure 3. Levels of serum pro-inflammatory cytokines.

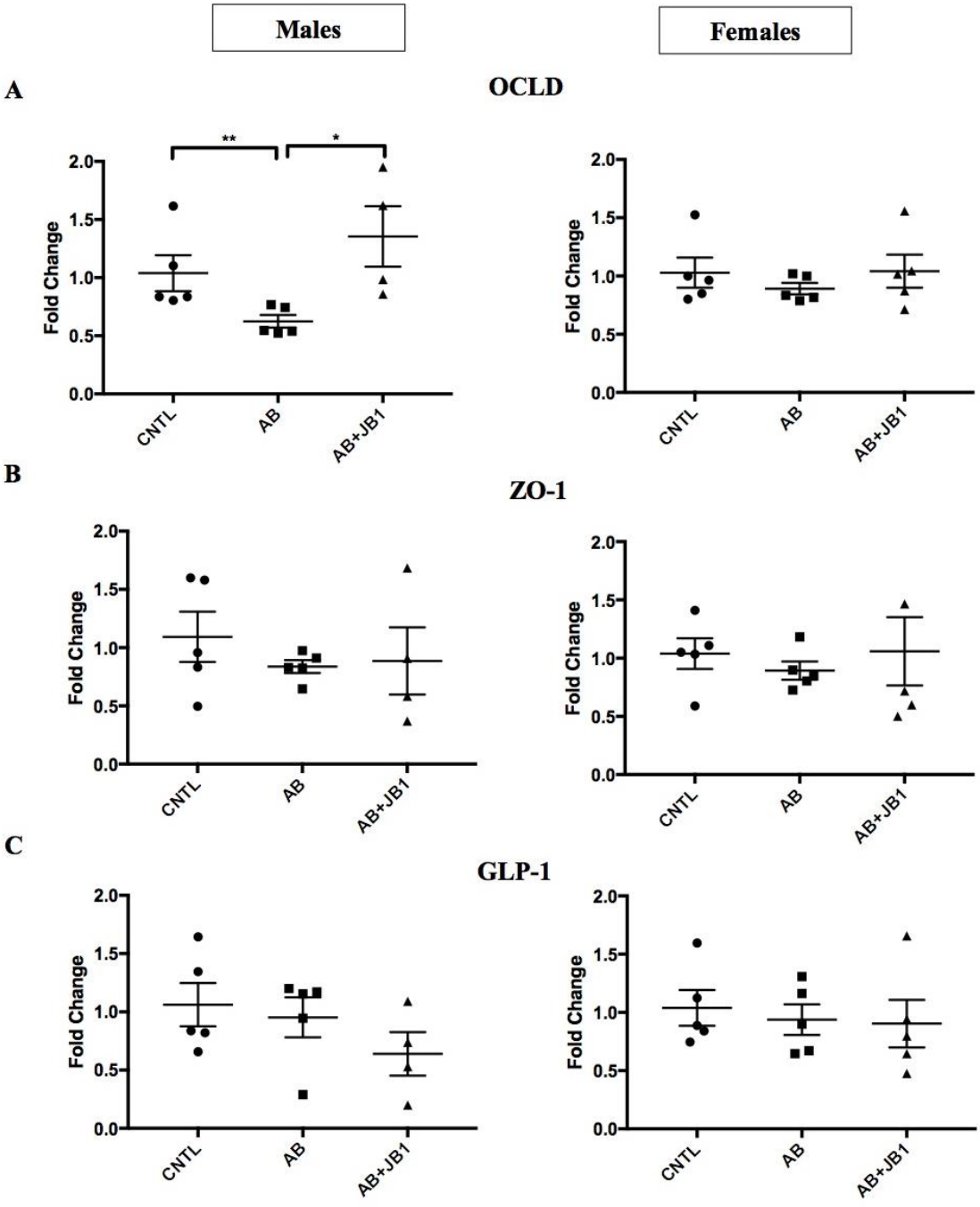
A-L are graphs from Experiment 1 (PND 70). **Males**: n = 8 CNTL, 8 AB, 8 AB+JB-1. **Females**: n = 8 CNTL, 8 AB, 8 AB+JB-1. **M-X** are graphs from Experiment 2 (PND 21). **Males**: n = 5 CNTL, 6 AB, 5 AB+JB-1. **Females**: n = 6 CNTL, 5 AB, 5 AB+JB-1.

AB alters mRNA expression of TJ proteins in the small intestines and brain (Figure 4)

AB treatment induced sex-dependent changes in gene expression of TJ proteins and enteroendocrine markers in the distal ileum. RNA expression of occludin (OCLD), a TJ component and adhesion protein, decreased with AB in adult males ($p = 0.008$) and this was attenuated by concurrent JB-1 feeding (AB vs. AB+JB-1: $p = 0.016$). With AB feeding, females showed a transient decrease in the expression of intestinal zona occludin-1 (ZO-1), another TJ protein component and barrier protein, which was attenuated by concurrent JB-1 feeding (AB vs. CNTL: $p = 0.043$; AB+JB-1 vs. CNTL: $p = 0.241$). There was also a decrease in GLP-1, a hormone that stimulates glucose-induced insulin, in AB-treated females at weaning, attenuated by JB-1 (AB vs. CNTL: $p = 0.036$; AB+JB-1 vs. CNTL: $p = 0.33$). There were no significant differences in mRNA expression of PYY, a hormone involved in energy homeostasis, at either time points for both sexes when calculated separately. However, when combined ($p = 0.028$), there was a significant decrease of PYY with AB ($p = 0.025$), which is ameliorated by concurrent JB-1 treatment at weaning (CNTL vs. AB+JB-1: $p= 0.219$).

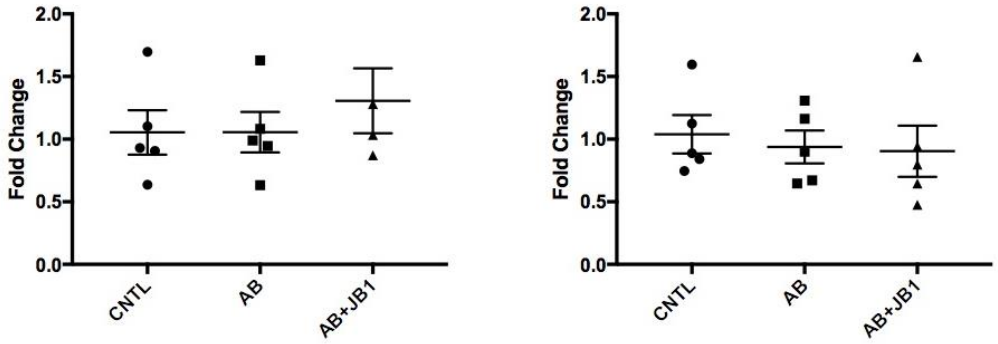
AB treatment drove male-specific changes in gene expression of blood-brain barrier (BBB) integrity proteins claudin-5 (CLDN-5) and OCLD in the frontal cortex and hippocampus. Some of these changes were prevented by simultaneous JB-1 feeding. These effects were age specific, as changes in RNA expression seen at weaning differed from those in adulthood. CLDN-5 was decreased in the frontal cortex ($p = 0.034$) at weaning but increased at adulthood in the hippocampus ($p = 0.012$) in AB male mice. OCLD was also

increased in adult male hippocampus (p = 0.003) with AB compared to AB+JB-1. In general, these markers were upregulated in various brain regions of adult males with early AB treatment. In females, OCLD expression in the hippocampus was reduced at weaning in the AB+JB-1 group compared to the controls (p = 0.022). AB alone did not drive statistically significant differences in CLDN-5 or OCLD expression in all three brain regions of female mice.


Males
Females
A
OCLD
B
ZO-1
C
GLP-1
Fold Change
2.0
1.5
1.0
0.5
0.0
**
*
CNTL
AB
AB+JB1

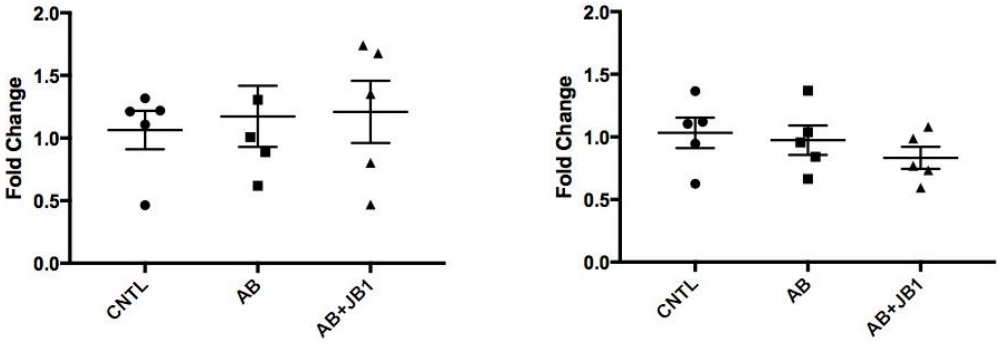
D

PYY



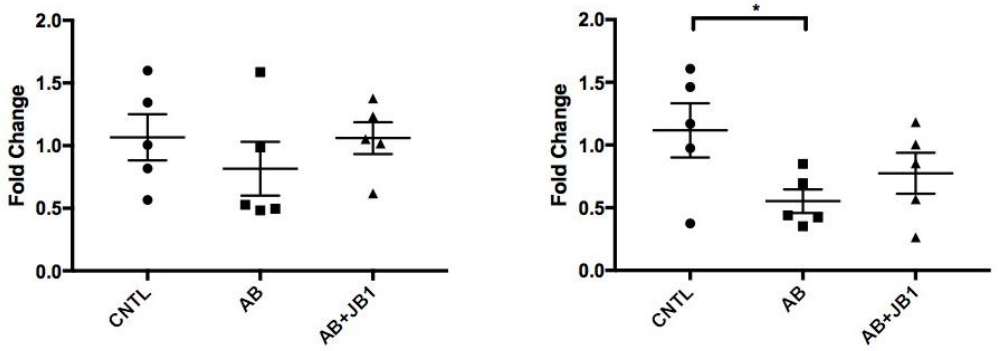
E

OCLD



F

ZO-1



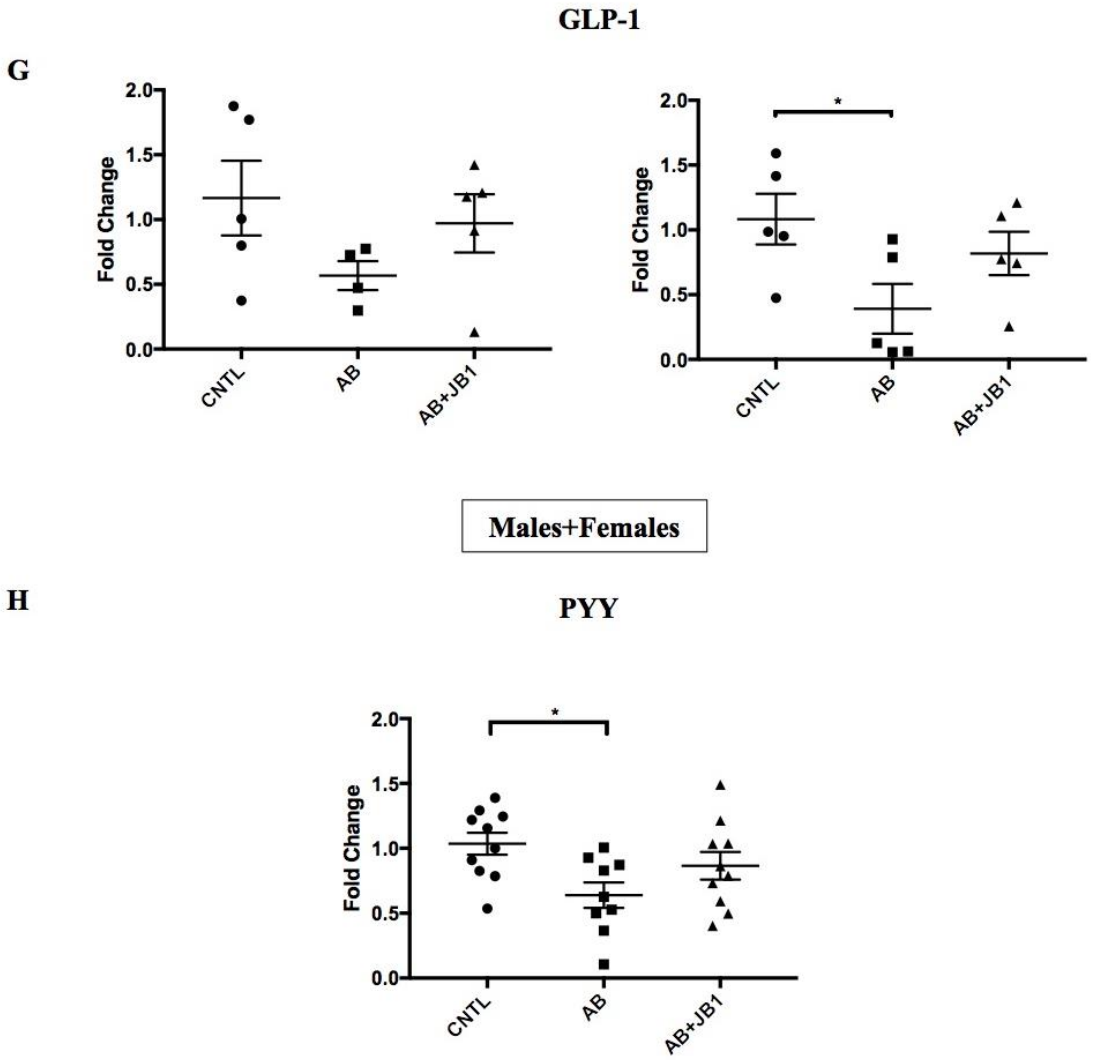
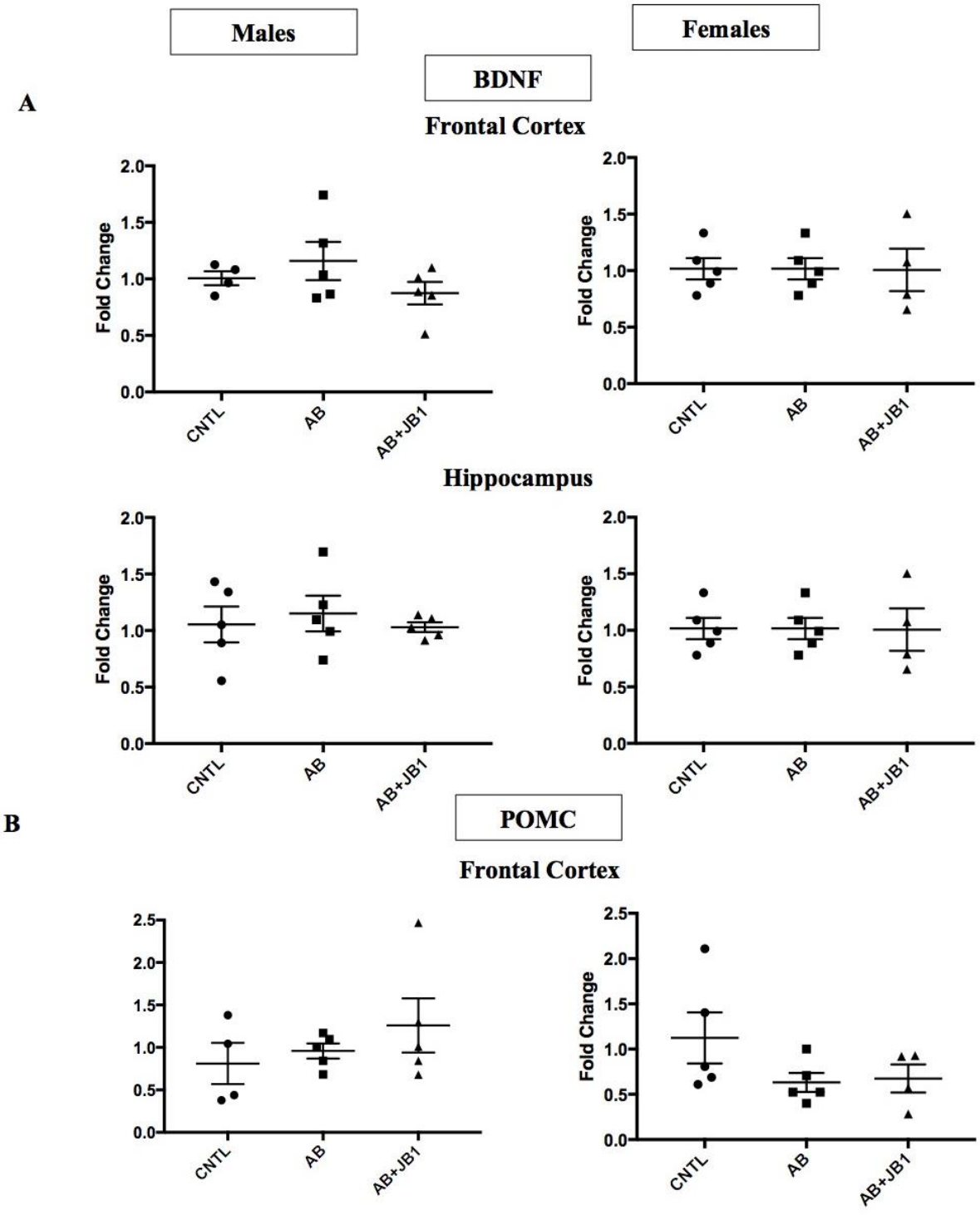


Figure 4. mRNA expression of ileum TJ and hormones.

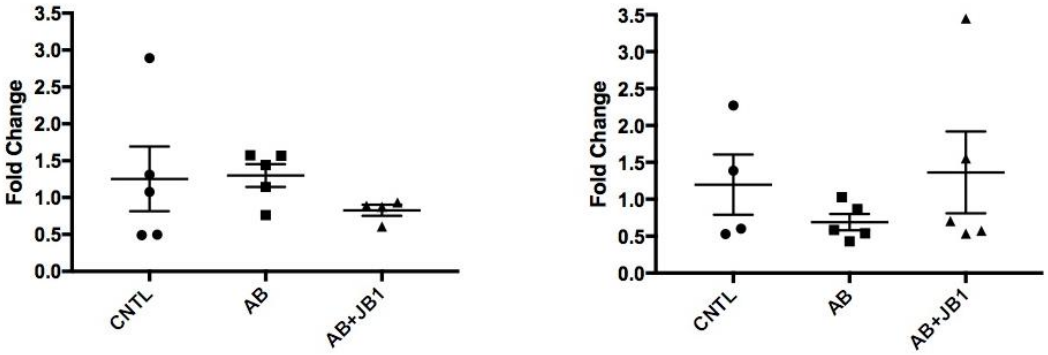
I-N are graphs from Experiment 2 (PND 21). **Males**: n = 5 CNTL, 5 AB, 5 AB+JB-1. **Females**: n = 5 CNTL, 5 AB, 5 AB+JB-1. **A-H** are graphs from Experiment 1 (PND 70). **Males**: n = 5 CNTL, 5 AB, 4 AB+JB-1. **Females**: n = 5 CNTL, 5 AB, 5 AB+JB-1.

AB alters mRNA expression of peptides in the brain (Figure 5)

AB administration induced hormone-related changes in the three selected brain regions related to behavior. Pro-opiomelanocortin (POMC), a hormone preprotein, was decreased at weaning in the frontal cortex of males (Tukey's *post-hoc:* $p = 0.025$, t-test: $p = 0.016$) and the hippocampus of females (Tukey's *post-hoc*: $p = 0.045$, t-test: $p = 0.039$) treated with AB. At weaning, females also showed altered BDNF, vasopressin receptor-1a (AVPR1a), and AVPR1b RNA expression. In the hippocampus, BDNF levels were downregulated with AB ($p = 0.013$), which was prevented by JB-1 (CNTL vs. AB+JB-1: $p = 0.460$). The two types of arginine vasopressin receptors (AVPR1a and AVRP1b) play a role in social and aggressive behavior. In the frontal cortex, AVPR1b was upregulated in the AB group compared to the AB+JB-1 group ($p = 0.011$). Males and females followed the same pattern of AVPR1a and AVPR1b expression in response to AB in the hippocampus at weaning: a decrease in AVPR1a compared to AB+JB-1 treatment (males: $p = 0.048$; females: $p = 0.038$) and a decrease in AVPR1b compared to controls (males: $p = 0.032$; females: $p = 0.028$). In adult females a decrease in oxytocin receptor (OTR), a G protein coupled receptor to oxytocin, was evident in the frontal cortex of AB compared to AB+JB-1 treated mice ($p = 0.029$). Oxytocin is involved in social behavior relating to maternal and mating behavior (Bredewold et al., 2014). With the exception of OTR expression, changes in hormone precursor peptides and receptors were transient and only significantly present at weaning.

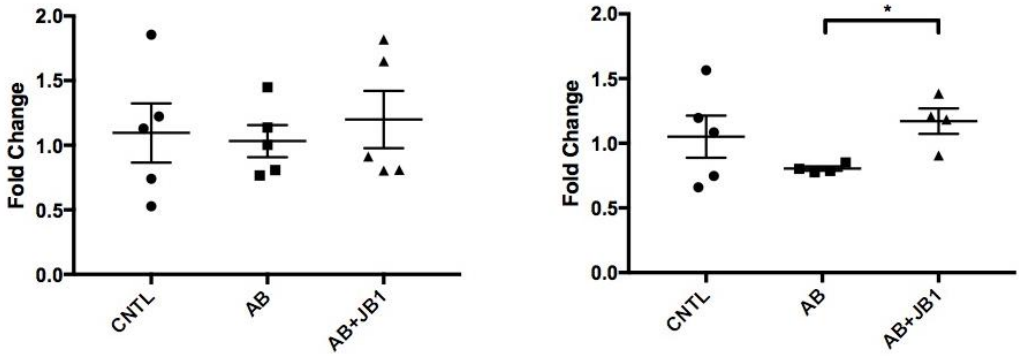

Males
Females
A
BDNF
Frontal Cortex
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1
Hippocampus
B
POMC
Frontal Cortex
2.5

Hippocampus

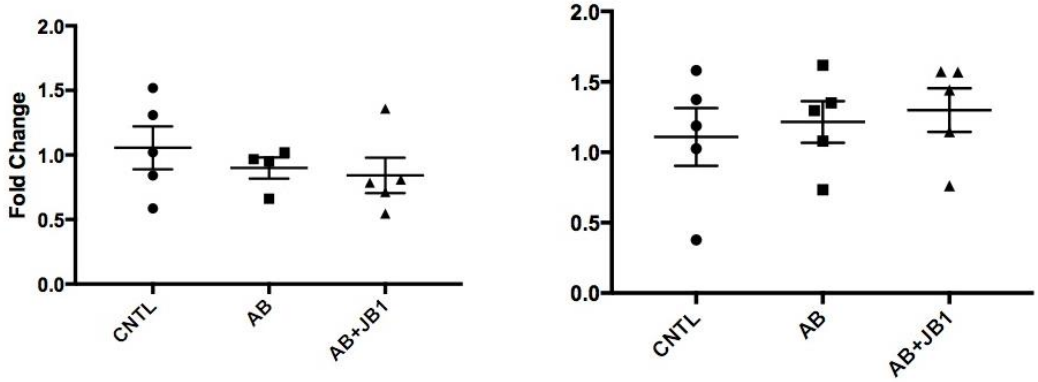


C **OTR**

Frontal Cortex



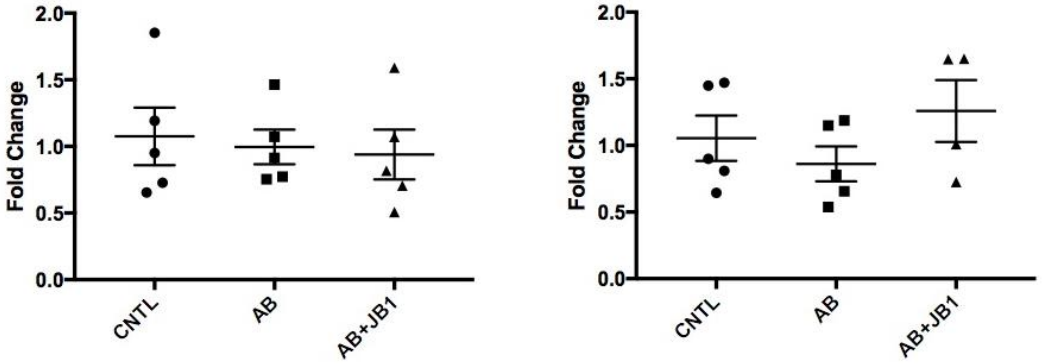
Hippocampus



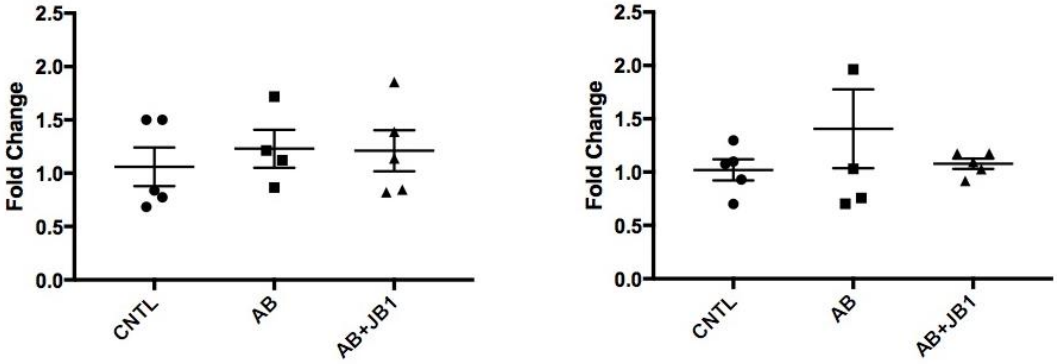
D

AVPR1a

Frontal Cortex



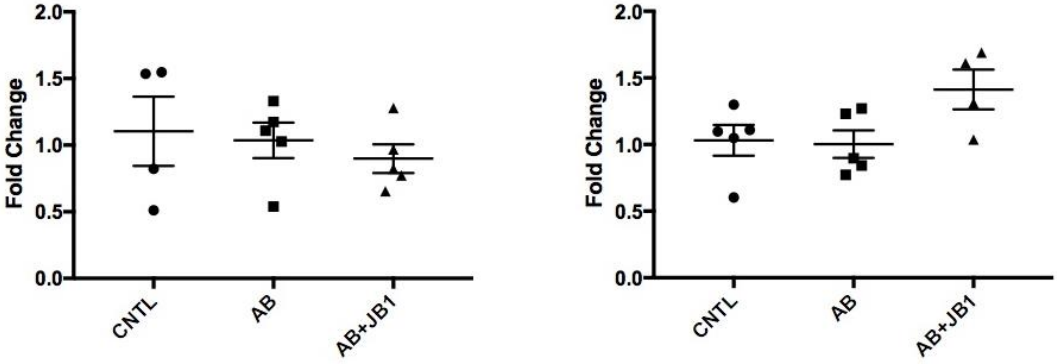
Hippocampus

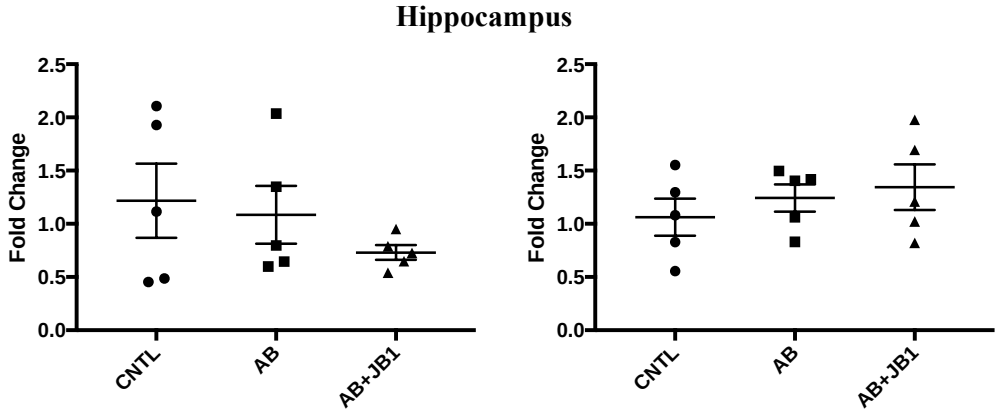


E

AVPR1b

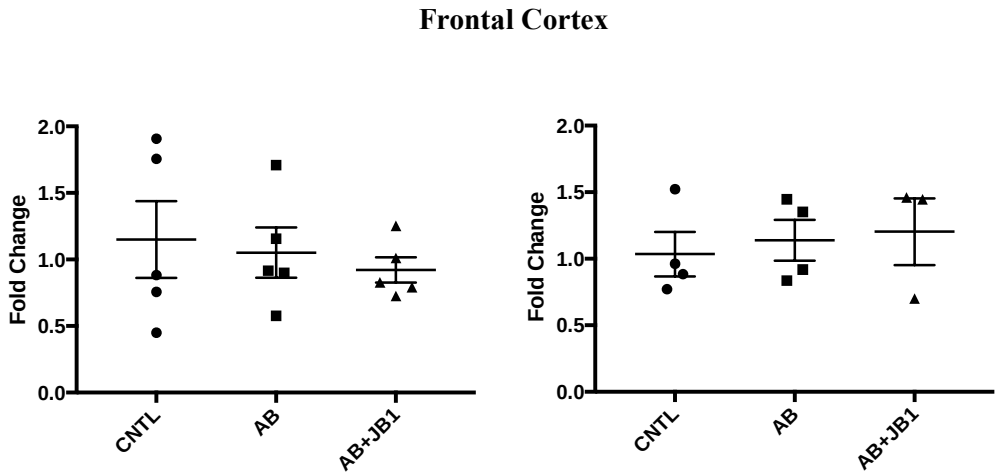
Frontal Cortex

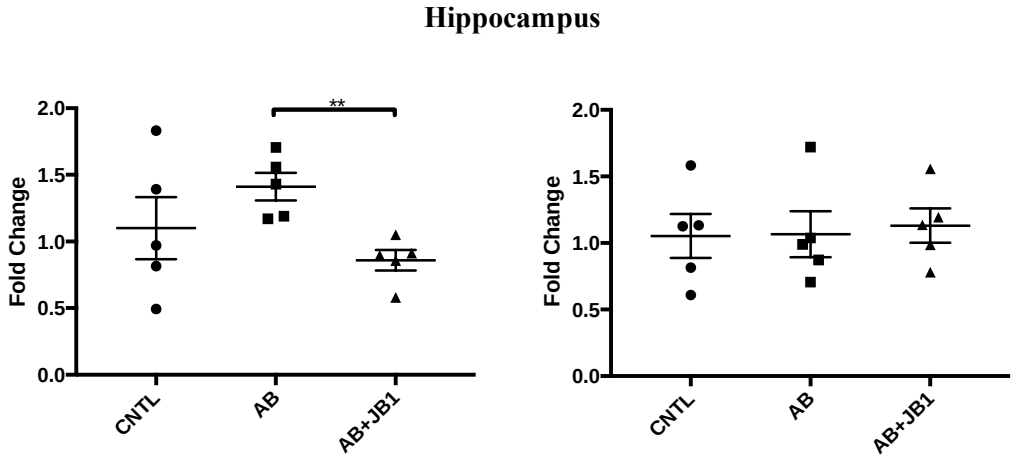



Hippocampus
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1

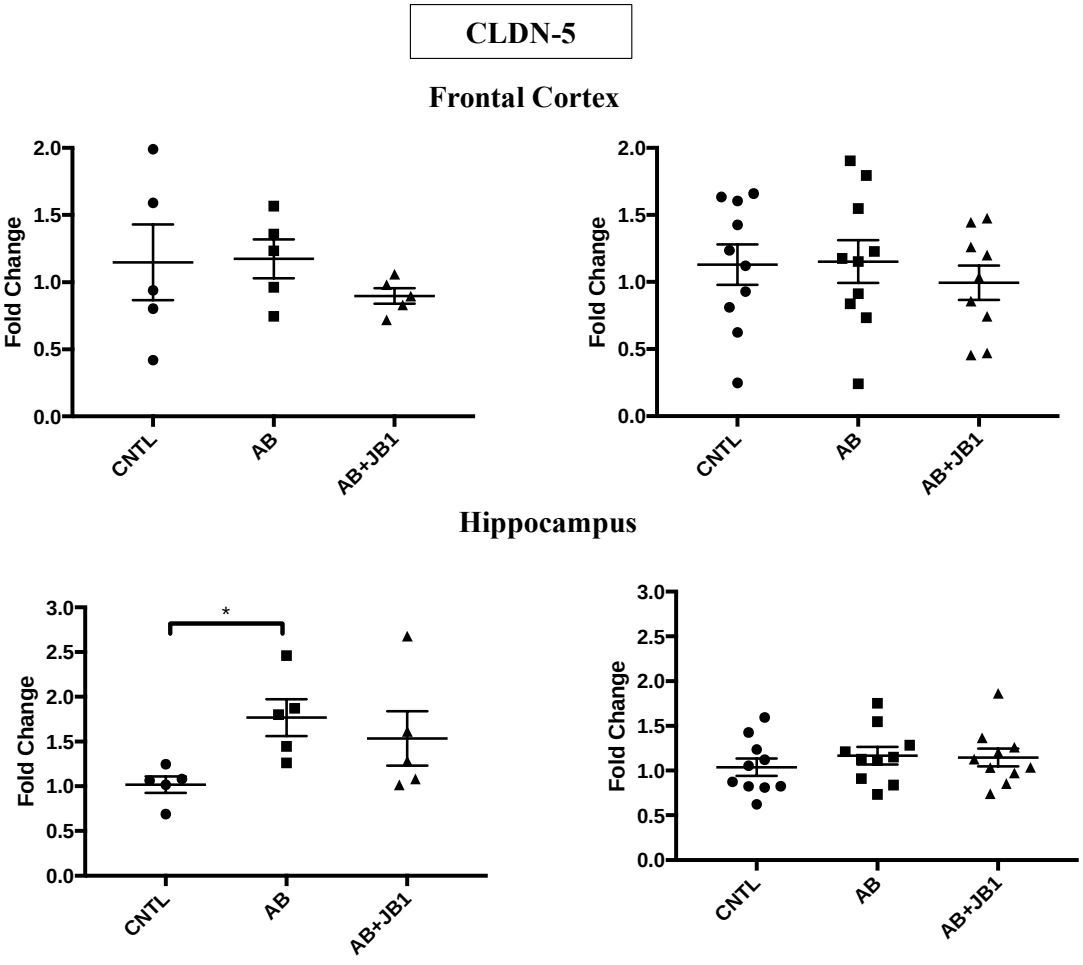
F

OCLD

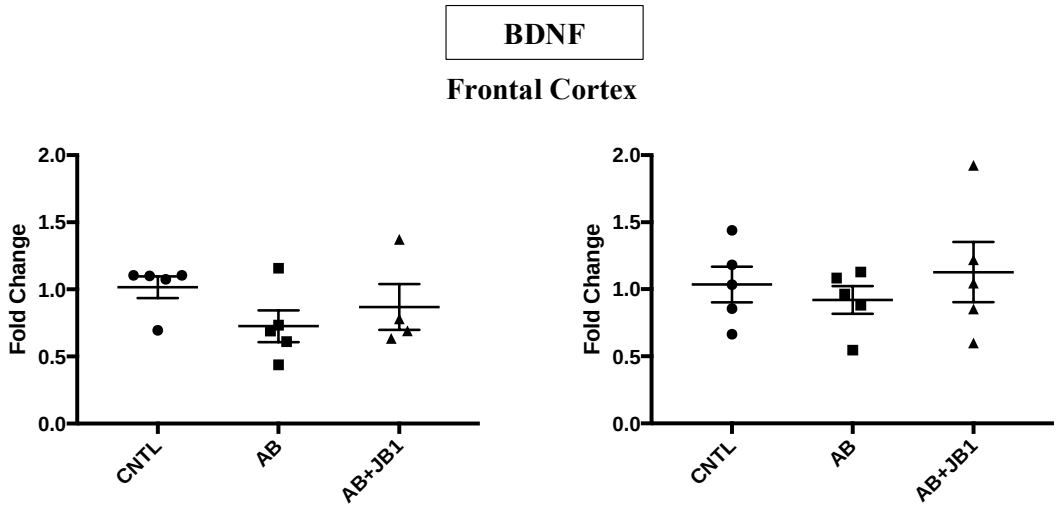

Frontal Cortex
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1


Hippocampus
**
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1

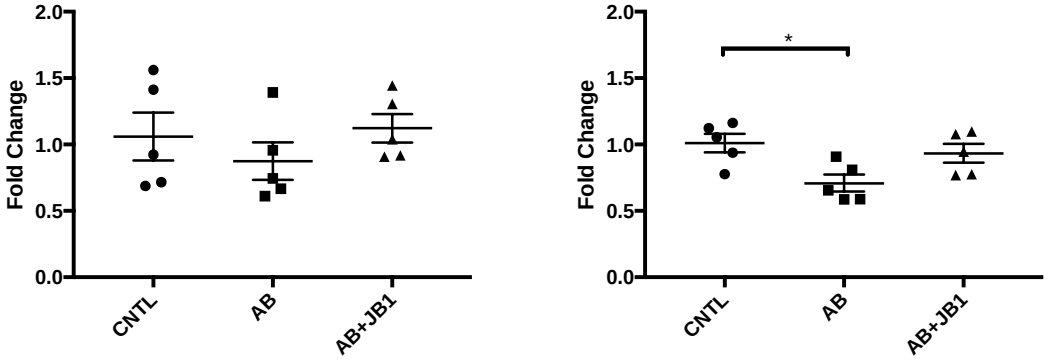
G


CLDN-5
Frontal Cortex
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1
Hippocampus
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*

H

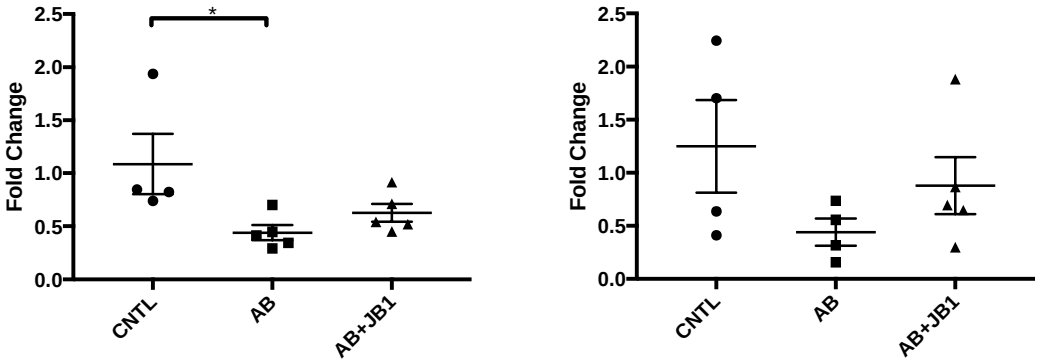

BDNF
Frontal Cortex
Fold Change
2.0
1.5
1.0
0.5
0.0
CNTL
AB
AB+JB1

Hippocampus

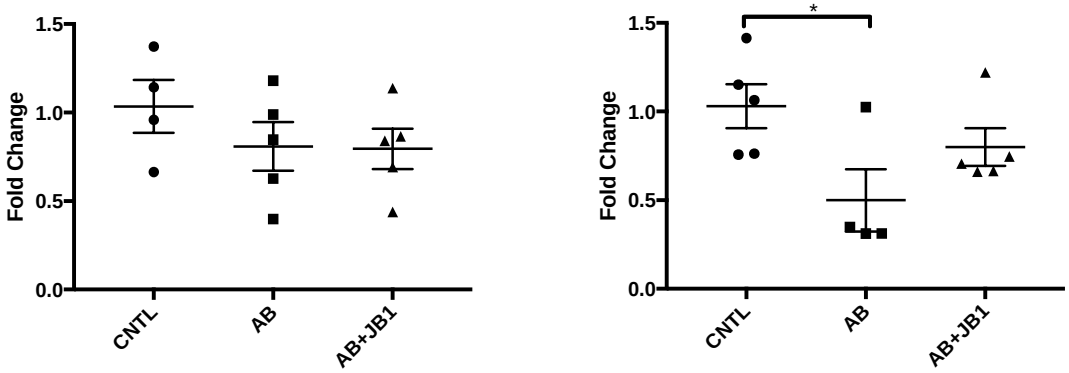


I **POMC**

Frontal Cortex

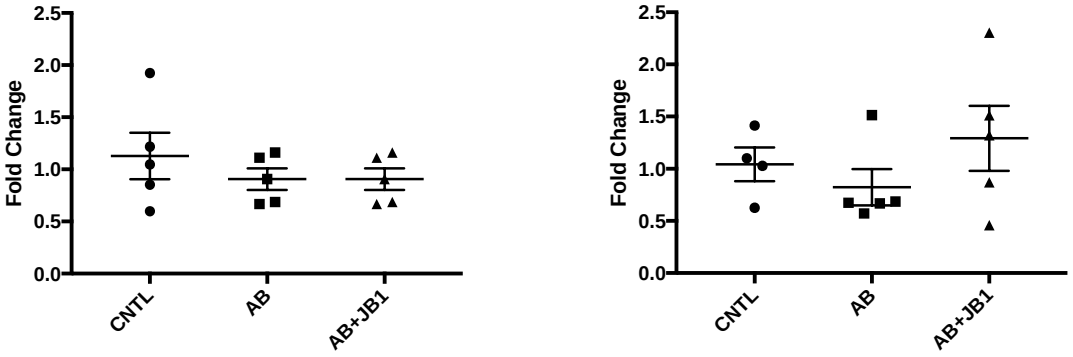


Hippocampus

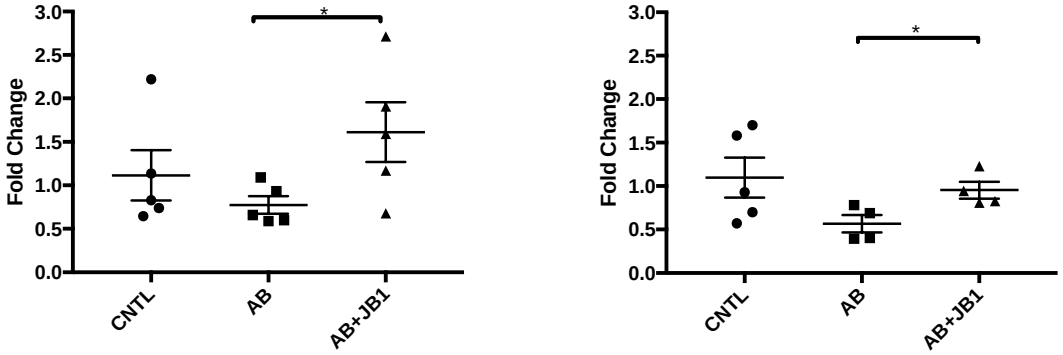


J **AVPR1a**

Frontal Cortex

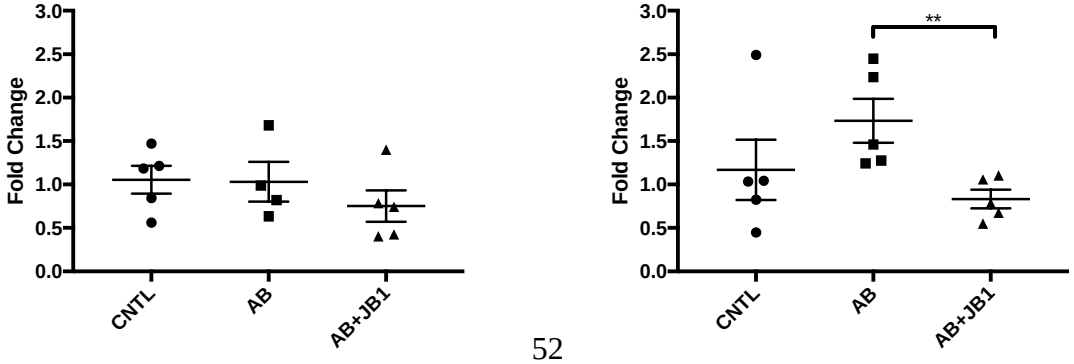


Hippocampus

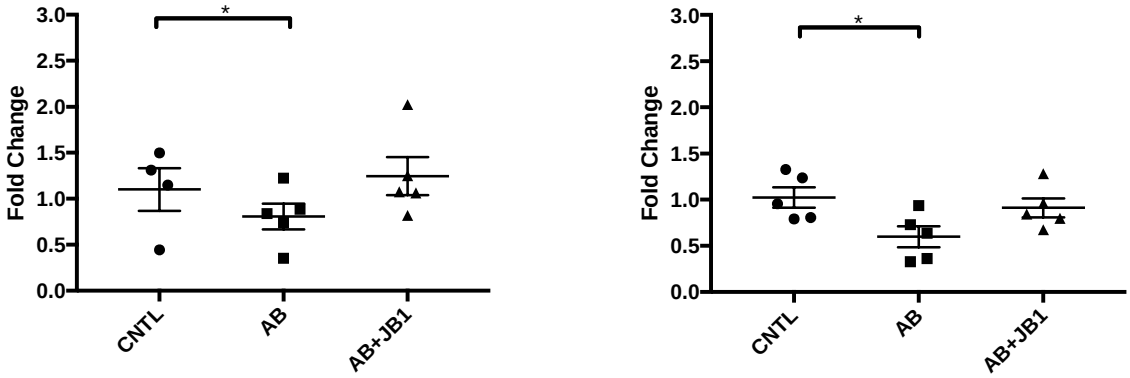


K **AVPR1b**

Frontal Cortex

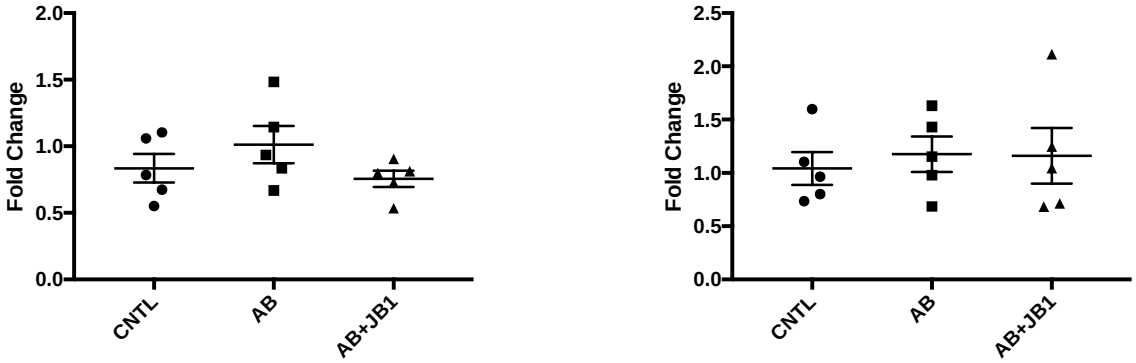


Hippocampus

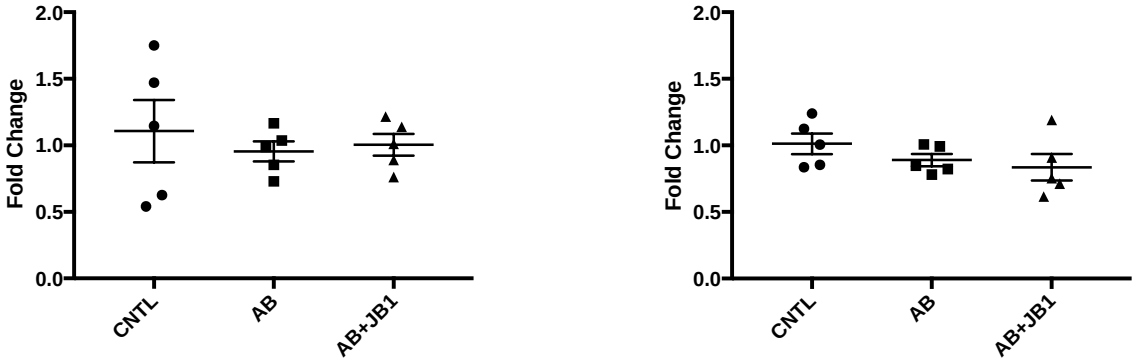


L **OTR**

Frontal Cortex



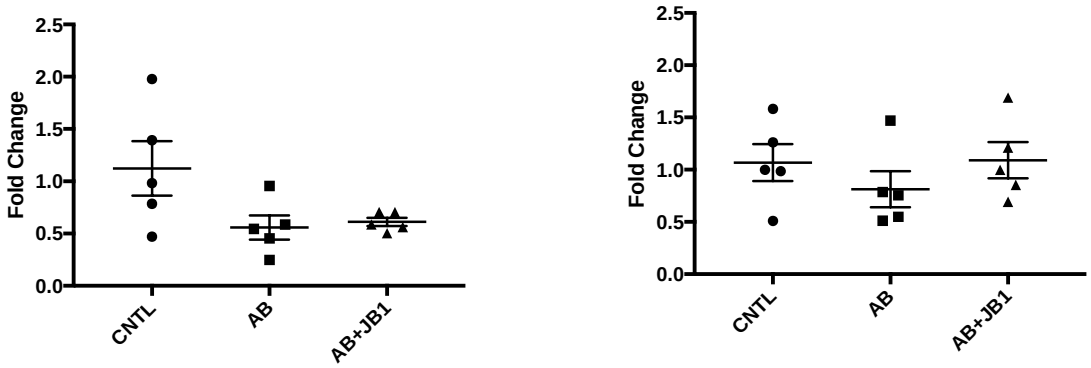
Hippocampus



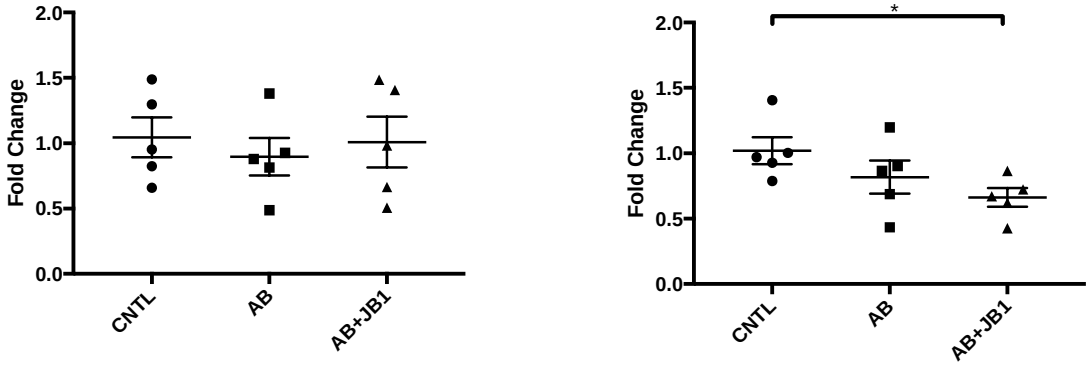
M

OCLD

Frontal Cortex



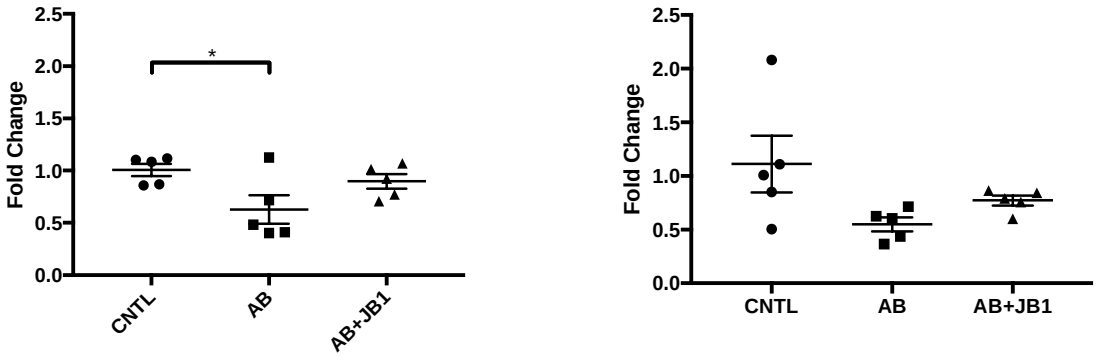
Hippocampus



N

CLDN-5

Frontal Cortex



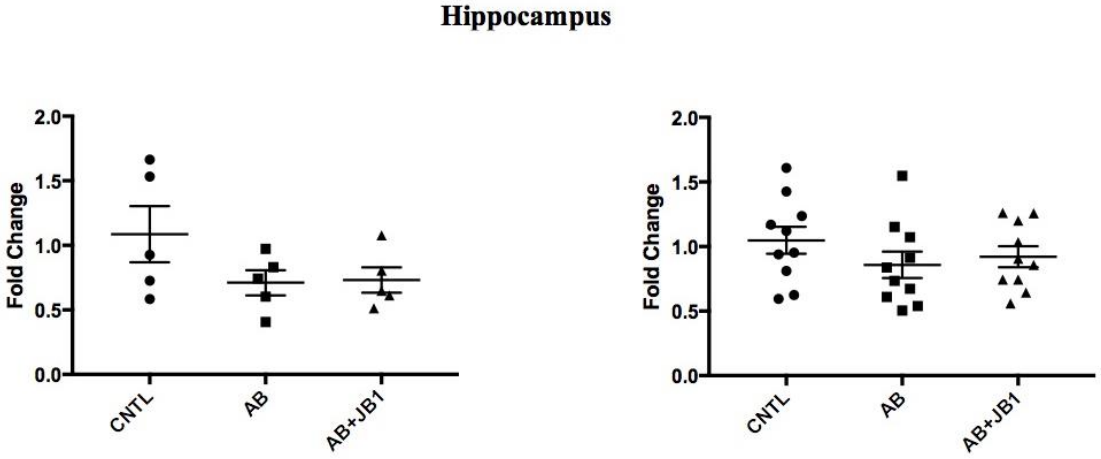


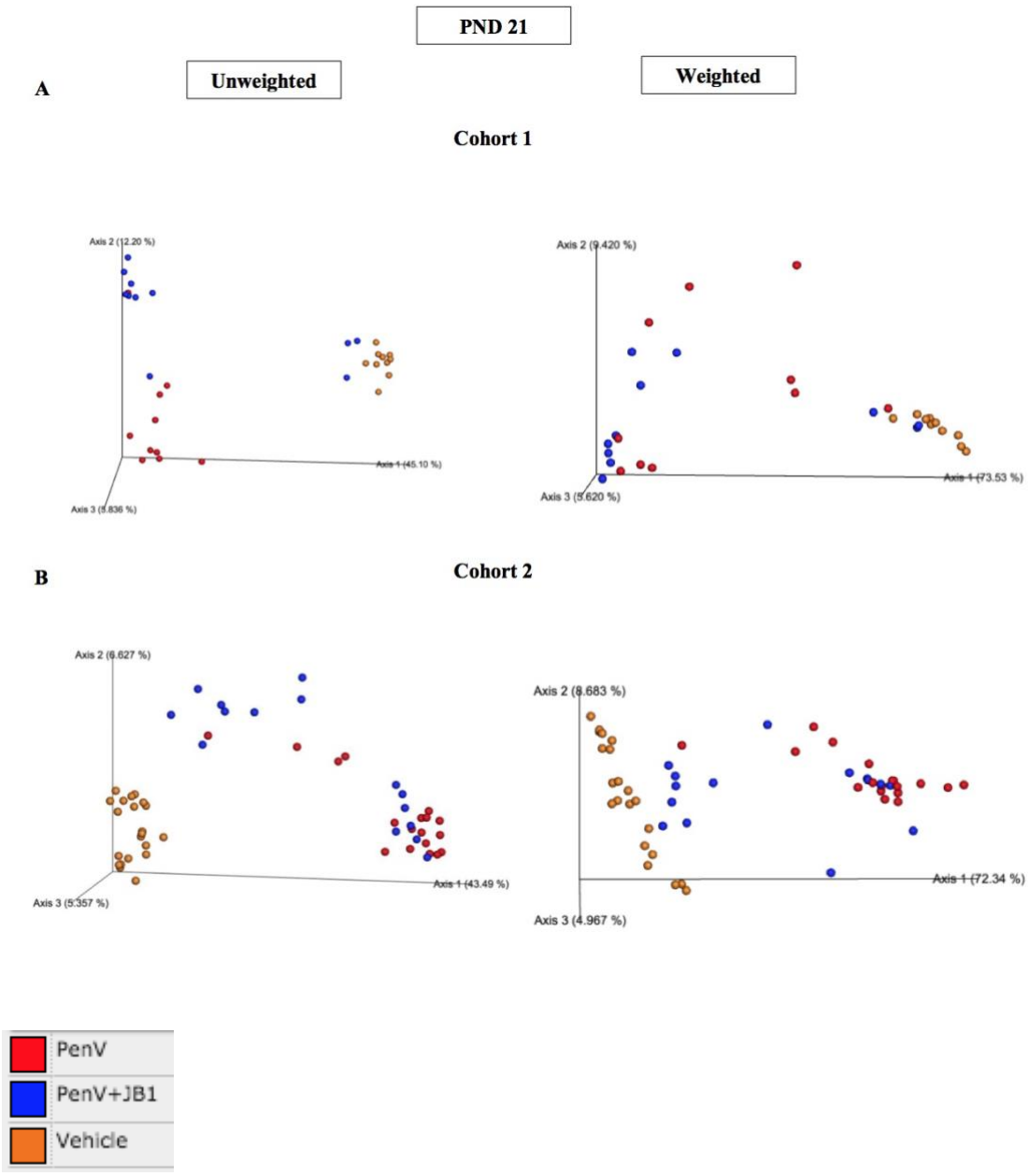
Figure 5. mRNA expression of TJ and other peptides in the frontal cortex and hippocampus.

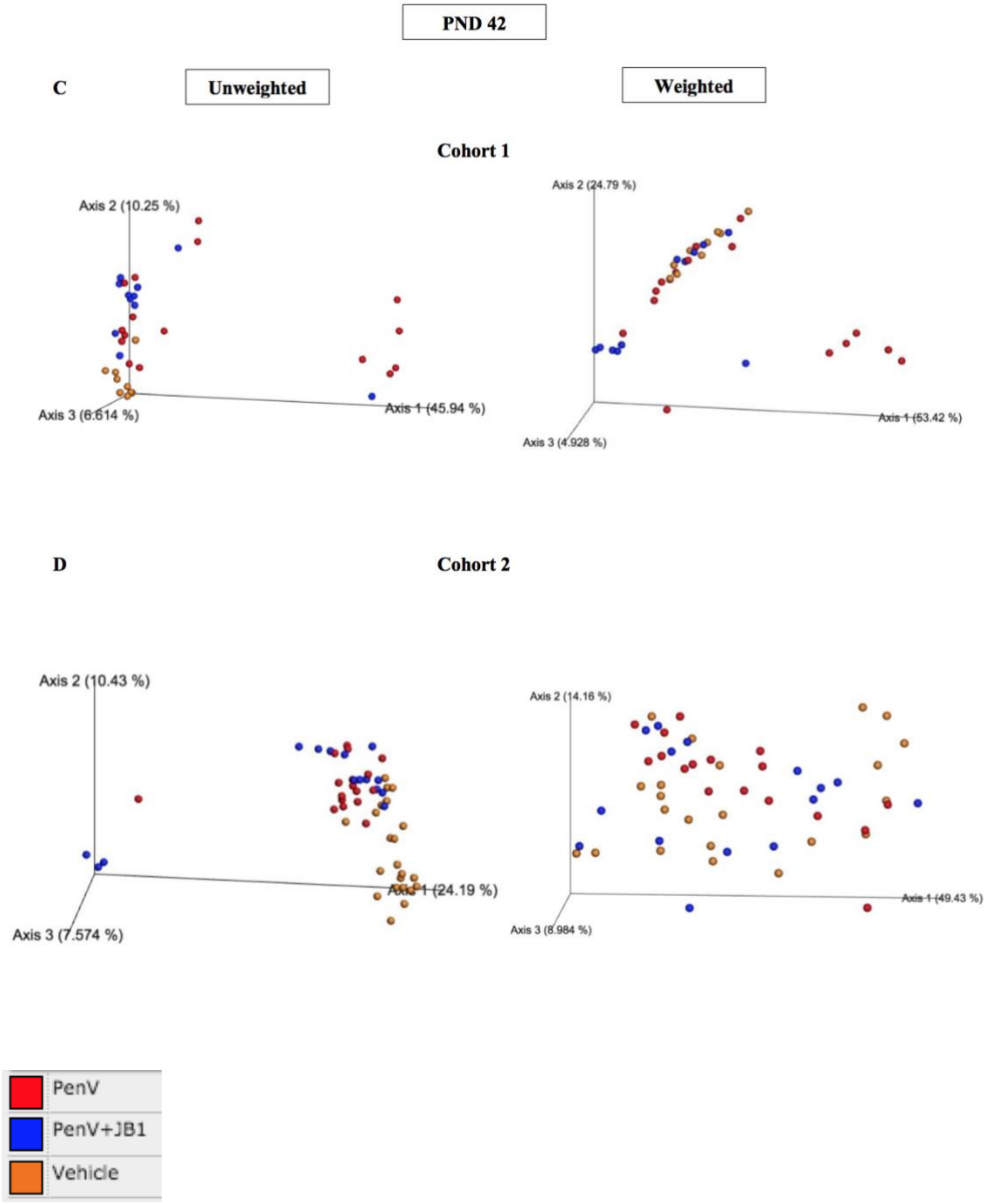
A-G are graphs from Experiment 1 (PND 70). **Males**: n = 5 CNTL, 5 AB, 5 AB+JB-1. **Females**: n = 5 CNTL, 5 AB, 5 AB+JB-1. **H-N** are graphs from Experiment 2 (PND 21). **Males**: n = 5 CNTL, 5 AB, 5 AB+JB-1. **Females**: n = 5 CNTL, 5 AB, 5 AB+JB-1.

AB administration leads to gut microbial changes (Figure 6)

At PND 21, 24 hours after treatment cessation, phylogenetic diversity and evenness of mice treated with AB (Faith's pd: q = 0.002; Pielou's ei: q = 0.001) and AB+JB-1 (Faith's pd: q = 0.002, Pielou's ei: q = 0.0009) was significantly different from controls. After 3 weeks of treatment, at PND 42, phylogenetic diversity of AB (Faith's pd: q = 0.0001) and AB+JB-1 (Faith's pd: q = 0.0004) groups remained statistically different from controls. However, evenness for both groups normalized (AB Pielou's ei: q = 0.12; AB+JB-1 Pielou's ei: q = 0.31) relative to controls. Treatment groups were not statistically different from each other at PND 21 (Faith's pd: q = 0.94, Pielou's ei: q = 0.29) or PND 42 (Faith's pd: q = 0.103, Pielou's ei: q = 0.40).

There were baseline differences in the microbiome between the two cohorts of the first experiment. Thus, UniFrac analyses of cohorts were done separately for beta diversity. Here, q values of weighted UniFrac analyses were considered for statistical significance. Microbial beta diversity of AB mice at PND 21 was significantly different from control mice in the first (unweighted: $q = 0.0015$, weighted: $q = 0.0015$) and second (unweighted: $q = 0.0015$, weighted: $q = 0.0015$) cohorts. The same was found for AB+JB-1 mice in the first (unweighted: $q = 0.002$, weighted: $q = 0.0015$) and second (unweighted: $q = 0.0015$, weighted: $q = 0.0015$) cohorts. In the first cohort, microbial composition of AB and AB+JB-1 mice (unweighted: $q = 0.0015$, weighted: $q = 0.681$) were not distinctly different at PND 21. For the second cohort they were (unweighted: $q = 0.008$, weighted: $q = 0.003$). At PND 42, the microbial composition of AB (unweighted: $q = 0.006$, weighted: $q = 0.034$) and AB+JB-1 (unweighted: $q = 0.003$, weighted: $q = 0.034$) groups were significantly different from controls in the first cohort. The difference between AB and AB+JB-1 microbial composition also remained significant (unweighted: $q = 0.051$, weighted: $q = 0.034$). In the second cohort, microbial structures of AB (unweighted: $q = 0.0015$, weighted: $q = 0.153$) and AB+JB-1 (unweighted: $q = 0.0015$, weighted: $q = 0.153$) groups normalized relative to controls at PND 42. Even the difference between AB and AB+JB-1 mice is diminished (unweighted: $q = 0.016$, weighted: $q = 0.41$).


PND 21
A
Unweighted
Weighted
Cohort 1
Axis 2 (12.20 %)
Axis 1 (45.10 %)
Axis 3 (8.836 %)
Axis 2 (9.420 %)
Axis 1 (73.53 %)
Axis 3 (5.620 %)
B
Cohort 2
Axis 2 (6.627 %)
Axis 1 (43.49 %)
Axis 3 (5.357 %)
Axis 2 (8.683 %)
Axis 1 (72.34 %)
Axis 3 (4.967 %)
PenV
PenV+JB1
Vehicle


PND 42
C
Unweighted
Weighted
Cohort 1
Axis 2 (10.25 %)
Axis 3 (6.614 %)
Axis 1 (45.94 %)
Axis 2 (24.79 %)
Axis 3 (4.928 %)
Axis 1 (53.42 %)
D
Cohort 2
Axis 2 (10.43 %)
Axis 3 (7.574 %)
Axis 1 (24.19 %)
Axis 2 (14.16 %)
Axis 3 (8.984 %)
Axis 1 (49.43 %)
PenV
PenV+JB1
Vehicle

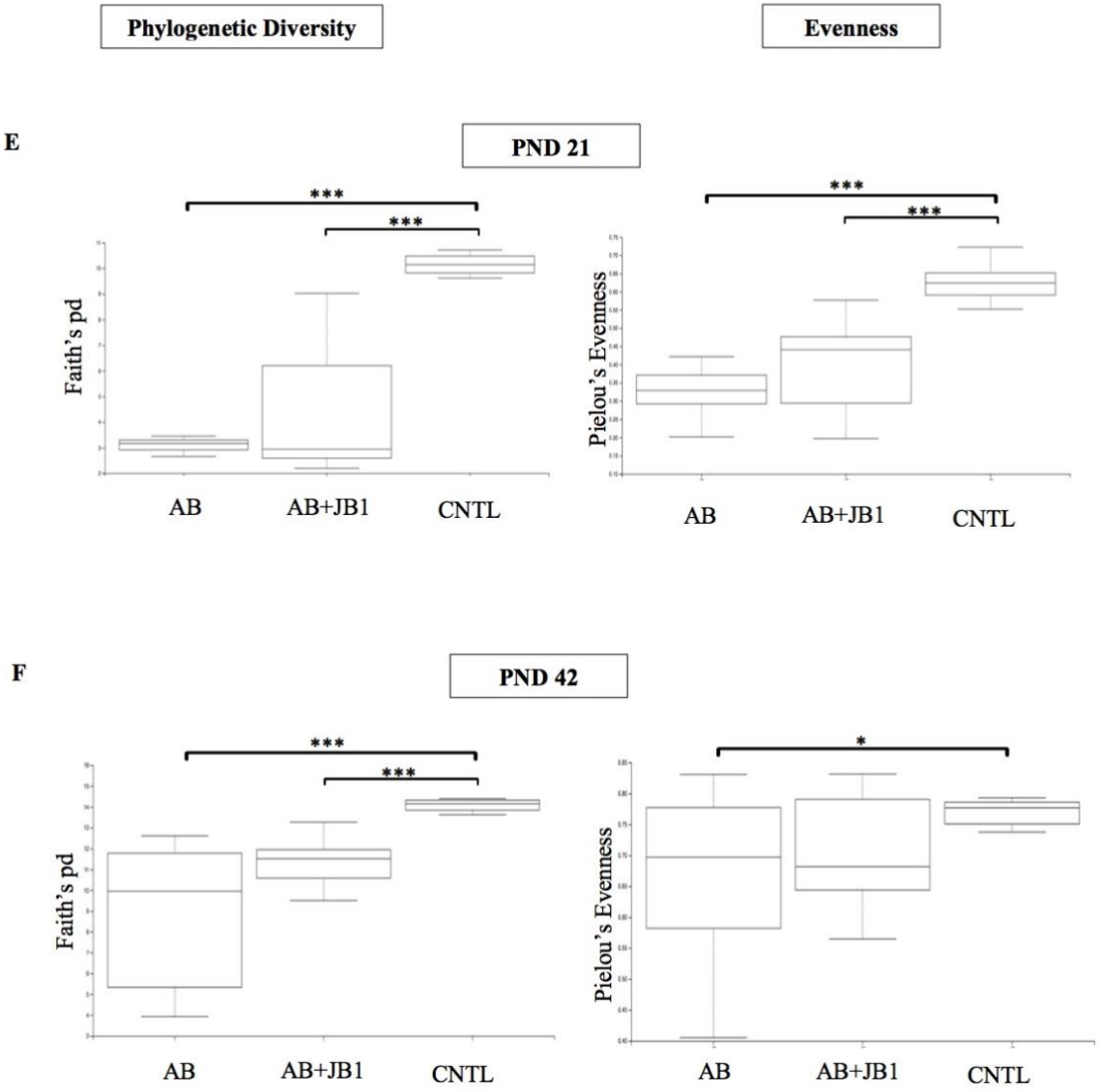


Figure 6. Gut microbiota diversity at PND 21 and 42.

A-D Beta diversity measured by weighted and unweighted UniFrac analysis. **Cohort 1**: n = 10 CNTL, 17 AB, 13 AB+JB-1. **Cohort 2**: n = 23 CNTL, 18 AB, 15 AB+JB-1. **E-F** Alpha diversity measures for phylogenetic diversity and evenness. **Cohorts 1+2**: n = 33 CNTL, 35 AB, 28 AB+JB-1.

Discussion

AB are commonly, sometimes unnecessarily, prescribed in pediatric care (Gonzales et al., 2001; Kutty et al., 2011). The consequences of these perturbations on the microbiota-gut-brain axis are being studied in GF and high-dose AB animal models. In this study, a clinically relevant dose of penicillin was used to examine the effects of early-life exposure to AB. The results suggest that low-dose AB, given postnatally, can alter long-term mouse physiology. These effects seem to be sex-specific, mostly appearing in males. The present study also found that social behavior was reduced with AB. AB decreased T_{reg} cells and increased activated DCs in the spleen at adulthood. In the brain and gut, various alterations in mRNA expression lasted until the adult stage in males and females. Administration of a probiotic with the AB attenuated some of lasting behavioral, immune, and mRNA expression changes.

The relationship between brain, behavior, and microbiome

In adult GF mouse models, social, depressive, and anxiety-like behaviors are altered (Neufeld et al., 2011; Heijtz et al., 2011; Desbonnet et al., 2014). Disruption of the microbial composition is also associated with changes in brain development and plasticity (Borre et al., 2014). It has been proposed that healthy microbial development in the postnatal period is critical for early neurodevelopment in humans (O'Mahony et al., 2017). Postnatal events that interrupt the pattern of normal microbial development are associated with behavioral deficits in animal models (Levine et al., 2014; Puceddu et al., 2015). Early-

life events that disturb the gut microbiota include stress and AB administration. In rodents, stress by maternal separation in early-life changes long-term behavioral outcomes through HPA activation (Lehmann et al., 2002; Lippmann et al., 2007). Cox et al. (2014) originally studied the metabolic and immune effects of LDP exposure in a mouse model. They found that LDP can alter long-term immune gene expression in the ileum and the metabolic profile, followed by dysbiosis in early life. Perinatal clinically relevant AB treatment diminishes gut bacterial richness and alters the microbial composition 3 weeks after treatment cessation (Leclercq et al., 2017). In the same study, AB-treated male mice exhibited reduced sociability and anxiety-like behavior with an increase in aggression. Our study focuses on postnatal disruption of the microbiota with a clinically relevant dose of AB administered from PND 14 to 21. We found a decrease in sociability in male mice exposed to AB, with no difference in anxiety-like behavior. We also found a lasting reduction in microbial phylogenetic diversity 3 weeks post treatment. The behavioral change associated with AB treatment in male mice is likely relevant to early distortion of gut microbial colonization with lasting consequences on the brain's neurochemistry. The CNS and microbiota develop in parallel and share similar critical time periods for maturation (Borre et al., 2014). Disturbance to the microbiota postnatally, during a critical window, is association with reduced mental health and increased risk of psychiatric disorders (Rapoport et al., 2012; Ben-Ari, 2013). Consistent with this, we found differences in mRNA expression in the brain. OCLD and CLDN-5, key markers for BBB integrity regulated by gut microbiota, had different patterns of expression at PND 21 and PND 70. In males, both CLDN-5 and OCLD increased in the hippocampus at adulthood. These

results are different from what has been found in GF mice, but consistent with Leclercq et al.'s (2017) study. In GF mice, there is an increased permeability of the BBB and reduced expression of TJs (Braniste et al., 2015). The increase in TJ expression might be a protective mechanism against neuroinflammation and damage to neuronal processes, such as those involved in learning and memory (Lecrecq et al., 2017). This is consistent with findings that several AB exhibit neuroprotective effects on BBB damage in models of inflammation (Mishra et al., 2009; Barichello et al., 2014). Another explanation for the increase in TJ expression is that AB might be exerting neuro-modulatory effects in this case. In females, there were no change in CLDN-5 and OCLD expression at adulthood. However, at weaning, there was a decrease in CLDN-5 expression in the frontal cortex, while no changes were observed in males. This may leave the female brain susceptible to neuromodulators, including cytokines (Schedlowski et al., 2014). The reduction in CLDN-5 in weaning females was associated with a decrease in BDNF expression in the hippocampus. Other than its role in learning and memory, BDNF has been reported to have anxiolytic and anti-depressive effects when expressed in the hippocampus (Deltheil et al., 2008). The sex-dependent change is consistent with the GF literature, which suggests that there is a sex-dependent change of hippocampal BDNF expression (Foster and McVey Neufeld, 2013).

Vasopressin and oxytocin are neuropeptides involved in various mammalian social behaviors in a sex-specific manner (Dumais and Veenema, 2016). Both hormones are implicated in various psychiatric conditions, such as autism and mood disorders (Yang et al., 2010; Yueng et al., 2014). AVPR1a and AVPR1b play a role in regulating maternal

care, social recognition, and aggression (Smith et al., 2014; Bredwold et al., 2014). In addition, AVPR1a has a role in pair bonding and social play behavior. While both receptors were decreased with AB treatment at weaning in male and female hippocampi, neither were changed in adulthood. The transient change in their mRNA expression may play a role in subsequent social behavior, as males exhibited less social behavior in adulthood, suggesting that males may be more susceptible to brain-behavior perturbations in early life. In Leclercq et al.'s (2017) study, increased aggressive behavior was associated with an upregulation of AVPR1b in the frontal cortex in early life of AB-fed male mice. Although the dose used was the same as in the present study, the treatment lasted for 14 days longer and began at an earlier gestational period. The window for effective long-term alternations to AVPR expression might be limited to an earlier period in mice or the result of more sustained AB exposure. In our study, a decrease in OTR was present in adult females, but not males, and was attenuated by concurrent JB-1 treatment. In prairie voles, injection of an oxytocin or OTR agonist has long-term behavioral and neurochemical changes in females but not males (Bales and Carter 2003; Yamamoto et al., 2006), suggesting that female rodents are more susceptible to disturbances to the oxytocin system.

The findings from the present study illustrate the ability of JB-1 to ameliorate AB-induced CNS changes in mRNA expression and social behavior. Probiotics have been used in animal models to reverse behavioral effects induced by stress or infection (Foster and McVey Neufeld, 2013). In clinical studies, probiotics show improved mental health scores in healthy patients (Benton et al., 2007; Messaoudi et al., 2011). Probiotics, specifically the psychoactive JB-1, have promise in treating patients with mood or anxiety disorders.

POMC is a preprotein that is cleaved in various ways to create up to ten different hormones involved in various cellular processes (Clark, 2016). POMC knockout mice exhibit an obese phenotype. Hence, POMC is characterized as an anti-obesity gene. POMC neurons in the hypothalamus play a central role in the hypothalamic melanocortin system, particularly in regulating energy homoeostasis (Mountjoy, 2010). POMC works in tandem with neuropeptide Y, a pro-obesity peptide under the control of leptin, to ensure a balance of energy consumption and production. The decrease of POMC expression at PND 21 in males and females, albeit in different brain regions, indicates an interruption of neuronal process involved in energy regulation mediated by dysbiosis. This effect was prevented by JB-1 treatment, indicating a potential role of the microbe in obesity-related neural processes. POMC expression in the hypothalamus is decreased in GF mice compared to specific-pathogen-free mice (Schéle et al., 2013). The clinically relevant dose of AB used in this experiment had a similar effect on POMC expression as in the GF model. However, assessment of more peptides involved in this system is required to get a more holistic comparison between GF and clinically relevant AB treatment models. Since PenV penetration into the cerebral spinal fluid is very low in the absence of infection (Grayson et al., 2017) these effects cannot be due to the direct effect of the AB on the brain.

The immune link to brain and behavior

The immune system is one of the mechanisms in which the microbiota-gut-brain axis communicates (Bengmark, 2013). The gut is a vital immune organ that provides defense against harmful pathogens while housing beneficial organisms. Studies of animal

models have shown that infectious organisms introduced to the host cause behavioral changes led by activation of gut-to-brain immune pathways (Lyte et al., 1998). Administration of pro-inflammatory cytokines to rodents is associated with depressive-like behaviors (Bilbo and Schwarz, 2012).

DCs are essential for T cell responses in adaptive immunity, as they monitor antigen presentation to naïve T cells. Hence, they play an important role in determining what type of T cell is differentiated. Dendritic MHCII+ cells present endotoxins, such as phagocytosed bacteria, to CD4+ T cells, which either become T_{reg} or effector T cells. T_{reg} cells specialize in suppressing autoreactive peripheral responses to pathogens (Austyn, 2018). In the spleen, we found a decrease in IL10+Foxp3+ T_{reg} cells and an increase in CD86+ and CD80+ activated DCs at PND 70 in male mice. These findings suggest a lasting pro-inflammatory milieu associated with early AB exposure, which can be explained by a compensatory increase of DCs in an attempt to convert T cells into IL10+ Foxp3+ T_{reg} cells. At the same time point, JB-1 attenuated the lasting effects of early-life AB on the immune profile by increasing IL10+ Foxp3+ T_{reg} cells, potentially through decreasing activated DCs.

Beneficial immunomodulatory microbes are thought to work through inducing T_{reg} cells and their secretion of anti-inflammatory cytokine IL-10 (Dinan et al., 2013). In support of this theory, a few studies have shown an upregulation of T_{reg} cells after probiotic administration. A mixture of probiotic fed to BALB/c mice for 20 days upregulated Foxp3+ T_{reg} cells in MLN (Kwon et al., 2009). Feeding JB-1 to male BALB/c mice increased Foxp3+ T_{reg} cell in Peyer's patch and MLN 3, 5, and 9 days after treatment (Karimi et al.,

2014). In humans, consumption of *Bifidobacterium infantis* 35624 is associated with an enhanced production and synthesis of IL-10 in peripheral blood (Bilbo and Schwarz, 2012). The microbe JB-1 seems to be anti-inflammatory in nature. In addition to upregulating T_{reg} cells 3 weeks post treatment, AB+JB-1 treatment lowered Th17 cells at weaning and Th1 in adulthood in male mice. AB reduced Th17 cells in the spleen, and concurrent JB-1 feeding further reduced Th17 cells. Simultaneous JB-1 and AB feeding was also associated with a decrease in splenic Th1 cells in males that lasted from adulthood to weaning. These changes suggest a dampening effect of JB-1 on the immune system. The decrease in these splenic cell population by JB-1 might be a direct consequence of the action of the microbe on luminal contents and their interaction with immune cells.

Increased presence of cytokines in the brain is associated with mood disorders (Biesmans et al., 2013). Cytokines from peripheral inflammation can reach the brain through a weakened BBB and other physiological routes, including humoral pathways (Schedlowski et al., 2014). Surprisingly, we found a decrease in pro-inflammatory serum cytokines in adult AB-treated female mice. JB-1 attenuated the decrease of serum IL-12 and TNFα in adult females, suggesting that JB-1 has the ability to interfere and inhibit the humoral mechanism by which PenV works through. The reduced levels of TNFα, a cytokine implicated in depression, may have contributed to the protection against behavioral changes in females, which was found in males (Dinan, 2009). At weaning, IL-2 levels were increased in females exposed to AB. DC cells produce IL-2, which might connect the increase in activated DC markers with AB exposure in females at the same time point as induced IL-2 (Granucci et al., 2001). An *in vivo* study showed that DCs with

impaired IL-2 production fail to expand T_{reg} cells (Cheng et al., 2012), which complements the notion that IL-2 has the ability to regulate T_{reg} development in the thymus (Yamazaki et al., 2007). In our study, activated DCs might be producing IL-2 as an immediate reaction to AB treatment in females, thus protecting the change to the splenic T_{reg} population, which was found in males. Cytokines play an important role in the microbiota-gut-brain axis, as they are involved in the development of mood disorders, inflammation, and the HPA response (Clapp et al., 2017). Deviations in cytokine levels can influence any or all of these processes.

Gut permeability and entero-endocrinology as potential influencers of brain-immune interactions

The TJ protein ZO-1 interacts with the transmembrane TJ protein OCLD to form the GI epithelial barrier (Fanning et al., 1998; Van Itallie et al., 2009). In previous studies, long- and short-term AB administration in adult mice and pups decreased expression of ZO-1 in the gut epithelium (Wang et al., 2014; Cheng et al., 2017). In our study, relative expression of OCLD decreased in adult males and ZO-1 transiently decreased in weaning females. These results suggest the presence of an altered OCLD to ZO-1 interaction associated with AB. However, both changes were reversed by concurrent JB-1 feeding. Gut barrier integrity can be compromised by AB, as indicated by the downregulation of TJ expression in the ileum at different time points. This may lead to the presence of cytokines in serum, and possibly the brain, thereby altering behavioral performance. The structure of the gut barrier is completed by the end of the first trimester of gestation (Kelly et al., 2015).

However, functional development of the epithelial barrier continues after birth and it greatly influenced by diet (Verhasselt, 2010). Underdevelopment of the GI epithelial barrier in infants predisposes them to immune diseases. The microbiota regulates the formation of a successful epithelial barrier, although the mechanism by which this occurs is not yet clear. Animal models of depression exhibit compromised gut barrier integrity associated with reduced microbial diversity and induced pro-inflammatory cytokines, including increased IL-6 and TNFα presence (Dinan, 2009; O'Mahony et al., 2011). The same cytokines have been implicated in other stress-related illnesses, like anxiety. Animal models of stress provide evidence of altered gut barrier permeability, which enables LPS and other molecules to circulate in the blood stream, resulting in activation of inflammatory cytokines (Kelly et al., 2015). A leaky gut initiates an immune response when microbiota or their components are translocated across the GI lining, which can be achieved by stress or AB use. This immune activation can influence brain function through neuronal or humoral routes of communication (Haulica et al., 2002).

The role of probiotics in epithelial barrier function and associated diseases such as IBS and IBD is currently an active area of research. *Lactobacillus helveticus* R0052 and *Lactobacills rhamnosus* R0011 prevent stress-induced dysfunction of the GI epithelium after maternal separation (Gareau et al., 2007). *Lactobacills rhamnosus* OLL2838 was shown to reduce epithelial lining defects in mice with DSS-induced colitis (Miyauchi et al., 2009). Consumption of *Lactobacillus plantarum* in humans alters ZO-1 localization in the gut, strengthening barrier integrity (Karczewski et al., 2010). The association between

stress-induced psychiatric illnesses, leaky gut syndrome, and inflammatory diseases indicates the interplay between immune function and the microbiota-gut-brain axis.

The enteroendocrine system is another identified pathway of the microbiota-gut-brain axis (Foster et al., 2017). EECs produce and secrete hormones into the gut lumen that then pass through the immune cell rich lamina propria to reach blood circulation or local ENS receptors (Holzer and Farzi, 2014). PYY and GLP-1 are neuroendocrine hormones secreted by L cells in the lower GI tract (Ekbald and Sundler, 2002; Holst, 2007). Both hormones are activated by fat receptors on L cells to inhibit gastric emptying through the vagus nerve (Strader and Woods, 2005; Murphy and Bloom, 2006). SCFAs, metabolic products of gut microbiota, can stimulate the secretion of PYY and GLP-1, allowing for microbial manipulation of their release (Cherbut el al., 1998). As GLP-1 is an incretin, it has the ability to reduce blood glucose (Marathe et al., 2013). In the present study, there was a reduction in GLP-1 after AB treatment at weaning female mice, which was attenuated by JB-1. When male and female data were combined, there was a statistically significant decrease in PYY at the same time point. JB-1 also ameliorates this change in PYY expression. In GF mice, intestinal satiety hormones PYY and GLP-1 are decreased (Duca et al., 2012). Here, clinically relevant AB treatment appears to transiently mimic GF enteroendocrine outcomes. Overall, AB affects the enteroendocrine system temporarily, as its effects do not persist into adulthood, and JB-1 has the ability to attenuate the changes imposed by AB administered postnatally.

Various probiotics are being studied in animal models and clinical trials as possible therapeutics for diabetes mellitus. In a randomized clinical trial, probiotic administration in

pregnant women reduced the incidence of gestational diabetes (Luoto et al., 2010). Administration of *Bifidobacterium animalis lactis* 420 dampened glucose intolerance and reduced translocation of gram negative bacteria in diabetic mice (Amar et al., 2011). Diabetes mellitus is associated with a pro-inflammatory milieu and increased barrier permeability. The beneficial effects of probiotics transcend one physiological system. In this study, JB-1 administration altered specific metabolic mRNA expression in the ileum and brain of male and female mice. The attenuation of intestinal PYY and GLP-1 and brain POMC with JB-1 feeding compared to AB-treated groups suggests that JB-1 leads to a reduction in appetite. However, this effect was only observed at weaning, soon after treatment cessation. More studies examining the effects of JB-1 consumption on intestinal satiety hormones are needed to develop a comprehensive understanding of the underlying mechanisms. If JB-1 is shown to be effective in manipulating these hormones, it may eventually be explored in a clinical setting to help prevent onset of diabetes in susceptible individuals, especially given that PYY and GLP-1 are being explored as therapeutic targets in obesity (De Silva and Bloom, 2012).

Sex differences in brain and behavior

In our experiment, female estrous cycles were not synchronized or taken into consideration for behavioral tests or other tissue analyses. Consistent with the literature, we found sex-dependent differences in behavioral performance in our study. In maternal separation rodent models, males show exaggerated fear-based and anxiogenic behavior, suggesting that they are more susceptible to early-life adversities related to mother-infant

interactions (Chocyk et al., 2015). Sucrose-exposed female rats show improved special memory in an object recognition test when their estrogen levels are highest in the pro-estrus part of the cycle (Abbott et al., 2016). This suggests that estrogen plays an active role in memory performance. In adult rats, extracellular release of oxytocin is increased in males but not females in response to social stimuli (Dumais et al., 2016). Adult male rats also show higher mRNA expression of OTR and higher OTR density in the ventromedial hypothalamus compared to females (Bale and Dorsa 1995; Dumais et al., 2013). Anxiety-like behavior in male, but not female, mice is decreased by stimulation of OXR-expressing interneurons (Nakajima et al., 2014). Similarly, in a vector-mediated knockout of OTR in the medial prefrontal cortex, only males show reduced anxiety-like behaviors (Li et al., 2016). These results demonstrate that oxytocin and OTR are differentially expressed in males and females, and that males are more susceptible to behavioral changes associated with this hormone. We found a reduction in OTR mRNA expression in the frontal cortex of AB-treated females at PND 70. Yet, this change was not associated with differences in social or anxiety-like behaviors. Males did not show a change in OTR or AVPR mRNA expression in the brain at the same time point. Vasopressinergic innervation extends to the lateral septum from the bed nucleus of the stria terminalis and medial amygdala (De Vries et al., 1983; Caffé et al., 1987). The lateral septum is a brain region that is vital for the regulation of emotion and social behavior (Sheehan et al., 2004). Blocking AVPR1a in the lateral septum does not alter preference for social novelty in either sexes (Veenema et al., 2013). However, it does alter social play in males and females in an opposite fashion (Bredewold et al., 2014). Sex-dependent changes are behavior specific (Bredewold and

Veenema, 2018). In our study, we found significant changes in social behavior for male mice only and no changes in anxiety-like behavior for either sexes. The sex-specific effect of the AB and JB-1 treatment is not fully understood. It is possible that microbial metabolites interact with various systems, including immune and neuroendocrine systems, signaling to the brain via the vagus nerve and driving these sex-specific effects (Leclercq et al., 2017).

Different lasting and transient changes in microbiota-gut-brain axis related results

There were distinct effects of AB at weaning and adulthood. Most effects at weaning did not persist into adulthood, but there were long-term effects of AB and JB-1 treatments. Both interventions influence components of the microbiota-gut-brain axis in an age-specific manner. The only long-term effect between weaning and adulthood identified in the present study was the lasting difference in phylogenetic diversity of AB and AB+JB-1 treated mice. This highlights the robust effect of clinically relevant AB exposure in this postnatal period.

Conclusion

Future studies are needed to yield a better understanding of the mechanism underlying the effects identified in the present study. A test of the viability of JB-1 in the gut would clarify whether the presence of JB-1 or its components, regardless of viability, triggers a signaling cascade or live JB-1 produces metabolites that activate communication pathways that induce its effects. To confirm that mRNA expression corresponds to protein presence in the ileum and brain, histological staining or Western blots are needed. PCR analysis for brain primers are underpowered; thus, more samples are needed to achieve power. There are several mRNA primers that would aid in proposing a better mechanism for the effects of AB. Testing for expression of inflammatory markers in the brain would provide a profile of its neuro-inflammatory status, which may be involved in behavioral changes mediated by serum cytokines. To further understand the neuroendocrine response to treatment, relative to POMC and PYY, a measure of neuropeptide Y expression in the brain is needed, this hormone links the two peptides. Neuropeptide Y and POMC have opposite effects: neuropeptide Y is an orexigenic and POMC is an anorexigenic. PYY appears to control the balance of both (Acuna-Goycolea and van den Pol, 2005). These experiments will be completed by future members of the Forysthe Lab.

An objective of this thesis project was to explore the effects of clinically relevant AB exposure and the potential therapeutic effects of JB-1 in the postnatal period. We confirm that postnatal AB treatment does alter immune and behavioral markers in adulthood, and we demonstrate that the long-term effects of AB are attenuated by

concurrent treatment with JB-1. Although JB-1 did not ameliorate the AB-induced shift in phylogenetic diversity that persisted into adulthood, we did not predict is that it does so in a sex-specific manner. Further investigation into why AB appear to have a greater long-term effect in males is warranted. Data from concurrent AB and JB-1 fed groups confirm the psychoactive and immuno-regulatory properties of JB-1 previously identified in the literature. JB-1 ameliorated most of the immune, behavioral, neurochemical, and enteroendocrine AB-induced changes.

Another objective of the project was to investigate the critical window in which AB exposure has lasting immune, nervous, and enteroendocrine effects mediated by dysbiosis. Our results suggest that the postnatal period, specifically PND 14 to 21, is a critical window for immune, brain, and entero-endocrinology development. In addition, these changes may be sensitive to altered microbial diversity at an early age. This study brings us closer to understanding how a clinically relevant disruption to the microbiota in early life can alter development and long-term health in a mouse model.

An important message of my research is to consider AB prescription and consumption more carefully in pediatric care. Excessive exposure to AB appears to play a role in disease development later in life. The work in the present thesis also suggests that probiotics may have potential as treatments to mitigate some of the apparent detrimental effects of AB.

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