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EFFECTS OF IRON DEFICIENCY UPON NEURODEVELOPMENT, METABOLISM, AND
INFLAMMATION

BY

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DISSERTATION

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Abstract

The first few years of life are a critical period in neurodevelopment. During this time, multiple developmental processes occur in tandem, rendering the brain especially vulnerable to environmental insults. The coordination of complex neuronal processes, including myelination and synaptogenesis, may be disrupted by factors such as malnutrition and infection, inflicting lasting consequences on developmental trajectory. Identifying the neurodevelopmental changes imposed by early life environmental insults is an important step towards developing mechanistic targets for therapeutic intervention.

Iron deficiency (ID) is the most common micronutrient deficiency in the world. Nearly 2 billion people worldwide suffer from anemia, and more than half of those cases are due to ID. Vulnerable populations in developing nations are most affected, with approximately 50% of pregnant women and 40% of young children suffering from anemia. ID infants are reported to display reduced positive affect and to be more difficult to soothe. ID in early life is of serious concern, as it is linked to lasting cognitive and behavioral changes that persist even after iron repletion. Low hemoglobin levels in infancy are correlated with increased likelihood of being placed in special education by age 10. At 19 years of age, adults who were severely ID anemic as infants displayed impaired executive function and memory. Previous animal studies of neonatal ID have found altered myelination and synaptogenesis.

As ID may lead to immunocompromise, ID and infections are often comorbid. Both early life ID and infection are separately associated with increased risk for neuropsychiatric disorders, including autism and schizophrenia, as well as negative cognitive changes. Microglia, the innate immune cells of the brain, can be activated by inflammatory signals from peripheral immune cells. In response, they produce inflammatory cytokines and reactive oxygen species, and perform

effector functions such as phagocytosis. Animal studies have found perinatal infection reduces neurogenesis, alters hippocampal dendrite arborization, and increases microglial activation, which may have long-term consequences on neurodevelopment.

Based on these data, we hypothesized ID would impair white matter development and myelination. Piglets were given either an iron-normal or ID diet for 28 days. Magnetic resonance imaging (MRI) and histology were used to measure structural and microstructural changes in the brain. Large areas of white matter reduction were observed in the hippocampus and other brain areas. White matter microstructural integrity was reduced both globally and in the corpus callosum. Histological staining showed that the width of the corpus callosum was reduced by ID. We next asked how ID altered peripheral and CNS immune activation in the context of an infection. An *in vitro* study testing oxidative respiratory capacity of an immortalized microglial cell line found ID reduced basal respiration, maximal respiration, and spare respiratory capacity. A piglet study using a 2x2 factorial design was then performed with diet (Normal/ID) and infection (Control/PRRSV) as factors. ID piglets displayed impaired viral clearance. ID reduced peripheral blood mononuclear cell cytokine and antimicrobial gene expression, including expression of interferon- γ , which is critical for clearance of the PRRS virus. Microglial activation state and phagocytic activity were increased by infection, but ID had no effect.

The last aim of this research evaluated neurodevelopmental and neurochemical changes caused by ID and infection in key brain regions, using the aforementioned 2x2 factorial design. ID altered dopamine metabolism in the hippocampus, while infection reduced dopamine turnover in the medial prefrontal cortex. Dopamine turnover in the amygdala was increased in infected ID piglets. Genes involved in neurodevelopment and oxidative metabolism were reduced by ID and infection in multiple brain regions. The medial prefrontal cortex displayed reduced expression of

key gene families in both ID and PRRSV infected piglets. Expression of inflammatory cytokines was generally increased in both infected groups and reduced in ID piglets.

Collectively, these data provide novel insight into the structural, molecular, and neurochemical changes following neonatal exposure to the common double burden of ID and infection.

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Chapter 1. General Introduction and Justification

Brain development is highly sensitive to environmental factors, including malnutrition, infection, and psychosocial stress. The perinatal period and first three years of life are a critical time for neurodevelopment. High rates of neurogenesis, myelination, and synaptogenesis occur simultaneously, resulting in tremendous brain growth. The hippocampus, crucial for learning and memory, begins its rapid growth spurt during the third trimester and continues through the first 1.5 years of life [1]. However, the coordination of multiple complex biological processes makes the brain especially vulnerable to environmental insults such as malnutrition [2] and inflammation [3]. The disruption of brain development during this critical period may lead to lifelong cognitive, behavioral, and emotional changes [2, 4, 5].

Iron deficiency (ID) is the most common micronutrient deficiency in the world – 2 billion people worldwide are estimated to suffer from anemia, with more than half due to ID [6]. Pregnant women and young children are especially vulnerable – in developing countries, 50% of pregnant women suffer from anemia. Early life ID is linked to lasting cognitive and behavioral changes that persist even after iron repletion. In humans, early life ID is linked to poorer cognition and altered emotionality, including deficits in attention and lower test scores, increased anxiety, and increased aggression. Adolescents who experienced early life ID report poorer motor, cognitive, and socio-emotional function, which is thought to be due to alterations in myelination, brain bioenergetics, and monoamine neurotransmission [7, 8]. Approximately nine years after iron repletion therapy, early life ID anemia was associated with poorer inhibitory control and slower reaction times [4]. At 19 years of age, persons who had experienced severe, chronic ID as infants displayed impaired performance on behavioral tasks testing inhibitory control, attentional shifting, planning, and

memory [9]. Animal studies have linked neonatal ID to altered neurotransmitter levels, myelination, and brain structural development, as well as cognitive deficits **reference**.

ID weakens the immune system, and in vulnerable populations, ID and infection are often comorbid. Respiratory infection is a leading cause of death in young children in developing countries, and a leading cause of hospitalization in children less than one-year-old in the United States. Early life infection is associated with impaired spatial learning. Both ID and early life infection are separately associated with increased risk for neurological disorders, including autism and schizophrenia, as well as learning and attentional deficits. In animal models, perinatal infection causes altered hippocampal dendrite arborization, microglial activation, and reduced neurogenesis. Peripheral infection can increase neuroinflammation through several mechanisms [10, 11], which may exert long-term consequences on developmental trajectory [3].

Despite often being comorbid and individually associated with long-term cognitive and neuropsychiatric consequences, intersectional effects upon brain development between ID and infection during early life have received little study [12, 13]. The bulk of animal research on ID has been performed using rodents, which have lissencephalic brains and reduced cerebral complexity compared to humans. The neonatal piglet is an ideal translational model for human infants, as it has a gyrencephalic brain and greater anatomical similarity to humans. The proposed research is expected to provide deeper understanding of the region-specific structural, molecular, and neurochemical brain changes caused by these common early life insults. A greater understanding of the effects of early life ID upon neurodevelopment is needed to foster identification of therapeutic targets. Developing therapeutic strategies to ameliorate long-term deficits arising from early life ID and infection would improve many lives and have a positive economic impact on developing nations.

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Chapter 2. Review of Literature

2.1 Mammalian Iron Distribution

Adequate iron reserves are imperative for the health and vitality of mammalian organisms. In ancient times, drinking water in which a sword had rusted was used to treat “weakness” – it was thought that the strength of the iron would overcome the patient’s frailty [1]. Iron is essential for proper functioning of mammalian cells, and is required for a variety of physiological applications, including aerobic respiration, synthesis of complex biomolecules including nucleic acids, lipids, and neurotransmitters, electron transport, and generation of reactive oxygen species, which are useful for both cellular signaling and antimicrobials. Iron is divided into several pools within the body. Heme-associated iron forms the largest iron pool in the human body, and is used to transport oxygen via hemoglobin and store oxygen via myoglobin [2, 3]. Approximately 1500-2500 mg of iron is stored in erythrocytes [3]. Other heme proteins include the cytochromes, which ferry electrons during aerobic respiration and perform intermediary metabolic and detoxification functions [2, 3]. The second largest iron pool is non-heme iron stored in ferritin proteins, a labile iron storage form that can quickly provide iron when more is needed for oxygen transport and other requirements [2], ranging from 200 mg to 1200 mg of iron storage [3]. Circulating transferrin in the plasma provides another readily accessible iron pool, transporting iron as needed from storage sites to peripheral locations through receptor-mediated uptake [3]. Under normal conditions, serum transferrin is 30-40% saturated, providing substantial buffering capacity [4]. Transferrin binds to the transferrin receptor, which is then endocytosed to an acidic intracellular compartment wherein the iron is released [5]. Other iron-containing proteins include fatty acid desaturases (lipid metabolism), iron-sulfur proteins in the TCA cycle (oxidative metabolism), tyrosine hydroxylase, tryptophan hydroxylase, and indoleamine 2,3-dioxygenase (neurochemical metabolism), as well as NADPH oxidase and nitric oxide synthase (host defense) [3].

2.2 Iron Absorption and Metabolism

As free iron can be toxic due to pro-oxidative properties, iron homeostasis is tightly regulated, with body levels of iron primarily controlled at the level of intestinal absorption. Within the body, iron is bound to carrier/storage proteins [2, 6, 7]. Body iron levels are tightly regulated and bound to proteins to maintain the levels necessary to function while avoiding iron excess, which can lead to oxidative stress and tissue damage [4, 6]. Dietary iron bioavailability is relatively low. Normally, dietary iron provides 1-2 mg per day of iron absorption (in the ferrous form) through proximal duodenal enterocytes via the Dmt1 transporter [4, 7], compensating for day-to-day iron loss due to sloughed skin cells or minor bleeding [8]. However, erythropoiesis requires iron to the order of 20 mg per day for adequate hemoglobin synthesis [4, 7], which is provided via phagocytosis of senescent erythrocytes by reticuloendothelial macrophages, as well as Kupffer cells and bone marrow macrophages, recycling the contained iron into circulation [2, 7]. The heme porphyrin ring is cleaved by heme oxygenase-1, yielding iron, CO, and biliverdin [9]. The iron is then ferried from the phagolysosome by the chaperone protein Nramp-1 (natural resistance associated macrophage protein), where it is either exported to circulation via ferroportin or stored in ferritin [7]. Continuous recycling of senescent erythrocytes by macrophages predominantly in the reticuloendothelial system (RES) and subsequent extracellular export of their component iron via ferroportin provides a steady supply of 20-25 mg of iron into the circulation, ensuring adequate iron for heme synthesis under normal conditions [7].

The primary regulator of iron metabolism is the peptide hormone hepcidin, which provides close control of the iron cycle and acts as a conduit for inflammatory signals to modulate the iron cycle [2, 4]. This is accomplished by binding to ferroportin, a transmembrane protein responsible for exporting intracellular iron that is primarily expressed in hepatocytes, macrophages, enterocytes, and the placenta [4]. After being bound by hepcidin, ferroportin proteins are

internalized and degraded by the proteasome, thus reducing the net export of iron into the blood by splenic macrophages, duodenal enterocytes and hepatocytes [2, 3], decreasing circulating iron. Binding of ferroportin by hepcidin also triggers the phosphorylation of Stat3, resulting in transcriptional changes to the inflammatory response [2]. Hepcidin plays a key role in ‘nutritional immunity’ by limiting availability of iron to extracellular pathogens. The bulk of hepcidin is produced by the liver, though macrophages and other cell types do produce small quantities under some conditions [4].

As a master regulator of iron homeostasis in the body, hepcidin expression is modulated by several factors, including iron surplus, iron deficiency, hypoxia, and inflammatory molecules including IL-6 [2-4]. Iron deficiency reduces hepcidin expression, leading to greater levels of iron absorption in the gut, and increased iron export from gut endothelial cells, circulating macrophages, and the RES [3, 10]. Similarly, hypoxia, which necessitates increased erythropoiesis, represses hepcidin synthesis in order to increase the available iron pool for heme synthesis through expression of mediators such as the hormones erythroferrone and platelet-derived growth factor-BB [3, 7, 11]. Hepatocyte hepcidin synthesis is triggered by inflammatory signals including IL-1 β , IL-6, IL-22, and Toll-Like Receptor (TLR) ligands [7], resulting in sequestering of circulating iron as part of an evolutionary strategy to prevent pathogen access to a growth-limiting nutrient [2, 3]. In response to IL-6 signaling, macrophages produce small, locally effective amounts of hepcidin [12].

2.3 Inflammation and Iron Homeostasis

The immune system plays a vital role in mammalian iron metabolism, modulating iron levels through cytokine signaling, cellular activity and hormones to limit the availability of iron to pathogens and potentiate certain immune effector pathways [12]. Macrophages are a focal point

of iron intake and export, phagocytizing senescent erythrocytes and acting as iron depots in the case of inflammation-induced iron sequestration, in addition to synthesis of iron-containing anti-microbial proteins. Aside from iron derived from erythrophagocytosis, macrophages also uptake iron through transferrin receptor-1 and Dmt1 [13, 14], and directly import ferritin and lactoferrin (Lf) via T cell immunoglobulin and mucin domain-containing molecule (Tim)-2 and scavenger receptor class A member (ScarA)-5 [7].

Hepcidin binds to ferroportin and induces its degradation. Cytokines affect iron metabolism independent of the hepcidin pathway [12]. Interferon- γ (IFN- γ) suppresses ferroportin expression, while ferritin expression is induced by TNF- α , IL-1 β , and IL-6 [12]. TNF- α , IL-1 β , and IL-6 also increase iron uptake by altering expression of transferrin receptor-1 [12], while IFN- γ increases Dmt-1 expression [15]. Additionally, TNF- α reduces duodenal iron absorption [16]. In this way, the body can quickly reduce the plasma iron pool in response to inflammation by shunting iron to circulating monocytes and macrophages, reducing macrophage iron export while promoting iron storage and leaving iron import unaffected [3, 7]. The anti-inflammatory cytokine IL-10 reverses this effect, promoting ferroportin expression and causing iron efflux [15]. Thus, iron metabolism differs between macrophages performing pro-inflammatory functions and those performing inflammation-resolving functions, with pro-inflammatory macrophages expressing high levels of iron storage proteins (ferritin) and low levels of iron export proteins (ferroportin), while the resolving macrophages are characterized by the reverse [7, 17]. Actively exporting iron may promote resolution of inflammation and tissue repair by providing a required cofactor for iron dependent enzymes, such as prolyl hydroxylase, which synthesizes collagen [4].

Iron also affects macrophage phenotype and cytokine expression [7], and low iron inhibits expression of nitric oxide synthase [18]. Bergman et al found peripheral blood mononuclear cells

from humans were deficient in IL-2 production [19]. Interestingly, NO also regulates iron metabolism; high concentrations of intracellular NO promote binding of Nrf2 to the ferroportin promoter, resulting in increased iron export and reduced macrophage iron – a useful tactic to restrict iron availability to intracellular bacteria that replicate within macrophages, such as salmonella [20].

Iron is a key factor for proper immune cell proliferation and function [4]. Iron deficiency is directly correlated to some infections [21] and is generally associated with increased risk of infection [22]. This is in part due to reduced phagocytic capacity of immune cells in persons with ID anemia [1]. Conversely, Walter et al found no difference in neutrophil phagocytic capacity in IDA infants compared to controls before or after iron repletion, but determined neutrophil bactericidal capacity was heavily impaired by IDA [23].

2.4 ID and Brain Development

ID during early life has profound effects upon brain development, including myelination [24-28], brain metabolism [25, 29], neurotransmitter tone [25, 30-32], and synaptogenesis [33, 34], in a regionally dependent fashion according to the brain areas undergoing development at the time of deficiency. Some effects, including impaired attention, cognition, and slowed response to auditory potentials, persist long after iron repletion therapy [35-37], and reduce achievement across the lifespan. Thus, it is imperative to better understand the changes wrought by early life ID. The pleiotropic effects of ID upon neurodevelopment are due to the multiple key biochemical processes the micronutrient is required for – including production of lipids for myelination, aerobic respiration to meet the energy demands of a growing brain, and synthesis of neurotransmitter precursors.

ID in the rat gestation/lactation period resulted in delayed hippocampal synaptic maturation and a reduction in LTP compared to control animals following iron repletion [38]. Gestational ID

was also found to impair dendrite arborization, resulting in less linear dendritic growth and tangled branches [39]. In the CA1 area of the hippocampus, genes associated with modulating actin and tubulin dynamics (necessary for dendrite growth) were reduced in tandem with reduced dendritic branching and smaller spine heads. Following iron repletion, the adult rats displayed persistently reduced transcription of the aforementioned genes and 25% fewer dendritic spines compared to controls [40].

The effects of ID upon neurotransmitter metabolism have been the subject of ongoing study. Iron is a required cofactor for both tryptophan and tyrosine hydroxylase, which are the rate limiting enzymes in the synthesis of serotonin and dopamine, respectively. ID has been found to alter extra- and intra-cellular neurotransmitter levels, in addition to receptor density. In one of the earlier studies looking at the effects of ID upon neurotransmitter metabolism, Chen et al found dopamine metabolism in the caudate/putamen to be profoundly altered, with extracellular dopamine increased by 30% in ID animals compared to controls and 40% when the animals were stimulated by darkness or food [41], suggesting decreased dopamine reuptake and degradation, a result and conclusion echoed by Beard et al [42]. Notably, these effects were determined to stem not from anemia, but ID by comparing a model of induced hemolytic anemia to a nutritionally ID model. Additionally, it was concluded that dopamine reuptake was blunted in ID rats [43].

Rat models of gestational/lactational ID show increased cellular dopamine in the prefrontal cortex (PFC) and striatum, reduced D2 dopamine receptor levels, and delayed development of dopamine related behaviors [44, 45], along with altered ratios of dopamine metabolites to dopamine, depending on brain region [46]. Rats that were only postnatally ID had reduced cellular dopamine levels in the PFC and striatum, accompanied by delays in ear development and motor tasks (bar holding, surface right reflex) [44]. Marginal ID induced during rat gestation/lactation

altered monoamine metabolism and transporter as well as receptor density before differences in brain iron concentration were even detectable. Sensorimotor tasks (bar holding, forelimb placing, grooming) were delayed in development and the rats displayed increased hesitancy in novel settings even after iron repletion, suggesting persistent dysfunctional dopamine metabolism in the hippocampus and striatum [47]. Thus, iron plays a key role in development of neurotransmitter tone, and perinatal ID may disrupt neurotransmitter ‘set points’ and density of related receptor and transporter proteins, resulting in persistent behavioral deficits.

2.5 Long-term neurobehavioral outcomes of ID

ID during infancy is linked to neurobehavioral problems as well as lasting deficits following iron therapy. Healthy children with IDA scored 6-15 points lower on intelligence tests, and performed more poorly in tests evaluating motor function and high-speed information processing [29]. IDA infants display starkly different socio-emotional behavior, and are judged as more wary, hesitant, solemn, and unhappy [29]. One study found lack of social interaction, social referencing, positive affect, and an inability to be soothed, as well as altered sleep patterns, in IDA infants [48]. In case-control studies, differences tended to persist after a full course of iron repletion therapy. Hemoglobin at 9 months of age correlated with IQ at 5 years of age [29], while another study in Yugoslavia found hemoglobin levels at 6 and 36 months indicated IQ at 4 years of age [49]. Formerly ID preschool children scored lower on IQ, perceptual speed, visual matching and motor integration, and gross and fine motor performance tests [29]. Low iron status at birth is indicative of impaired mental and psychomotor development – children in the lowest quartile of umbilical cord ferritin concentration performed more poorly on all cognitive tests and had significantly worse language ability, fine motor skills, and tractability scores compared to the two median quartiles [50]. At 4 years of age, formerly IDA children displayed starkly delayed auditory and visual response. Like ID infants, formerly ID children exhibit altered sleep architecture [51].

Demonstrating that later childhood outcome is affected by ID, Hurtado et al found anemia during infancy increased risk for placement into special education by 10 years of age [52]. Children aged 11-14 who suffered chronic, severe ID in infancy were more than twice as likely to have repeated a grade or been placed in special education compared to controls, and scored lower in mathematics and writing exams. According to parent and teacher assessments, these formerly ID children were assessed as having increased anxiety, depression, and attention issues compared to controls [35]. 10 year old, formerly IDA children exhibited slower reaction time and poorer inhibitory control in a go/no-go cognitive task [53]. Inhibitory control is a key component of cognition, and is associated with decision-making and addictive behavior. The performance of IDA children on the go/no-go task is consistent with deficits in myelination and PFC-striatal circuit function, wherein dopamine is the primary neurotransmitter. Echoing the previous study, 19 year olds who were IDA as infants performed poorly compared to controls on frontostriatal-mediated executive function tasks, including inhibitory control, set-shifting, and planning, along with a hippocampal dependent memory task [37]. An observational study focusing on formerly ID persons at 25 years of age evaluated education, employment, marital status, and physical and mental health. In each measure, a much higher proportion of the formerly ID group compared to controls did not complete high school and were not pursuing further education or training. The formerly ID group reported poorer emotional health, more negative emotions, increased feelings of dissociation/detachment, and were more than twice as likely to be single [36]. This study points to the possibility of a substantial loss of potential in adulthood stemming from severe early life ID. Recently, Algarin et al evaluated adults who were IDA as infants with fMRI, and found altered patterns of functional connectivity in brain networks involved with memory, social cognition, and self-referential processes [54]. Additionally, altered behavior stemming from ID may lead to

additional environmental insults that further affect development, i.e. inability of an ID infant to be soothed and elicit caregiver interaction may result in less maternal care and attention, increasing the burden of the direct effects of early life ID.

2.6 Parity between the porcine and human immune systems

Pigs are monogastric omnivores with an immune system that exhibits a great deal of anatomical and physiological similarity to the human immune system, thus providing an excellent translational model for inflammatory stimuli and infections [55, 56]. A comparison of the murine and porcine immune systems to the human immune system found pigs closely resembled humans on more than 80% of assessments, whereas mice exhibited close similarity to humans on less than 10% of the outcomes measured [56, 57]. Anatomically, the porcine thymus is similar to that of humans, and unlike the mouse, pigs have tonsils [55]. However, pig lymphoid tissue differs from the human at some points – porcine lymph nodes are inverted [55], pigs have ileal Peyer's patches, and pig placentation is epitheliochorial as opposed to human hemochorial placentation [55, 58].

Excepting differences in some host defense polypeptides, the proteins of the porcine immune system share considerable structural and functional similarities to their human homologs [58], and exhibits similarity in response dynamics [55]. The pig has ten different Toll-like receptors (TLRs), the same number found in humans. The mouse has 12 TLRs [56]. Porcine TLRs generally display more than 80% homology compared to human TLRs [59, 60]. Porcine TLR distribution is also more similar to humans. TLR7 and TLR9 are primarily expressed on porcine and human plasmacytoid dendritic cells, whereas both conventional and plasmacytoid dendritic cells express TLR7 and TLR9 in the mouse [61]. Pig have a direct equivalent to IL-8, while mice lack one [62]. While murine macrophages exhibit profound induction of iNOS following lipopolysaccharide (LPS) stimulation, porcine macrophages display a response more similar to human macrophages, with no evidence of NO production following LPS treatment [63].

As with humans, in pigs 50-70% of peripherally circulating leukocytes is composed of neutrophils [62]. Porcine T cells are quite similar to human T cells. Briefly, Th1 cells produce IL-2 and IFN- γ , activating both cytotoxic T cells and macrophages to perform immune effector functions. Th2 cells secrete cytokines to promote antibody production and wound repair. Th17 cells produce IL-17 and are involved in regulating a variety of immune functions. Regulatory T cells (Treg) perform immune modulation and maintain immune tolerance. Functional equivalents for all cytokines and cells involved in the human Th1/Th2/Th17/Treg axis have been characterized in the pig [58, 64].

2.7 Effects of early life inflammation and infection upon brain development

Immune activation during the perinatal period is associated with several neurological disorders, including autism, schizophrenia, and epilepsy [65-67]. This is of great concern, as acute respiratory infection is the leading cause of hospitalization for children <1 year old [68]. Inflammation in the vulnerable period of early life is known to increase risk for later CNS disorders and alter behavior in animal models as well [69, 70]. This is an indirect effect – pro-inflammatory signals produced by peripheral immune cells reach the brain via the blood or direct neural signaling via the vagus nerve, activating microglia, the resident neuroimmune cells [71], and increasing pro-inflammatory cytokine expression in the brain [72]. In addition to microglia, other glia and neurons are sensitive to cytokine signals, and inflammatory signaling is known to influence the mental state [73]. Early life microglial activation may result in increased phagocytosis of neural precursor cells [74, 75] and increased synaptic pruning [76].

Peripheral immune challenge may cause architectural, physiological, and neurochemical changes in the developing brain. Mice repeatedly administered LPS in the first week of life exhibited cerebellar hypoplasia, neuronal loss, gliosis in the brain parenchyma, and delayed myelination due to altered proliferation patterns of oligodendrocyte progenitors [77].

Inflammation reduced neurogenesis, but not cellular proliferation, in the adult rat hippocampus [78]. Postnatal infection reduced gray matter, altered white matter distribution, and reduced white matter in the primary visual cortex in the piglet [79]. Additionally, postnatal infection reduced neurogenesis by 24%, decreased progenitor cell survival in the hippocampus and altered granule cell morphology in the dentate gyrus [80], with reduced nerve growth factor (NGF) and myelin basic protein (MBP) expression in the striatum [81]. In the hippocampus, postnatal infection resulted in reduced expression of plasticity-associated genes and upregulated genes involved in the microglial sensome [82]. Infected piglets performed more poorly in a spatial learning task and displayed increased latency to choice. This was accompanied by a 43% increase in activated hippocampal microglia [81]. *Ex vivo*, microglia from postnatally infected piglets displayed increased phagocytic and chemotactic activity, along with increased TNF α production [82].

A set of studies by Harvey et al examining the effects of maternal ID and LPS administration upon pup outcome found additive effects, but no interaction. Pups from ID dams displayed delayed acquisition in forelimb grasp and startle reflex acquisition [83], along with increased metabolic activity in the ventral hippocampus [84]. Pups from LPS dams displayed early grip acquisition, altered acoustic startle response, reduced locomotor activity, and more social activity [83, 84].

2.8 Brain Plasticity

Synaptic plasticity, the changes in synaptic strength based on use or disuse, is a key process for brain function and adaptation across the lifespan. Long-term alterations in synaptic strength are typically enacted by structural as well as functional changes [85]. Presynaptic adaptations to change synaptic strength include modulating the release of neurotransmitters, while postsynaptic alterations include changing the density and type of neurotransmitter receptors and their connection to intracellular signaling networks [85]. Neurotrophic factors are key mediators in

synaptic and overall brain plasticity, regulating synaptogenesis and withdrawal, neurogenesis, myelination, dendritic arborization, neurotransmitter receptor dynamics, as well as somatic and local dendritic protein synthesis in neurons [85-89]. Long-term potentiation (LTP) is thought to be the cluster of cellular processes allowing learning to occur [85].

The neurotrophin family includes NGF, N3, N4, and brain-derived neurotrophic factor (BDNF). BDNF is expressed in multiple brain regions, and has pleiotropic effects depending upon cell type, brain region, and developmental stage [90]. First identified and isolated from pig brains by Barde et al in 1982 [91], BDNF is known to have effects upon LTP, receptor trafficking, and alteration of receptor and signaling molecule density [85], and is a key factor in proper brain development and maintenance of brain plasticity, modulating neurogenesis, synaptogenesis and dendrite arborization, and preventing apoptosis [85, 92]. Early life cognitive deficits, later cognitive decline, and neurodegenerative disorders are associated with impaired or reduced BDNF signaling, and with impaired synaptogenesis and neurogenesis [90, 93]. The expression of BDNF and its corresponding receptors, tropomyosin-related kinase B (TrkB) and p75, occur across the lifespan, though p75 expression in the CNS is extinguished following early development [92]. p75 is a member of the tumor necrosis factor receptor family.

2.9 BDNF formation and secretion

The proBDNF protein is generated in the endoplasmic reticulum. proBDNF is converted to mature BDNF (mBDNF) at several locations: the Golgi apparatus wherein it is packaged into vesicles with the PC1 enzyme, by tissue-type plasminogen activator activity, or by extracellular metalloproteinases following secretion [92]. BDNF secretion is largely activity-dependent – excitatory synaptic activity, neuropeptides, and neurohormones induce expression and transport to the axonal or dendritic terminal for release [94]. At the post-synapse, excitatory neurotransmitters bind AMPA and NMDA, inducing calcium influx, activation of CaMK, PKC, and MAPKs,

resulting in CREB and NF- κ B induction of BDNF expression [95]. It should be noted that *Bdnf* mRNA has been found in dendrites, where local translation occurs following appropriate stimuli [92].

BDNF binds to the TrkB and p75 receptors, with mBDNF having high affinity for TrkB and proBDNF having high affinity for p75. TrkB and p75 have different signaling mechanisms and downstream effects [96]. proBDNF largely has inverse effects compared to BDNF, resulting in axon and dendrite pruning [89]. Binding and activation of TrkB receptor by mBDNF dimerizes and autophosphorylates the receptor, leading to activation of the ERK, PI3K, and PLC- γ intracellular signaling pathways. These pathways modulate protein expression resulting in enhanced plasticity and cell survival, as well as metabolic processes, including enhanced glucose uptake, mitochondrial biogenesis, and activation of mTOR, the master regulator of protein synthesis [89, 90, 92]. Enhanced protein expression is required for cytoskeletal additions needed for extension of neurites.

Increased methylation of DNA promoters induces transcriptional repression, typically through the blocking action of methyl-CpG binding proteins. Chronic stress exposure causes hypermethylation of BDNF promoter IV [97, 98], reducing BDNF expression and indicating a potential mechanism by which early life stress exerts lasting effects on brain development and behavior. Indeed, fetal-neonatal ID rats display altered methylation at the BDNF-IV promoter along with altered histone methylations [99], resulting in reduced hippocampal BDNF expression along with reduced neurogenesis. TrkB receptor density was not increased to compensate for reduced BDNF concentration [100]. Formerly ID pigs have reduced BDNF in the ventral hippocampus and increased BDNF levels in the dorsal hippocampus, with reduced TrkB density

throughout the hippocampus [101]. Overall, little data exists examining the effects of ID upon BDNF.

2.10 Effects of BDNF upon Neuron Structure

BDNF has profound effects upon neuron structure, modulating neurite outgrowth and arborization via TrkB and retraction via p75 [85, 89]. BDNF directs axonal growth cones via local regulation of cytoskeletal protein translation [102]. Additionally, BDNF is a powerful regulator of neuronal protein synthesis, primarily through activation of mTOR via PI3K, thus increasing production of structural proteins related to synaptic strength (i.e. PSD95) and neurotransmitter-related proteins (i.e. AMPA subunits), thereby enhancing synaptic functionality [85]. Enhancement of cytoskeletal dynamics by BDNF signaling increases extension of dendritic and axonal filopodia, which are synaptic precursors and mature into dendritic spines and presynapses [89], promoting synaptogenesis and increasing spine density [90]. Reduced BDNF levels, as observed in animals exposed to high stress, is associated with decreased spine density and dendritic atrophy in the hippocampus and medial PFC [90], as well as hypertrophy of neurons in the amygdala [103]. The integral role of BDNF in neuronal health and synaptic dynamics, especially neurodevelopment, places BDNF as a key factor in the etiology of cognitive and affective disorders later in life [90].

2.11 Neurotransmission, and Cell Signaling

BDNF signaling modulates both excitatory and inhibitory neurotransmission at the pre- and post- synapse, altering receptor production and dynamics [104]. Mice with a knock-in at *Bdnf* promoter IV (BDNF-KIV), which drives activity-dependent BDNF expression, displayed impaired inhibitory transmission and reduced GABAergic neurons in the medial PFC [105], an area that plays an important role in regulating the amygdala. In the hippocampus, BDNF regulates glutamate release and promotes expression and externalization of both AMPA and NMDA

glutamate receptors [90]. NMDA activation is involved in the intracellular signaling cascade leading to induction of LTP. BDNF-KIV mice exhibit depressive behavior, inflexible learning, and reduced inhibition, with disturbed monoaminergic receptor gene expression in the PFC and hippocampus. In the PFC, BDNF-KIV leads to reduced expression of serotonin receptors 1b, 2a, 5b, and dopamine receptor 2, while tryptophan hydroxylase and dopamine receptor 4 are increased. Hippocampal serotonin 5b receptor expression was reduced [88]. Consolidation of fear extinction memory in the rat requires BDNF [106], and decreased BDNF amygdala levels are associated with increased anxiety-like behavior in rats [107].

Region-specific alterations of BDNF in the developing brain may imbalance the regulatory communication between different brain regions. The medial PFC, hippocampus, and amygdala neural circuitry regulates mood and affective behaviors [90]. BDNF modulation of neurogenesis and synaptic dynamics result in behavioral differences [90]. Altered PFC and hippocampal regulation of the amygdala may reduce organismal stress resilience and increase risk for anxiety and depression [108]. Conversely, BDNF injection into the medial PFC reduced a conditioned fear response, while hippocampal injection facilitated fear extinction learning [109]. Behavioral data suggest hippocampal-striatal communication is impaired by maternal/neonatal ID, which enhances stimulus-response (reward) learning at the expense of spatial information, signifying reduced hippocampal control of learning [110].

Glucocorticoids are steroid hormones that promote adaptation to stress. However, chronic glucocorticoid exposure leads to maladaptive measures in the brain, including neuronal death, reduced dendritic arborization, and impaired induction of LTP [85]. BDNF is typically reduced during stress while glucocorticoids are increased, and the two appear to have reciprocal effects upon brain plasticity, with BDNF promoting neurite outgrowth and dendritic arborization, while

glucocorticoids have the opposite effect and reduce LTP [111]. However, glucocorticoids and BDNF may have synergistic effects in responding to acute stress, which become dysregulated in chronic stress paradigms [112]. In acute stress, BDNF signaling may be increased by glucocorticoids, potentiating consolidation of long-term fear memories [113]. By the same token, low BDNF levels reduce glucocorticoid receptor (GR) sensitivity, resulting in glucocorticoid resistance. Arango-Lievano et al show that BDNF improves GR sensitivity, and coordinated action of BDNF and glucocorticoids is essential for continued neuroplasticity and appropriate adaptation during the stress response [114]. One study found perinatal ID is associated with increased corticosterone and altered GR receptor density in the hippocampus, which correlated with reduced BDNF and impaired spatial memory [115]. BDNF and glucocorticoid signaling are interrelated and their tandem activity may be important for effectively adapting to stress.

BDNF modulates neuronal energy metabolism by increasing metabolic substrate uptake and increasing mitochondrial biogenesis. TrkB binding activates mTOR through the PI3K pathway, increasing both GLUT3 and MCT2 production and externalization to the cell membrane, allowing increased uptake of glucose and lactate, respectively [92]. Additionally, BDNF increases activity of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), the master regulator of mitochondrial biogenesis and a driver of increased metabolic capacity. Increased metabolic capacity supports neuronal functions related to plasticity such as synaptogenesis. PGC-1 α knockdown reduced synapse count in mice and extinguished BDNF-mediated synaptogenesis in cultured hippocampal neurons [116].

2.12 Energy Metabolism and Immunometabolism

The metabolic state of a cell has profound implications for phenotype and longevity. In the case of immune cells, metabolism is inextricably linked with immune function; energy metabolism does not simply provide substrates and energy to the cell, but regulates gene expression,

polarization, and effector functions [117, 118]. A brief review of pertinent metabolic pathways is in order. Glycolysis is an anaerobic cytosolic process that yields pyruvate and produces little energy. However, the intermediate metabolic products of glycolysis can be diverted to support cellular anabolism and synthesis of various biomolecules, as with the Pentose Phosphate Pathway. Glycolysis is an essential part of rapid cellular expansion. The Krebs cycle (TCA cycle) occurs in the mitochondrion and produces NADH and FADH₂, reducing intermediates which ferry electrons to the electron transport chain (ETC) to drive oxidative phosphorylation. Carbohydrates, lipids, and protein all converge into the Krebs cycle for energy production: glucose is converted into pyruvate and enters the TCA cycle as acetyl-CoA, β -oxidation of fatty acids provide acetyl-CoA along with NADH and FADH₂, which are fed into the TCA cycle and ETC, respectively, and proteins are deaminated and enter the TCA cycle at various steps as metabolic intermediates. Anabolic reactions, such as fatty acid synthesis, rely on TCA cycle intermediates for substrate.

Glucose enters the cell via glucose transporters. There several types of glucose transporters (GLUTs) present in the brain. The most abundant isoform, GLUT3, is present throughout the neuropil, primarily on axons and dendrites, and its density is linked to local glucose demands [119]. GLUT1 is located in the blood-brain barrier and in astrocytes. GLUT2 is expressed by hypothalamic neurons and acts as a sensor of systemic glucose, similar to its role in the pancreas, but regulates appetite instead of insulin release. GLUT4, an insulin-regulated glucose transporter predominantly expressed by muscle and adipose tissue, is expressed in the hippocampus and amygdala, and is associated with hippocampus-dependent cognitive functions [119]. GLUT5, a fructose transporter, is exclusively expressed in microglia [119, 120], and is part of the microglial sensome [121], a family of receptors unique to microglia that sense endogenous and pathogen-related ligands [122].

Switching to a ‘glycolytic’ phenotype is necessary for macrophage immune cell polarization to an inflammatory state [118, 123, 124]. Experiments performed in the 1950s determined that inhibition of glycolysis impaired neutrophil respiratory burst and phagocytic activity [125, 126]. Upregulation of glycolysis directly increases expressions of inflammatory markers through regulatory activity of glycolytic enzymes [118]. Hexokinase (the initial step of glycolysis) promotes activation of the NLRP3 inflammasome [127]. GAPDH, which conditionally represses IFN- γ mRNA expression by binding to its promoter, is removed by increased glycolysis, allowing IFN- γ expression [128, 129]. GAPDH also binds TNF α mRNA and prevents its translation when glycolysis is low, but releases the mRNA for translation when glycolysis is increased [130]. Pyruvate kinase increases IL-1 β production via promotion of NLRP3 inflammasome activation [131]. In addition to direct promotion of the inflammatory response, glycolysis also rapidly furnishes ATP and biosynthetic precursors for the antimicrobial response [118].

In pro-inflammatory macrophages, the TCA cycle is blocked at two sections, leading to accumulation of succinate and citrate. Citrate is repurposed for fatty acid synthesis in order to provide substrate for prostaglandin, itaconate (an anti-microbial), and NO production [118]. Succinate is a key driver of the inflammatory shift, switching the ETC from energy production to ROS production, further polarizing the cell to an inflammatory phenotype [132]. This inhibition of oxidative phosphorylation functions as a roadblock, preventing inflammatory to anti-inflammatory polarization, suggesting that the appearance of anti-inflammatory macrophages in a previously inflamed area is due to recruitment and polarization of new cells, rather than shifting of pro-inflammatory cells to an anti-inflammatory phenotype [133]. This roadblock functions due to the reliance of anti-inflammatory macrophages upon oxidative phosphorylation. Oxidative

phosphorylation and fatty acid oxidation are associated with non-inflammatory immune cells that survive longer, including T_{reg} and memory T cells and anti-inflammatory macrophages. Oxidative metabolism generates intermediates necessary for maintaining the anti-inflammatory phenotype [118].

Ultimately, macrophages must meet wildly disparate phenotypic demands and thus have high metabolic flexibility to maintain phenotypic plasticity. Broadly, M1 cells rely on glycolysis to fuel rapid response and destruction of pathogens, whereas M2 macrophages use β -oxidation and oxidative phosphorylation for long-term energy production [118, 123]. As the signaling cascades for inflammation and metabolism are so thoroughly interlinked, metabolic phenotype reflects the inflammatory phenotype - increased glycolysis promotes an inflammatory phenotype, and oxidative phosphorylation promotes alternative activation. However, it is important to note that much immunometabolism work has been done *in vitro*, and recent advances in the field indicate that metabolic phenotype, like inflammatory phenotype, consists of many points on a continuum, rather than two extremes [124].

2.13 Conclusions

ID is the most common micronutrient deficiency in the world, and the mounting evidence linking early life ID to cognitive deficits stress the need for studies to improve understanding of the molecular effects of early life ID upon brain development, the mechanisms by which these effects occur, and how some deficits persist into adulthood. Understanding these effects in the context of infection, a likely condition for chronically ID infants, is crucial to advance research to the point of developing therapeutics to serve as adjunct interventions alongside iron repletion therapy to reduce the long-term deficits associated with ID, and not just cure the acute ID.

Chapter 3. Postnatal iron deficiency alters brain development in piglets¹

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3.1 Abstract

Background: Cognitive deficits associated with postnatal iron deficiency (ID) suggest abnormal brain development, but little is known in animals with a gyrencephalic brain.

Objective: The objective was to assess the impact of ID on brain development in piglets.

Methods: Male and female Yorkshire piglets were reared from postnatal day (PD) 2 until PD 29 or 30 using milk replacer adequate (CON) or deficient (100 vs. 10 mg/kg) in iron and subjected to magnetic resonance imaging (MRI) to assess brain macrostructure, microstructure, and metabolites in the dorsal hippocampi and intervening space. After MRI, brains were collected for histology. Hematocrit, hemoglobin, and liver iron were measured to determine iron status.

Results: Hematocrit and hemoglobin in ID piglets were less than CON after PD 14 ($P < 0.001$), and at the study end liver iron in ID piglets was less than CON ($P < 0.001$). Brain region volumes were not affected by ID, but changes in brain composition were evident. ID piglets had less white matter in 78,305 voxels, with large clusters in the hippocampus and cortex. ID piglets had less gray matter in 13,625 voxels primarily in cortical areas, and more gray matter in 28,017 voxels, most notably in olfactory bulbs and hippocampus. The major effect of ID on white matter was supported by lower fractional anisotropy values in the corpus callosum (0.300 vs. 0.284, $P = 0.006$)

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and in whole brain white matter (0.313 vs. 0.307, $P = 0.002$) in ID piglets. In coronal brain sections, corpus callosum width was less ($P = 0.043$) in ID piglets. Inositol was lower ($P = 0.01$) and phosphocholine was higher ($P = 0.03$) in hippocampus of ID piglets.

Conclusions: Postnatal ID in piglets affects brain development, especially white matter. If the effects of ID persist, it might explain the lasting detrimental effects on cognition.

3.2 Introduction

Iron deficiency (ID)⁴ is the most common micronutrient deficiency in the world, affecting approximately 2 billion people in both developing and industrialized nations [134]. ID affects all age groups, but children age 0 to 5 y are most vulnerable due to the rapid brain growth and development that occurs during the first year of life [135]. During this early sensitive period iron is needed for neurodevelopmental processes, including energy metabolism, neurotransmission, and myelination [29, 135-137]. Disrupting neurodevelopment in the neonate can result in cognitive impairment and behavioral problems that endure for many years, if not a lifetime [35]. Young adults that experienced ID as infants had poorer executive function, pattern recognition, and spatial memory [37]. Furthermore, children age 10 y had slower reaction times and impaired inhibitory control if they experienced IDA during infancy [53]. Early life ID has been linked to poorer math and writing abilities, increased hesitancy and anxiety, reduced attention and planning ability, increased visual and auditory latencies, reduced language skills, and reduced motor skills [29, 136]. Many outcomes related to ID suggest white matter development is vulnerable. Oligodendrocytes synthesize and maintain myelin in the central nervous system [134] and are the principle cells in the brain that stain for iron under normal conditions [27]. Myelination is a metabolically intensive process, and synthesis and maintenance of myelin requires substantial energy turnover by the oligodendrocytes [138]. Iron is a key cofactor for production of energy and substrates such as fatty acids needed for myelination [27]. Most research in this area has used rodent models. Myelination in the lissencephalic rat and mouse brain occurs postnatally. However, myelination in the gyrencephalic human brain initiates before birth and continues into the postnatal period [139]. As such, the effects of postnatal ID on development of the human brain are not fully understood.

The present study investigated the effects of postnatal ID on brain development in a neonatal piglet model. The piglet has a gyrencephalic brain with similar growth and myelination patterns to humans [140, 141]. A recent study reported poor performance in a hippocampal-dependent spatial T-maze task and lower iron concentration in the hippocampus when piglets were fed an ID diet from PD 2-28 [142]. No effects of ID on whole brain and hippocampal volume in piglets were found by MRI [142], but sophisticated tools for assessing brain macrostructure, microstructure, and metabolites in piglets by MRI were not available at the time. Therefore, in the present study we used a newly developed MRI toolset for piglets [143] to assess the impact of ID on gray and white matter volume in discrete brain areas, white matter integrity, and several metabolites indicative of neural and glial health. We report substantial developmental alterations due to ID.

3.3 Methods

Animals, housing, and feeding

Twenty naturally farrowed domestic Yorkshire piglets (12 males, 8 females) from 3 litters were obtained from the University of Illinois swine herd and assigned to either the control diet (CON) or the ID diet controlling for litter of origin, body weight, and sex. Piglets assigned to CON received an intramuscular iron dextran injection (200 mg, Uniferon), shortly after birth (standard swine industry practice to ward off ID). Piglets remained with the sow and littermates for 24-48h to receive colostrum and then were delivered to the biomedical animal facility. As sow milk has a low iron concentration ($\sim 1.79 \mu\text{mol/mL}$), it was reasoned that intake of colostrum would have minimal effects on piglet iron status [144]. Piglets were individually housed in cages as previously described, and were provided a toy (plastic Jingle-Ball; Bio-Serv) and towel for environmental enrichment [81]. A 12h light/dark cycle was maintained with light from 0700 to 1900h. Animals were weighed each morning. All animal care and experimental procedures were performed in

accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Diets

Powdered sow milk replacer diets were formulated at Test Diet (Richmond, IN) to the same specifications used previously [142]. Piglets were assigned to either the CON diet (100 mg Fe/kg solids) or ID diet (10 mg Fe/kg solids) controlling for body weight, sex, and litter of origin; besides iron, all diets met the NRC recommended requirements [145]. Piglets were fed 4 times daily for a total of 300 mL/kg body weight each day as described previously [142]. Piglets consumed their entire meal, though by the end of study several ID piglets took longer to finish their meals. No extra water was provided.

Iron Status Assessment

Blood samples were taken from the jugular vein weekly starting at PD 7 and used to assess hematocrit and hemoglobin as described previously [142]. The criterion for anemia in swine is hemoglobin less than 8 g/dL [145]. To assess iron reserves at the end of the study (PD 29 or 30), piglets were intracardially perfused with PBS to flush blood from vasculature, 1 g of liver tissue was taken and iron concentration was analyzed in wet tissue via inductively coupled plasma mass spectrometry as previously described [142].

Magnetic Resonance Imaging

On PD 29 or 30, piglets were anesthetized using a telazol:ketamine:xylazine solution [50 mg tiletamine plus 50 mg of zolazepam reconstituted with 2.5 mL ketamine (100 g/L) and 2.5 mL xylazine (100 g/L); Fort Dodge Animal Health] and anesthesia was maintained via inhalation of isoflurane (98% oxygen/2% isoflurane). After the piglet was fully anesthetized, it was restrained

to prevent movement and placed in the MRI scanner. A MRI-compatible pulse oximeter was used to monitor piglet vital signs during the MRI procedure.

A Siemens MAGNETOM Trio 3T imager with 32-channel head coil was used to conduct all MRI (Siemens, Erlangen, Germany). For macrostructural analysis, anatomical data were acquired via a T1-weighted, magnetization-prepared, rapid gradient-echo (T1 MPRAGE) sequence using the following parameters: repetition time, TR = 1900 ms; echo time, TE = 2.49 ms; inversion time, TI = 900 ms; flip angle = 9°, and slice thickness = 0.7 mm. A final voxel size of 0.7 mm³ isotropic was used across the entire head from the snout to the cervical/thoracic spinal cord joint as described previously [140]. All methods utilized for brain region volume estimation and voxel-based morphometry (VBM) analysis were performed as previously described by Radlowski et al [146] and Conrad et al [79, 143]. Clusters consisting of less than 20 edge-connected clusters were not considered for analysis. Corresponding anatomical regions were assigned to voxel clusters of significant difference using a digital piglet brain atlas [143]. Cortical regions of significant voxel clusters were estimated using a brain atlas for adult pigs [147].

Microstructural data were acquired using diffusion tensor imaging (DTI) as previously described [148]. Briefly, the following parameters were used: repetition time, TR = 5000 ms; echo time, TE = 91 ms; averages = 3; diffusion weightings = 2; b-value of 1000 s/mm². Forty slices 2.00 mm thick were collected utilizing a matrix size of 100 × 100 and a final voxel size of 2.0 mm isotropic throughout the brain. Analysis was performed using the diffusion toolbox in the FSL software package as described previously [146].

Brain metabolites were measured via Magnetic Resonance Spectroscopy (MRS) as previously described [146]. A single 12 × 25 × 12 mm voxel was placed across the left and right dorsal horns of the hippocampi, including the intervening space consisting of non-hippocampal

tissue. A spin echo chemical shift sequence was performed using the following parameters: echo time, TE = 30 ms; repetition time, TR = 3000; 128 averages; FOV (field of view) = 200 mm. Water-suppressed and non-water suppressed data were collected. All MRS data were analyzed using the LC Model 6.3 fitting program [146].

Tissue Preparation

After MRI, anesthesia was maintained and piglets were euthanized by intracardial perfusion with PBS (phosphate-buffered saline) followed by perfusion with 4% paraformaldehyde (PFA)/PBS solution. Brains were removed, weighed, and stored in 4% PFA for two months. Following analysis of MRI data, the corpus callosum was selected for further evaluation and a tissue block containing the posterior half of the corpus callosum and surrounding tissue was dissected from the right hemisphere of each brain. Tissue was sunk in 30% sucrose/PBS solution for cryoprotection. Tissue was then placed in a plastic 'boat,' coated with optimal cutting temperature (OCT) medium, and frozen in an acetone bath cooled with dry ice. The resulting block was sectioned at 30 μ m and stored in cryoprotectant at -20° C until staining.

Immunohistochemical Staining

Sections were stained while free-floating. After being initially washed in tris-buffered saline (TBS), sections were placed in 2x sodium citrate solution (SSC) at 70°C for 2 hours and rinsed in 2x SSC for 15 minutes, then rinsed in TBS. Sections were blocked in 4% normal goat serum (NGS), 0.3% Triton X-100 TBS solution for 1 hour at room temperature before being placed in antibody solution (TBS, 3% NGS, 0.3% Triton X-100, 1:1000 rat anti-Myelin Basic Protein [MBP], [Abcam #ab7349]), incubated at 4°C overnight, and rinsed in TBS before incubation in secondary antibody solution (3% NGS, 0.3% Triton X-100, 1:1000 goat anti-rat IgG [Jackson ImmunoResearch Laboratories], and 3% NGS) for 1 hour. With 10 minutes remaining in secondary

antibody staining period, NucBlue Fixed Cell Stain ReadyProbe Reagent (DAPI stain) was added according to manufacturer instructions. Tissue was then washed 3x in TBS and mounted with ProLong Gold Antifade medium. Tissues were imaged using a Zeiss LSM 700 Confocal microscope with Imaris imaging software. Nine tissue slices were imaged per pig, and 3 z-stacks were taken in the corpus callosum of each slice, providing 27 z-stacks per pig for analysis. Z-stacks were taken beginning when the first slice signal was visible to where the last slice signal was visible. Z-stacks were then cut in half, and the 'top' half (closer to the microscope aperture) was collapsed into a maximum intensity projection. Axiovision software was used to semi-automatically quantify fluorescing area and intensity in each image, with the base threshold set from the highest image in the dataset and applied to all images. To demonstrate antibody specificity, western blotting was done using the MBP antibody with purified porcine MBP purchased from Sigma (cat. # SRP5204) and porcine brain protein lysates. The purified porcine MBP from Sigma contained only the 21.5 kDa MBP isotype. Western blotting revealed 3 bands with molecular weights between 17 and 23 kDa (kilodaltons), in porcine brain lysates, one of which corresponds to the single band seen in the column loaded with 2 μ g purified MBP with the predicted molecular weight. Other bands present in the porcine brain lysate column is consistent with the four forms of MBP present in the pig central nervous system (**Supplemental Figure 3.1**). No other bands were visible in porcine brain lysates. When lower amounts of purified MBP were loaded, no bands were visible.

Black-Gold Staining

Black-Gold II staining was used to detect myelin and performed according to kit instructions (Millipore). Tissue slices 30 microns thick were mounted on slides and dried. Tissue was rehydrated for 2 minutes in MilliQ water and placed in 0.3% Black-Gold II solution preheated

to 60°C. After incubation for 12 minutes, slides were checked for sufficient staining and returned to Black-Gold II solution for 2 minute intervals if needed. Slides were rinsed in MilliQ water and fixed in 1% sodium thiosulfate for 3 minutes at 60°C, rinsed in MilliQ water, dehydrated in graded ethanol solutions and mounted. Slides were visualized using the Hamamatsu Nanozoomer Digital Pathology System to capture a single layer at 40x. Hamamatsu NDP.view2 software was used for analysis and quantification of tissue. Measurements of the corpus callosum were taken in reference to the ventral anterior cingulate cortex as a landmark.

Statistical Analysis

Sex did not affect body weight or iron status so data from males and females were pooled by treatment for final analysis. Body weight was analyzed via a 2-factor (treatment $\times$ time) repeated-measures ANOVA with Bonferroni post hoc tests using GraphPad Prism 5. Hemoglobin and hematocrit were analyzed using the MIXED procedure of the SAS software package (SAS Institute). Liver iron concentration, gross brain region volumes, DTI, MRS, IHC, and Black-Gold II data were analyzed using a two-sided t-test within GraphPad Prism 5. Significance was accepted at $\alpha=0.05$. Data are presented as means $\pm$ SEM.

VBM statistical analysis was performed using a t-test comparing ID and CON groups with no covariates and a false discovery rate (FDR) corrected alpha of 0.05 instead of an uncorrected alpha of 0.01 in order to provide more rigorous statistical validation. The statistical non-parametric methods toolbox was used for VBM analysis [149]. Global normalization was performed with an ANCOVA. Pseudo-t statistic maps were generated to visualize regional differences in gray or white matter volume between ID and CON groups. To consider a cluster significant, a threshold of at least 20 contiguous voxels was set.

Ten piglets per treatment group were studied. For some measurements n is <10 due to missing blood samples or motion artifacts during MRI. For histology, a subgroup from each treatment was selected for analysis ($n = 6-7$).

3.4 Results

Body Weight and Iron Status

Piglet body weight was affected by time ($P < 0.001$) and a diet $\times$ time interaction ($P < 0.001$) with ID piglets weighing 0.68 kg and 1.02 kg less than CON piglets on PD 27 ($P < 0.05$) and 28 ($P < 0.001$), respectively (**Supplemental Figure 3.2**). Piglet hematocrit was affected by diet ($P < 0.001$), time ($P < 0.001$), and a diet $\times$ time interaction ($P < 0.001$). Hematocrit in ID piglets decreased during the study and was lower than CON on PD 7 ($P = 0.002$), 14, 21, and at the end of study (PD29 or 30; $P < 0.001$; **Figure 3.1A**). Piglet blood hemoglobin was affected by diet ($P < 0.001$), time ($P = 0.003$), and a diet $\times$ time interaction ($P < 0.001$). Blood hemoglobin in ID piglets was lower than CON on PD 14, 21, and the end of the study ($P < 0.001$); **Figure 3.1B**). At the end of the study, liver iron concentration was markedly lower in piglets provided the ID diet ($P < 0.001$; **Figure 3.1C**). Collectively, these data show that iron status was significantly and progressively reduced by the ID diet.

Magnetic Resonance Imaging

Brain region volumes did not differ between ID and CON piglets as measured by MRI (**Supplemental Table 3.1**), nor did brain weights taken immediately after sacrifice (data not shown). VBM, however, revealed widespread changes in brain composition. **Figure 3.2** uses a color scale on piglet brain atlas images to highlight all brain areas where ID piglets had significantly less white matter compared to CON piglets ($P < 0.05$ FDR). Areas of greatest difference are shown in red and lesser but still significant areas are shown in blue. Areas with less white matter in ID piglets map to six distinct centers (**Table 3.1**). A large cluster of 78,024 voxels

representing reduced white matter extended through much of the central portion of the brain, with centers of high significance in the left cortex, right cortex, and left hippocampus. Additionally, clusters of voxels with less white matter volume were found in the cerebellum and left insular cortex of ID piglets. There were no brain areas with more white matter in ID piglets compared to CON.

Iron deficiency also resulted in alterations in gray matter volume compared to CON piglets, with 14 distinct centers with less gray matter as determined by VBM (Table 3.1). Gray matter volume was less primarily in the occipital lobe, with smaller clusters of voxels with less gray matter volume in the prefrontal cortex, thalamus, and areas of the cortex (Table 3.1, **Supplemental Figure 3.3**). There were 16 centers with more gray matter (Table 3.1, **Supplemental Figure 3.4**), with the primary areas of difference located in the olfactory bulbs and hippocampus (**Figure 3.3**). Of the total voxels affected by ID, 65% represented less white matter, 12% represented less gray matter, and 23% signified more gray matter. Collectively, these data indicate ID preferentially reduced white matter.

White matter tract development was evaluated by DTI. Globally, fractional anisotropy (FA), a marker of white matter microstructural integrity and health, was lower in the white matter of ID piglets compared to CON piglets ($P = 0.002$; **Figure 3.4**). Corpus callosum FA was lower in ID piglets compared to CON piglets ($P = 0.006$; Figure 3.4). Consistent with reduced FA values, radial diffusivity, the sum of the two minor eigenvectors of diffusion, was higher in the left hippocampus ($P < 0.001$), right hippocampus ($P = 0.01$), and thalamus of ID piglets ($P = 0.003$; **Figure 3.5A**); no other brain regions differed significantly in radial diffusivity. Mean diffusivity values were greater in the left hippocampus ($P < 0.001$), right hippocampus ($P = 0.009$), and thalamus ($P = 0.002$; Figure 3.5B) in ID piglets compared to CON piglets; no other brain regions

differed significantly in mean diffusivity. Axial diffusivity, the major eigenvector of water diffusion, was higher in left hippocampus ($P < 0.001$), right hippocampus ($P = 0.011$), and thalamus ($P = 0.004$; Figure 3.5C) of ID piglets; no other brain regions differed significantly in axial diffusivity. These data further highlight the detrimental effects of ID on white matter development and integrity.

Absolute metabolite concentrations in the dorsal horns of the hippocampi and intervening space were analyzed via MRS (**Table 3.2**). Phosphocholine was significantly higher in ID piglets compared to CON piglets ($P = 0.035$). Inositol, an osmolyte and astrocyte marker, was significantly lower in ID piglets compared to CON piglets ($P = 0.01$). Glutamate, N-acetyl-aspartate, creatine, and phosphocreatine did not differ significantly between the two groups.

Histology

Immunohistochemistry was used to evaluate MBP content in the corpus callosum. Mean intensity, total intensity, and total area were evaluated; no significant differences were found between the two groups (data not shown). Based on findings from the MRI data, the corpus callosum was selected for further histological analysis. To assess the thickness of the corpus callosum, sections were stained with Black-Gold II (**Figure 3.6A**). Consistent with less white matter, thickness of the corpus callosum was lower in ID piglets compared to CON piglets ($P = 0.043$; **Figure 3.6B**). No differences were found in mean intensity between the two groups (data not shown).

3.5 Discussion

The present study evaluated the effects of ID on neurodevelopment using MRI and histology. Clinical criteria for anemia in swine including hematocrit and hemoglobin were reached by the end of the study [145], and were consistent with previous studies [142]. VBM demonstrated

ID caused widespread reduction in white matter volume. ID also altered gray matter development, resulting in areas with both more and less gray matter volume compared to CON, a logical outcome as no significant difference in overall brain volume was observed. Thus, similar to what was reported previously using MRI with piglets [142], brain development in terms of overall volume or weight is at least partially protected during ID. However, the more detailed data provided by the VBM toolset found brain composition to be profoundly altered. Additionally, DTI revealed lower fractional anisotropy, the gold standard for assessing myelin integrity and health, in both the corpus callosum and globally across white matter in ID piglets. Together, these findings show ID alters brain composition and white matter development.

Notably, hippocampal white matter volume was less in ID piglets, which may play a role in the impaired learning and memory observed with this model [142]. In the study by Rytych et al. [142], no difference was found in hippocampal volume between ID piglets and CON. However, VBM for piglets was not available at that time. Using the VBM tool newly adapted to the piglet [143], we were able to detect large changes in white matter and gray matter across several brain regions including the hippocampus. One limitation of this study was the reporting ability of the SnPM toolbox. Because areas with less white matter were widespread and overlapping, the ability to resolve specific brain areas within a cluster with the SnPM toolbox was limited. For example, a large single cluster of 78,024 voxels with less white matter volume encompassed several brain areas (Table 3.1). Manual inspection shows the cluster encompasses portions of the internal capsule, hippocampus, fornix, thalamus, and midbrain. Early life ID is associated with reduced display of emotions and increased anxiety, which may be affected by alterations in white matter development in these areas [150]. Gray matter was lower primarily in the occipital lobe, indicating visual cortex development may be affected by ID.

One of the primary functions of the thalamus is to act as a ‘switchboard’ for incoming sensory information and the appropriate cortices. The lower thalamic white matter (Figure 3.2), in context with the aforementioned lower white matter fractional anisotropy, may play a role in the increased visual and auditory latencies seen in children that have experienced early life ID [151]. Iron deficiency reduced FA in the corpus callosum, the largest white matter structure in the brain, which may contribute to impaired interhemispheric communication. Importantly, impairment of the corpus callosum microstructure correlates with decreased cognitive function [152]. While the thalamus and hippocampi of ID piglets did not significantly differ from CON piglets in terms of fractional anisotropy, radial diffusivity, a measure of the minor diffusion eigenvectors, was greater in ID piglet hippocampi. Greater radial diffusivity is associated with reduced myelination, which allows increased diffusion perpendicular to the primary direction of flow – in this case, the axon. Mean diffusivity, a measure of total diffusivity, was also greater, suggesting less structural organization or cellularity in the ID hippocampus and thalamus. Alternatively, early ID in the piglet may result in greater diffusivity due to reduced dendritic arborization in the hippocampus similar to findings by Jorgenson et al using the rat model [39].

Inositol and phosphocholine were significantly altered by ID. Inositol is an osmolyte and is also used as an astrocyte marker. That it was lower may indicate decreased astrocyte activity in the hippocampus due to ID. Higher phosphocholine in ID piglets may indicate altered myelination patterns, as phosphocholine readings are increased in cases of myelin damage [153].

After MRI, brain tissue was collected and preserved, allowing MRI data to be compared to histology. This approach is potentially important for interpreting MRI data from human infants that experience ID. After analysis, no differences were found by IHC evaluation of corpus callosum MBP. Based on the MRI data, we expected to find reduced MBP staining in the corpus

callosum. It is possible that reduced myelination resulted in increased exposure of the MBP epitope and thus confounded the results. Following this observation, the histological myelin stain Black-Gold II was utilized for a larger-scale examination of myelination in the brain. Width of the corpus callosum was found to be lower in ID piglets, further supporting DTI findings that corpus callosum development is altered by postnatal ID. It must be considered that the gross volume of the corpus callosum was not found to differ between the two treatments via MRI. However, these measurements were taken under two different conditions – one *in vivo*, and one *ex vivo*, following fixation, freezing, adherence to a slide, and dehydration. This emphasizes the importance of using multiple approaches for quantification when possible.

Although the present study did not go beyond PD 30 or involve an iron repletion phase, a recent study using a similar piglet model reported long-lasting cognitive deficits due to early postnatal ID [154]. Several studies in rodents provide insight into the underlying mechanisms. For example, Felt et al. found rats that were IDA as pups exhibited impaired performance in both striatal and hippocampal centered tasks as adults, even after normalization of iron status [155]. Others showed ID in the prenatal period affected axonal maturation and dendritic complexity in the adult hippocampus [156]. Iron is a cofactor for neurotransmitter synthesis, and early life ID resulted in altered monoamine metabolism and dopamine receptor (D₂) density in rats [47, 157]. Furthermore, rats that experienced early life ID had less subcortical myelination, despite being fed an iron sufficient diet after weaning [158]. Altered neuronal structure, myelination, and changes in neurotransmitter tone may contribute to acute [142] and chronic [154] behavioral deficits observed in piglets exposed to ID but this has not been investigated.

The markedly higher gray matter volume in the olfactory bulb of ID piglets was an unexpected finding, although there is some evidence metals may interact with olfaction. A study

of professional welders determined occupational metal exposure is associated with olfactory dysfunction [159]. Further, rats fed an ID diet after weaning displayed altered olfactory behavior, including prolonged exploratory time towards attractive odorants compared to controls [160]. Further studies in piglets are warranted given the dramatic effects of ID on the olfactory bulb tissue composition and the importance of olfaction to pig behavior.

The current study in neonatal piglets modeled alterations in brain development caused by early life ID from birth to 4-6 months of age in the human infant [140]. As the piglet is born with low iron but quickly becomes deficient unless supplemented, this models the situation wherein an infant is born with reduced iron stores and becomes deficient in the ensuing months of early childhood, a pressing concern, as the human infant is unable to effectively regulate iron absorption until approximately 9 months of age [161]. These results in the piglet suggest brain development, particularly white matter development, is profoundly altered by ID. More research is needed to develop effective therapies for iron repletion and recuperation of developmental losses.

3.6 Acknowledgments

B.J.L. and R.W.J. designed research; B.J.L. conducted research; A.T.M. and A.J.S. provided essential materials; B.L. and E.C.R. analyzed data; and B.L. and R.W.J. wrote the paper. R.W.J. had primary responsibility for final content. All authors read and approved the final manuscript.

3.7 Tables and Figures

Table 3.1: Brain areas differing in gray or white matter volume between CON and ID piglets as shown by voxel-based morphometry analysis ¹

Tissue Type	Comparison	Anatomic Region	Cluster information			Local Maxima Coordinates		
			Voxels, <i>n</i>	FDR-corrected <i>P</i> -value	Pseudo- <i>t</i>	x	y	z
White Matter	ID < CON	Pyriform Cortex	78024	0.002	8.52	-11	9	-6
		Pyriform Cortex	-	0.003	8.01	12	10	-6
		Left Hippocampus	-	0.002	7.54	-12	-6	0
		Cerebellum	64	0.037	2.48	1	-15	-8
		Insular Cortex	184	0.044	1.38	-8	36	2
		Pons	33	0.047	0.85	7	-8	-16
	ID > CON	No significant differences						
Gray Matter	ID < CON	Dorsal posterior cingulate cortex	10690	0.007	10.9	1	-15	12
		Parahippocampal cortex	-	0.007	9.7	8	-19	7
		Parahippocampal cortex	-	0.007	9.36	-6	-18	8
		Dorsal anterior cingulate cortex	176	0.025	4.89	1	29	9
		Thalamus	224	0.04	4.29	0	8	-1
		Insular Cortex	170	0.046	4.02	14	28	9
		Insular Cortex	216	0.033	3.91	17	13	14
		Insular Cortex	188	0.048	3.82	15	20	10
		Premotor Cortex	664	0.033	3.74	1	17	18
		Fusiform Gyrus	201	0.04	3.61	17	-9	-2
		Auditory Cortex	517	0.007	3.29	-21	1	11
		Insular Cortex	554	0.046	2.97	18	16	8
		Insular Cortex	-	0.042	2.82	14	27	6
		Amygdala	25	0.04	2.47	-15	10	3
	ID > CON	Right Olfactory Bulb	11486	0.009	10.9	6	27	-8
		Left Olfactory Bulb	-	0.009	10.8	-4	27	-8
		Left Olfactory Bulb	-	0.016	6.9	5	36	-5
		Left Hippocampus	15262	0.009	7.6	-8	-3	8
		Right Hippocampus	-	0.013	6.31	10	-3	7
		Medulla	-	0.026	6.1	0	-25	-18
		Primary Visual Cortex	100	0.026	3.87	-7	-4	19
		Fusiform Gyrus	136	0.046	3.81	13	-12	9
		Perirhinal Cortex	116	0.021	3.53	5	-9	9
		Anterior Entorhinal Cortex	208	0.024	3.17	15	3	-4

Table 3.1, continued

Ventral Anterior Cingulate Cortex	562	0.047	3.13	2	21	8
Ventral Anterior Cingulate Cortex	-	0.043	2.77	3	12	13
Insular Cortex	-	0.048	0.94	10	16	13
Perirhinal Cortex	59	0.024	2.98	-5	-10	8
Premotor Cortex	60	0.033	2.66	-4	5	19
Primary Visual Cortex	28	0.33	2.66	6	1	20

¹ Data from non-parametric permutation tests presented as n voxels, FDR-corrected P -value, and pseudo-t value with a threshold at $P < 0.05$ FDR corrected and a minimum cluster size of 20 voxels are shown. The analysis is based on 10 piglets for each treatment ($n = 10$). Comparison column lists the directionality of comparison, i.e. the first set of comparisons describes areas where ID piglet brains had less white matter than CON brains. “Cluster size” denotes the number of voxels composing an area of significant difference. Effects of ID were widespread, often resulting in multiple overlapping clusters of voxels that read as a single combined cluster volume. In such instances the volume of the cluster is displayed once with other significant centers in the cluster listed with a “-” symbol. “Local maxima coordinates” correspond to a digital piglet brain atlas used for analysis [143]. CON, control; FDR, false discovery rate; ID, iron deficient; VBM, voxel-based morphometry.

Table 3.2: Comparison of dorsal hippocampal metabolites between CON and ID piglets via magnetic resonance spectroscopy¹

Metabolite	CON	ID	<i>P</i> -value	Functional Importance
Glu	5.22 ± 0.14	5.34 ± 0.23	0.68	Excitatory Neurotransmitter
PCh	1.49 ± 0.03	1.62 ± 0.03	0.03	Cell membrane/myelin marker
Inositol	9.14 ± 0.26	8.18 ± 0.21	0.01	Glial Cell Marker, Osmolyte
NAA	4.93 ± 0.14	4.89 ± 0.09	0.80	Neuronal Marker
Cr+PCr	4.05 ± 0.06	3.88 ± 0.08	0.12	Metabolic Activity

¹ Data are presented in absolute units as means ± SEM, *n* = 9-10. Phosphocholine was increased in ID hippocampi compared to controls while inositol was reduced. CON, control; Cr, creatine; Glu, Glutamate; ID, iron deficient; MRS, magnetic resonance spectroscopy; NAA, N-acetylaspartic acid; PCh, phosphocholine; PCr, phosphocreatine.

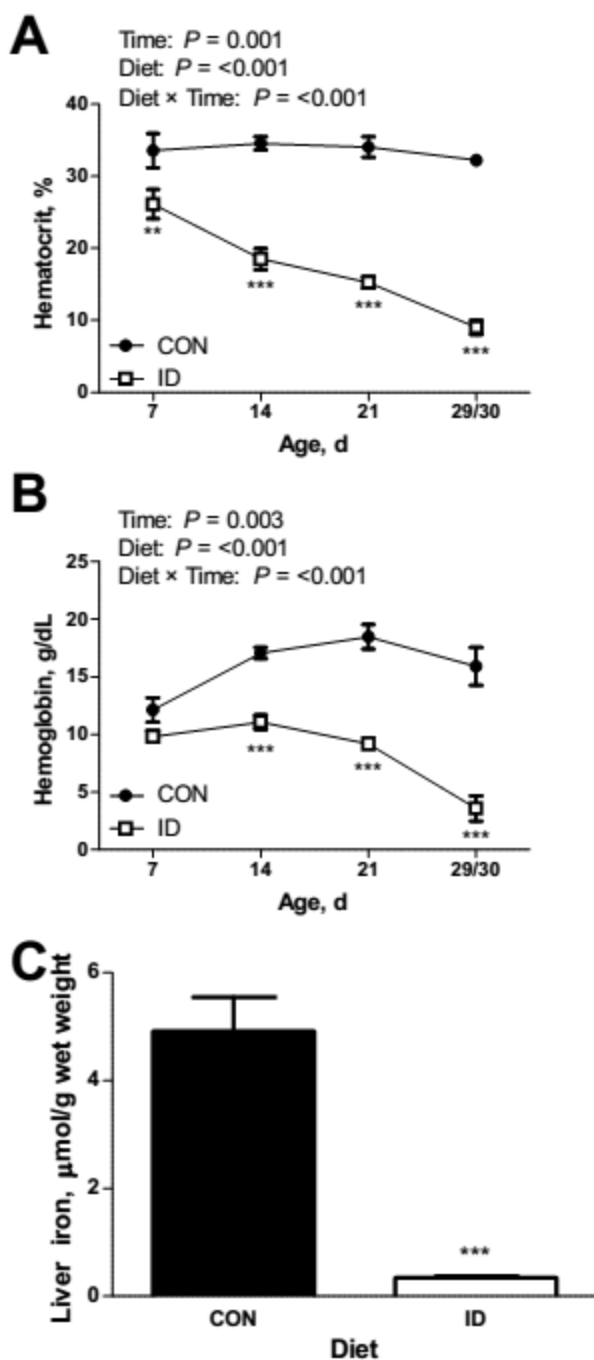


Figure 3.1: Hematocrit (A) and hemoglobin (B) concentration and liver iron of piglets fed ID or CON diet from PD 2 through PD 29 or 30. Values are means $\pm$ SEM ($n = 3-10$ due to missing samples). Asterisks indicate different from CON at that time: ** $P < 0.01$; *** $P < 0.001$. CON, control; d, day; ID, iron deficient.

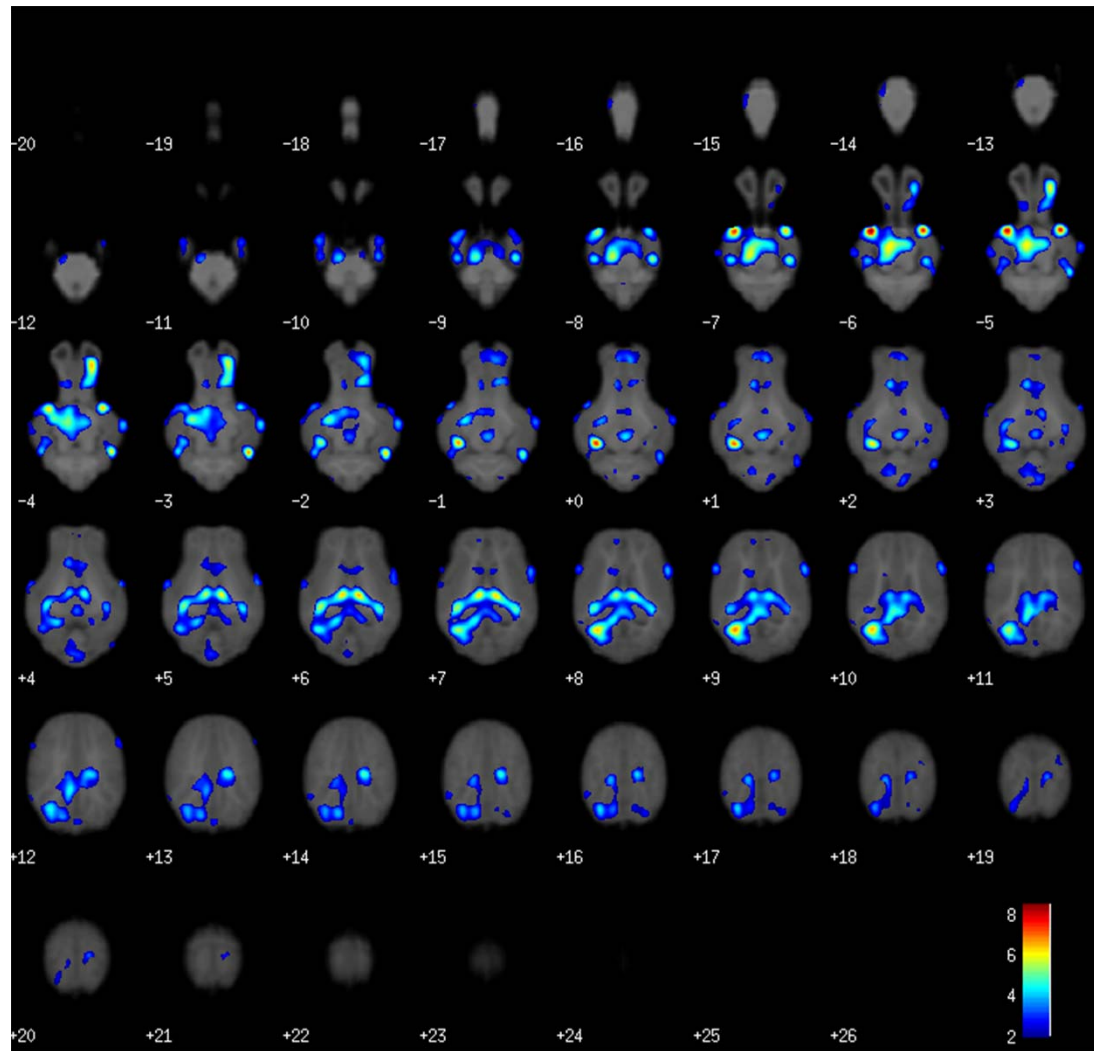


Figure 3.2: Heatmap showing brain areas with less white matter in piglets fed ID diet compared to CON diet from PD 2 through PD 29 or 30. The heatmap is overlaid on axial piglet brain atlas sections of 1 mm slice thickness. Increasing slice number (from [-] to [+]) indicates inferior to superior directionality. The color corresponds to the level of significance (pseudo t-statistic). CON, control; ID, iron deficient.

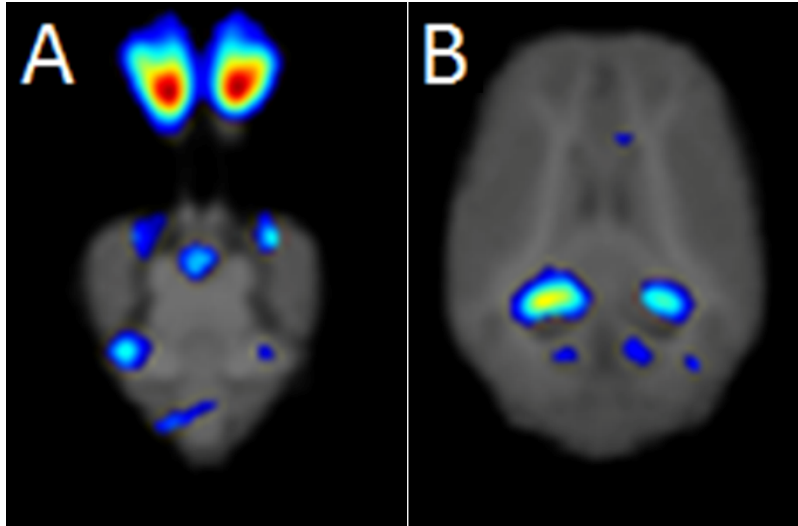


Figure 3.3: Heatmap showing higher gray matter in olfactory bulbs (A) and hippocampus (B) of piglets fed ID diet compared to CON diet from PD 2 through PD 29 or 30. The color corresponds to the level of significance (pseudo t-statistic). CON, control; ID, iron deficient.

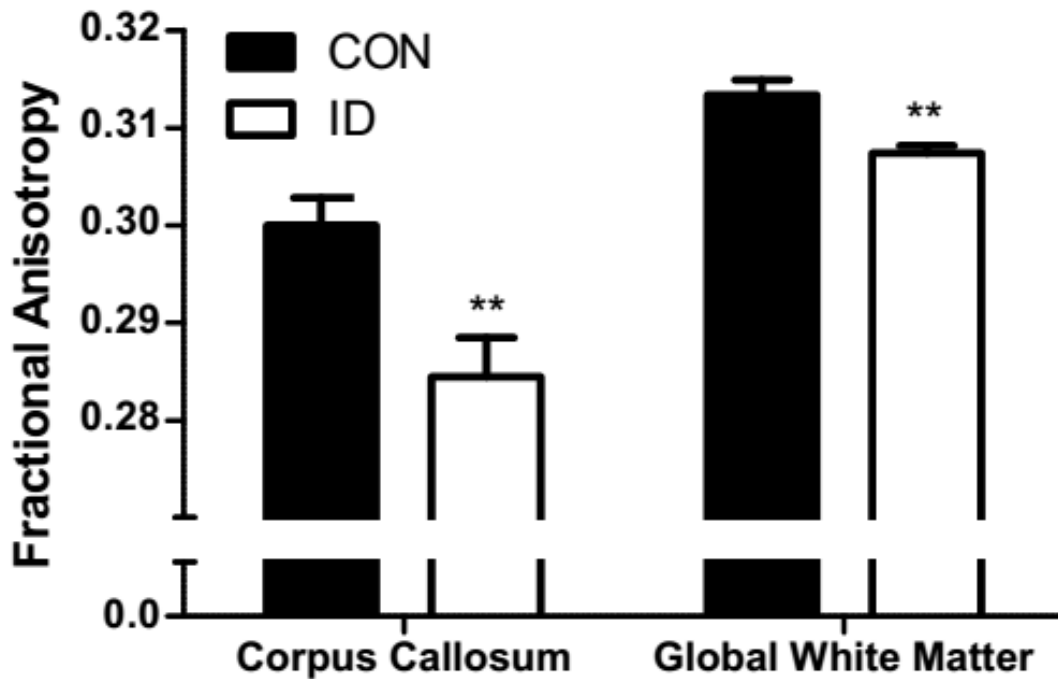


Figure 3.4: Fractional anisotropy of corpus callosum and global white matter in piglets fed ID or CON diet from PD 2 through PD 29 or 30. Values are means $\pm$ SEM, $n = 9-10$. Asterisks indicate different from CON for that brain region: ** $P < 0.01$. CON, control; ID, iron deficient.

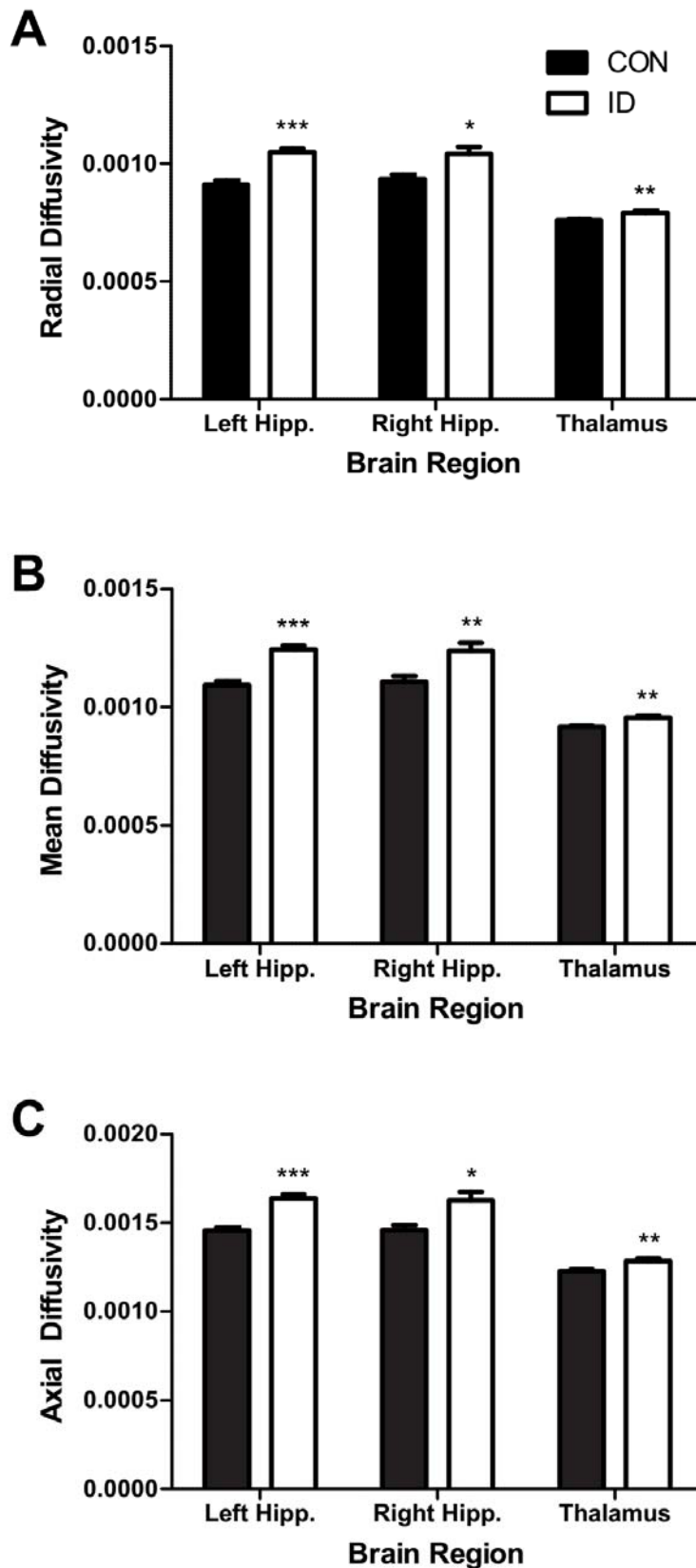


Figure 3.5: Radial diffusivity (A), mean diffusivity (B), and axial diffusivity (C) of the left hippocampus, right hippocampus, and thalamus of piglets fed ID or CON diet from PD 2 through PD 29 or 30. Values are means $\pm$ SEM, $n = 9-10$. Asterisks indicate different from CON for a given brain region: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. CON, control; Hipp, hippocampus; ID, iron deficient.

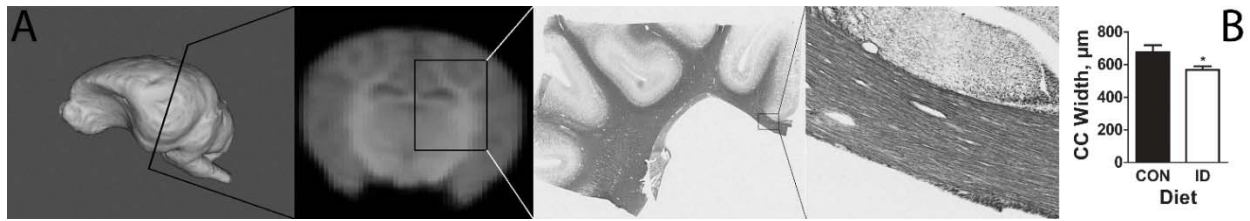
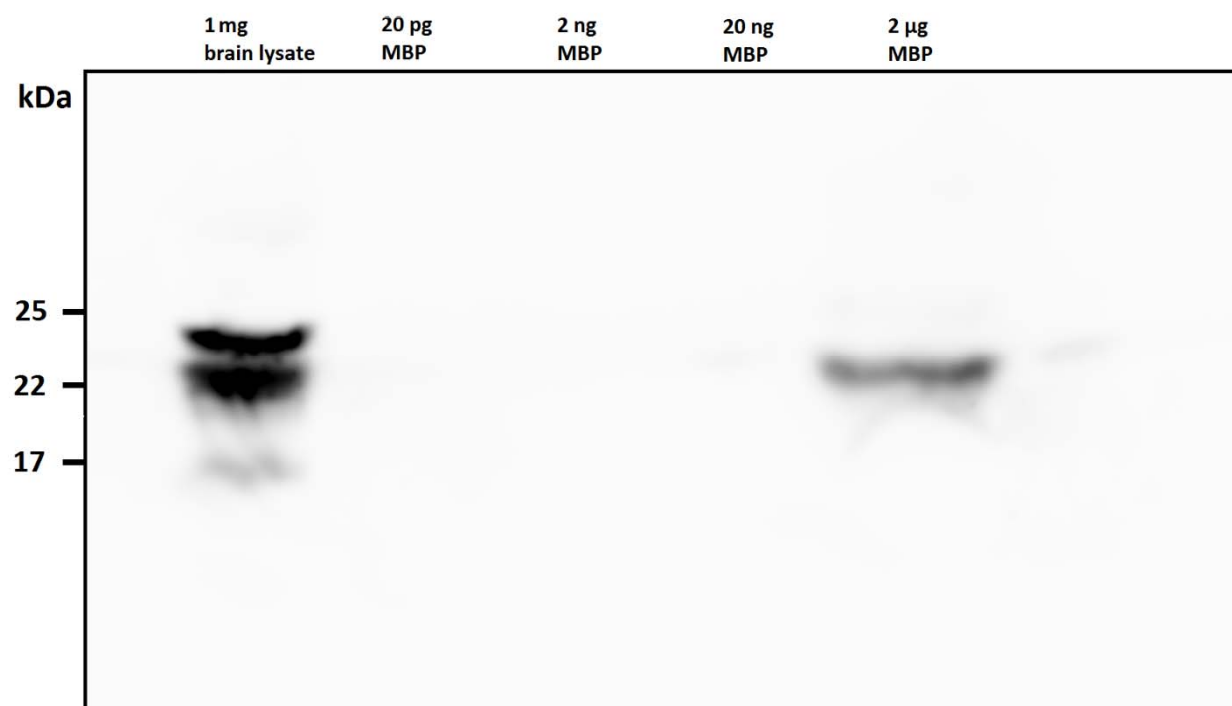


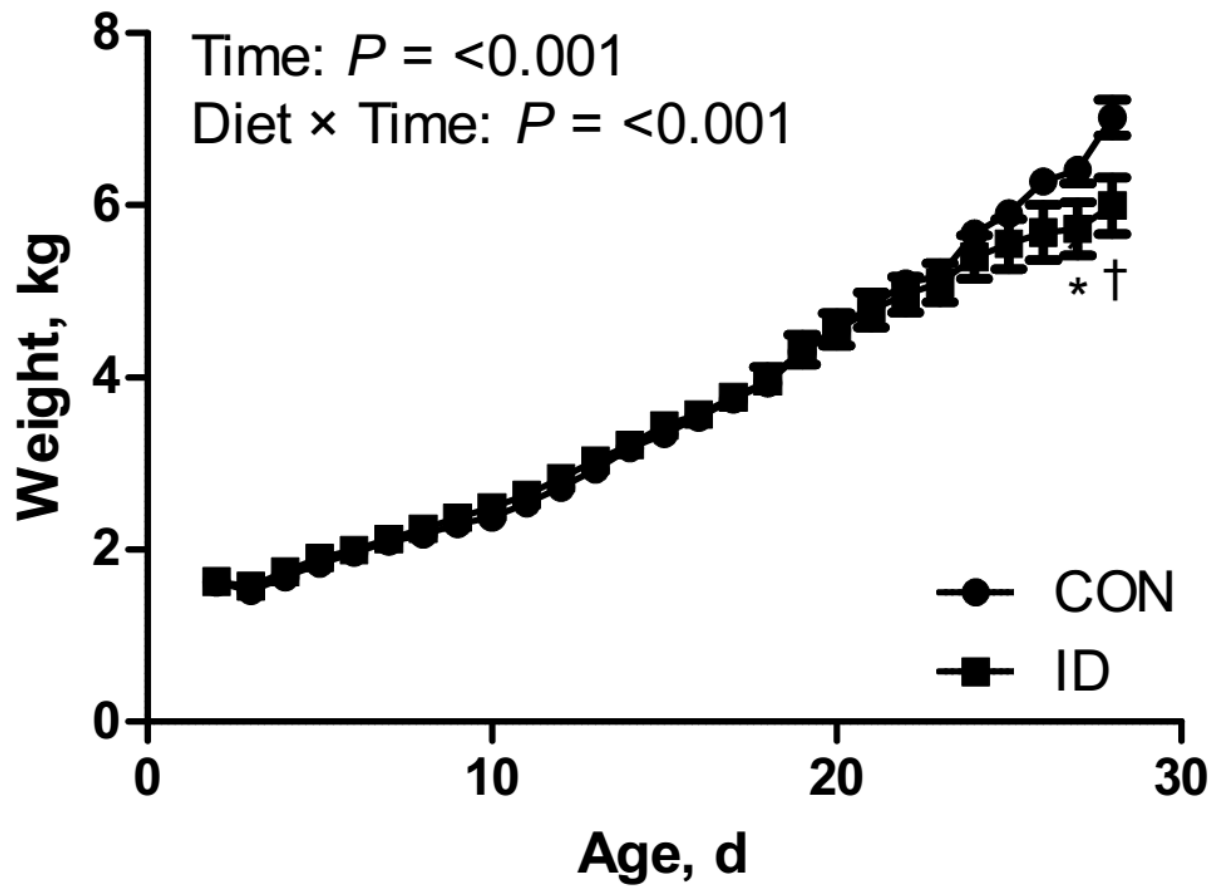
Figure 3.6: Three-dimensional rendering of piglet brain, MRI coronal section, and tissue sections stained with Black Gold highlighting where thickness of corpus callosum was assessed by histology (A). Corpus callosum width in piglets fed ID or CON diet from PD 2 through PD 29 or 30 (B). Values are means $\pm$ SEM, $n = 6-7$. Asterisk indicates different from CON: $*P < 0.05$. CC, corpus callosum; CON, control; ID, iron deficient; MRI, magnetic resonance imaging.

3.8 Supplemental Data



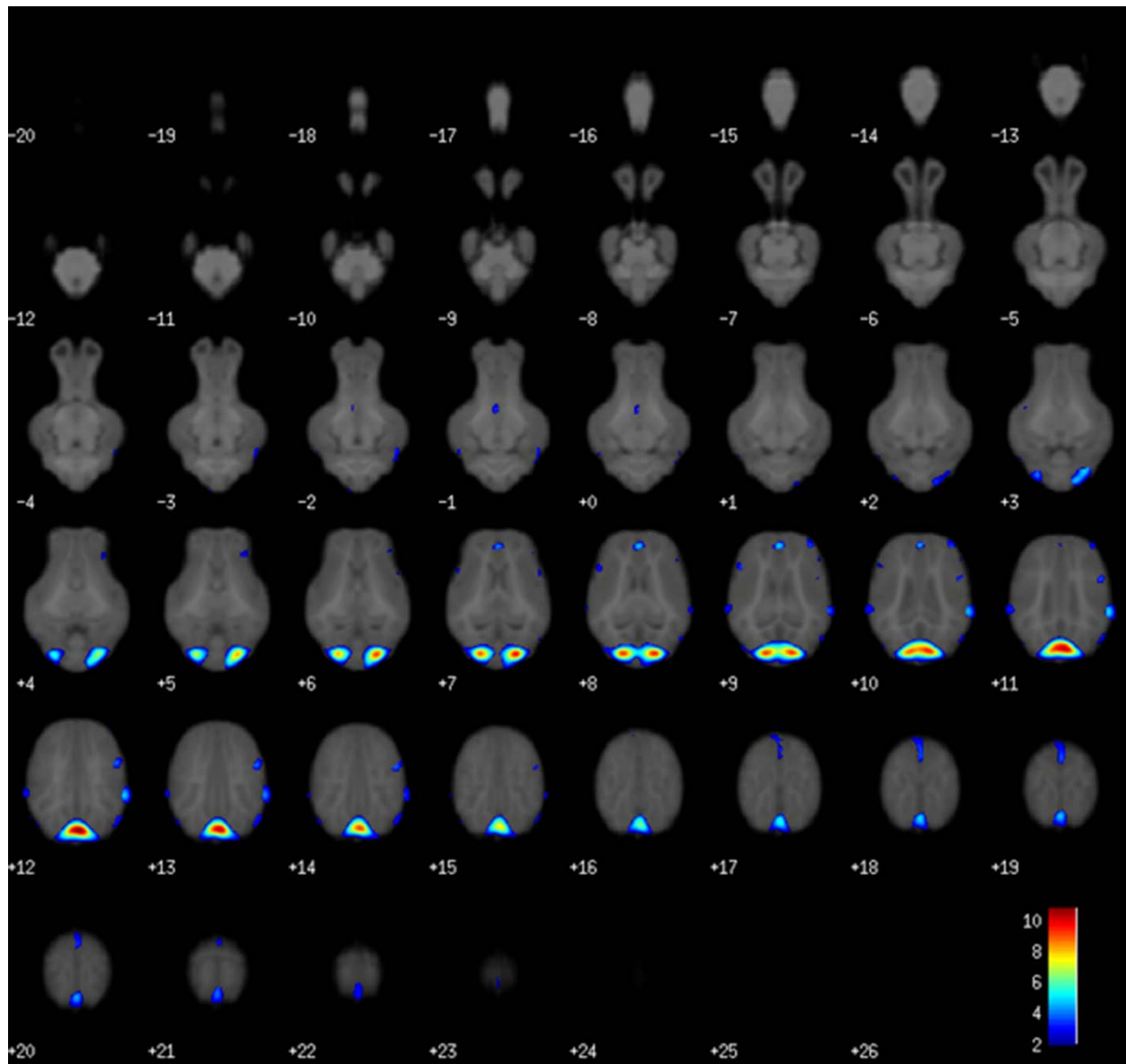
Supplemental Figure 3.1: Western blot demonstrating antibody specificity to MBP. From left to right, lanes contain 1 mg protein from porcine brain lysate, 20 pg purified porcine MBP, 2 ng purified porcine MBP, 20 ng purified porcine MBP, 2 μg purified porcine MBP. kDa, kilodaltons; MBP, myelin basic protein.

Supplemental Data



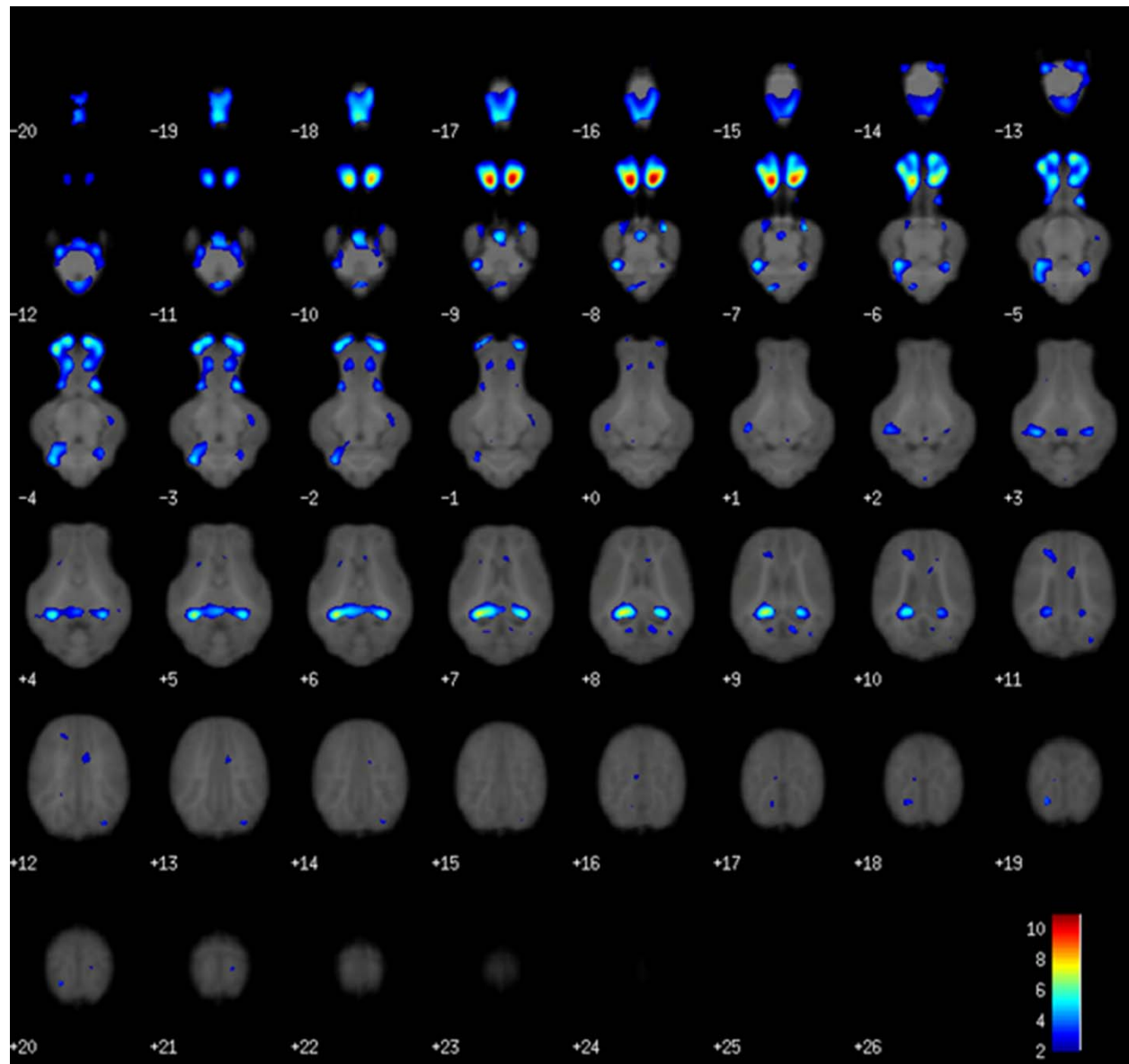
Supplemental Figure 3.2: Body weight of piglets provided CON or ID milk replacer from PD 2-28. Data are presented as means $\pm$ SEM, $n = 10$. Symbols indicate differences between CON and ID piglets at a given time: * $P < 0.05$; † $P < 0.001$. CON, control; d, day; ID, iron deficient; PD, postnatal day.

Supplemental Data



Supplemental Figure 3.3: Heatmap demonstrating areas of gray matter reduction in ID piglet brains compared to CON piglet brains. Voxel-based morphometric analysis showed decreased gray matter volumes in the brains of ID piglets in the occipital lobe and cortex compared to CON. Occipital lobe is visible from steps +9 to +13, and cortex is visible from steps +7 to +10. CON, control; ID, iron deficient.

Supplemental Data



Supplemental Figure 3.4: Heatmap demonstrating areas of gray matter increase in ID piglet brains compared to CON piglet brains. Voxel-based morphometric analysis showed increased gray matter volumes in the hippocampus and olfactory bulbs of ID piglets compared to CON. Hippocampus visible on steps +4 to +10, olfactory bulbs visible on steps -11 to -2. CON, control; ID, iron deficient.

Supplemental Data

Supplemental Table 3.1: Estimated volumes by brain region in ID piglets compared to CON piglets¹

Brain Region	CON (mm ³)	ID (mm ³)	<i>P</i> -value
Whole Brain	68128 ± 1639	65331 ± 1364	0.21
White Matter	15373 ± 515	16035 ± 274	0.27
Gray Matter	35052 ± 1040	35041 ± 608	0.99
Hippocampus	1226 ± 47	1256 ± 27	0.58
Corpus Callosum	1027 ± 26	1030 ± 27	0.95
Internal Capsule	4763 ± 107	4918 ± 69	0.24
Left Cortex	17729 ± 369	17383 ± 327	0.49
Right Cortex	16796 ± 369	16356 ± 314	0.38
Thalamus	3031 ± 54	3071 ± 40	0.56
Hypothalamus	558 ± 9.5	550 ± 9.9	0.59
Cerebral Aqueduct	131 ± 4.3	119 ± 3.9	0.06
Fourth Ventricle	139 ± 4.7	141 ± 2.2	0.81
Lateral Ventricle	1104 ± 45	1061 ± 28	0.43
Third Ventricle	197 ± 3.3	200 ± 4.3	0.51
Caudate	577 ± 17	566 ± 13	0.62
Putamen	1190 ± 35	1250 ± 19	0.15
Cerebellum	4982 ± 165	4975 ± 109	0.97
Midbrain	3041 ± 58	3065 ± 44	0.75
Medulla	2495 ± 68	2624 ± 63	0.18
Olfactory Bulb	2972 ± 58	2910 ± 40	0.39
Pons	880 ± 19	895 ± 19	0.58

¹ Values are means ± SEM, n = 20. CON, control; ID, iron deficient.

Chapter 4. Iron deficiency alters peripheral inflammation and iron metabolism during infection, but not microglial activation or activity

4.1 Abstract

During the postnatal period the developing brain is vulnerable to insults including nutrient insufficiency and infection that may lead to disrupted development and cognitive dysfunction. Since ID often presents with immunodeficiency, the objective of this study was to investigate peripheral viremia and inflammation as well as microglial phenotype and function when ID and respiratory infection occur simultaneously in a neonatal piglet model.

On postnatal day 2 (PD 2) male and female piglets were divided into four groups and fed either control or ID milk replacer. Piglets were inoculated with either vehicle or porcine reproductive and respiratory syndrome virus (PRRSV; P-129) on PD 8. Baseline blood samples were collected prior to inoculation (PD 7) and repeated once weekly. Rectal temperatures, feeding score and sickness behaviors were measured daily until PD 28. Hematocrit, hemoglobin, and serum iron were reduced by ID but not PRRSV infection. PRRSV-infected piglets displayed viremia by PD 14; however, those fed control diet had lower viral titer at PD 28, while circulating virus remained elevated in those fed an ID diet, suggesting that ID either impaired immune function necessary for viral clearance or increased viral replication. ID piglets infected with PRRSV displayed reduced sickness behavior compared to those fed control diet on PD 13-15 and 18-20. While ID piglet sickness behavior progressively worsened, piglets fed control diet displayed improved sickness score after PD 21. Microglia isolated from PRRSV piglets had increased MHCII expression and phagocytic activity *ex vivo* compared to uninfected piglets. ID did not alter microglial activation or phagocytic activity. Similarly, microglial cytokine expression was increased by PRRSV but unaffected by ID, in stark contrast to PBMC cytokine expression, which was increased by infection and generally decreased by ID.

Taken together, these data suggest that ID decreases peripheral immune function leading to decreased viral clearance, but immune activity in the brain is protected from acute ID.

4.2 Introduction

Iron deficiency (ID) and infection often present as comorbid conditions in vulnerable populations, such as infants and young children in developing countries. Respiratory infection is a leading cause of death in developing nations and hospitalization for children younger than one year old in the United States [162, 163]. Iron is important for immune function, and associated with phagocytic activity [1, 7] and cytokine production [19], with ID contributing to immunocompromise [21, 22]. The pig immune system is highly similar to human immune system, whereas the mouse immune system has little parity with the human [56, 57]. Thus, the pig provides an excellent model of peripheral immune dynamics. The brain is sensitive to peripheral inflammatory signals through both direct signaling via activation of peripheral afferent nerves, and humoral signaling at the choroid plexus and circumventricular organs. These signals are subsequently amplified and propagated by microglia, leading to a variety of downstream effects, including sickness behavior, a cluster of adaptive behaviors expressed during illness including lethargy, anorexia, and social withdrawal [71]. Besides their role as the innate immune cells of the brain, microglia perform roles in neurodevelopment, including phagocytosis of synapses and neural progenitor cells [74-76]. Modulation of microglial activation by peripheral immune signals may increase microglial synaptic pruning and phagocytic activity.

The combined effects of ID and infection on neuroinflammation have been the subject of minimal research, with no postnatal studies to date. Two previous studies used rats to model prenatal ID and postnatal immune challenge with bacterial endotoxin [83, 84]. The present study uses the neonatal piglet as a translational model of infants developing ID during the early postnatal period and subsequently contracting a respiratory infection. Piglets were fed either control or ID

diet for six days and then infected with porcine reproductive and respiratory syndrome virus (PRRSV), which leads to interstitial pneumonia. ID often presents with immunodeficiency, thus increasing vulnerability to infection. Examining their combined effects provides a translational model of a serious public health problem – the double burden of early life ID and infection. The first three years of life are crucial to brain development - multiple brain processes, including myelination and development of the monoamine neurotransmitter systems, begin prenatally and undergo the majority of their development in this timeframe [164], rendering the brain vulnerable to environmental insults during this critical period. Individually, both early life ID and postnatal infection are associated with altered neurodevelopment [26, 29, 80].

The present study examined the effects and potential interactions of postnatal ID and infection on iron metabolism, peripheral inflammation, neuroinflammation, and microglial phenotype and activity. We hypothesized ID-PRRSV piglets would display a blunted inflammatory cytokine response, impaired ability to clear the infection, and altered iron metabolism in the liver, spleen, lung, and peripheral blood mononuclear cells (PBMCs). Furthermore, we expected microglia from ID infected piglets would be less activated, less phagocytic and would have altered cytokine profiles resulting from blunted peripheral inflammatory cytokine production. Since microglia are key effectors during neurodevelopment, performing functions such as dendritic pruning, understanding their phenotype when confronted with these insults provides useful translational data.

4.3 Methods

Animals, housing, and experimental design. Two cohorts that included 12 crossbred domestic piglets (N=24) born of dams maintained on an iron-adequate diet throughout gestation were received on postnatal day (PD) 2 from the University of Illinois swine farm. Upon arrival piglets

were assigned to one of four treatment groups (n=6) comprising the 2 x 2 factorial arrangement of diet (control or ID) and infection (control or PRRSV). After controlling for sex, weight, and litter of origin, piglets evenly distributed to four groups: uninfected and control diet (CN), uninfected and ID diet (CID), PRRSV infected and control diet (PN), and PRRSV infected with ID diet (PID). Piglets assigned to the control diet received an intramuscular iron dextran injection (200 mg, Uniferon). From PD 2 to 28 piglets were housed individually in cage racks as previously described [165] and were provided a toy (plastic Jingle-Ball; Bio-Serv) for environmental enrichment. Ambient heating was provided to maintain a room temperature of at least 27° C, and supplemental heating was provided by an in-cage heating pad (K&H Lectro-Kennel Heat Pad). A 12h light/dark cycle was maintained with light from 0700 to 1900h. All animal care and experimental procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee.

Diets. Nutritionally complete sow milk replacer diets were formulated according to previous specifications [165]. Piglets were assigned to either an iron-sufficient control diet (100 mg Fe/kg solids) or the ID diet (10 mg Fe/kg solids). Aside from iron levels, all diets met the NRC recommended guidelines [145]. Piglets were fed 4 times per day for a total of 300 mL/kg bodyweight. Extra water and swine Blue-Lite (TechMix LLC, Stewart, MN) were provided on a case-by-case basis to piglets displaying grievous symptoms of infection such as severe diarrhea per veterinary staff recommendation.

Inoculation with PRRSV and daily assessment of food intake, sickness behavior, temperature, and body weight. On PD 8, piglets were inoculated intranasally with either 1 mL of PRRSV (strain P129-BV4, obtained from the School of Veterinary Medicine at Purdue

University, West Lafayette, Indiana) or 1 mL sterile PBS sham as we have described previously [82]. PRRSV is a single-stranded RNA virus that produces a chronic interstitial pneumonia in piglets and a set of sickness behaviors that includes fever, reduced appetite, and lethargy [166, 167]. Animals were weighed daily before feeding. Sickness behavior was scored for each piglet upon entering the room and before feeding using a 4-criterion scale quantifying if the piglet displayed lack of appetite, loose stool, panting, or lolling tongue. If a behavior was present, it was marked as a 1; a piglet displaying all 4 behaviors would receive a score of 4, whereas a piglet displaying no sickness behaviors would be scored 0. Rectal temperatures were taken daily. Piglets were assigned a feeding score based on their willingness to complete the first meal of the day (1 = no attempt to eat; 2 = attempted to eat but stopped shortly after; 3 = ate continuously for at least 1 minute). PRRSV serum viral load was analyzed by the Veterinary Diagnostic Laboratory via serum multiplex rRT-PCR (University of Illinois, Urbana, IL).

Assessment of ID and anemia. Blood samples were taken weekly from the jugular vein using both serum and plasma Vacutainer tubes. An aliquot of whole blood was sent to the Veterinary Diagnostic Laboratory for complete blood count (CBC). The remaining blood was centrifuged at 1300 xg at 4° C for 15 min to collect serum or plasma and stored at -80° C. White blood cell counts were saved for later analysis. Serum iron was assessed at the Microanalysis Laboratory using inductively-coupled plasma mass spectroscopy (ICP-MS; University of Illinois, Urbana, IL).

Piglet necropsy and brain dissection. Piglets were sacrificed at PD 28. After anesthetic induction with an intramuscular injection of a telazol:ketamine:xylazine cocktail (50 mg of tiletamine with 50 mg of zolazepam reconstituted with 2.5 mL ketamine (100 g/L) and 2.5 mL xylazine (100 g/L); Fort Dodge Animal Health, Fort Dodge, IA) at a dose of 0.03 mL/kg BW, piglets were sacrificed via intracardiac (i.c.) injection of sodium pentobarbital (86 mg/kg BW; Fatal Plus, Vortech

Pharmaceuticals). Blood was collected for CBC, serum, plasma, and peripheral blood mononuclear cell (PBMC) isolation. Liver, spleen, and lung were collected and snap frozen in liquid nitrogen. Piglet brains were removed, weighed, and specific regions of interest were dissected, snap frozen, and stored at -80° C.

Microglial Isolation. Microglia were isolated from cerebellar tissue based on CD11b expression. Isolation was performed using Miltenyi Biotec GentleMACS C-tubes, Neural Tissue Dissociation Kits (P), GentleMACS Octo Dissociator with Heaters, CD11b MicroBeads, MACS MS columns, and the OctoMACS separator (Miltenyi Biotec, San Diego, CA) according to manufacturer's instructions, with minor modifications. Dissected cerebellar tissue was placed in C-tubes containing Enzyme Mix 1 & 2 (volume doubled from manufacturer specification due to tissue amount). C-tubes were incubated at room temperature for 20 minutes and attached to the GentleMACS Octo Dissociator with Heaters for mechanical digestion. The tissue slurry was then forced through a 40 µm Falcon cell strainer to separate the tissue into individual cells (Thermo Fisher Scientific). The cell suspension was then added to 30% Percoll PLUS and PBS solution and centrifuged to remove myelin. CD11b MicroBeads were added to the solution and the mixture was then run through MS columns and rinsed, retaining CD11b+ cells in the column while other material passed through. Columns were then removed from the magnet and washed into an Eppendorf tube to obtain the CD11b+ cell fraction, which was centrifuged at 300 xg for 10 minutes at 4° C to obtain a cell pellet.

Flow Cytometry. Cerebellar CD11b+ cells were re-suspended in flow cytometry buffer (1% BSA, 20 mM glucose PBS solution). Cells were stained with CD45 antibody (AbD Serotec, Raleigh, NC) to identify microglia, and MHCII (Antibodies Online, Atlanta, GA) as a marker for immune cell activation. CD11b+ cells expressing intermediate levels of CD45 were considered porcine

microglia [81]. Flow cytometry was performed using a core facility FACS Aria II (BD Biosciences, San Jose, CA). Gating was set according to forward scatter, side scatter, and autofluorescence of an unstained control.

Phagocytic activity. Microglial phagocytic activity was assessed using the Vybrant Phagocytosis Assay Kit (ThermoFisher, Waltham, MA). Cells were diluted to approximately 6×10^5 cells/mL in cell culture medium consisting of DMEM with 10% FBS, and 1% penicillin streptomycin, plated at 5 well replicates per sample (150 μ L/well), and incubated for 24 h at 37° C. After incubation, cell culture medium was replaced with fresh medium. 100 μ L fluorescein-labeled BioParticles were then added to each well. After incubation for 90 minutes, the medium was removed by aspiration and 100 μ L of 1x trypan blue suspension was added for 10 minutes at room temperature to quench fluorescence of any remaining BioParticles that were not phagocytosed. The plate was read at an excitation of 485 nm and an emission of 528 nm.

Quantitative Real-Time PCR. Circulating peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus gradient according to manufacture protocol (GE Healthcare, Uppsala, Sweden) and stored in TRIzol Reagent (Thermofisher Scientific, Grand Island, NY) at -80° C until RNA isolation. RNA from microglia, PBMCs, spleen, lung, and liver were isolated using the TRIzol method according to manufacturer protocol. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). Quantitative real-time PCR was performed using the Applied Biosystems Taqman Gene Expression Assay Protocol. Genes of interest related to inflammation, anti-microbial functions, and iron metabolism, were expressed as fold change relative to the endogenous control gene (RPL19).

Statistical assessment. Statistics were performed in SAS (SAS Institute, Cary, NC) or GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA). All data were checked for normality, and, where

applicable, homogeneity of variance. Two-way ANOVA was used to analyze all data, with feeding score, sickness behavior, body weight, temperature, viremia, and CBC measures analyzed as repeated measures using the SAS Proc MIXED function. If significant main effects or interactions were found, Least Significant Difference (LSD) post-hoc tests were performed to parse differences between individual groupings. While the current study is not powered to adequately assess sex differences, sex was included in Proc MIXED analyses. As sex only had significant effects upon temperature, it was not included in subsequent statistical models.

4.4 Results

Body Weights, Feeding Score, and Temperature

Weights were not affected by either infection or diet until day 20 (Figure 4.1A). Two-way, repeated measures ANOVA determined main effects of infection ($p = 0.001$) and day ($p < 0.001$) upon weight, as well as several interactions (infection x day, $p < 0.001$; diet x day, $p = 0.001$; infection x diet x day, $p < 0.001$). Two-way ANOVA of PD 27 and PD 28 body weights found effects of infection ($p = 0.003$ and $p = 0.023$, respectively). Feeding score (Figure 4.1B) was determined daily following the first meal, with a score of 3 signifying the piglet finished its meal, 2 that the meal was eaten but not finished, and 1 signifying no attempt to eat. Two-way, repeated ANOVA of feeding scores found effects of infection ($p < 0.001$), diet ($p = 0.006$), and day ($p < 0.001$). Both diet x day ($p = 0.007$) and infection x day ($p < 0.001$) interactions were present. Rectal temperature (Figure 4.1C) was taken daily before feeding. Temperature was affected by infection ($p < 0.001$), sex ($p < 0.002$), day ($p < 0.001$), and interactions between diet x day ($p = 0.02$) and infection x day ($p < 0.001$).

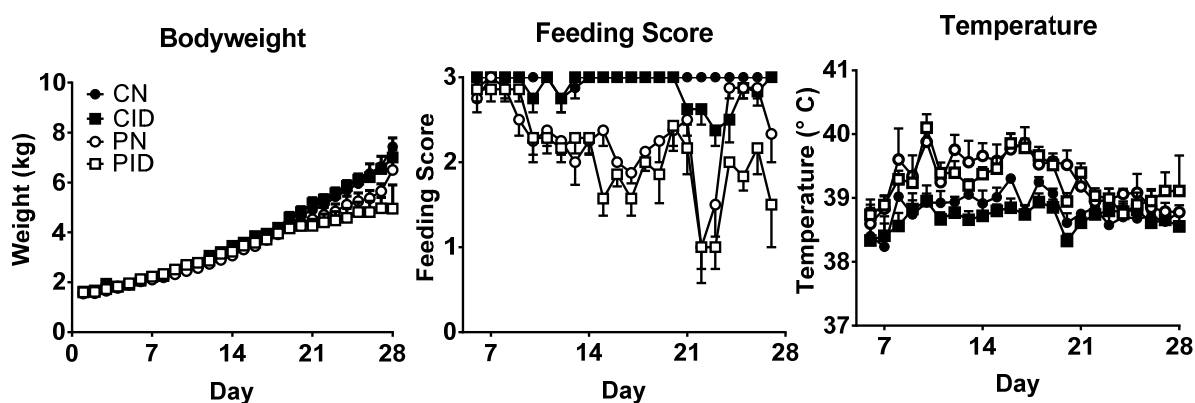


Figure 4.1: Daily measures of piglet bodyweights (A), feeding scores (B), and temperature. Bodyweight was affected by infection ($p = 0.001$), day ($p < 0.001$) infection x day ($p < 0.001$) diet x day ($p = 0.001$) infection x diet x day ($p = 0.001$). Feeding score was altered by infection ($p < 0.001$), diet ($p = 0.006$), day ($p < 0.001$), diet x day ($p = 0.007$), infection x day ($p < 0.001$), infection x diet x day ($p = 0.053$). Temperature was altered by infection ($p < 0.001$), day ($p < 0.001$), diet x day ($p = 0.02$), and diet x day ($p < 0.001$). Data are presented as means $\pm$ SEM ($n=6-8$).

Hemoglobin, hematocrit, and serum iron, are reduced by diet, but not infection.

Piglets fed ID diet developed microcytic anemia. Hemoglobin and hematocrit, standard measures of anemia, were reduced by main effects of diet, but not infection (Figure 4.2A and 4.2B). Main effects of diet ($p < 0.001$) and day were presented after statistical analysis of hemoglobin, as well as interactions between infection x day ($p < 0.002$) and diet x day ($p < 0.001$). Post-tests showed PN hemoglobin levels to be different from CN hemoglobin at PD 21. Hematocrit was reduced by diet ($p < 0.001$). Effects of day ($p < 0.001$) and diet x day ($p < 0.001$) were also present. As with hemoglobin, PN hematocrit was different from CN values at PD 21 ($p = 0.001$). Red blood cell (RBC) count (Figure 4.2C) was reduced by infection ($p = 0.006$), diet ($p < 0.001$), and day (0.002), with interactions between infection x day ($p = 0.001$), and diet x day ($p = 0.037$). Notably, the CID group maintained a higher RBC count than the PID group at PD 14 and PD 21, but dipped to PID levels by PD 28. PN piglet RBC count decreased on PD 21, and was similar to

CID RBC count. By PD 28, PN RBC count increased slightly but still differed from controls. This was expected as PRRSV can contribute to anemia [168]. Erythrocyte mean cell volume (MCV) was affected by infection ($p = 0.001$), diet ($p < 0.001$), and day ($p < 0.001$). Interactions of infection x day ($p = 0.001$) and diet x day ($p < 0.001$) were present (Figure 4.2D). Both ID groups displayed less MCV (Figure 4.2D). Platelet levels (Figure 4.2E) were increased by diet ($p = 0.004$), with a main effect of day ($p < 0.001$) and a diet x day interaction ($p = 0.005$). Serum iron at PD 28 (Figure 4.2F) was reduced in ID groups by a main effect of diet ($p < 0.001$). Infection had no effect on serum iron.

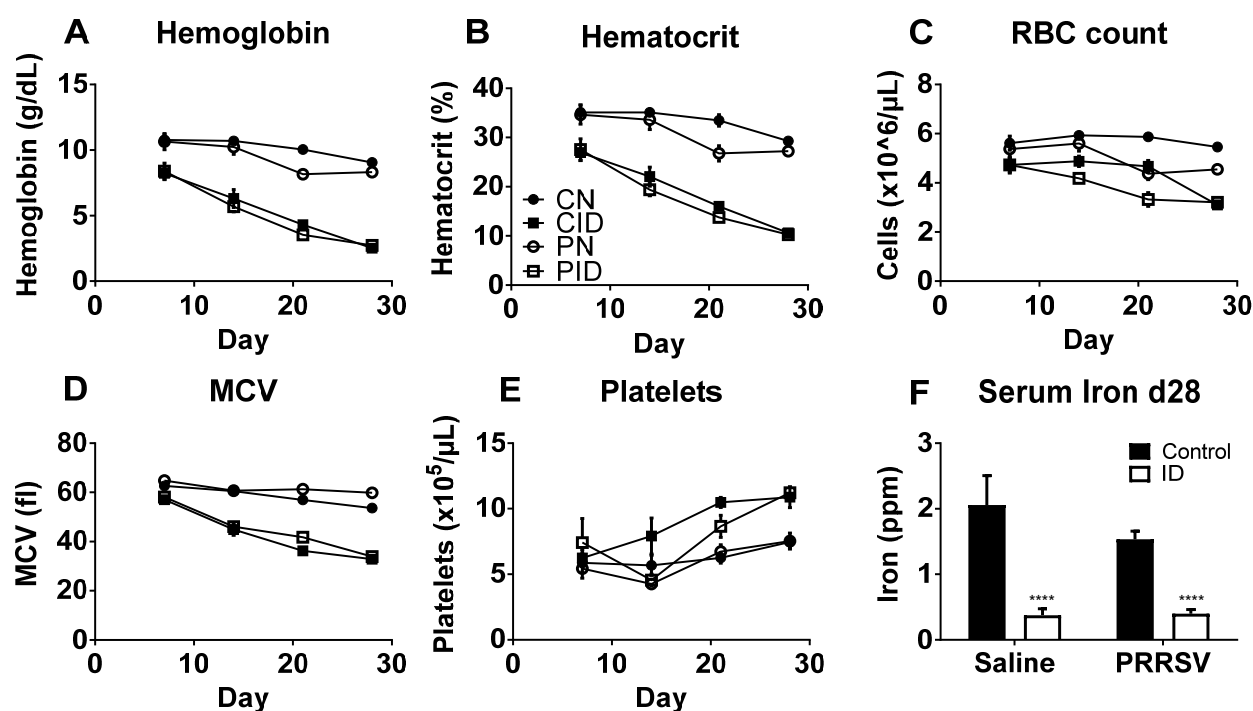


Figure 4.2. Whole blood hemoglobin (A), hematocrit (B), red blood cell (RBC) count (C), erythrocyte mean cell volume, (D), platelet count (E), at PD 7, 14, 21, and 28. Serum iron at PD 28 (F). Data are presented as means $\pm$ SEM ($n=6-8$). Error bars were not rendered on data points for which the error bars would be shorter than the height of the symbol. **** denotes $p < 0.001$.

ID impairs peripheral immunity and viral clearance

Viral burden by tissue was assessed by quantifying abundance of PRRS virus RNA. Serum viral load, or viremia (Figure 4.3A), was increased by ID at PD 28 ($p = 0.007$). On PD 14 and 21, there were not differences in viremia between PRRSV-infected groups (not shown). Lung viral load was not affected by dietary group (Figure 4.3B). However, spleens harvested from PID piglets contained higher quantities of virus than their PN counterparts ($p = 0.007$, Figure 4.3C). Sickness behavior was scored daily before the first feeding, and largely affected by PRRSV (Figure 4.3D). Main effects of infection ($p < 0.001$) and day ($p = 0.001$) were found by 2-way repeated measures ANOVA. Interactions of infection x day ($p < 0.001$), diet x day ($p = 0.015$), and treatment x diet x day ($p = 0.03$) were present. Generally, the PID piglets displayed less sickness behavior in the first 3 weeks of the study than the PN piglets, until PD 21, where this trend inverted. During the last week of the study, as the PN piglets began to resolve the infection and displayed less sickness behavior, the PID piglets displayed increasingly severe sickness behavior. Analysis of the last week of sickness behavior found main effects of infection ($p < 0.001$) and diet ($p = 0.041$), and a trend for a treatment x diet interaction ($p = 0.052$). White blood cell count (Figure 4.3E) was heavily modulated by the experimental interventions, with infection ($p = 0.001$), diet ($p = 0.001$), and day ($p < 0.001$) effects. Additionally, there were interactions of infection x day ($p < 0.001$) and diet x day ($p = 0.014$). While all groups were similar at the baseline PD 7 blood draw, PID white blood cell count decreased nearly 50% one week later, while the other groups remained relatively unchanged. However, this reversed by PD 21, with both PN and PID white blood cell counts sharply increasing on PD 21, likely as the immune response is marshalled, before falling at PD 28. PN white blood cell counts were still elevated by PD 28, while PID crashed to baseline, uninfected levels by PD 28. ID reduced white blood cell count, which is most clearly illustrated on PD 21. Post-hoc tests determined WBC count of each group differed from every other group at

PD 21, with each ID group displaying a suppressed WBC count in comparison to their corresponding infection group.

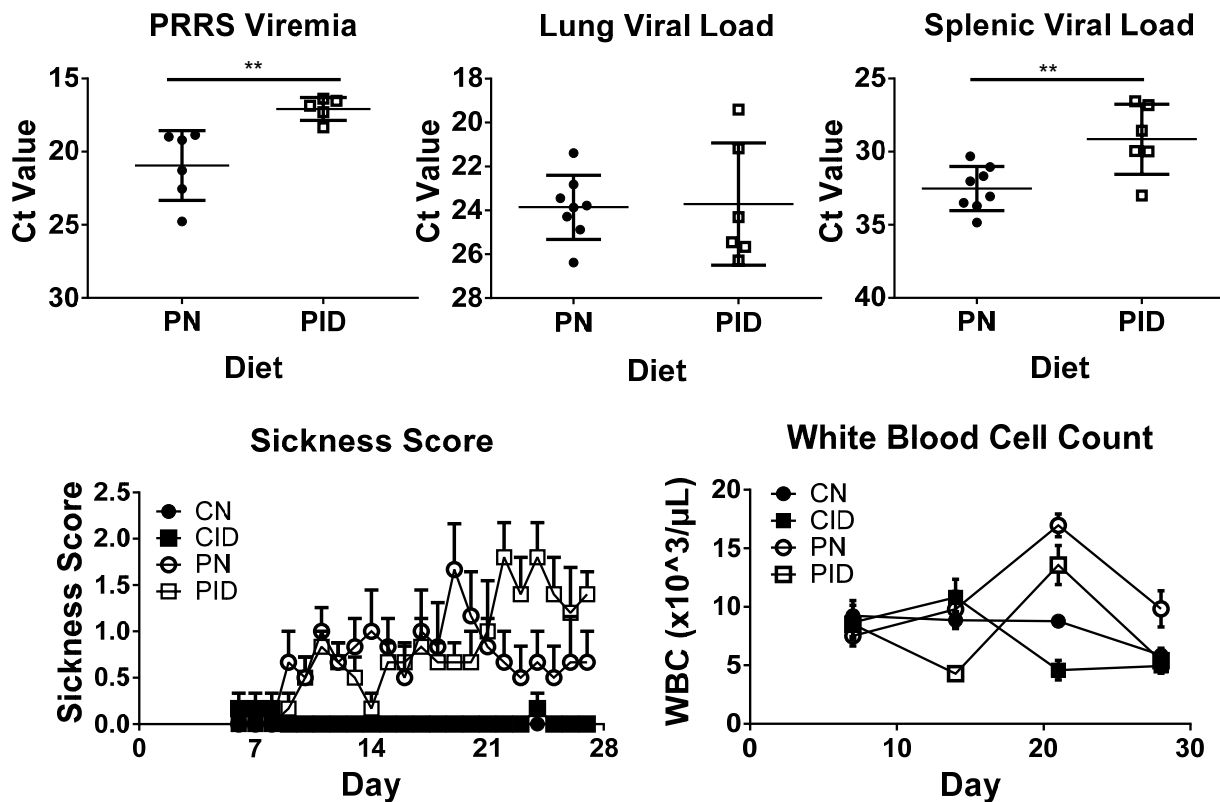


Figure 4.3: Viral load by tissue, sickness behavior, and white blood cell (WBC) count. Viremia (serum viral load at termination of study, A) differed between PID and PN groups ($p = 0.007$) at end of study. Lung (B) and spleen (C) viral load at study termination. In A-C, the y-axis is inverted to visually convey increased virus, as a lower Ct value indicates higher levels of RNA. Sickness behavior (D) was recorded from PD 6 until study termination. White blood cell count (E) from weekly blood draws beginning at PD 7 and lasting until study termination. Data are presented as means $\pm$ SEM ($n=6-8$). Error bars were not rendered on data points for which the error bars would be shorter than the height of the symbol. ** denotes $p < 0.01$.

Both ID and infection heavily modulate PBMC and liver expression of inflammatory, anti-microbial, iron metabolism, and especially anti-viral genes.

ID and diet caused widespread changes in peripheral gene expression. Specific genes of interest are highlighted in Figure 4.4. In PBMC (Figure 4.4A), $\text{TNF}\alpha$ and $\text{IL-1}\beta$ expression were

both depressed by ID diet ($p < 0.001$ and $p < 0.001$, respectively). Notably, trending effects of infection ($p = 0.06$) as well as infection x diet ($p = 0.07$) were present on *TNF α* . A similar trend was present for PBMC *IL-1 β* expression (infection, $p = 0.06$; diet x infection, $p = 0.087$). Conversely, liver *TNF α* expression was increased in PN, but reduced to baseline by the ID diet, whereas liver *IL-1 β* expression was increased by ID, with CID and PN *IL-1 β* expression remaining roughly equal (Figure 4.4C). PID piglet *IL-1 β* expression in liver was approximately double that of PN (infection, $p = 0.001$; diet, $p < 0.001$; diet x infection $p = 0.048$). Interferon- γ (IFN- γ) is a keystone cytokine in the anti-viral response, and is imperative for proper immune response against PRRSV infection. *IFN- γ* expression was heavily upregulated in PBMC of PN piglets (Figure 4.4B), but reduced to baseline CN expression in PID piglets (post-hoc test PN:PID, $p < 0.001$). Effects of diet ($p = 0.001$), infection ($p = 0.001$), and diet x infection ($p = 0.004$) modulated PBMC *IFN- γ* expression. However, in the liver, only infection had an effect upon *IFN- γ* expression ($p < 0.001$), with no differences due to diet within infection groups (Figure 4.4D). Liver expression of *NF- κ B*, a key transcription factor in the inflammatory response, was altered by infection ($p = 0.042$) and diet x infection ($p = 0.004$); the upregulation of *NF- κ B* expression seen in PN piglets was not present in PID piglets. PBMC cytokine expression during infection is depressed by dietary ID, especially production of the antiviral cytokine *IFN- γ* . Liver cytokine production was differentially modulated by diet during infection, with interactive effects producing both reduced *TNF α* expression and increased *IL-1 β* expression in PID piglets compared to PN piglets, while PID liver *IFN- γ* expression (Figure 4.4D) was not depressed by ID as in PBMC (Figure 4.4B).

Antimicrobial gene expression was differentially altered by PRRSV infection and ID. PBMC expression of *CYBA*, a component of NADPH oxidase, a protein used to generate the macrophage oxidative burst, was downregulated by a main effect of diet ($p < 0.001$, Figure 4.4A).

Liver *CYBA* expression was altered by diet ($p < 0.001$), infection ($p = 0.006$), and diet x infection ($p = 0.013$, Figure 4.4C). Liver *CYBA* expression was upregulated in PN piglets, but dropped to baseline expression levels in PID piglets. *Nos2*, also known as inducible nitric oxide, is upregulated during infection to produce nitric oxide reactive oxygen species (ROS) to destroy pathogens. PBMC *Nos2* expression was altered by infection ($p = 0.002$), diet ($p < 0.001$), and diet x infection ($p = 0.001$). While PN expression was sharply increased, PID *Nos2* expression was depressed to baseline CN levels by the ID diet. Liver *Nos2* expression was unchanged across all groups. Thus, anti-microbial gene expression is reduced in ID piglets, and the upregulation seen in infected animals fed a normal diet is ablated in infected animals fed an ID diet.

Iron metabolism was profoundly altered by infection. Ferritin (FTH1), the primary intracellular iron storage protein, was sharply upregulated in PBMC of PN piglets, implicating the iron sequestration response of the monocyte response to infection. However, ferritin expression in PBMC of PID piglets did not differ from control pigs (Figure 4.4A). Ferritin expression in PBMC was modulated by diet ($p = 0.002$), infection ($p = 0.013$), and diet x infection ($p = 0.005$). Conversely, liver ferritin expression was only altered by diet ($p = 0.002$), and increased by ID diet compared to normal diet (Figure 4.4C). Transferrin receptor (TF-R) allows cellular uptake of transferrin, and is the primary method by which body cells acquire iron from the circulation. PBMC *TF-R* expression was upregulated by ID diet (main effect, $p = 0.002$), and reduced by infection (main effect, $p = 0.008$). Similarly, liver *TF-R* expression was also upregulated by diet ($p < 0.001$), though effects of infection ($p = 0.04$) and a diet x infection interaction ($p = 0.013$) were present. PN did not differ from CN, though PID displayed an attenuated upregulation compared to CID. Liver transferrin (TF, Figure 4.4C), the primary chaperone for extracellular iron in the body, showed increased expression in CID piglets, but reduced expression in PID piglets.

Both CN and PN groups displayed unchanged expression levels. Effects of infection ($p = 0.001$) and diet x infection ($p < 0.001$) altered liver *TF* expression. In PID compared to PN piglets, the iron sequestration response to infection appears to be impaired in PBMC and liver, the two primary depots for iron storage.

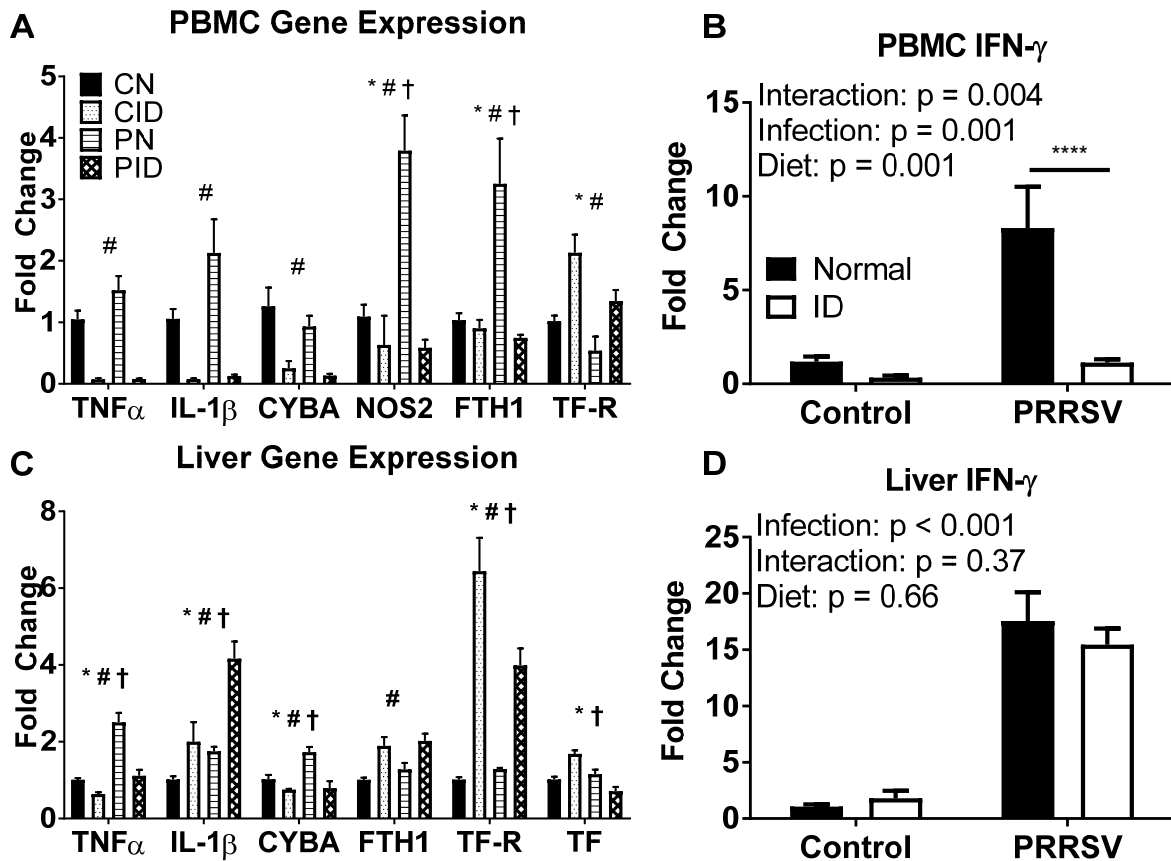


Figure 4.4: Expression of key genes altered by ID, infection, and interactions between the two in PBMC (A and B) and liver (C and D). For (A) and (C), * denotes a main effect of infection, # denotes main effect diet, and † signifies an interaction between diet and infection. **** denotes $p < 0.001$. PBMC, peripheral blood mononuclear cells. Data are presented as means $\pm$ SEM ($n=6-8$).

Gene expression of cytokines, pathogen/danger sensors, anti-microbial genes, and iron metabolism genes are modulated by both PRRSV infection and diet in peripheral blood mononuclear cells and the liver, though the lung and spleen display blunted effects and are modulated only by PRRSV infection

A complete representation of peripheral genes assessed in this study is presented in Table 4.1. Notably, diet, PRRSV infection, and diet x infection effects are strongly present in gene expression of PBMC and liver tissue. Conversely, the spleen and lung display far fewer significant effects, with only infection reaching significance, excepting the spleen transferrin receptor (TF-R), which is altered by diet ($p < 0.001$), infection ($p < 0.001$), and diet x infection ($p = 0.001$). Spleen TF-R displays equivalent upregulation in CID and PN piglets (9 and 12 fold, respectively), while PID spleen tissue displays a massive upregulation of TF-R by 50 fold. Generally, inflammatory and iron metabolism gene expression in the spleen and lung appear to be insulated from ID and were primarily modulated by PRRSV infection.

Table 4.1: Relative Abundance of mRNA in peripheral tissues¹

Peripheral Blood Mononuclear Cells											
	CN		CID		PN		PID		Significant Effects		
Cytokines	FC	SEM	FC	SEM	FC	SEM	FC	SEM	PRRSV	Diet	Interaction
TNF α	1.05	0.14	0.07	0.02	1.52	0.23	0.08	0.01		****	
IL-1 β	1.06	0.16	0.08	0.01	2.13	0.55	0.13	0.02		****	
IL-6	1.07	0.17	0.19	0.08	3.25	2.30	0.13	0.02			
IL-10	1.07	0.16	0.09	0.02	2.15	0.56	0.17	0.03		****	
IFN- γ	1.18	0.28	0.33	0.12	8.30	2.20	1.13	0.18	***	***	**
Toll-like receptors											
TLR3	1.04	0.11	0.30	0.14	0.47	0.12	0.12	0.03	**		****
TLR4	1.03	0.10	0.08	0.02	1.74	0.86	0.08	0.01		**	
TLR7	1.01	0.06	0.09	0.02	2.63	1.31	0.11	0.01		****	
Major histocompatibility complex											
SLA-DRA	1.05	0.15	0.08	0.02	1.39	0.17	0.07	0.01		****	
SLA-DRB	1.05	0.14	0.06	0.01	0.93	0.16	0.05	0.01		****	
Anti-Microbial											
CYBA	1.26	0.30	0.25	0.12	0.93	0.18	0.13	0.03		****	
NOS2	1.09	0.19	0.63	0.47	3.79	0.57	0.59	0.13	**	****	**
Iron metabolism											
FTH1	1.03	0.11	0.90	0.14	3.26	0.73	0.75	0.05	*	**	**
FTL	1.00	0.03	2.65	0.29	2.48	0.22	1.68	0.15		*	****
TF-R	1.02	0.09	2.13	0.30	0.54	0.23	1.34	0.18	**	***	
HIF α	1.06	0.16	0.45	0.22	0.73	0.15	0.13	0.02	*	***	

Table 4.1, continued

Liver	CN		CID		PN		PID		Significant Effects		
<u>Cytokines</u>	FC	SEM	FC	SEM	FC	SEM	FC	SEM	PRRSV	Diet	Interaction
TNF α	1.01	0.04	0.64	0.04	2.50	0.25	1.11	0.16	****	****	**
IL-1 β	1.02	0.08	2.00	0.51	1.75	0.12	4.16	0.45	***	****	*
IL-4	1.03	0.10	0.47	0.12	0.49	0.12	0.25	0.06	**	**	
IL-6	1.06	0.18	0.76	0.11	0.88	0.14	0.49	0.05		*	
IL-10	1.08	0.20	0.79	0.09	1.81	0.20	0.99	0.09	**	**	
IFN- γ	1.08	0.19	1.80	0.67	17.54	2.57	15.45	1.44	****		
NF κ B	1.01	0.07	1.12	0.06	1.49	0.14	1.03	0.04	*		**
<u>Toll-like receptors</u>											
TLR3	1.02	0.10	0.71	0.12	0.86	0.10	0.97	0.13			
TLR4	1.01	0.05	0.90	0.06	1.21	0.12	0.74	0.13		**	
TLR7	1.05	0.17	0.96	0.18	1.81	0.23	0.82	0.12		**	*
<u>Major histocompatibility complex</u>											
SLA-DRA	1.01	0.07	0.64	0.07	2.03	0.18	0.95	0.11	****	****	**
SLA-DRB	1.03	0.13	0.59	0.08	1.50	0.19	0.92	0.11	**	**	
<u>Anti-Microbial</u>											
CYBA	1.03	0.11	0.75	0.02	1.73	0.14	0.79	0.18	**	****	*
NOS2	1.03	0.11	0.94	0.13	1.07	0.19	0.79	0.13			
<u>Iron metabolism</u>											
FTH1	1.01	0.06	1.89	0.23	1.28	0.17	2.02	0.19		***	
FTL	1.01	0.05	1.38	0.11	1.30	0.08	1.38	0.05		*	
TF-R	1.01	0.07	6.44	0.87	1.28	0.04	3.99	0.44	*	****	*
TF	1.01	0.07	1.68	0.09	1.15	0.12	0.71	0.11	***		****
HIF α	1.04	0.12	0.97	0.07	1.00	0.14	0.95	0.07			
<u>Acute Phase Response Proteins</u>											
HAMP	1.38	0.44	0.00	0.00	0.65	0.23	0.01	0.00		**	
CRP	1.32	0.45	0.60	0.11	3.87	1.84	4.06	1.67	*		

Table 4.1, continued

Spleen	CN		CID		PN		PID		Significant Effects		
<u>Cytokines</u>	FC	SEM	FC	SEM	FC	SEM	FC	SEM	PRRSV	Diet	Interaction
TNF α	1.09	0.15	0.78	0.15	0.81	0.10	0.60	0.09			
IL-1 β	1.35	0.40	1.26	0.19	1.74	0.33	2.73	0.43	*		
IL-6	1.07	0.16	0.70	0.15	0.68	0.06	0.76	0.14			
IL-10	1.17	0.28	0.69	0.13	0.81	0.06	0.72	0.07			
IFN- γ	1.06	0.13	0.99	0.14	6.86	1.35	10.04	1.24	****		
NF κ B	1.04	0.11	1.06	0.11	1.01	0.11	0.84	0.08			
<u>Toll-like receptors</u>											
TLR3	1.33	0.34	0.88	0.29	3.10	1.31	1.61	0.46			
TLR4	1.03	0.08	0.95	0.16	0.77	0.10	0.72	0.09	*		
TLR7	1.02	0.08	0.68	0.10	0.99	0.09	0.75	0.12	**		
<u>Anti-Microbial</u>											
CYBA	1.21	0.24	0.86	0.16	3.02	1.23	1.52	0.62			
NOS2	1.95	0.48	1.58	0.48	2.45	1.22	1.36	0.62			
<u>Iron metabolism</u>											
FTH1	1.03	0.10	1.30	0.20	3.86	1.67	1.98	0.25			
FTL	1.12	0.24	0.84	0.09	2.62	1.06	1.94	0.40	*		
TF-R	1.27	0.32	8.90	4.32	12.15	1.78	69.60	12.45	****	****	***
HIF α	1.13	0.18	0.84	0.15	2.03	1.14	1.30	0.27			

Table 4.1 continued

Lung	CN		CID		PN		PID		Significant Effects		
<u>Cytokines</u>	FC	SEM	FC	SEM	FC	SEM	FC	SEM	PRRSV	Diet	Interaction
TNF α	1.03	0.10	2.07	0.65	3.65	1.17	2.42	0.59			
IL-1 β	1.24	0.35	1.21	0.26	9.18	3.37	8.04	2.34	**		
IL-4	1.05	0.13	0.65	0.17	1.23	0.46	0.76	0.26			
IL-6	1.10	0.19	1.16	0.17	0.83	0.24	1.49	0.78			
IL-10	1.03	0.10	1.67	0.36	2.15	0.63	2.81	0.61			
IFN- γ	1.05	0.11	2.04	0.63	24.13	10.36	20.22	5.80	**		
NF κ B	1.03	0.10	1.20	0.14	1.63	0.21	1.69	0.33	*		
<u>Toll-like receptors</u>											
TLR3	1.29	0.29	0.64	0.25	2.42	1.07	1.27	0.38			
TLR4	1.00	0.07	1.19	0.17	1.90	0.65	1.32	0.20			
TLR7	1.05	0.13	1.28	0.22	1.75	0.32	2.02	0.34	*		
<u>Major histocompatibility complex</u>											
SLA-DRA	1.03	0.11	1.31	0.25	2.89	0.28	2.49	0.25	****		
SLA-DRB	1.04	0.11	1.32	0.33	1.80	0.23	1.94	0.28	*		
<u>Anti-Microbial</u>											
CYBA	1.31	0.33	1.04	0.25	3.01	1.14	1.50	0.57			
NOS2	1.04	0.12	1.14	0.13	1.05	0.50	0.62	0.24			
<u>Iron metabolism</u>											
FTH1	1.03	0.10	1.15	0.11	4.16	1.70	2.02	0.25	*		
FTL	1.01	0.07	0.95	0.07	3.30	1.32	2.47	0.48	*		
TF-R	1.19	0.26	1.85	0.79	3.00	0.63	4.84	1.51	**		
HIF α	1.15	0.20	0.71	0.13	2.01	1.05	1.19	0.26			

¹Statistical effects determined by 2-way ANOVA. * signifies $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$,

**** $p < 0.001$. CN = control, normal diet; CID = control, ID diet; PN = PRRSV-infected, normal diet; PID = PRRSV infected, ID diet.

Microglial gene expression is altered by infection, but insulated from the effects of ID

Microglial expression of anti-microbial and iron metabolism genes was altered by PRRSV infection, but not ID (Figure 4.5). Infection increased CYBA expression (main effect, $p = 0.043$). Inflammatory cytokine expression was not significantly affected. Expression of HIF α was increased by infection (main effect, $p = 0.041$). HIF α is a transcription factor involved in regulating cellular responses to infection and hypoxia and modulates many cellular processes, including angiogenesis, cell proliferation, and energy and iron metabolism. Ferritin and transferrin receptor expression were upregulated by infection (main effects, $p < 0.001$ and $p = 0.005$, respectively). Anti-microbial and iron metabolism genes were upregulated by infection, but microglial gene expression appears largely insulated from the effects of ID.

Microglial Gene Expression

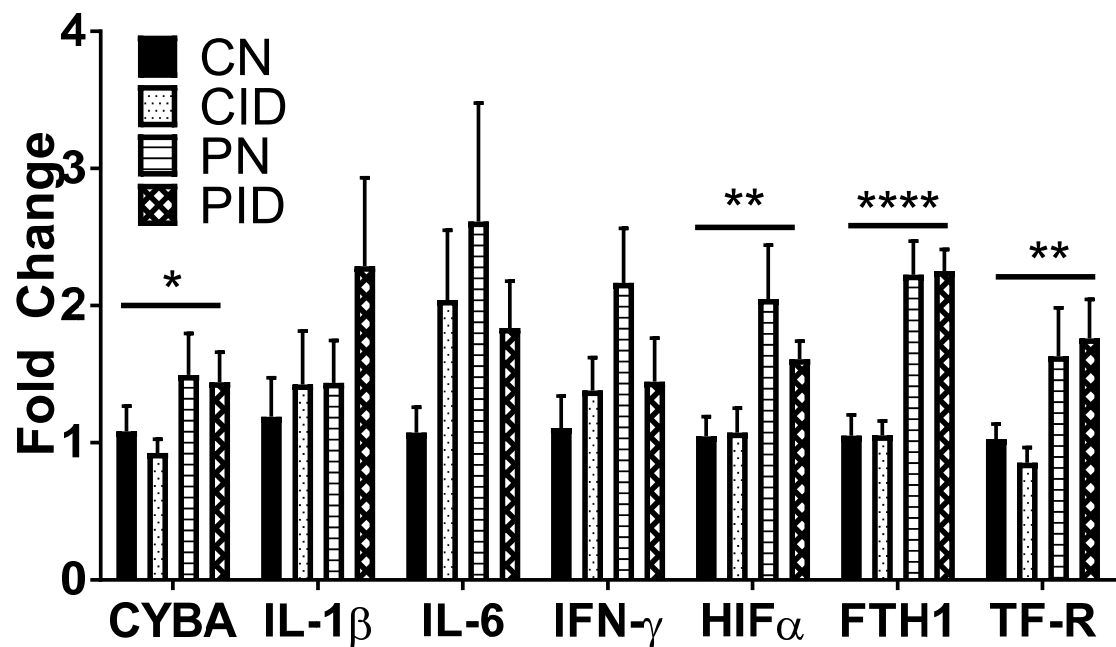


Figure 4.5: Microglial gene expression. Data are presented as means $\pm$ SEM (n=6-8). Asterisks denote main effect of PRRSV infection, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$

Infection, but not ID, alters microglial activation and phagocytic activity

Flow cytometry and measurement of phagocytic activity were used to assess microglial phenotype. CD11b⁺ cells expressing both CD45 and MHCII were considered activated microglia. Percentage of activated microglia was averaged for each group (Figure 4.6A). A two-way ANOVA found a main effect of infection ($p < 0.001$); no other effects were present. Representative plots of control and PRRSV-infected piglet microglia are depicted by Figure 4.6C and Figure 4.6D, respectively. The percentage of CD45⁺ and MHCII⁺ cells are presented in the upper-right corner of the representative plots. Microglial phagocytosis (Figure 4.6B) was assessed using the Vybrant Phagocytosis Assay Kit. A main effect of infection ($p = 0.002$) increased phagocytic activity. No other effects were present, and dietary groups did not differ. Lower case letters denote significantly different groups as determined by Fisher's LSD ($p < 0.05$). Data are presented as means $\pm$ SEM ($n = 6-8$).

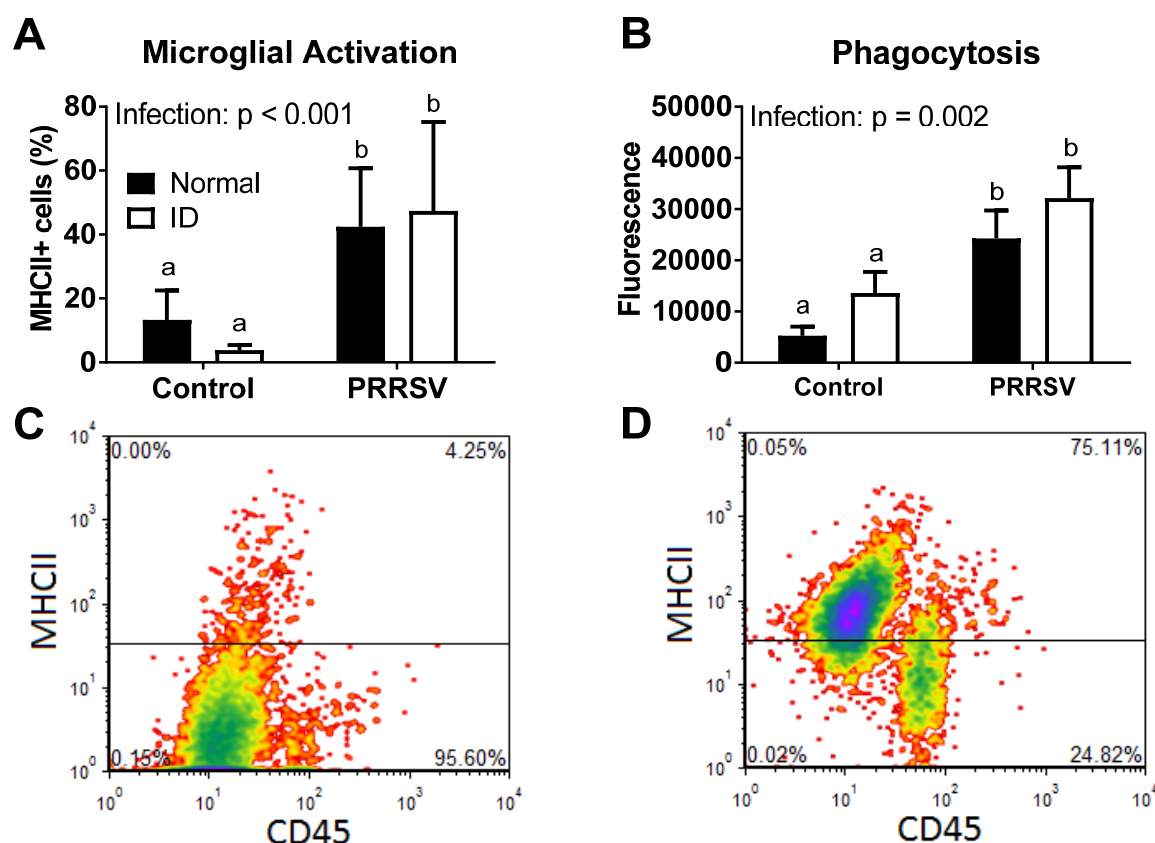


Figure 4.6: Microglial activation and phagocytic activity. Cells positive for CD45 and MHCII were considered activated (A). Representative plots of CD11b+ cells from an uninfected piglet (C) and CD11b+ cells from a PRRSV-infected piglet (D). Microglial phagocytic activity (B) was altered by a main effect of infection ($p=0.002$). Lower case letters denote significantly different groups as determined by Fisher's LSD ($p<0.05$). Data are presented as means $\pm$ SEM ($n=6-8$).

4.5 Discussion

The present study was designed to evaluate how the link between peripheral inflammation and neuroinflammation is affected by iron deficiency. ID is the most common micronutrient deficiency in the world. As ID often presents with immunodeficiency, the double burden of ID and infection in early life is of serious concern, especially in developing nations. Though there is evidence ID alters immune cell function [1, 19, 21], more work is required to determine specifically how this leads to immunocompromise. To date, there has been no work studying the interactions of ID and infection upon neuroinflammation in the postnatal period. To address this

question, the current work used a translational neonatal piglet model raised to a developmental time point analogous to that of a 4-6 month old human infant [140].

Our data show that while infection did not alter measures of anemia in the context of ID, ID reduced viral clearance from both the serum and spleen, suggesting a direct effect of ID upon immune cells, thereby reducing their efficacy. White blood cell counts were reduced in ID piglets compared to piglets fed normal diet; reduced immune cell proliferation may be one route by which ID weakens the immune system. Interestingly, PID piglets displayed a pattern of sickness behavior largely inverse to that of infected piglets fed a normal diet (Figure 4.3). PID piglets demonstrated fewer stereotypical sickness behaviors during the first three weeks of the study, displaying greater appetite and activity levels compared to PN piglets. However, this phenomenon inverted during the last week of the study, wherein the condition of PID piglets sharply declined. During this final week, as PN piglets were recovering steadily (displaying increased appetite, energy levels, and vocalization), PID piglets displayed a sharp increase in sickness behaviors which persisted to the end of the study. While there are examples of nutrition attenuating the sickness behavior response [169], there is little data to be found on malnutrition exacerbating sickness behavior.

There are several possible mechanisms which may contribute to the atypical illness progression observed in PRRSV piglets. The heightened viral load compared to piglets fed a normal diet indicates impaired immune function and may cause comparatively increased tissue damage, contributing to the observed decline in PID piglet condition. ID had marked effects on PBMC cytokine expression, reducing $\text{TNF}\alpha$, $\text{IL-1}\beta$, $\text{IFN-}\gamma$, and IL-10 . While the first three pro-inflammatory cytokines promote the sequestration of iron by immune cells, the anti-inflammatory cytokine IL-10 promotes resolution of inflammation and also promotes iron export from immune cells, reversing the iron sequestration response to inflammation [12]. The generally reduced

cytokine expression in PID piglets compared to PN piglets indicates a blunted infection response. Changes in IFN- γ expression, which was reduced to basal levels in PID piglets compared to an 8-fold increase in PN piglets, are notable as IFN- γ is a key mediator in antiviral immunity and imperative for clearance of the PRRS virus [170]. Interestingly, directional changes in expression differed across tissues - while IL-1 β expression was decreased in PID PBMCs, it increased in the liver. PBMC expression of Toll-like Receptors 3 and 7, which detect double- and single-stranded RNA, respectively, was reduced by ID, which may further depress the immune response to viral infection. ID reduced the antimicrobial capacity of PBMCs, as expression of both NADPH oxidase and NOS2, which are reliant upon iron to generate reactive oxygen species to destroy pathogens, was reduced, matching previous findings showing iron chelation reduced iNOS activity in a murine macrophage cell line [18]. Thus, ID impairs both the initial inflammatory response to infection as well as subsequent resolution of the inflammatory response in PBMCs as well as liver tissue. These data are valuable as the porcine immune system is highly similar to the human immune system [56], and the pig has functional equivalents to all cells and cytokines involved in the Th1/Th2/Th17/Treg axis [64].

The brain is sensitive to peripheral inflammatory signals, which promote neuroinflammation, activation of microglia, and sickness behavior [73]. During neurodevelopment, microglia perform synaptic pruning and phagocytosis of neural precursor cells [74, 76], which may be modulated by early life environmental insults. While microglial activation and phagocytic activity were increased due to postnatal infection in agreement with previous results [82], ID did not alter microglial activation or phagocytic activity. Microglial gene expression was also altered by infection alone, with no diet effects. Cytokine and antimicrobial gene expression was increased by infection, as were genes involved in iron sequestration, suggesting microglia were insulated from

the effects of iron deficiency, at least at the observed time point. The brain is ‘iron privileged’ compared to the periphery, e.g. in the case of deficiency, the brain is depleted of iron more slowly. It is possible that continued ID would eventually affect microglial physiology. Previous data from our lab has found the iron content of cortical tissue from ID piglets did not differ from that of controls, though hippocampi from ID piglets showed significantly reduced iron levels [171]. Thus, microglial phenotype and cytokine expression may be unaffected due to increased iron concentrations in the local tissue compared to the periphery. Therefore, the altered sickness behavior observed in PID piglets likely results from the altered expression of cytokines in the periphery.

To date, this is the first study how postnatal ID in the context of infection affects microglia and neuroinflammation. Despite drastically altered sickness behavior and abundant differences in the peripheral immune response of PID piglets, microglial activation state, activity, and cytokine expression were unaffected by ID. Thus, at the time point assessed in this study, microglia do not appear to be involved with neurodevelopmental changes associated with ID, though further study is required.

4.6 Acknowledgements

This work was supported by NIH grant HD069899. We thank Dr. William Van Alstine for provision of PRRS virus.

Chapter 5. Effects of ID and infection upon neurodevelopment, neurotransmission, and bioenergetics

5.1 Abstract

Since iron deficiency (ID) often presents with immunodeficiency, this study used a neonatal piglet model to investigate negative impacts on neurodevelopment and neuroinflammation caused by concurrent postnatal ID and respiratory infection. We hypothesized that ID would reduce immune activation and cytokine expression, reduce expression of genes associated with neurodevelopment, and impair both energy and monoamine metabolism.

A two-level factorial design was used for this experiment. On postnatal day 2 (PD 2) male and female piglets were divided into four groups and fed either iron normal (N) or ID sow milk replacer. Piglets were inoculated with vehicle or porcine reproductive and respiratory syndrome virus (PRRSV) on PD 8. Rectal temperatures, feeding score and sickness behaviors were measured daily until piglets were harvested on PD 28. Tissues were collected from the medial prefrontal cortex, striatum, hippocampus, hypothalamus, and amygdala for monoamine analysis and Fluidigm gene expression panels. The Seahorse XFp analyzer was used for metabolic analysis of ID BV-2 cells.

Brain weight was reduced by PRRSV infection but not ID. Expression of genes playing roles in neurodevelopment, energy and neurotransmitter metabolism, iron metabolism, white matter development, inflammation, and the microglial sensome were disrupted across tissues in a region-specific manner by main effects of ID and infection as well as interactions. ID alone typically downregulated gene expression. Dopamine metabolism was differentially affected by ID and PRRSV in key brain regions including the hippocampus, medial prefrontal cortex, striatum and amygdala. Hippocampal dopamine turnover was increased by ID. ID reduced immune cell basal oxidative respiration and spare immune capacity.

ID and infection altered expression of key gene families in multiple brain areas. Impaired dopamine metabolism may contribute to increased fear and anxiety and reduced stress resilience reported with postnatal ID and infection. ID severely impaired immune cell metabolic flexibility.

5.2 Introduction

The early postnatal period is a critical window in brain development, and environmental insults can disrupt the delicate confluence of neurodevelopmental processes, leading to lasting alterations in the brain and behavior. ID and early life infection both have the potential to interfere with neurodevelopmental domains through disrupted synaptogenesis, neurite outgrowth, neurotransmitter tone, and receptor density [32, 44, 47, 70, 80, 156]. These have been shown to result in altered behavior [47, 84, 142]. Adolescents who experienced early life ID report poorer motor, cognitive, and socio-emotional function, which is thought to be due to alterations in myelination, neurometabolism, and monoamine neurotransmission [29, 136]. Nine years after iron repletion therapy, early life ID anemia was associated with poorer inhibitory control and slower reaction times [53]. While early life ID and perinatal immune challenge are both associated with later neurological and behavioral disorders, including schizophrenia and autism, the interactive effects of concurrent ID and infection upon brain development during early life have not been examined.

Neurotrophic factors play a key role in orchestrating brain development and maintaining brain health and function throughout the lifespan. Brain-derived neurotrophic factor (BDNF) is required for brain plasticity and maintenance of neuronal health [100, 101, 172]. BDNF helps maintain brain plasticity by increasing synaptogenesis and neurogenesis [85, 89]. Environmental insults can reduce neurotrophin levels, which may alter neurodevelopment if the insult occurs in the perinatal period [164]. BDNF in the context of ID has been the focus of little study – a study in the rat found a persistent reduction in brain BDNF [172], and a reduction of hippocampal BDNF

was found the pig [101]. Maternal ID is associated with impaired spatial memory in rats [115]. Early life ID is associated with increased anxiety in humans, and causes altered emotionality and fear response in nonhuman primates and increased anxiety in humans [35, 173]. At 19 years of age, persons who had experienced severe, chronic ID as infants displayed impaired performance on both fronto-striatal (inhibitory control, set shifting, planning) and hippocampal-reliant (recognition memory) behavioral tasks, functions associated with dopaminergic transmission [37]. Monoamines are key regulators of mood, cognition, and learning. Greater understanding of the mechanisms by which ID alters brain development in the short-term and how deficits persists develop additional targets for therapeutic intervention.

Cell longevity, and in some cases cell phenotype, are partly regulated by cellular energy metabolism. Neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, are linked to alterations in brain energy metabolism [174]. However, energy metabolism plays an even greater role in immune cells – energy metabolism is interlinked with immune function; regulating gene expression, polarization, and immune effector functions [117, 118]. While M1 cells use aerobic glycolysis to fuel rapid response and substrate generation for destruction of pathogens, M2 macrophages rely on β -oxidation and mitochondrial oxidative phosphorylation for long-term energy production [118, 123].

The current study builds upon previous work showing impaired spatial memory and white matter development in ID piglets [142, 165], and provides deeper understanding of the region-specific neurochemical, metabolic, neurodevelopmental, and inflammatory changes to the brain caused by the common early life insults of ID and infection. We expected ID to alter monoamine metabolism and reduce associated gene expression, which would be exacerbated by infection.

Additionally, we hypothesized that postnatal ID and infection would reduce expression of genes involved with neurodevelopmental and energy metabolism.

5.3 Methods

Animals care, diet, PRRSV inoculation, and daily measures. Animal care, diet, inoculation, and daily measures were performed as described in the previous chapter. Briefly, two cohorts of piglets were received on postnatal day (PD) 2 from UIUC swine farms and split into 4 groups of 6 piglets, and randomized according to sex, weight, and litter. Groups were Control, Normal Diet (CN); Control, Iron Deficient Diet (CID); PRRSV-infected, Normal Diet (PN); PRRSV infected, Iron Deficient diet (PID). After facility intake, piglets were raised until PD 28. Piglets were fed a nutritionally complete sow milk replacer (Test Diet, Richmond, IN) as described previously that was either iron-normal or iron-deficient by a factor of 10 [165]. Piglets were housed individually in cage racks as previously described [165], and provided environmental enrichment. Room temperature was maintained at 27° C minimum, and supplemental heating was provided by an in-cage heating pad (K&H Lectro-Kennel Heat Pad). A 12h light/dark cycle was maintained with light from 0700 to 1900h. All animal care and experimental procedures were performed in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. Piglets were inoculated with either PRRS virus (strain P129-BV4) or a sterile PBS sham. Food intake, sickness behavior, temperature, and body weight were measured daily as described previously.

Blood draws and tissue collection. Weekly blood draws were performed to assess anemia and collect serum and plasma as described in the previous chapter. An aliquot of whole blood was sent to the UIUC Veterinary Medicine Diagnostic lab for complete blood count (CBC). Piglet sacrifice and tissue collection were performed as described previously. The following brain regions were

collected: amygdala, medial prefrontal cortex (mPFC), striatum, hypothalamus, and hippocampus. Additional tissue punches from regions of interest were taken for HPLC, specifically the amygdala, anterior mPFC, dorsolateral striatum, anterior hypothalamus, and ventral hippocampus. All tissue punches were taken by the same dissector with experience in neuroanatomy so as to reduce variation in tissue punch location. The cerebellum was removed and used for microglial isolation.

Cell culture. Cell culture experiments were performed with a BV-2 immortalized murine microglia cell line plated in either 12-well plates (1×10^6 cells/well) or Seahorse miniplates (3.5×10^4 cells/well) in serum free DMEM with 4.5 g/L glucose, L-glutamine, sodium pyruvate, and Pen/Strep (Corning Celgro, Manassas, VA). Cells were allowed to adhere for 2 hours and treated for 48 hours with the iron chelator deferoxamine mesylate (DFO) from Sigma-Aldrich (Darmstadt, Germany) or sterile PBS vehicle. Cells were incubated at 37° C in a 5% CO₂ incubator.

Measurement of Neurotransmitters using High-Pressure Liquid Chromatography. Brain tissue punches were collected at necropsy and stored at -80° C until use were digested in .1 N perchloric acid and 25 mM ascorbic acid. Samples were spun at 12,000 xg for 10 minutes at 4° C. The supernatant was then removed and filtered through HPLC grade filter tubes at 12,000 xg for 5 minutes at 4° C. Sample solutions were stored at -80° C until analyzed using high-pressure liquid chromatography (HPLC) using an ESA 540 unit. The following monoamines were measured: dopamine (DA), the dopamine metabolites 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), serotonin (5-HT), and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA). HPLC data were analyzed as ratios (DOPAC:DA, HVA:DA, and 5-HIAA:5-HT) to provide insight into monoamine neurotransmitter metabolism and account for any differences in tissue weight before processing.

Quantitative Real-Time PCR. RNA was isolated using Trizol reagent (Thermofisher Scientific, Grand Island, NY) according to manufacturer protocol. cDNA will be synthesized using the high-capacity RT kit (Applied Biosystems, Grand Island, NY, USA). cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). qPCR was performed using the Taqman Gene Expression Assay protocol at the Keck Functional Genomics Center (Urbana, IL). All genes were analyzed using the delta-delta CT method and normalized to *RPL19* expression.

Fluidigm High-Throughput qPCR. The Fluidigm 96.96 high-throughput PCR array was performed by the Keck Functional Genomics Center (Urbana, IL). 15 μ L of each sample and primer were provided to the Keck Center. Following pre-amplification, samples and primers were loaded in the Fluidigm 96.96 Access Array and placed in the Fluidigm Thermal Cycler for massive parallel PCR. Data were processed using the Fluidigm Biomark SP1 software suite (Fluidigm Corporation, San Francisco, CA).

Oxygen Consumption Rate. The Agilent Seahorse Extracellular Flux XFp Analyzer (Seahorse Bioscience, US) was used for cellular bioenergetic analyses. Cell preparation and the Mito Stress Test were performed according to manufacturer protocol. Cells were seeded in Seahorse cell culture miniplates in 80 μ L culture medium (3.5×10^4 cells/well) and allowed to adhere for 2 hours at 37° C. Cells were then treated with DFO and incubated for 48h (37° C, humidified 5% CO₂). 24h before the assay, the sensor cartridge was hydrated with SeaHorse XF Calibrant and placed in a CO₂-free incubator. After incubation, cells were washed twice with assay medium (Seahorse Bioscience, US) and assay medium was added to each well for a final volume of 180 μ L per well. Plate injection ports were loaded with oligomycin (final well concentration 1 μ M), FCCP (0.5 μ M), and rotenone/antimycin A (0.5 μ M) per manufacture instructions (Mito Stress Test, Seahorse

Biosciences, US). Following calibration, the sensor cartridge was added to the miniplate and the standard Mito Stress Test assay was run with three replicates assessed per group.

Statistical Assessment. Data was analyzed with GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA), SAS (SAS Institute, Cary, NC) and MatLab (MathWorks, Natick MA). All data was checked for normality and analyzed using Student's t-test or 2-way mixed model ANOVA as appropriate. When significant interactions were found, post-hoc comparisons were made using Fisher's LSD.

5.4 Results

Brain Weights

Brain weights were reduced by a main effect of PRRSV infection ($p = 0.012$, Figure 5.1).

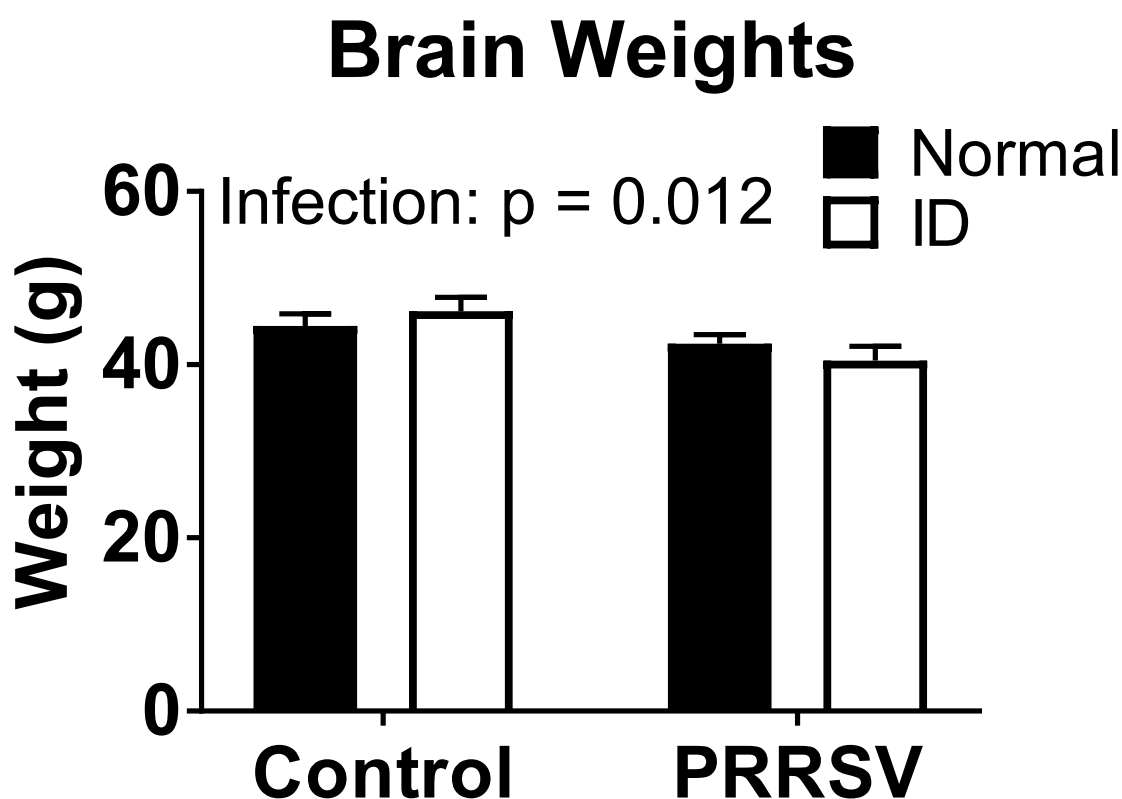


Figure 5.1: Wet brain weights of piglets. Data represented as means $\pm$ SEM ($n = 6-8$).

Fluidigm Hippocampus

Region-specific gene expression was assessed via Fluidigm 96.96 array (Fluidigm Corporation, San Francisco, CA). Neurodevelopmental genes were broadly modulated by PRRSV infection and interactions between ID and infection (Figure 5.2). *Ntrk2*, a neurotrophin receptor gene, genes associated with synaptic pruning and response to viral infection, and epigenetic modifiers *Hdac3* and *Dnmt1* were decreased by ID and PRRSV infection individually, but displayed increased expression in PID piglets (Figure 5.2). ID reduced expression of *Drd2*. *Htr4* expression was reduced in ID piglets, increased in PN piglets and further increased in PID piglets. Transferrin receptor (Tfrc) expression was increased by ID. Genes involved in energy metabolism were modulated by infection and interactions, and were primarily decreased in CID piglets and increased in PID piglets (Figure 5.2). *mTOR*, a master regulator of metabolism and protein synthesis, was decreased in CID and increased by PID. *Taco1* (translational activator of cytochrome C oxidase), which is directly associated with cytochrome C oxidase production, was reduced by the CID condition but increased in the PID group. *Ucp2*, a marker for mitochondrial function, was reduced in CID but increased in PID (Figure 5.2). Expression of *Acadm*, a dehydrogenase for medium length acyl chains that performs the rate limiting step of beta-oxidation, was reduced in CID, PN, and PID, with CID displaying the most profound reduction in expression. A main effect of infection altered expression of *Scd5*, which catalyzes the rate-limiting step in formation of monounsaturated fatty acids (Figure 5.2). Hippocampal inflammatory genes were modulated by interactive and infection effects, and generally displayed a pattern of reduced expression in CID but increased expression in infected groups (Figure 5.3). Notably, *TNF α* and *IL-8* was increased compared to PN, while *IFN γ* was reduced compared to PN. Similarly, microglial sensome gene expression was broadly altered by effects of infection and diet x infection

interactions. Affected genes displayed reduced expression in CID, a slight increase in PN, and a larger increase in PID. *Cd74*, a marker for activation microglial activation, was decreased by ID but increased by PN and further increased by PID (Figure 5.2). *P2ry12* was reduced by CID, slightly reduced by PN, and increased by PID. All Fluidigm values are presented in Appendix A.

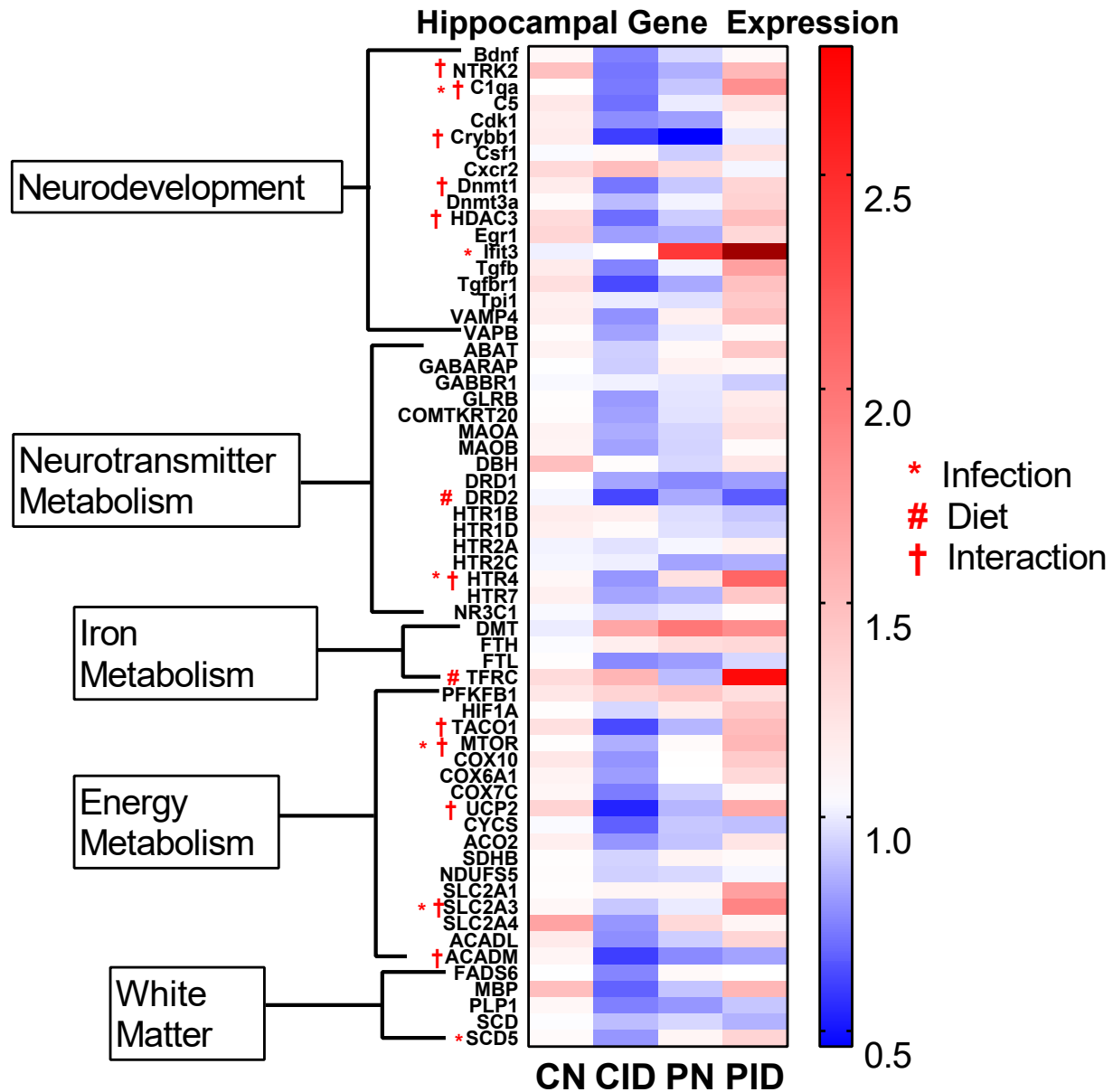


Figure 5.2: Heat map of hippocampal gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. Dark red indicates fold change beyond the upper limit of the scale. More intense color signifies a greater change. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

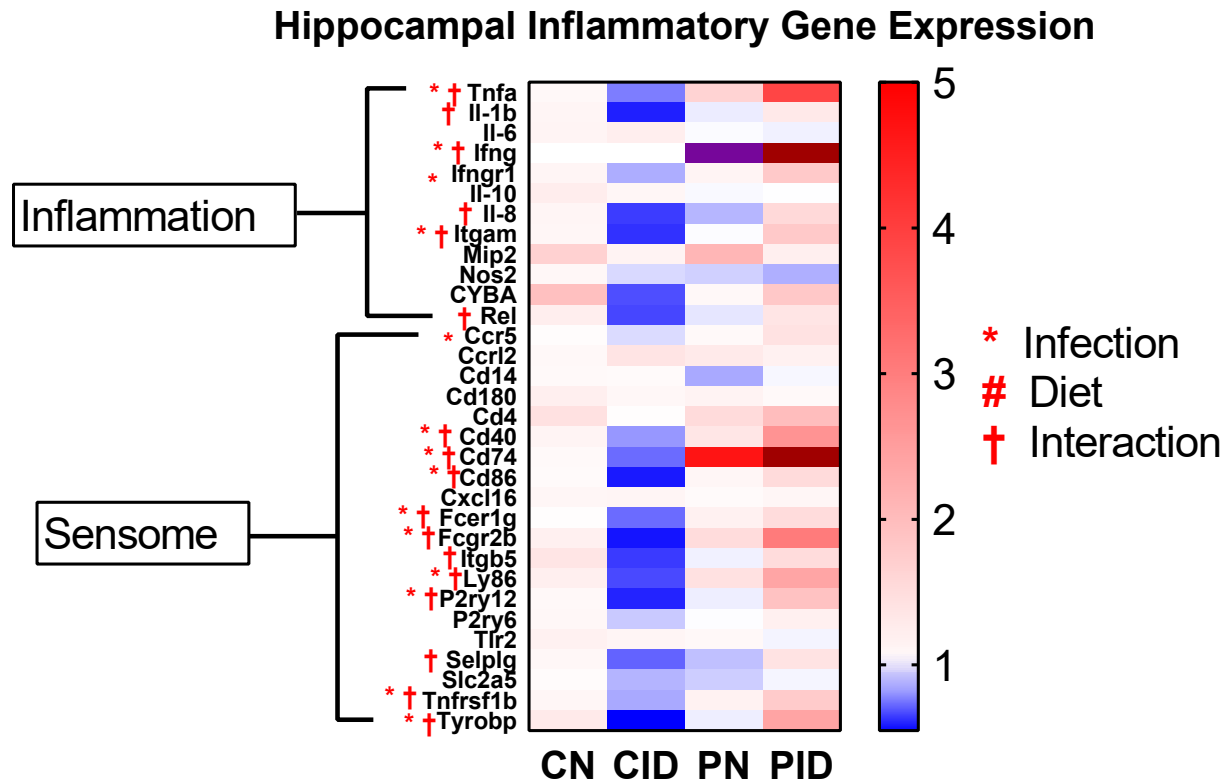


Figure 5.3: Heat map of hippocampal inflammatory gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale, and purple indicates even higher expression than dark red. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

Amygdala

Neurodevelopmental gene expression in the amygdala displayed a trend of reduced expression in CID (Figure 5.4). Main effects of infection increased expression of neurodevelopmental genes, whereas the single diet effect reduced expression of *Crybb1*. Affected genes associated with neurotransmitter metabolism displayed reduced expression (Figure 5.4). Only diet effects altered genes associated with energy metabolism; of those, genes associated with aerobic respiration were reduced while expression of the neuronal glucose transporter was increased (Figure 5.4). Expression of *Scd5*, which plays a role in white matter development and maintenance, was reduced by infection. Inflammation and cytokine expression, especially *Ifn γ* , was increased by infection (Figure 5.5). Sensome gene expression was broadly increased by infection. Non-significant decreases in sensome and inflammatory gene expression were present in CID.

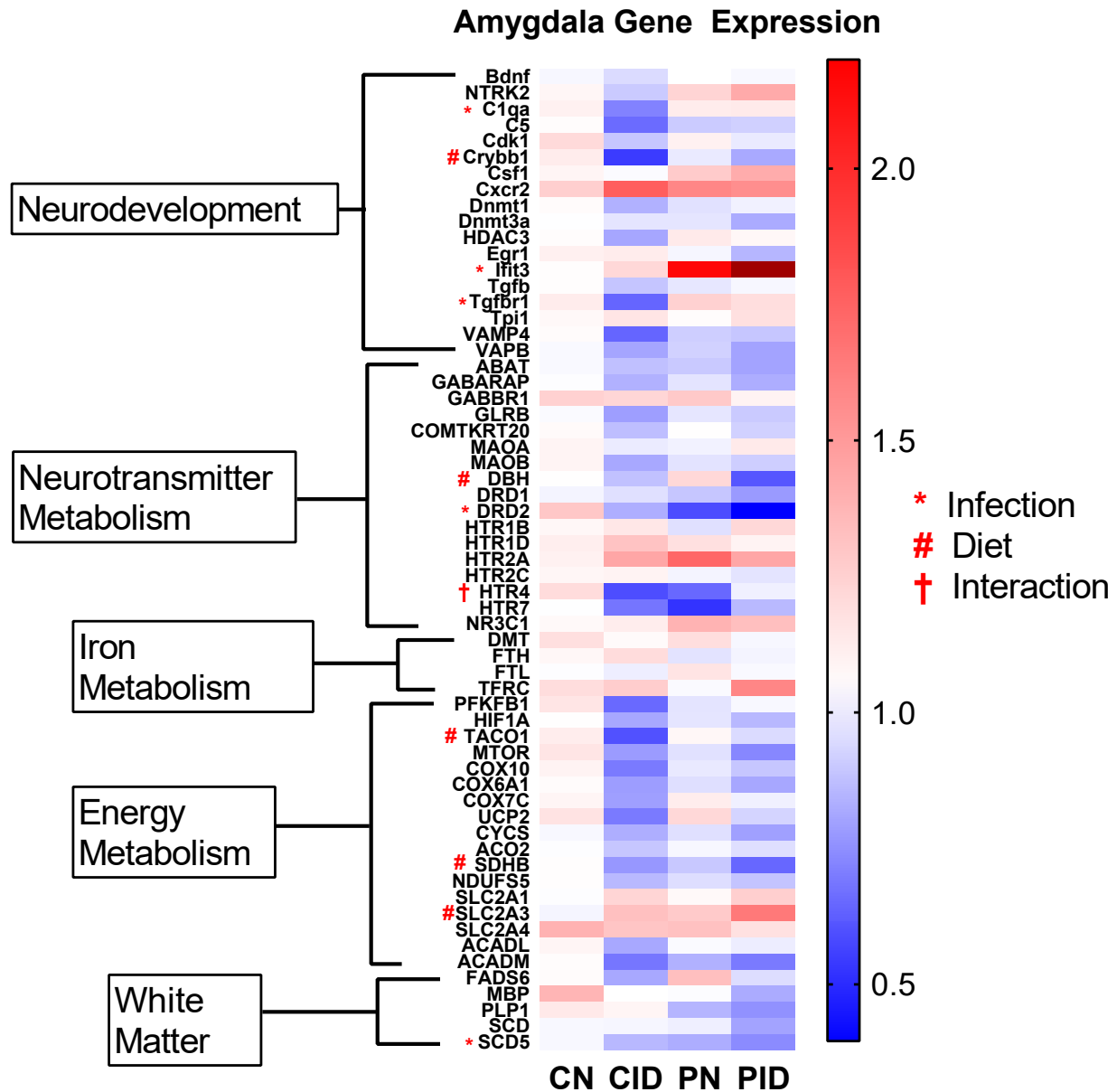


Figure 5.4: Heat map of amygdala gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. Dark red indicates fold change beyond the upper limit of the scale. More intense color signifies a greater change. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

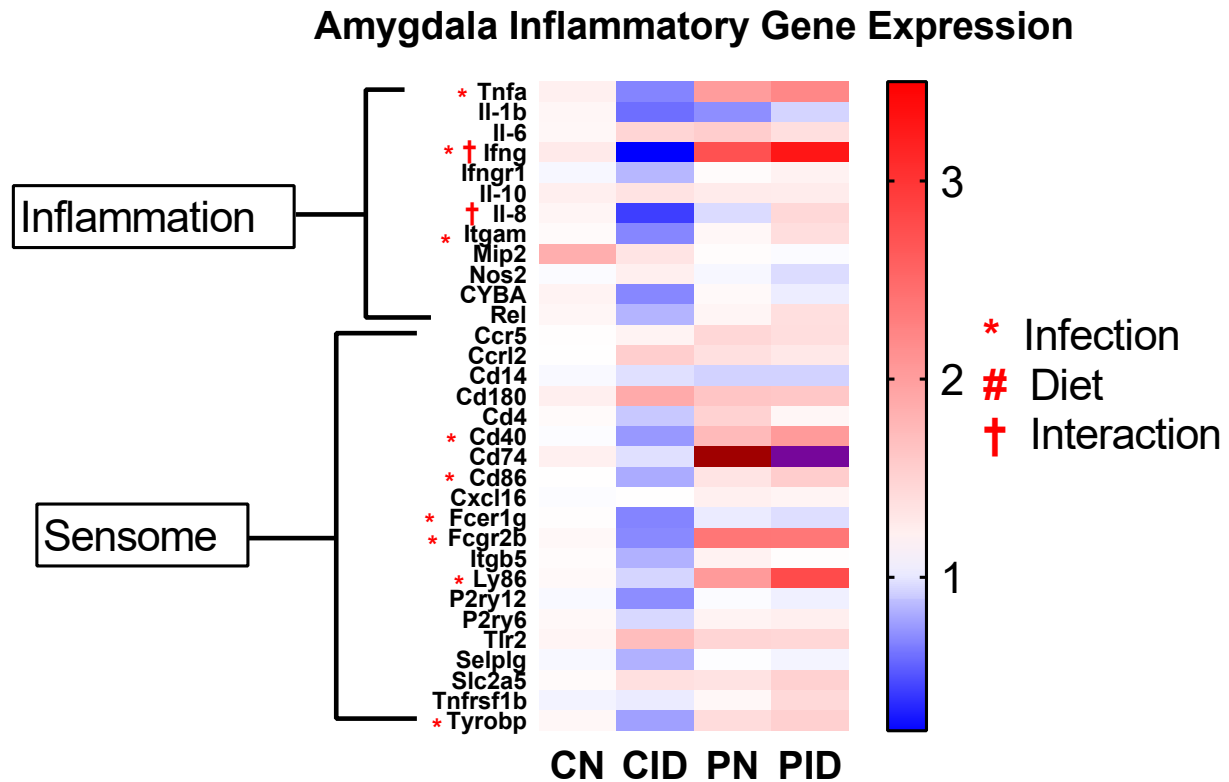


Figure 5.5: Heat map of amygdala inflammatory gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. Dark red indicates fold change beyond the upper limit of the scale, and purple indicates even higher expression than dark red. More intense color signifies a greater change. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

mPFC

Medial prefrontal cortex expression of genes associated with neurodevelopment, neurotransmitter metabolism, energy metabolism, and white matter were broadly reduced by main effects of diet and infection (Figure 5.6). Unlike other regions, mPFC gene expression was broadly downregulated by both insults, with primarily additive effects and only two interactions. Neurodevelopmental genes, including genes associated with epigenetic modification (*Dnmt3a*, *Hdac3*), were primarily reduced by main effects of infection. *Bdnf* was reduced by a main effect of diet. Genes responsible for catabolism of GABA and monoamines were downregulated by infection (Figure 5.6). ID reduced expression of a serotonin receptor (*Htr2A*). Expression of transferrin receptor (*Tfrc*) was reduced by infection (Figure 5.6). Both diet and infection effects reduced expression of genes associated with oxidative phosphorylation, the Krebs cycle, mitochondrial function, and glucose uptake (Figure 5.6). Expression of ‘white matter’ genes associated with myelination and unsaturated fatty acid production were reduced by infection. Expression of inflammatory and sensome genes in the mPFC was primarily increased by main effects of infection, though two genes were reduced by a main effect of ID (*P2ry12* and *Selplg*) and one interaction was present (*Cd86*, Figure 5.7).

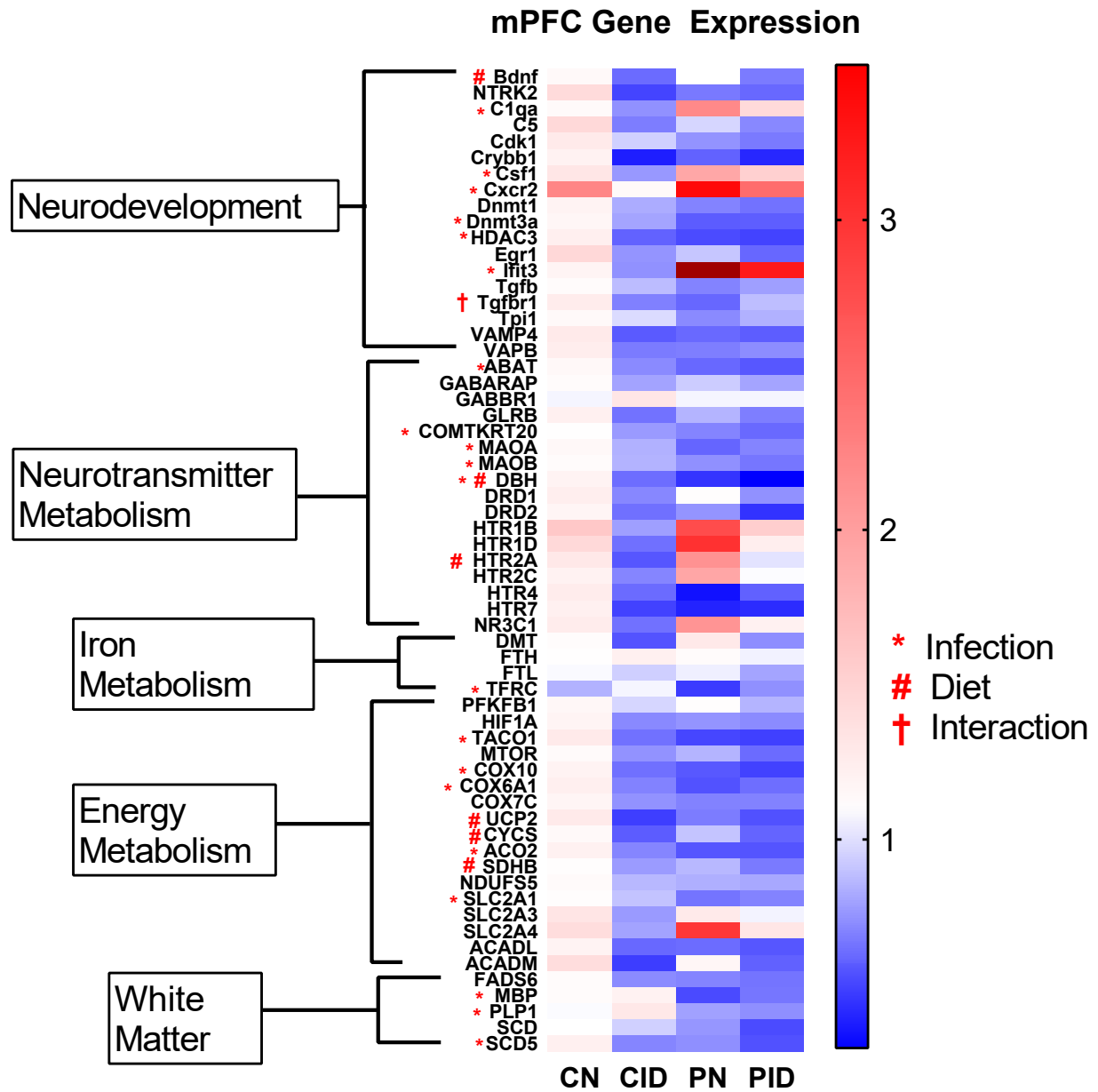


Figure 5.6: Heat map of medial prefrontal cortex gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

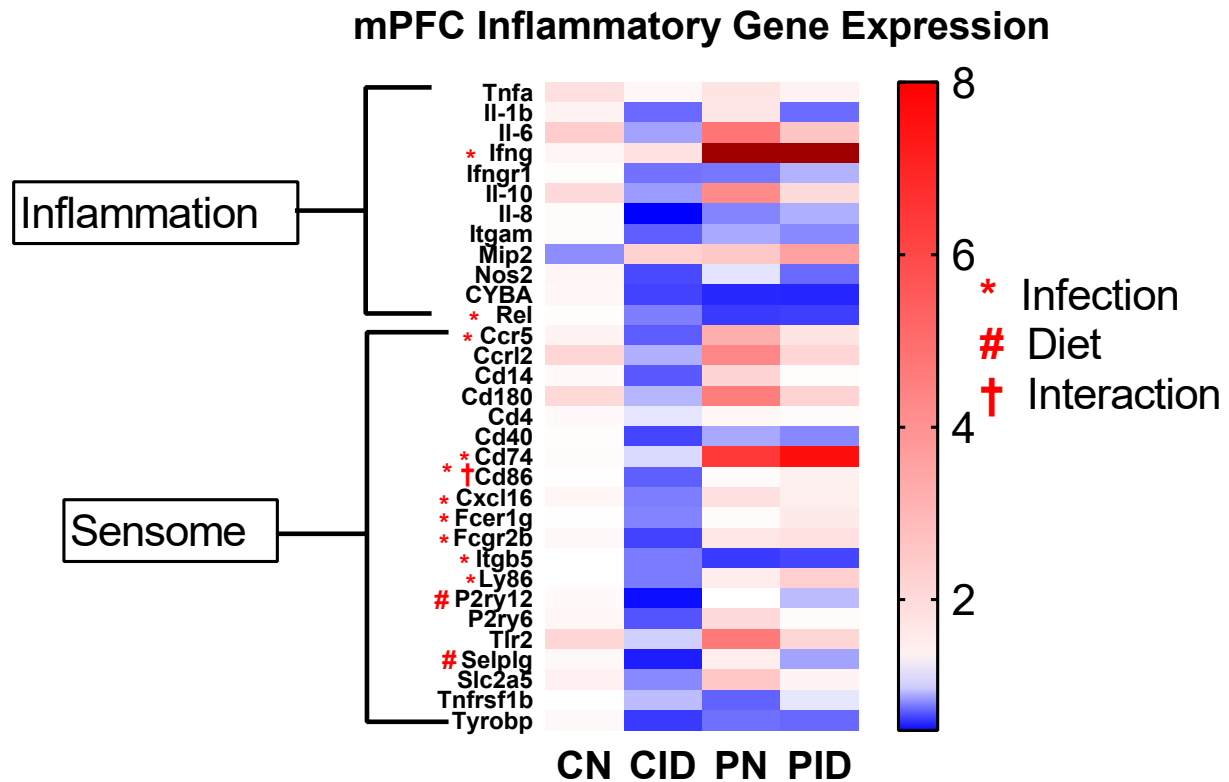


Figure 5.7: Heat map of medial prefrontal cortex inflammatory gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

Striatum

Striatal gene expression was almost exclusively modulated by interactive effects. Neurodevelopmental genes were profoundly affected by diet x infection interactions, following a pattern of reduced expression in CID and PN groups, and overexpression in PID (Figure 5.8). Some genes displayed increased expression in CID and PN piglets, but reduced expression in PID (*Dnmt1*, *Abat*, *Tgfbr1*, *Vamp*, *Htr4*). This pattern was also displayed in genes involved with oxidative metabolism, inverting the pattern observed in the hippocampus. Three serotonin receptor subtypes and the glucocorticoid receptor displayed reduced expression in both CID and PN groups, but slight overexpression in the PID group (Figure 5.8). *Dbh*, which synthesizes norepinephrine from dopamine, was increased in ID but not PID. *Tfrc* expression was increased in CID piglets but reduced in PID piglets (Figure 5.8). Expression of several genes associated with energy metabolism, and specifically oxidative phosphorylation, beta-oxidation, and glucose uptake was reduced by interactive effects, following a pattern of reduced expression in CID, increased expression in PN, and strongly reduced expression in PID (Figure 5.8). Genes associated with white matter were modulated by interactions and less expressed in PID. Inflammatory and sensome gene expression was modulated by infection and interaction effects (Figure 5.9). Infection effects increased expression of inflammatory and sensome genes, while interactions resulted in reduced expression in CID and PN, and increased expression in PID.

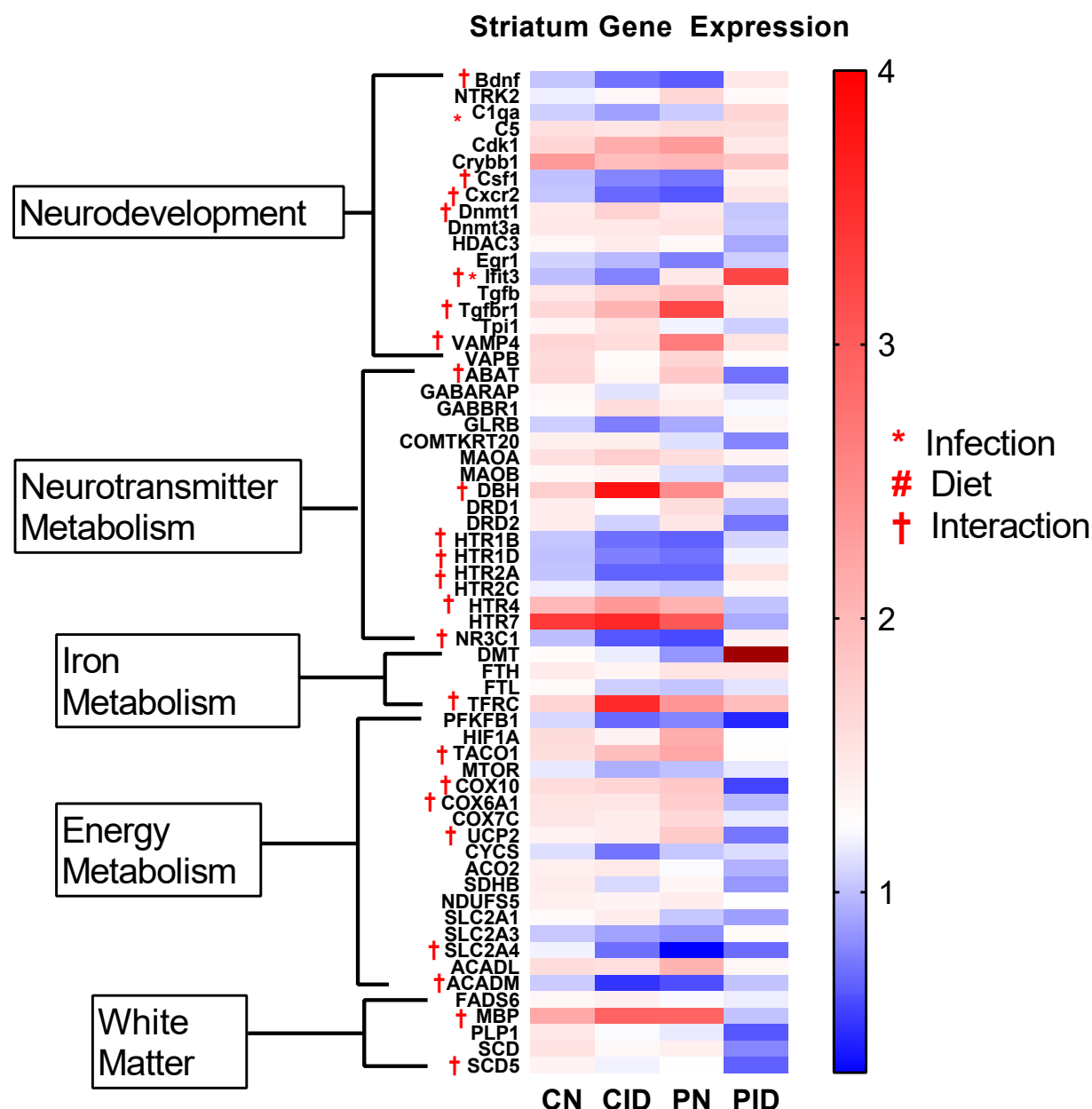


Figure 5.8: Heat map of striatum gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

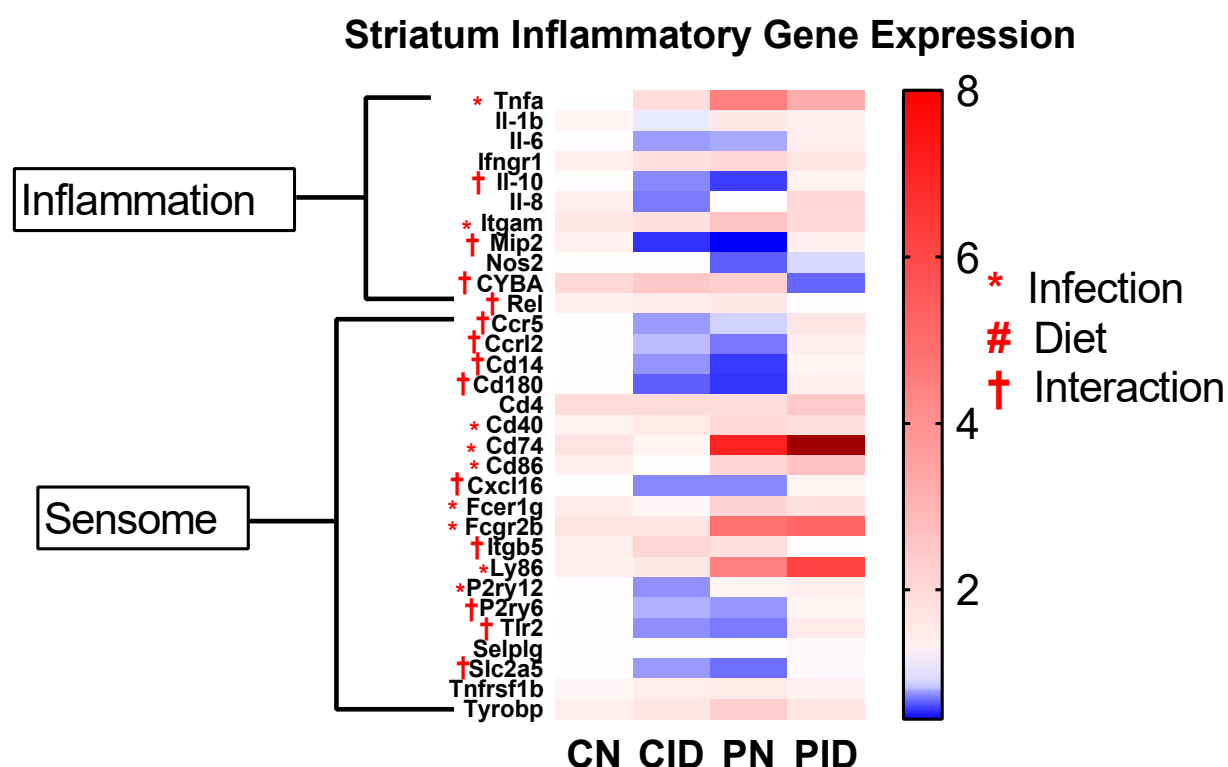


Figure 5.9: Heat map of striatum inflammatory gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

Hypothalamus

Neurodevelopmental gene expression in the hypothalamus was primarily modulated by infection, which increased expression (Figure 5.10). The exception to this is expression of the methyl transferase *Dnmt3a*, which was reduced by infection. Two genes associated with neurotransmitters were modulated by interactions. *Gabbr1* was decreased in PID and *Htr4* was increased by ID and infection individually but reduced to baseline in PID. Expression of genes for iron uptake was increased by diet effect. Genes associated with energy metabolism were increased by ID, with the single infection effect reducing expression of *Ndufs5*, the electron acceptor for complex I of the ETC (Figure 5.10). Expression of all glucose transporters measured was increased in CID and PID, and *Slc2A4* (*Glut4*) displayed a sharp increase in PN due to an interaction (Figure 5.10). Expression of genes associated with white matter was reduced by infection (Figure 5.11). Sensome and inflammatory gene expression was increased by ID and PRRSV infection, individually, while the single interaction reduced *Nos2* expression in PID (Figure 5.11). Both diet and infection main effects increased expression of inflammatory and sensome genes.

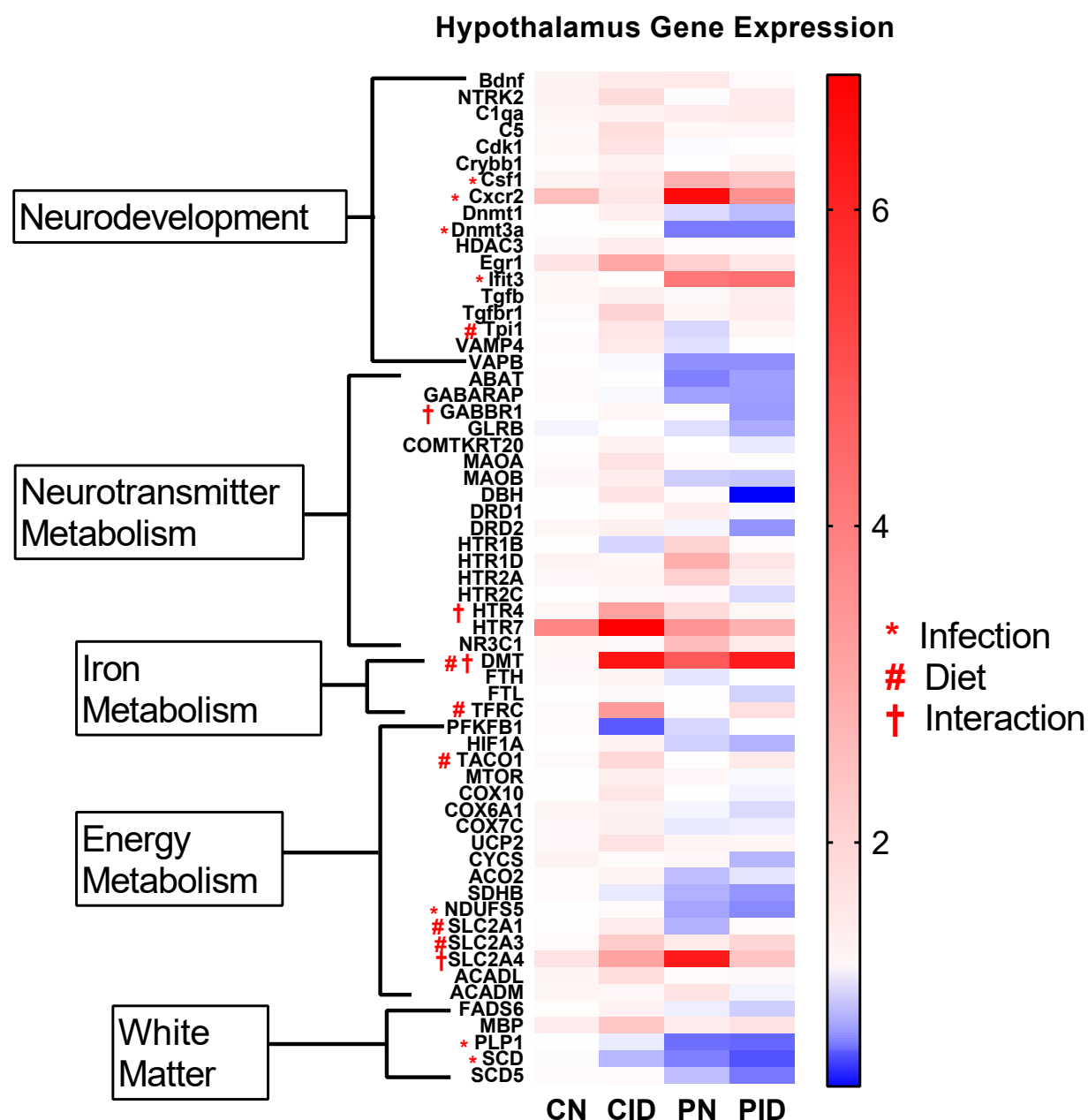


Figure 5.10: Heat map of hypothalamus gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

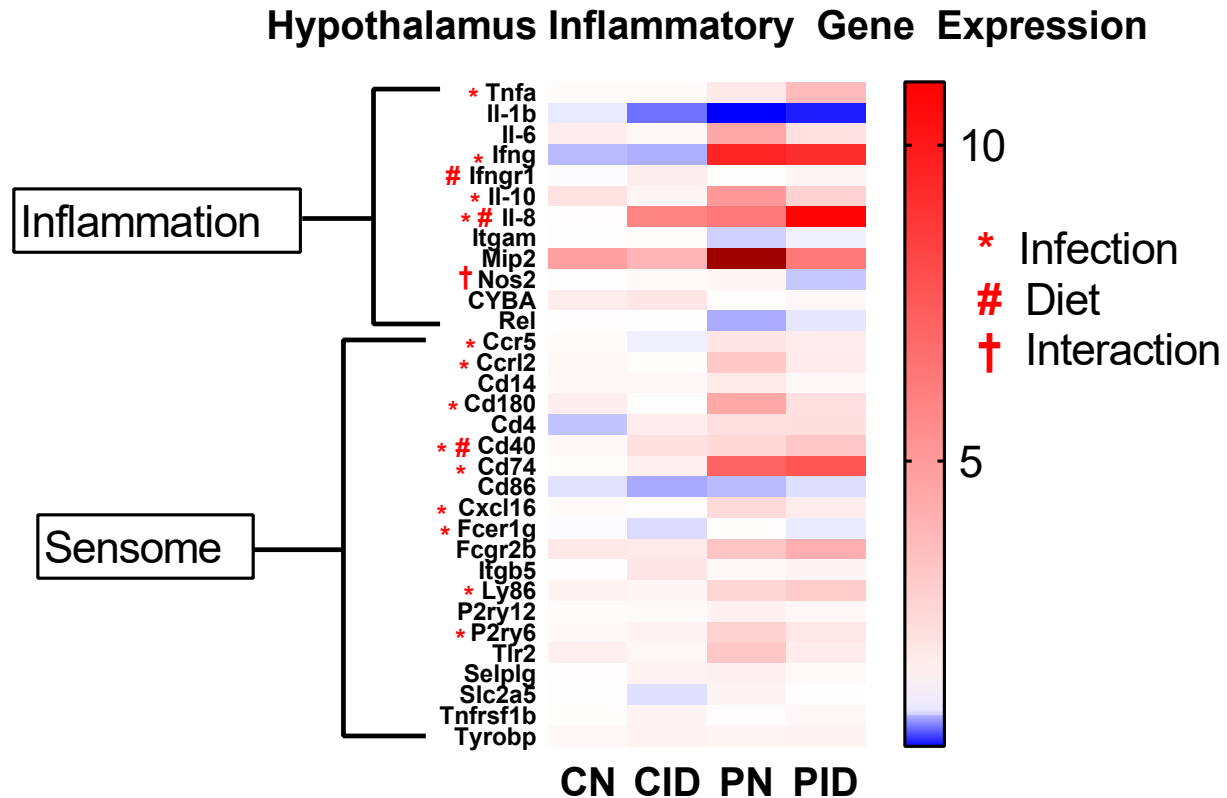


Figure 5.11: Heat map of hypothalamus inflammatory gene expression. Data means are represented. Blue signifies decreased expression, red signifies increased expression. More intense color signifies a greater change. Dark red indicates fold change beyond the upper limit of the scale. * denotes a main effect of PRRSV infection ($p < 0.05$), # a main effect of ID diet ($p < 0.05$), † denotes an interaction ($p < 0.05$). Values are presented in Appendix A.

Mitochondrial function is impaired in ID microglia

Oxidative respiration of BV-2 cells treated with an iron chelator was measured using an Agilent Seahorse XFp analyzer. ID BV-2 cells exhibited half the basal respiration rate of controls ($p = 0.004$, Figure 5.12B). The maximal respiration rate of ID cells was 3.5-fold less than controls ($p = 0.001$ Figure 5.12C). Spare respiratory capacity of ID cells was dramatically reduced, with a 6-fold reduction compared to controls ($p < 0.001$, Figure 5.12D). BV-2 expression of the iron storage gene *Fth* and the inflammatory cytokine *Il-1 β* were reduced by ID treatment ($p = 0.001$, $p = 0.023$, Figure 5.13). ID also decreased expression of the microglia-specific sensome gene *Tmem119* ($p < 0.001$, Figure 5.13). *TNF α* expression was unaffected.

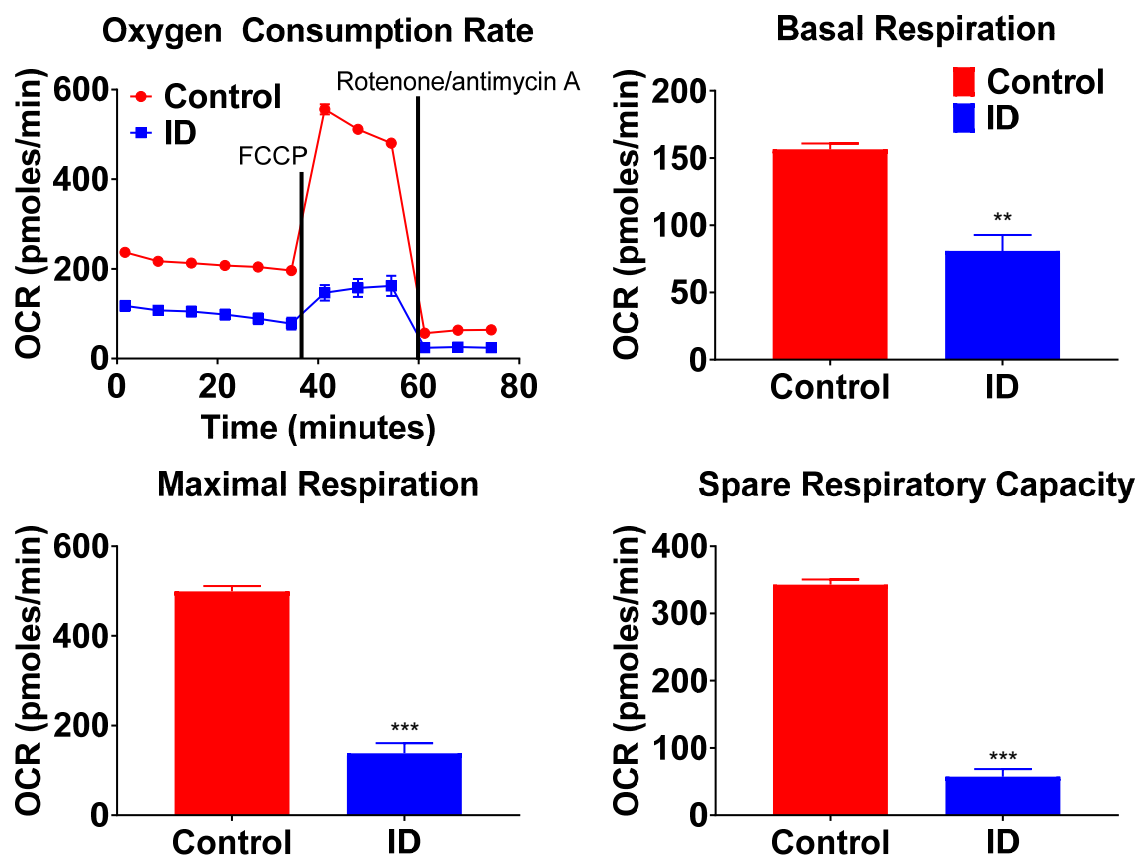


Figure 5.12: Aerobic Respiration of BV-2 cells is reduced by ID. (A) Oxygen consumption rate (OCR) curves from the Mito Stress Test. (B) Basal respiration rate of ID and control cells. (C) Maximal respiration rate of ID and control cells. (D) Spare respiratory capacity of ID and control cells. Data are presented as means $\pm$ SEM (n=3). Error bars were not rendered on data points for which the error bars would be shorter than the height of the symbol. ** denotes $p < 0.01$, *** denotes $p < 0.001$.

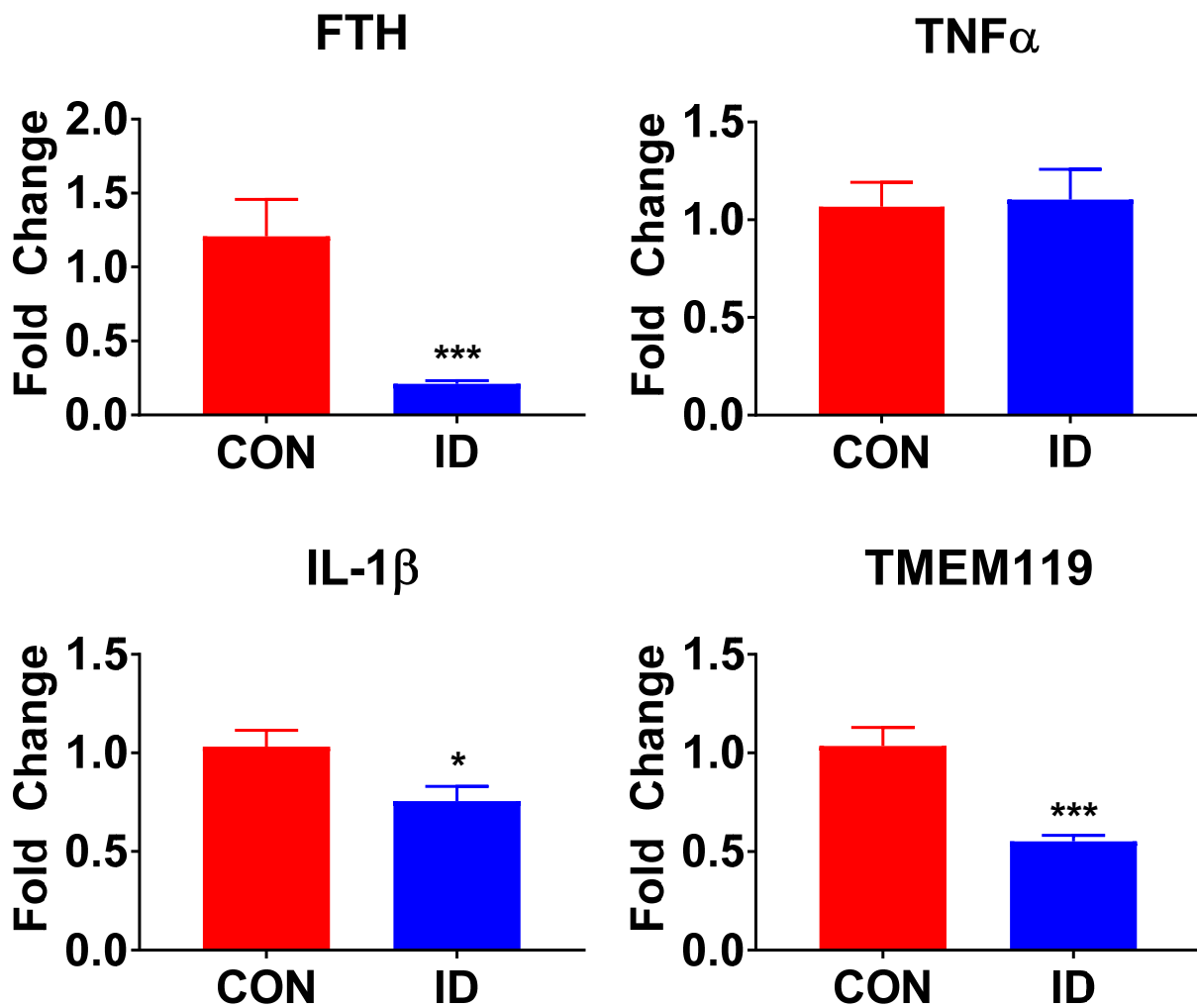


Figure 5.13: Gene Expression of ID BV-2 cells. Expression of iron storage and inflammation related genes by BV-2 cells treated with vehicle or iron chelator to induce ID. CON, control; FTH, ferritin; ID, iron deficient. Data are presented as means $\pm$ SEM (n=10-11). * denotes $p < 0.05$, *** denotes $p < 0.001$.

Early life ID and infection cause significant alterations in dopamine metabolism across several key brain regions

HPLC was used to determine concentrations of monoamine neurotransmitters and metabolites. Both ID and infection changed dopamine metabolism in the brain regions assessed. Serotonin concentration and metabolism were unaffected by either ID or PRRSV infection. The hippocampus DOPAC:DA ratio was increased by ID ($p = 0.011$), indicating reduced levels of dopamine compared to its degraded form (Figure 5.14A). There was also a trending effect of

infection ($p = 0.06$) upon dopamine turnover. No significant alterations were present in HVA:DA or 5-HIAA:5-HT levels (Figure 5.14B,C).

The amygdala displayed the greatest changes in dopamine metabolism (Figure 5.14D, E). Main effects of infection ($p = 0.002$), diet ($p = 0.038$) and a diet x infection interaction ($p = 0.005$) altered DOPAC:DA levels. Post-hoc LSD test found PID DOPAC:DA to be higher than PN levels ($p = 0.001$). HVA:DA levels in the amygdala (Figure 5.14E) were modulated by infection ($p = 0.026$). DOPAC:DA levels were unchanged in the hypothalamus (Figure 5.14G). However, hypothalamic HVA:DA (Figure 5.14H) was altered by a diet x infection interaction ($p = 0.006$). Post-tests determined CN was lower than CID ($p = 0.039$) while PN was higher than PID ($p = 0.048$), illustrating an interesting interaction of infection and ID upon hypothalamic dopamine metabolism.

Striatal dopamine metabolism was modulated by infection and an interaction (Figure 5.14J,K). The striatum is important for motor learning, motor control, and procedural memory. Striatal DOPAC:DA levels were altered by infection ($p = 0.011$). Demonstrating a similar trend, striatal HVA:DA levels were affected by a diet x infection interaction ($p = 0.002$). PID HVA:DA was lower than PN ($p = 0.003$). Dopaminergic metabolism in the mPFC was solely altered by infection (Figure 5.14M,N). Both DOPAC:DA and HVA:DA levels were modulated by a main effect of infection ($p = 0.009$ and $p = 0.008$, respectively).

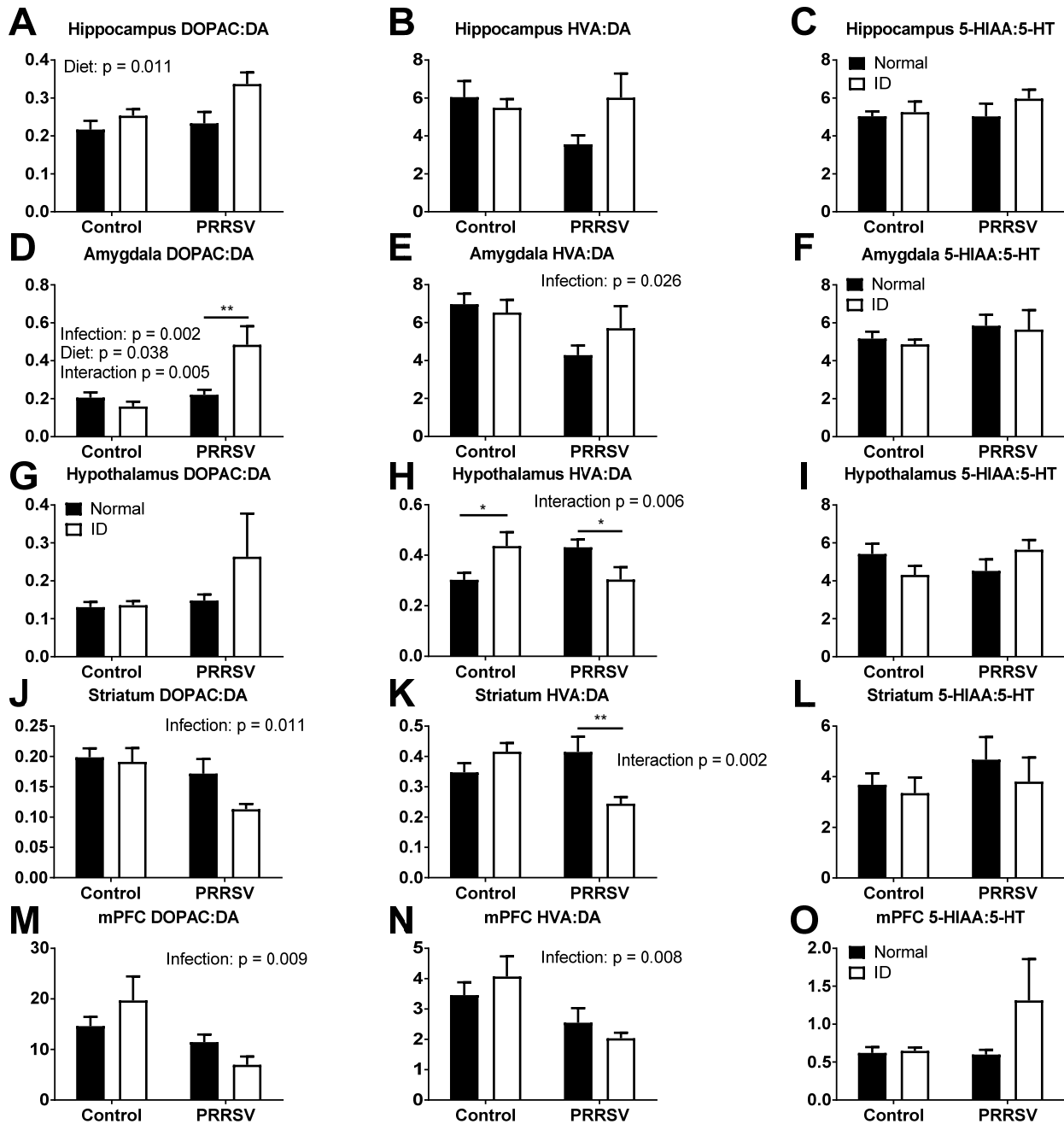


Figure 5.14: Neurotransmitter and metabolite ratios as determined by HPLC. All data compared using 2-way ANOVA. Main effects shown in table for each individual graph. * denotes $p < 0.05$, ** denotes $p < 0.01$. Data are presented as means $\pm$ SEM ($n=6-8$). Black bars denote normal diet, white bars denote ID diet.

5.5 Discussion

The early postnatal period is a critical point during which neurodevelopmental processes are particularly vulnerable to environmental insults [164]. The present study was designed to determine the molecular, neurochemical, and metabolic changes that occur resulting from the early life insults of iron deficiency and respiratory infection, and whether additional interactions occur from the double burden of comorbid ID and infection. We demonstrated that ID and infection interact to produce broad neurodevelopmental changes in a region-specific manner. Transcription profiles of genes related to neurodevelopment, energy metabolism, neurotransmitter reception and metabolism, white matter development, inflammation, and the microglial sensome were profoundly altered by postnatal ID and infection. The pattern of expression changes varied by brain region and treatment – while ID tended to reduce expression of all genes measured, PRRSV infection decreased expression in the mPFC and hypothalamus, and had mixed effects in the remaining brain regions. Interestingly, the combination of ID and PRRSV infection resulted in either further reduced expression, or overexpression, depending on brain region. The global reduction in expression observed in the mPFC suggests it is especially vulnerable to environmental insults in the postnatal period.

Neurodevelopmental gene expression was altered across brain regions by infection as well as interactions between ID and infection. The genes assessed were largely unaffected by ID alone, with the notable exception of reduced *Bdnf* expression in the mPFC. CID and PN piglets exhibited reduced *Bdnf* expression in the striatum. BDNF is a key neurotrophic factor driving a range of neurodevelopmental processes, including neuronal survival, neurogenesis, and synaptogenesis [85, 89] by binding to its primary receptor *Ntrk2*, which was downregulated in CID piglets (Figure 5.2). Epigenetic regulators (*Dnmt1*, *Dnmt3a*, *HDAC3*) expression was differentially modified by both treatments in a region-specific manner. While epigenetic regulator expression was generally

reduced by each individual treatment condition, their expression was increased in hippocampus and striatum of PID piglets and decreased in the hypothalamus and striatum, indicating region-specific changes in methyl transferases. Epigenetic remodeling may account for the long-lasting effects of early life environmental insults that persist long after the disease condition has been corrected [99, 175]. Perinatal ID in rats has been shown to alter methylation patterns at specific loci resulting in reductions in *Bdnf* expression [100, 172]. Altered neurodevelopmental gene expression profiles in the hippocampus, including reduced *Ntrk2*, clarify the reduced learning and memory performance of ID piglets in a hippocampal oriented T-maze task [171]. Expression of *mTOR*, the master regulator of protein metabolism, was decreased in CID piglets but increased in PID piglets. This may be related to the similar trends observed in *Ntrk2* expression as BDNF is a potent activator of mTOR in the brain. Reduced mTOR expression could be especially impactful in the developmental period, as reduced protein synthesis may lead to reduced synaptogenesis and growth. ID induced via iron chelation treatment was recently found to inhibit hippocampal deposition of amyloid beta protein in a mouse model of Alzheimer's [176]. The reduction in amyloid beta deposition in that case may be due to reduced overall protein synthesis due to mTOR downregulation as a result of ID.

We have previously shown white matter to be reduced in the brains of ID piglets [165]. Genes related to white matter development were downregulated by infection and interactive effects in all brain regions assessed, particularly *Scd* or *Scd5*, the rate-limiting enzyme for monounsaturated fatty acid synthesis, which requires iron as a cofactor. Unsaturated fatty acids are key components required for proper white matter development. Reduced expression of genes needed to synthesize key white matter components provides a partial explanation as to how white matter development is affected by early life insults.

ID is known to alter neurotransmitter levels [31, 42, 136], and cognitive and behavioral differences associated with early life ID may be due to lasting changes in neurotransmitter tone and receptor density. The rate-limiting step of dopamine and serotonin synthesis requires iron as a cofactor [177]. As a result, we expected altered concentrations of dopamine, serotonin, and their metabolites along with altered receptor expression. Diet alone altered dopamine metabolism in the hippocampus while both treatments modulated it in the amygdala, increasing the ratio of DOPAC, (the initial product of dopamine catabolism) to dopamine in PID piglets compared to the other groups, indicating either increased dopamine degradation or reduced dopamine production. The hypothalamic ratio of the dopamine catabolite HVA to dopamine was again increased by each treatment separately, but in the PID group the ratio was reduced to control levels (Figure 5.10). This case, along with the similar trend observed in the striatum (Figure 5.8), likely stem from a combination of reduced brain iron and neuroinflammation. Dopamine metabolism was altered in the striatum, a key structure involved in procedural memory and motor learning that requires dopaminergic input for proper functioning. While serotonin metabolism was not changed (Figure 5.8), expression of serotonin receptors was profoundly altered in the striatum; receptors associated with sexual behavior, aggression; obsessive-compulsive disorder, depression, and anxiety were reduced in CID and PN piglets, but remained near baseline in PID piglets. Conversely, *Htr4*, which is involved with learning and memory as well as regulation of anxiety, was increased in CID but decreased in PID piglets. Expression of the glucocorticoid receptor was also reduced in the striatum, which may further contribute to altered stress response and increased anxiety associated with early life ID [114]. Signaling between the hippocampus and striatum is key for reward-driven learning and memory [110], and the altered hippocampal and striatal neurotransmitter tone and receptor density observed in this study explains the aberrant learning and memory observed in ID

piglets [142, 154]. In the mPFC, the decreased dopamine turnover (Figure 5.14 M-N) and the diet/infection induced decrease in expression of genes essential to dopamine metabolism and catabolism demonstrate reduced dopamine catabolism and indicate a potential compensatory action to preserve dopamine levels in the face of reduced dopamine production resulting from environmental insults.

Perinatal ID is associated with altered emotionality and increased anxiety [35]. The hippocampus and mPFC exert top-down regulation of amygdala activity [108]; in the present study, the hippocampus and mPFC exhibited altered dopamine metabolism as a result of diet and infection, respectively. Moreover, both the hippocampus and mPFC displayed alterations in neurotransmitter related gene expression resulting from ID and infection, which was particularly pronounced in the mPFC. These changes are underlined by the alterations in amygdala dopamine metabolism, and likely contribute to the lack of positive affect and social interaction in ID infants [48]. Long-term alterations of neurotransmitter tone in these circuits may lead to the increased negative emotionality and feelings of detachment reported by adults who were ID as children [36]. Impairment of fronto-striatal mediated executive functions reported in formerly IDA adults [37] may stem from altered dopamine metabolism and serotonergic receptor expression observed in the mPFC and striatum found in this study. Altered dopamine metabolism in the striatum and mPFC is associated with increased risk for addiction. Together, these changes offer a potential mechanistic explanation for the alterations in emotional behavior, attentional regulation, and inhibitory control associated with perinatal ID.

Properly functioning metabolism is key for brain health [92, 178], and since iron is a required cofactor for many enzymes involved in aerobic metabolism, we expected to see broad changes in expression of related genes. In all brain areas measured, expression of genes involved

with oxidative metabolism was altered, though vulnerability to the treatments varied in a region-specific manner. The hippocampus and striatum were primarily affected by interactions between iron deficiency and infection, while the mPFC, amygdala, and hypothalamus were almost exclusively altered by main effects of either diet or infection. The most reliably affected genes were glucose transporters and those related to iron containing proteins related to oxidative metabolism, particularly cytochrome *c* oxidase and succinate dehydrogenase, key enzymes in the electron transport chain and Krebs cycle, respectively. This set of genes was decreased by ID, except in the striatum, where expression was increased by ID.

Based on these results, we evaluated mitochondrial function of ID upon a microglial cell line using the Agilent Seahorse to model how cellular respiration is affected by ID. ID cells exhibit profoundly impaired mitochondrial respiratory capacity, exhibiting severely reduced basal and maximal respiration rates, resulting in a 6-fold reduction in spare respiratory capacity. ID severely handicaps metabolic flexibility, rendering the cell less capable of adapting to environmental changes such as reduced energy availability, which is relevant to neuronal survival and function as neurons are especially dependent on oxidative metabolism of glucose. Metabolic resilience is necessary for the brain to function optimally across the lifespan, particularly during exposure to stressors [178]. Additionally, the inflammatory phenotype of immune cells such as microglia is closely tied to the metabolic state of the cell and reduced metabolic flexibility will impair the ability of the cell to adequately respond to changing environmental demands, such as shifting to a pro inflammatory phenotype during infection. Most specific to ID, the shift to an anti-inflammatory phenotype post-infection necessitates a shift to oxidative metabolism of glucose and fatty acids [118, 123]. Thus, ID immune cells may be less able to effect an anti-inflammatory tissue repair phenotype following injury or infection.

Inefficient oxidative respiration necessitates a metabolic skewing towards glycolysis, requiring increased glucose uptake via increased glucose transporter density. Expression of *Slc2a3* (Glut3), the primary neuronal glucose transporter, was modulated by diet, infection, and interactive effects in the hippocampus and amygdala, and was especially increased in the PID group, suggesting that either inflammation is promoting greater neuronal glucose uptake, or the iron sequestration resulting from infection is exacerbating the effects of dietary ID. Glucose transporter expression in the hypothalamus, which is also responsible for energy sensing and appetite control, was increased by diet, and, in the case of Glut4, which is sparingly expressed in the brain [119], expression was increased in CID and PID, but most strongly in the PN group.

Across all brain areas, microglial sensome genes were upregulated due to infection, along with *IFN- γ* . Interestingly, sensome genes displayed consistently decreased expression in CID piglets, yet expression in PID piglets was consistently higher in PID piglets compared to PN piglets, suggesting PID microglia may have increased sensitivity to inflammatory stimuli while microglia in CID animals were less sensitive. However, some sensome genes, including *P2ry12*, are downregulated during microglial activation [179], indicating ID microglia may be biased towards a more pro-inflammatory phenotype, which may result from having greater glycolytic flux resulting from impaired oxidative respiration. However, as noted in the previous chapter, microglial activation as measured by presence of MHCII was not altered by ID.

Overall, the dual early life burdens of ID and respiratory infection profoundly affect multiple neurodevelopmental domains, including neurotransmitter tone, expression of key genes for neurodevelopment, energy metabolism, neurotransmitter metabolism and reception, white matter development, and inflammation. The molecular and neurochemical changes observed provide deeper understanding of the mechanisms by which early life ID and infection result in

cognitive and behavioral changes. Further, ID severely impairs oxidative respiration *in vitro*, which is reflected in the altered expression of metabolic genes *in vivo*. Reduced metabolic flexibility may contribute to the reduced efficacy of PID piglets to clear the infection. These data provide insight as to how two common developmental insults interact to alter neurodevelopment, identifying especially affected neurodevelopmental domains as potential treatment targets.

5.6 Acknowledgments

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Chapter 6. Summary and Significance

The objective of this research was to determine how postnatal iron deficiency alters brain development and how these alterations are modulated by infection. Recent research has demonstrated the gravity of the perinatal period upon developmental trajectory. Environmental insults such as malnutrition and infection can have lasting detrimental ‘programming’ effects upon cognitive, emotional, and behavioral domains. Understanding the neurodevelopmental changes caused by early life environmental insults is an important step towards identifying mechanistic targets for therapeutic intervention. Previously, our lab has shown learning and memory deficits in both PRRSV infected piglets and ID piglets, and changes in hippocampal neurogenesis, neuron morphology, and brain structure in piglets infected with PRRSV. This dissertation describes potential mechanisms behind those observations and other deficits associated with postnatal ID and infection.

The aim of the first study was to determine the effects of ID upon brain structure, myelination, and hippocampal metabolites using magnetic resonance imaging and immunohistochemistry. Large areas of white matter reduction were observed, including in the hippocampus, a key structure for learning and memory, which sheds light on previously observed learning and memory deficits in the ID piglet. Fractional anisotropy, a marker for myelination and white matter integrity, was reduced in white matter of ID piglets, and specifically in the corpus callosum, which is the largest white matter structure in the brain and needed for interhemispheric communication. Collectively, this study demonstrated postnatal ID altered brain tissue composition and severely reduced white matter volume and microstructural integrity, which may contribute to previously observed learning and memory deficits, as well as increased visual and auditory latencies reported by other investigators.

As there have been no studies examining the interactions between ID and infection in the postnatal period, the second study aimed to determine how ID alters peripheral inflammation, neuroinflammation, and microglial phenotype in the context of infection to lay a foundation for subsequent studies. ID piglets had increased virus in serum and spleen, showing ID impaired viral clearance. Additionally, ID blunted peripheral blood mononuclear cell and liver cytokine expression, including PBMC production of interferon- γ , which is essential for clearance of PRRS virus. However, microglial activation state, phagocytic activity, and cytokine expression were only affected by infection and not ID. While the peripheral infection response was impaired by ID, microglia were unaffected. Based on gene expression data from peripheral immune cells, it is likely microglia were able to retain sufficient iron to continue responding normally to peripheral inflammatory signals.

The third arm of this research targeted neurodevelopmental, neurochemical, and inflammatory changes resulting from ID and infection in key brain regions. Fluidigm panels were used to evaluate expression of key gene families, and high-pressure liquid chromatography was used to quantify neurotransmitters and their corresponding metabolites. Genes involved in neurodevelopment and white matter component synthesis were altered separately by ID and infection as well as by interactions between the two. Genes involved in energy and neurotransmitter metabolism were modulated by ID, infection, and treatment interactions. As expected, inflammatory and microglial sensome genes were increased by infection. ID tended to reduce expression of the key gene families evaluated, though the PID group rarely exhibited additive effects of PN and ID. Instead, PID gene expression was often affected by interactions, indicating that the double hit of early life ID and infection may impose additional burdens on neurodevelopment beyond those of each individual insult. The alterations in neurotransmitter

metabolism and related gene expression may indicate lasting changes in neurotransmitter tone and receptor density and interfere with the wiring of brain circuitry involved in learning and memory as well as emotionality and anxiety. Cell culture work revealed that ID severely impairs cellular metabolic flexibility.

These data provide insight into the structural, molecular, and neurochemical changes following neonatal exposure to ID and infection. Further work is needed to bridge the gap and generate therapeutics that may be beneficial to those who have experienced early life infection and ID. Useful future directions in this line of research include utilizing recently refined transgenic techniques to generate pigs with region-specific knockdown of iron uptake receptors to explore how ID affects individual tissues without causing global ID and anemia. Studies comparing ID pigs to iron-sufficient pigs with pharmacologically induced hemolytic anemia would be useful in separating the effects of ID from the effects of anemia/hypoxia. Additionally, it has been proposed that the lasting effects of early life ID stem from epigenetic changes. To verify this, brain methylation should be measured following treatment of ID. If changes in methylation patterns are detected, a study would be performed wherein iron repletion therapy with or without a methyl donor such as folate or choline is given to formerly ID animals to verify if adjunct therapies may mitigate the lasting effects of ID.

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Appendix A. Fluidigm Data

Fluidigm p-values

Gene	Effect	p-value
Hippocampus		
1_ABAT	Infection	0.109
1_ABAT	Diet-Infection	0.204
1_ABAT	Diet	0.988
10_Cd14	Infection	0.396
10_Cd14	Diet-Infection	0.640
10_Cd14	Diet	0.684
11_Cd180	Diet	0.640
11_Cd180	Diet-Infection	0.798
11_Cd180	Infection	0.902
12_Cd4	Infection	0.102
12_Cd4	Diet-Infection	0.347
12_Cd4	Diet	0.841
13_Cd40	Infection	0.001
13_Cd40	Diet-Infection	0.015
13_Cd40	Diet	0.474
14_Cd74	Infection	0.000
14_Cd74	Diet-Infection	0.034
14_Cd74	Diet	0.784
15_Cd86	Infection	0.001
15_Cd86	Diet-Infection	0.005
15_Cd86	Diet	0.293
16_Cdk1	Diet-Infection	0.153
16_Cdk1	Diet	0.598
16_Cdk1	Infection	0.607
17_COMTKRT20	Diet-Infection	0.119
17_COMTKRT20	Infection	0.197
17_COMTKRT20	Diet	0.831
18_COX10	Infection	0.099
18_COX10	Diet-Infection	0.135
18_COX10	Diet	0.764
19_COX6A1	Infection	0.158
19_COX6A1	Diet-Infection	0.165
19_COX6A1	Diet	0.631
2_ACADL	Diet-Infection	0.077
2_ACADL	Infection	0.177
2_ACADL	Diet	0.775

Gene	Effect	p-value
20_COX7C	Diet-Infection	0.128
20_COX7C	Infection	0.256
20_COX7C	Diet	0.416
21_Crybb1	Diet-Infection	0.003
21_Crybb1	Infection	0.413
21_Crybb1	Diet	0.554
22_Csfl	Diet	0.109
22_Csfl	Diet-Infection	0.267
22_Csfl	Infection	0.693
23_Cxcl16	Infection	0.835
23_Cxcl16	Diet	0.846
23_Cxcl16	Diet-Infection	0.980
24_Cxcr2	Diet-Infection	0.332
24_Cxcr2	Infection	0.512
24_Cxcr2	Diet	0.763
25_CYBA	Diet-Infection	0.068
25_CYBA	Infection	0.204
25_CYBA	Diet	0.501
26_CYCS	Diet	0.064
26_CYCS	Diet-Infection	0.069
26_CYCS	Infection	0.250
27_DBH	Diet-Infection	0.622
27_DBH	Diet	0.869
27_DBH	Infection	0.914
28_DMT	Infection	0.086
28_DMT	Diet-Infection	0.506
28_DMT	Diet	0.782
29_Dnmt1	Diet-Infection	0.048
29_Dnmt1	Infection	0.117
29_Dnmt1	Diet	0.748
3_ACADM	Diet-Infection	0.053
3_ACADM	Diet	0.171
3_ACADM	Infection	0.992
30_Dnmt3a	Diet-Infection	0.135
30_Dnmt3a	Infection	0.150
30_Dnmt3a	Diet	0.946
31_DRD1	Infection	0.202
31_DRD1	Diet-Infection	0.314
31_DRD1	Diet	0.603
32_DRD2	Diet	0.008
32_DRD2	Diet-Infection	0.240
32_DRD2	Infection	0.948

Gene	Effect	p-value
33_Egr1	Diet-Infection	0.067
33_Egr1	Infection	0.355
33_Egr1	Diet	0.608
34_FADS6	Infection	0.160
34_FADS6	Diet	0.233
34_FADS6	Diet-Infection	0.258
35_Fcer1g	Infection	0.011
35_Fcer1g	Diet-Infection	0.048
35_Fcer1g	Diet	0.614
36_Fcgr2b	Infection	0.001
36_Fcgr2b	Diet-Infection	0.012
36_Fcgr2b	Diet	0.588
37_FTH	Infection	0.107
37_FTH	Diet	0.687
37_FTH	Diet-Infection	0.791
38_FTL	Diet-Infection	0.139
38_FTL	Diet	0.432
38_FTL	Infection	0.980
39_GABARAP	Infection	0.209
39_GABARAP	Diet	0.471
39_GABARAP	Diet-Infection	0.656
4_ACO2	Diet-Infection	0.098
4_ACO2	Infection	0.249
4_ACO2	Diet	0.826
40_GABBR1	Infection	0.711
40_GABBR1	Diet	0.756
40_GABBR1	Diet-Infection	0.832
41_GLRB	Diet-Infection	0.091
41_GLRB	Infection	0.115
41_GLRB	Diet	0.717
42_HDAC3	Diet-Infection	0.055
42_HDAC3	Infection	0.125
42_HDAC3	Diet	0.802
43_HIF1A	Infection	0.059
43_HIF1A	Diet-Infection	0.275
43_HIF1A	Diet	0.856
44_HTR1B	Infection	0.389
44_HTR1B	Diet	0.655
44_HTR1B	Diet-Infection	0.743
45_HTR1D	Infection	0.487
45_HTR1D	Diet	0.714
45_HTR1D	Diet-Infection	0.808

Gene	Effect	p-value
46_HTR2A	Infection	0.415
46_HTR2A	Diet-Infection	0.510
46_HTR2A	Diet	0.766
47_HTR2C	Infection	0.245
47_HTR2C	Diet	0.693
47_HTR2C	Diet-Infection	0.891
48_HTR4	Infection	0.024
48_HTR4	Diet-Infection	0.057
48_HTR4	Diet	0.926
49_HTR7	Diet-Infection	0.111
49_HTR7	Infection	0.611
49_HTR7	Diet	0.896
5_Bdnf	Diet-Infection	0.116
5_Bdnf	Infection	0.234
5_Bdnf	Diet	0.462
50_Ifit3	Infection	0.000
50_Ifit3	Diet	0.183
50_Ifit3	Diet-Infection	0.245
51_Ifn γ	Infection	0.001
51_Ifn γ	Diet	0.154
51_Ifn γ	Diet-Infection	0.621
52_Ifn γ 1	Infection	0.021
52_Ifn γ 1	Diet-Infection	0.065
52_Ifn γ 1	Diet	0.805
53_Il-10	Infection	0.602
53_Il-10	Diet	0.825
53_Il-10	Diet-Infection	0.981
54_Il-1b	Diet-Infection	0.019
54_Il-1b	Infection	0.069
54_Il-1b	Diet	0.462
55_Il-6	Infection	0.364
55_Il-6	Diet-Infection	0.746
55_Il-6	Diet	0.785
56_Il-8	Diet-Infection	0.046
56_Il-8	Infection	0.206
56_Il-8	Diet	0.591
57_Itgam	Diet-Infection	0.010
57_Itgam	Infection	0.013
57_Itgam	Diet	0.736
58_Itgb5	Diet-Infection	0.044
58_Itgb5	Infection	0.086
58_Itgb5	Diet	0.550

Gene	Effect	p-value
59_Ly86	Infection	0.002
59_Ly86	Diet-Infection	0.022
59_Ly86	Diet	0.766
6_C1qa	Diet-Infection	0.004
6_C1qa	Infection	0.014
6_C1qa	Diet	0.401
60_MAOA	Diet-Infection	0.132
60_MAOA	Infection	0.236
60_MAOA	Diet	0.971
61_MAOB	Diet-Infection	0.234
61_MAOB	Infection	0.409
61_MAOB	Diet	0.604
62_MBP	Diet-Infection	0.063
62_MBP	Infection	0.273
62_MBP	Diet	0.610
63_Mip2	Diet	0.180
63_Mip2	Diet-Infection	0.427
63_Mip2	Infection	0.676
64_MTOR	Infection	0.013
64_MTOR	Diet-Infection	0.043
64_MTOR	Diet	0.622
65_NDUFS5	Diet-Infection	0.441
65_NDUFS5	Infection	0.671
65_NDUFS5	Diet	0.889
66_Nos2	Infection	0.483
66_Nos2	Diet	0.525
66_Nos2	Diet-Infection	0.926
67_NR3C1	Diet-Infection	0.568
67_NR3C1	Infection	0.927
67_NR3C1	Diet	0.979
68_NTRK2	Diet-Infection	0.037
68_NTRK2	Infection	0.163
68_NTRK2	Diet	0.899
69_P2ry12	Diet-Infection	0.001
69_P2ry12	Infection	0.002
69_P2ry12	Diet	0.810
7_C5	Diet-Infection	0.116
7_C5	Infection	0.127
7_C5	Diet	0.428
70_P2ry6	Diet-Infection	0.243
70_P2ry6	Infection	0.325
70_P2ry6	Diet	0.946

Gene	Effect	p-value
71_PFKFB1	Infection	0.474
71_PFKFB1	Diet	0.779
71_PFKFB1	Diet-Infection	0.969
72_PLP1	Diet-Infection	0.178
72_PLP1	Diet	0.363
72_PLP1	Infection	0.822
73_Rel	Diet-Infection	0.051
73_Rel	Infection	0.112
73_Rel	Diet	0.593
75_SCD	Diet	0.255
75_SCD	Diet-Infection	0.739
75_SCD	Infection	0.981
76_SCD5	Infection	0.037
76_SCD5	Diet-Infection	0.123
76_SCD5	Diet	0.683
77_SDHB	Infection	0.208
77_SDHB	Diet	0.480
77_SDHB	Diet-Infection	0.697
78_Selplg	Diet-Infection	0.005
78_Selplg	Infection	0.051
78_Selplg	Diet	0.711
79_SLC2A1	Infection	0.062
79_SLC2A1	Diet-Infection	0.209
79_SLC2A1	Diet	0.213
8_Ccr5	Infection	0.017
8_Ccr5	Diet-Infection	0.077
8_Ccr5	Diet	0.468
81_SLC2A3	Diet-Infection	0.010
81_SLC2A3	Infection	0.013
81_SLC2A3	Diet	0.115
82_SLC2A4	Diet	0.437
82_SLC2A4	Diet-Infection	0.757
82_SLC2A4	Infection	0.892
83_Slc2a5	Diet-Infection	0.360
83_Slc2a5	Diet	0.568
83_Slc2a5	Infection	0.966
84_TACO1	Diet-Infection	0.023
84_TACO1	Infection	0.093
84_TACO1	Diet	0.687
85_TFRC	Diet	0.041
85_TFRC	Diet-Infection	0.092
85_TFRC	Infection	0.284

Gene	Effect	p-value
86_Tgfb	Diet-Infection	0.042
86_Tgfb	Infection	0.050
86_Tgfb	Diet	0.920
87_Tgfb1	Diet-Infection	0.041
87_Tgfb1	Infection	0.136
87_Tgfb1	Diet	0.474
88_Tlr2	Diet	0.619
88_Tlr2	Infection	0.648
88_Tlr2	Diet-Infection	0.843
89_Tnfa	Infection	0.001
89_Tnfa	Diet-Infection	0.029
89_Tnfa	Diet	0.521
9_Ccr12	Diet-Infection	0.369
9_Ccr12	Diet	0.864
9_Ccr12	Infection	0.965
90_Tnfrsf1b	Infection	0.015
90_Tnfrsf1b	Diet-Infection	0.049
90_Tnfrsf1b	Diet	0.594
91_Tpi1	Diet-Infection	0.188
91_Tpi1	Infection	0.256
91_Tpi1	Diet	0.388
92_Tyrobp	Diet-Infection	0.006
92_Tyrobp	Infection	0.012
92_Tyrobp	Diet	0.804
93_UCP2	Diet-Infection	0.014
93_UCP2	Infection	0.091
93_UCP2	Diet	0.675
94_VAMP4	Infection	0.092
94_VAMP4	Diet-Infection	0.126
94_VAMP4	Diet	0.632
95_VAPB	Diet-Infection	0.262
95_VAPB	Infection	0.274
95_VAPB	Diet	0.488

Amygdala

Gene	Effect	p-value
1_ABAT	Diet	0.175
1_ABAT	Infection	0.233
1_ABAT	Diet-Infection	0.962
10_Cd14	Infection	0.517
10_Cd14	Diet	0.736

Gene	Effect	p-value
10_Cd14	Diet-Infection	0.919
11_Cd180	Diet-Infection	0.383
11_Cd180	Diet	0.698
11_Cd180	Infection	0.899
12_Cd4	Infection	0.170
12_Cd4	Diet	0.369
12_Cd4	Diet-Infection	0.774
13_Cd40	Infection	0.000
13_Cd40	Diet-Infection	0.122
13_Cd40	Diet	0.427
14_Cd74	Infection	0.000
14_Cd74	Diet-Infection	0.542
14_Cd74	Diet	0.967
15_Cd86	Infection	0.001
15_Cd86	Diet-Infection	0.168
15_Cd86	Diet	0.651
16_Cdk1	Diet	0.304
16_Cdk1	Diet-Infection	0.802
16_Cdk1	Infection	0.953
17_COMTKRT20	Diet	0.148
17_COMTKRT20	Infection	0.553
17_COMTKRT20	Diet-Infection	0.907
18_COX10	Diet	0.109
18_COX10	Diet-Infection	0.289
18_COX10	Infection	0.534
19_COX6A1	Diet	0.079
19_COX6A1	Diet-Infection	0.636
19_COX6A1	Infection	0.896
2_ACADL	Diet	0.321
2_ACADL	Diet-Infection	0.530
2_ACADL	Infection	0.584
20_COX7C	Diet	0.139
20_COX7C	Infection	0.142
20_COX7C	Diet-Infection	0.500
21_Crybb1	Diet	0.031
21_Crybb1	Diet-Infection	0.103
21_Crybb1	Infection	0.264
22_Csfl	Infection	0.164
22_Csfl	Diet-Infection	0.728
22_Csfl	Diet	0.770
23_Cxcl16	Infection	0.745
23_Cxcl16	Diet	0.761

Gene	Effect	p-value
23_Cxcl16	Diet-Infection	0.870
24_Cxcr2	Diet-Infection	0.424
24_Cxcr2	Diet	0.799
24_Cxcr2	Infection	0.992
25_CYBA	Diet	0.144
25_CYBA	Diet-Infection	0.428
25_CYBA	Infection	0.437
26_CYCS	Diet	0.097
26_CYCS	Infection	0.707
26_CYCS	Diet-Infection	0.828
27_DBH	Diet	0.025
27_DBH	Diet-Infection	0.175
27_DBH	Infection	0.284
28_DMT	Diet	0.560
28_DMT	Infection	0.579
28_DMT	Diet-Infection	0.729
29_Dnmt1	Diet-Infection	0.372
29_Dnmt1	Diet	0.449
29_Dnmt1	Infection	0.653
3_ACADM	Diet	0.088
3_ACADM	Infection	0.394
3_ACADM	Diet-Infection	0.469
30_Dnmt3a	Diet	0.291
30_Dnmt3a	Infection	0.345
30_Dnmt3a	Diet-Infection	0.562
31_DRD1	Infection	0.097
31_DRD1	Diet	0.564
31_DRD1	Diet-Infection	0.919
32_DRD2	Infection	0.023
32_DRD2	Diet	0.106
32_DRD2	Diet-Infection	0.994
33_Egr1	Infection	0.278
33_Egr1	Diet-Infection	0.575
33_Egr1	Diet	0.839
34_FADS6	Diet	0.053
34_FADS6	Infection	0.105
34_FADS6	Diet-Infection	0.914
35_Fcrlg	Diet	0.109
35_Fcrlg	Diet-Infection	0.215
35_Fcrlg	Infection	0.238
36_Fcgr2b	Infection	0.000
36_Fcgr2b	Diet	0.205

Gene	Effect	p-value
36_Fcgr2b	Diet-Infection	0.248
37_FTH	Diet	0.268
37_FTH	Infection	0.336
37_FTH	Diet-Infection	0.512
38_FTL	Infection	0.208
38_FTL	Diet-Infection	0.352
38_FTL	Diet	0.356
39_GABARAP	Diet	0.087
39_GABARAP	Infection	0.751
39_GABARAP	Diet-Infection	0.837
4_ACO2	Diet	0.431
4_ACO2	Diet-Infection	0.688
4_ACO2	Infection	0.773
40_GABBR1	Diet-Infection	0.277
40_GABBR1	Infection	0.713
40_GABBR1	Diet	0.939
41_GLRB	Diet	0.124
41_GLRB	Diet-Infection	0.373
41_GLRB	Infection	0.693
42_HDAC3	Infection	0.093
42_HDAC3	Diet	0.211
42_HDAC3	Diet-Infection	0.422
43_HIF1A	Diet	0.120
43_HIF1A	Diet-Infection	0.710
43_HIF1A	Infection	0.922
44_HTR1B	Diet-Infection	0.651
44_HTR1B	Diet	0.711
44_HTR1B	Infection	0.763
45_HTR1D	Diet-Infection	0.495
45_HTR1D	Infection	0.605
45_HTR1D	Diet	0.987
46_HTR2A	Diet-Infection	0.308
46_HTR2A	Infection	0.428
46_HTR2A	Diet	0.849
47_HTR2C	Infection	0.446
47_HTR2C	Diet-Infection	0.831
47_HTR2C	Diet	0.849
48_HTR4	Diet-Infection	0.029
48_HTR4	Diet	0.424
48_HTR4	Infection	0.894
49_HTR7	Infection	0.198
49_HTR7	Diet-Infection	0.345

Gene	Effect	p-value
49_HTR7	Diet	0.745
5_Bdnf	Diet	0.439
5_Bdnf	Diet-Infection	0.758
5_Bdnf	Infection	0.847
50_Ifit3	Infection	0.000
50_Ifit3	Diet	0.206
50_Ifit3	Diet-Infection	0.426
51_Ifng	Infection	0.001
51_Ifng	Diet-Infection	0.030
51_Ifng	Diet	0.158
52_Ifngr1	Infection	0.089
52_Ifngr1	Diet-Infection	0.341
52_Ifngr1	Diet	0.602
53_Il-10	Diet-Infection	0.620
53_Il-10	Infection	0.815
53_Il-10	Diet	0.860
54_Il-1b	Diet-Infection	0.162
54_Il-1b	Diet	0.439
54_Il-1b	Infection	0.537
55_Il-6	Diet-Infection	0.436
55_Il-6	Diet	0.889
55_Il-6	Infection	0.895
56_Il-8	Diet-Infection	0.020
56_Il-8	Infection	0.104
56_Il-8	Diet	0.165
57_Itgam	Infection	0.026
57_Itgam	Diet-Infection	0.148
57_Itgam	Diet	0.296
58_Itgb5	Infection	0.186
58_Itgb5	Diet	0.247
58_Itgb5	Diet-Infection	0.721
59_Ly86	Infection	0.000
59_Ly86	Diet-Infection	0.305
59_Ly86	Diet	0.918
6_C1qa	Infection	0.043
6_C1qa	Diet	0.145
6_C1qa	Diet-Infection	0.225
60_MAOA	Diet-Infection	0.539
60_MAOA	Infection	0.653
60_MAOA	Diet	0.690
61_MAOB	Diet	0.227
61_MAOB	Diet-Infection	0.465

Gene	Effect	p-value
61_MAOB	Infection	0.695
62_MBP	Diet	0.302
62_MBP	Diet-Infection	0.751
62_MBP	Infection	0.767
63_Mip2	Diet	0.776
63_Mip2	Diet-Infection	0.849
63_Mip2	Infection	0.943
64_MTOR	Diet	0.084
64_MTOR	Infection	0.612
64_MTOR	Diet-Infection	0.970
65_NDUFS5	Diet	0.225
65_NDUFS5	Diet-Infection	0.615
65_NDUFS5	Infection	0.952
66_Nos2	Infection	0.528
66_Nos2	Diet-Infection	0.650
66_Nos2	Diet	0.971
67_NR3C1	Infection	0.308
67_NR3C1	Diet-Infection	0.851
67_NR3C1	Diet	0.954
68_NTRK2	Infection	0.057
68_NTRK2	Diet-Infection	0.404
68_NTRK2	Diet	0.955
69_P2ry12	Diet	0.084
69_P2ry12	Infection	0.137
69_P2ry12	Diet-Infection	0.341
7_C5	Diet-Infection	0.106
7_C5	Diet	0.118
7_C5	Infection	0.480
70_P2ry6	Infection	0.267
70_P2ry6	Diet-Infection	0.764
70_P2ry6	Diet	0.790
71_PFKFB1	Diet-Infection	0.160
71_PFKFB1	Diet	0.311
71_PFKFB1	Infection	0.354
72_PLP1	Infection	0.117
72_PLP1	Diet-Infection	0.593
72_PLP1	Diet	0.594
73_Rel	Infection	0.063
73_Rel	Diet-Infection	0.298
73_Rel	Diet	0.809
75_SCD	Infection	0.211
75_SCD	Diet	0.314

Gene	Effect	p-value
75_SCD	Diet-Infection	0.320
76_SCD5	Infection	0.030
76_SCD5	Diet	0.095
76_SCD5	Diet-Infection	0.840
77_SDHB	Diet	0.004
77_SDHB	Infection	0.139
77_SDHB	Diet-Infection	0.816
78_Selplg	Diet	0.298
78_Selplg	Infection	0.377
78_Selplg	Diet-Infection	0.673
79_SLC2A1	Diet	0.064
79_SLC2A1	Infection	0.695
79_SLC2A1	Diet-Infection	0.905
8_Ccr5	Infection	0.273
8_Ccr5	Diet-Infection	0.764
8_Ccr5	Diet	0.918
81_SLC2A3	Diet	0.034
81_SLC2A3	Infection	0.120
81_SLC2A3	Diet-Infection	0.795
82_SLC2A4	Diet	0.567
82_SLC2A4	Diet-Infection	0.714
82_SLC2A4	Infection	0.883
83_Slc2a5	Diet	0.540
83_Slc2a5	Infection	0.594
83_Slc2a5	Diet-Infection	0.644
84_TACO1	Diet	0.028
84_TACO1	Infection	0.131
84_TACO1	Diet-Infection	0.214
85_TFRC	Diet	0.209
85_TFRC	Diet-Infection	0.321
85_TFRC	Infection	0.523
86_Tgfb	Diet-Infection	0.460
86_Tgfb	Diet	0.619
86_Tgfb	Infection	0.636
87_Tgfbr1	Infection	0.046
87_Tgfbr1	Diet	0.087
87_Tgfbr1	Diet-Infection	0.276
88_Tlr2	Diet-Infection	0.379
88_Tlr2	Diet	0.772
88_Tlr2	Infection	0.990
89_Tnfa	Infection	0.001
89_Tnfa	Diet	0.193

Gene	Effect	p-value
89_Tnfa	Diet-Infection	0.231
9_Ccr12	Diet-Infection	0.578
9_Ccr12	Diet	0.923
9_Ccr12	Infection	0.968
90_Tnfrsf1b	Infection	0.076
90_Tnfrsf1b	Diet-Infection	0.387
90_Tnfrsf1b	Diet	0.451
91_Tpi1	Diet	0.304
91_Tpi1	Infection	0.602
91_Tpi1	Diet-Infection	0.984
92_Tyrobp	Infection	0.012
92_Tyrobp	Diet	0.308
92_Tyrobp	Diet-Infection	0.410
93_UCP2	Diet	0.054
93_UCP2	Infection	0.252
93_UCP2	Diet-Infection	0.744
94_VAMP4	Diet	0.066
94_VAMP4	Diet-Infection	0.082
94_VAMP4	Infection	0.416
95_VAPB	Diet	0.068
95_VAPB	Infection	0.506
95_VAPB	Diet-Infection	0.663

mPFC		
Gene	Effect	p-value
1_ABAT	Infection	0.024
1_ABAT	Diet	0.204
1_ABAT	Diet-Infection	0.383
10_Cd14	Diet	0.137
10_Cd14	Infection	0.207
10_Cd14	Diet-Infection	0.894
11_Cd180	Infection	0.081
11_Cd180	Diet	0.356
11_Cd180	Diet-Infection	0.761
12_Cd4	Diet	0.703
12_Cd4	Infection	0.809
12_Cd4	Diet-Infection	0.924
13_Cd40	Diet	0.099
13_Cd40	Diet-Infection	0.166
13_Cd40	Infection	0.929
14_Cd74	Infection	0.000

Gene	Effect	p-value
14_Cd74	Diet-Infection	0.368
14_Cd74	Diet	0.543
15_Cd86	Infection	0.009
15_Cd86	Diet-Infection	0.033
15_Cd86	Diet	0.849
16_Cdk1	Infection	0.133
16_Cdk1	Diet-Infection	0.659
16_Cdk1	Diet	0.912
17_COMTKRT20	Infection	0.020
17_COMTKRT20	Diet	0.134
17_COMTKRT20	Diet-Infection	0.524
18_COX10	Infection	0.008
18_COX10	Diet	0.064
18_COX10	Diet-Infection	0.266
19_COX6A1	Infection	0.048
19_COX6A1	Diet-Infection	0.097
19_COX6A1	Diet	0.692
2_ACADL	Infection	0.090
2_ACADL	Diet	0.103
2_ACADL	Diet-Infection	0.199
20_COX7C	Infection	0.116
20_COX7C	Diet-Infection	0.258
20_COX7C	Diet	0.296
21_Crybb1	Diet	0.064
21_Crybb1	Diet-Infection	0.166
21_Crybb1	Infection	0.300
22_Csfl	Infection	0.012
22_Csfl	Diet	0.385
22_Csfl	Diet-Infection	0.793
23_Cxcl16	Infection	0.027
23_Cxcl16	Diet	0.181
23_Cxcl16	Diet-Infection	0.947
24_Cxcr2	Infection	0.047
24_Cxcr2	Diet	0.461
24_Cxcr2	Diet-Infection	0.497
25_CYBA	Infection	0.105
25_CYBA	Diet	0.244
25_CYBA	Diet-Infection	0.332
26_CYCS	Diet	0.013
26_CYCS	Diet-Infection	0.453
26_CYCS	Infection	0.765
27_DBH	Infection	0.003

Gene	Effect	p-value
27_DBH	Diet	0.031
27_DBH	Diet-Infection	0.886
28_DMT	Diet	0.217
28_DMT	Diet-Infection	0.439
28_DMT	Infection	0.807
29_Dnmt1	Infection	0.052
29_Dnmt1	Diet	0.410
29_Dnmt1	Diet-Infection	0.636
3_ACADM	Diet	0.068
3_ACADM	Diet-Infection	0.490
3_ACADM	Infection	0.797
30_Dnmt3a	Infection	0.009
30_Dnmt3a	Diet-Infection	0.330
30_Dnmt3a	Diet	0.604
31_DRD1	Diet	0.151
31_DRD1	Infection	0.818
31_DRD1	Diet-Infection	0.874
32_DRD2	Diet	0.071
32_DRD2	Diet-Infection	0.402
32_DRD2	Infection	0.468
33_Egr1	Diet	0.316
33_Egr1	Infection	0.363
33_Egr1	Diet-Infection	0.753
34_FADS6	Infection	0.053
34_FADS6	Diet	0.168
34_FADS6	Diet-Infection	0.300
35_Fcrlg	Infection	0.031
35_Fcrlg	Diet-Infection	0.122
35_Fcrlg	Diet	0.975
36_Fcgr2b	Infection	0.003
36_Fcgr2b	Diet-Infection	0.152
36_Fcgr2b	Diet	0.287
37_FTH	Diet-Infection	0.409
37_FTH	Diet	0.841
37_FTH	Infection	0.980
38_FTL	Diet	0.235
38_FTL	Diet-Infection	0.574
38_FTL	Infection	0.592
39_GABARAP	Diet	0.151
39_GABARAP	Diet-Infection	0.680
39_GABARAP	Infection	0.796
4_ACO2	Infection	0.015

Gene	Effect	p-value
4_ACO2	Diet-Infection	0.212
4_ACO2	Diet	0.346
40_GABBR1	Diet-Infection	0.219
40_GABBR1	Diet	0.235
40_GABBR1	Infection	0.438
41_GLRB	Diet	0.075
41_GLRB	Diet-Infection	0.449
41_GLRB	Infection	0.696
42_HDAC3	Infection	0.013
42_HDAC3	Diet	0.093
42_HDAC3	Diet-Infection	0.129
43_HIF1A	Diet	0.262
43_HIF1A	Infection	0.283
43_HIF1A	Diet-Infection	0.289
44_HTR1B	Infection	0.139
44_HTR1B	Diet	0.232
44_HTR1B	Diet-Infection	0.764
45_HTR1D	Diet	0.095
45_HTR1D	Infection	0.137
45_HTR1D	Diet-Infection	0.814
46_HTR2A	Diet	0.046
46_HTR2A	Infection	0.225
46_HTR2A	Diet-Infection	0.942
47_HTR2C	Diet	0.125
47_HTR2C	Infection	0.428
47_HTR2C	Diet-Infection	0.805
48_HTR4	Infection	0.107
48_HTR4	Diet-Infection	0.203
48_HTR4	Diet	0.433
49_HTR7	Diet-Infection	0.096
49_HTR7	Infection	0.299
49_HTR7	Diet	0.722
5_Bdnf	Diet	0.050
5_Bdnf	Infection	0.724
5_Bdnf	Diet-Infection	0.817
50_Ifit3	Infection	0.000
50_Ifit3	Diet-Infection	0.428
50_Ifit3	Diet	0.583
51_Ifng	Infection	0.001
51_Ifng	Diet	0.473
51_Ifng	Diet-Infection	0.553
52_Ifngr1	Diet-Infection	0.072

Gene	Effect	p-value
52_Ifngr1	Infection	0.654
52_Ifngr1	Diet	0.752
53_Il-10	Infection	0.094
53_Il-10	Diet	0.324
53_Il-10	Diet-Infection	0.813
54_Il-1b	Diet	0.278
54_Il-1b	Diet-Infection	0.927
54_Il-1b	Infection	0.976
55_Il-6	Infection	0.057
55_Il-6	Diet	0.330
55_Il-6	Diet-Infection	0.756
56_Il-8	Diet	0.120
56_Il-8	Diet-Infection	0.475
56_Il-8	Infection	0.965
57_Itgam	Diet	0.215
57_Itgam	Diet-Infection	0.284
57_Itgam	Infection	0.943
58_Itgb5	Infection	0.027
58_Itgb5	Diet-Infection	0.176
58_Itgb5	Diet	0.352
59_Ly86	Infection	0.002
59_Ly86	Diet-Infection	0.066
59_Ly86	Diet	0.941
6_C1qa	Infection	0.001
6_C1qa	Diet	0.068
6_C1qa	Diet-Infection	0.956
60_MAOA	Infection	0.015
60_MAOA	Diet-Infection	0.302
60_MAOA	Diet	0.865
61_MAOB	Infection	0.025
61_MAOB	Diet	0.296
61_MAOB	Diet-Infection	0.833
62_MBP	Infection	0.006
62_MBP	Diet-Infection	0.821
62_MBP	Diet	0.834
63_Mip2	Infection	0.092
63_Mip2	Diet-Infection	0.185
63_Mip2	Diet	0.601
64_MTOR	Diet	0.081
64_MTOR	Infection	0.251
64_MTOR	Diet-Infection	0.915
65_NDUFS5	Infection	0.293

Gene	Effect	p-value
65_NDUFS5	Diet	0.472
65_NDUFS5	Diet-Infection	0.647
66_Nos2	Diet	0.086
66_Nos2	Infection	0.518
66_Nos2	Diet-Infection	0.906
67_NR3C1	Diet	0.105
67_NR3C1	Infection	0.156
67_NR3C1	Diet-Infection	0.917
68_NTRK2	Diet-Infection	0.121
68_NTRK2	Diet	0.153
68_NTRK2	Infection	0.491
69_P2ry12	Diet	0.032
69_P2ry12	Infection	0.175
69_P2ry12	Diet-Infection	0.194
7_C5	Diet	0.176
7_C5	Infection	0.790
7_C5	Diet-Infection	0.792
70_P2ry6	Infection	0.116
70_P2ry6	Diet	0.140
70_P2ry6	Diet-Infection	0.916
71_PFKFB1	Diet-Infection	0.519
71_PFKFB1	Diet	0.844
71_PFKFB1	Infection	0.849
72_PLP1	Infection	0.013
72_PLP1	Diet-Infection	0.315
72_PLP1	Diet	0.797
73_Rel	Infection	0.027
73_Rel	Diet-Infection	0.267
73_Rel	Diet	0.476
75_SCD	Infection	0.003
75_SCD	Diet	0.113
75_SCD	Diet-Infection	0.378
76_SCD5	Diet	0.096
76_SCD5	Infection	0.105
76_SCD5	Diet-Infection	0.737
77_SDHB	Diet	0.047
77_SDHB	Infection	0.190
77_SDHB	Diet-Infection	0.951
78_Selplg	Diet	0.040
78_Selplg	Infection	0.251
78_Selplg	Diet-Infection	0.557
79_SLC2A1	Infection	0.014

Gene	Effect	p-value
79_SLC2A1	Diet-Infection	0.421
79_SLC2A1	Diet	0.901
8_Ccr5	Infection	0.009
8_Ccr5	Diet	0.156
8_Ccr5	Diet-Infection	0.964
80_SLC2A2	Diet	0.596
81_SLC2A3	Diet	0.503
81_SLC2A3	Infection	0.618
81_SLC2A3	Diet-Infection	0.647
82_SLC2A4	Diet	0.222
82_SLC2A4	Infection	0.477
82_SLC2A4	Diet-Infection	0.893
83_Slc2a5	Infection	0.112
83_Slc2a5	Diet	0.231
83_Slc2a5	Diet-Infection	0.756
84_TACO1	Infection	0.022
84_TACO1	Diet-Infection	0.217
84_TACO1	Diet	0.433
85_TFRC	Infection	0.013
85_TFRC	Diet	0.271
85_TFRC	Diet-Infection	0.273
86_Tgfb	Infection	0.087
86_Tgfb	Diet-Infection	0.325
86_Tgfb	Diet	0.977
87_Tgfbr1	Diet-Infection	0.049
87_Tgfbr1	Infection	0.373
87_Tgfbr1	Diet	0.917
88_Tlr2	Infection	0.066
88_Tlr2	Diet	0.335
88_Tlr2	Diet-Infection	0.636
89_Tnfa	Diet-Infection	0.565
89_Tnfa	Diet	0.872
89_Tnfa	Infection	0.947
9_Ccr12	Infection	0.054
9_Ccr12	Diet	0.320
9_Ccr12	Diet-Infection	0.796
90_Tnfrsf1b	Diet-Infection	0.100
90_Tnfrsf1b	Infection	0.246
90_Tnfrsf1b	Diet	0.302
91_Tpi1	Infection	0.063
91_Tpi1	Diet-Infection	0.462
91_Tpi1	Diet	0.586

Gene	Effect	p-value
92_Tyrobp	Diet-Infection	0.162
92_Tyrobp	Diet	0.225
92_Tyrobp	Infection	0.566
93_UCP2	Diet	0.017
93_UCP2	Diet-Infection	0.254
93_UCP2	Infection	0.443
94_VAMP4	Diet	0.123
94_VAMP4	Diet-Infection	0.136
94_VAMP4	Infection	0.179
95_VAPB	Diet-Infection	0.235
95_VAPB	Infection	0.235
95_VAPB	Diet	0.328

Striatum		
Gene	Effect	p-value
1_ABAT	Diet-Infection	0.015
1_ABAT	Diet	0.112
1_ABAT	Infection	0.837
10_Cd14	Diet-Infection	0.011
10_Cd14	Infection	0.573
10_Cd14	Diet	0.584
11_Cd180	Diet-Infection	0.012
11_Cd180	Diet	0.935
11_Cd180	Infection	0.944
12_Cd4	Diet	0.255
12_Cd4	Infection	0.303
12_Cd4	Diet-Infection	0.681
13_Cd40	Infection	0.022
13_Cd40	Diet-Infection	0.204
13_Cd40	Diet	0.372
14_Cd74	Infection	0.000
14_Cd74	Diet	0.075
14_Cd74	Diet-Infection	0.344
15_Cd86	Infection	0.001
15_Cd86	Diet-Infection	0.576
15_Cd86	Diet	0.631
16_Cdk1	Diet-Infection	0.056
16_Cdk1	Infection	0.580
16_Cdk1	Diet	0.818
17_COMTKRT20	Diet-Infection	0.098
17_COMTKRT20	Infection	0.217

Gene	Effect	p-value
17_COMTKRT20	Diet	0.918
18_COX10	Diet-Infection	0.001
18_COX10	Diet	0.121
18_COX10	Infection	0.163
19_COX6A1	Diet-Infection	0.024
19_COX6A1	Diet	0.566
19_COX6A1	Infection	0.881
2_ACADL	Diet-Infection	0.066
2_ACADL	Infection	0.263
2_ACADL	Diet	0.929
20_COX7C	Diet-Infection	0.096
20_COX7C	Infection	0.470
20_COX7C	Diet	0.936
21_Crybb1	Diet-Infection	0.347
21_Crybb1	Infection	0.384
21_Crybb1	Diet	0.478
22_Csfl	Diet-Infection	0.007
22_Csfl	Diet	0.392
22_Csfl	Infection	0.486
23_Cxcl16	Diet-Infection	0.018
23_Cxcl16	Infection	0.572
23_Cxcl16	Diet	0.943
24_Cxcr2	Diet-Infection	0.015
24_Cxcr2	Infection	0.664
24_Cxcr2	Diet	0.943
25_CYBA	Diet-Infection	0.009
25_CYBA	Infection	0.145
25_CYBA	Diet	0.205
26_CYCS	Infection	0.228
26_CYCS	Diet	0.279
26_CYCS	Diet-Infection	0.280
27_DBH	Diet-Infection	0.003
27_DBH	Diet	0.272
27_DBH	Infection	0.598
28_DMT	Diet-Infection	0.056
28_DMT	Diet	0.084
28_DMT	Infection	0.200
29_Dnmt1	Diet-Infection	0.036
29_Dnmt1	Diet	0.722
29_Dnmt1	Infection	0.787
3_ACADM	Diet-Infection	0.008
3_ACADM	Diet	0.216

Gene	Effect	p-value
3_ACADM	Infection	0.857
30_Dnmt3a	Diet-Infection	0.069
30_Dnmt3a	Infection	0.835
30_Dnmt3a	Diet	0.914
31_DRD1	Diet-Infection	0.124
31_DRD1	Infection	0.519
31_DRD1	Diet	0.541
32_DRD2	Diet-Infection	0.070
32_DRD2	Diet	0.098
32_DRD2	Infection	0.984
33_Egr1	Infection	0.381
33_Egr1	Diet-Infection	0.546
33_Egr1	Diet	0.915
34_FADS6	Diet-Infection	0.289
34_FADS6	Diet	0.477
34_FADS6	Infection	0.980
35_Fcrlg	Infection	0.013
35_Fcrlg	Diet-Infection	0.643
35_Fcrlg	Diet	0.836
36_Fcgr2b	Infection	0.000
36_Fcgr2b	Diet	0.331
36_Fcgr2b	Diet-Infection	0.526
37_FTH	Infection	0.193
37_FTH	Diet-Infection	0.456
37_FTH	Diet	0.538
38_FTL	Diet	0.663
38_FTL	Infection	0.833
38_FTL	Diet-Infection	0.917
39_GABARAP	Diet-Infection	0.326
39_GABARAP	Infection	0.338
39_GABARAP	Diet	0.804
4_ACO2	Diet-Infection	0.115
4_ACO2	Infection	0.518
4_ACO2	Diet	0.887
40_GABBR1	Diet-Infection	0.107
40_GABBR1	Diet	0.435
40_GABBR1	Infection	0.855
41_GLRB	Diet-Infection	0.126
41_GLRB	Infection	0.317
41_GLRB	Diet	0.793
42_HDAC3	Diet-Infection	0.060
42_HDAC3	Infection	0.582

Gene	Effect	p-value
42_HDAC3	Diet	0.923
43_HIF1A	Diet-Infection	0.058
43_HIF1A	Infection	0.231
43_HIF1A	Diet	0.484
44_HTR1B	Diet-Infection	0.041
44_HTR1B	Diet	0.750
44_HTR1B	Infection	0.756
45_HTR1D	Diet-Infection	0.024
45_HTR1D	Diet	0.801
45_HTR1D	Infection	0.926
46_HTR2A	Diet-Infection	0.021
46_HTR2A	Infection	0.700
46_HTR2A	Diet	0.974
47_HTR2C	Diet	0.375
47_HTR2C	Diet-Infection	0.412
47_HTR2C	Infection	0.469
48_HTR4	Diet-Infection	0.016
48_HTR4	Infection	0.652
48_HTR4	Diet	0.874
49_HTR7	Diet-Infection	0.052
49_HTR7	Infection	0.337
49_HTR7	Diet	0.931
5_Bdnf	Diet-Infection	0.019
5_Bdnf	Diet	0.725
5_Bdnf	Infection	0.836
50_Ifit3	Infection	0.000
50_Ifit3	Diet-Infection	0.000
50_Ifit3	Diet	0.070
51_Ifngr1	Diet-Infection	0.052
51_Ifngr1	Infection	0.129
51_Ifngr1	Diet	0.466
52_Il-10	Diet-Infection	0.015
52_Il-10	Diet	0.844
52_Il-10	Infection	0.978
53_Il-1b	Infection	0.186
53_Il-1b	Diet	0.339
53_Il-1b	Diet-Infection	0.910
54_Il-6	Diet-Infection	0.073
54_Il-6	Infection	0.690
54_Il-6	Diet	0.854
55_Il-8	Diet-Infection	0.215
55_Il-8	Infection	0.253

Gene	Effect	p-value
55_Il-8	Diet	0.527
56_Itgam	Infection	0.045
56_Itgam	Diet-Infection	0.144
56_Itgam	Diet	0.608
57_Itgb5	Diet-Infection	0.008
57_Itgb5	Infection	0.706
57_Itgb5	Diet	0.844
58_Ly86	Infection	0.000
58_Ly86	Diet	0.193
58_Ly86	Diet-Infection	0.896
59_MAOA	Diet-Infection	0.099
59_MAOA	Diet	0.419
59_MAOA	Infection	0.715
6_C1qa	Infection	0.036
6_C1qa	Diet-Infection	0.051
6_C1qa	Diet	0.512
60_MAOB	Diet-Infection	0.235
60_MAOB	Infection	0.487
60_MAOB	Diet	0.635
61_MBP	Diet-Infection	0.004
61_MBP	Infection	0.556
61_MBP	Diet	0.892
62_Mip2	Diet-Infection	0.014
62_Mip2	Diet	0.779
62_Mip2	Infection	0.880
63_MTOR	Diet-Infection	0.535
63_MTOR	Infection	0.576
63_MTOR	Diet	0.944
64_NDUFS5	Diet-Infection	0.232
64_NDUFS5	Infection	0.470
64_NDUFS5	Diet	0.670
65_Nos2	Infection	0.099
65_Nos2	Diet-Infection	0.256
65_Nos2	Diet	0.615
66_NR3C1	Diet-Infection	0.007
66_NR3C1	Diet	0.872
66_NR3C1	Infection	0.924
67_NTRK2	Diet-Infection	0.120
67_NTRK2	Infection	0.152
67_NTRK2	Diet	0.889
68_P2ry12	Infection	0.024
68_P2ry12	Diet	0.233

Gene	Effect	p-value
68_P2ry12	Diet-Infection	0.253
69_P2ry6	Diet-Infection	0.042
69_P2ry6	Diet	0.731
69_P2ry6	Infection	0.771
7_C5	Infection	0.297
7_C5	Diet-Infection	0.350
7_C5	Diet	0.450
70_PFKFB1	Diet	0.054
70_PFKFB1	Infection	0.092
70_PFKFB1	Diet-Infection	0.349
71_PLP1	Diet-Infection	0.067
71_PLP1	Infection	0.206
71_PLP1	Diet	0.323
72_Rel	Diet-Infection	0.037
72_Rel	Diet	0.706
72_Rel	Infection	0.980
74_SCD	Diet-Infection	0.061
74_SCD	Diet	0.424
74_SCD	Infection	0.689
75_SCD5	Diet-Infection	0.035
75_SCD5	Diet	0.171
75_SCD5	Infection	0.316
76_SDHB	Diet-Infection	0.173
76_SDHB	Diet	0.349
76_SDHB	Infection	0.925
77_Selplg	Diet-Infection	0.437
77_Selplg	Infection	0.606
77_Selplg	Diet	0.679
78_SLC2A1	Diet-Infection	0.137
78_SLC2A1	Infection	0.188
78_SLC2A1	Diet	0.563
79_SLC2A3	Diet-Infection	0.166
79_SLC2A3	Diet	0.683
79_SLC2A3	Infection	0.787
8_Ccr5	Diet-Infection	0.015
8_Ccr5	Infection	0.088
8_Ccr5	Diet	0.752
80_SLC2A4	Diet-Infection	0.024
80_SLC2A4	Infection	0.118
80_SLC2A4	Diet	0.793
81_Slc2a5	Diet-Infection	0.022
81_Slc2a5	Infection	0.829

Gene	Effect	p-value
81_Slc2a5	Diet	0.861
82_TACO1	Diet-Infection	0.016
82_TACO1	Infection	0.494
82_TACO1	Diet	0.916
83_TFRC	Diet-Infection	0.011
83_TFRC	Diet	0.090
83_TFRC	Infection	0.710
84_Tgfb	Diet-Infection	0.072
84_Tgfb	Infection	0.367
84_Tgfb	Diet	0.721
85_Tgfb1	Diet-Infection	0.006
85_Tgfb1	Infection	0.423
85_Tgfb1	Diet	0.506
86_Tlr2	Diet-Infection	0.019
86_Tlr2	Infection	0.541
86_Tlr2	Diet	0.828
87_Tnfa	Infection	0.036
87_Tnfa	Diet-Infection	0.095
87_Tnfa	Diet	0.963
88_Tnfrsf1b	Diet-Infection	0.102
88_Tnfrsf1b	Infection	0.210
88_Tnfrsf1b	Diet	0.428
89_Tpi1	Diet-Infection	0.135
89_Tpi1	Diet	0.350
89_Tpi1	Infection	0.508
9_Ccr12	Diet-Infection	0.044
9_Ccr12	Infection	0.757
9_Ccr12	Diet	0.863
90_Tyrobp	Diet-Infection	0.089
90_Tyrobp	Infection	0.092
90_Tyrobp	Diet	0.742
91_UCP2	Diet-Infection	0.010
91_UCP2	Diet	0.248
91_UCP2	Infection	0.759
92_VAMP4	Diet-Infection	0.037
92_VAMP4	Infection	0.128
92_VAMP4	Diet	0.587
93_VAPB	Diet-Infection	0.249
93_VAPB	Infection	0.289
93_VAPB	Diet	0.801

Gene	Effect	p-value
Hypothalamus		
Gene	Effect	p-value
1_ABAT	Infection	0.015
1_ABAT	Diet-Infection	0.256
1_ABAT	Diet	0.738
10_Cd14	Diet-Infection	0.513
10_Cd14	Infection	0.591
10_Cd14	Diet	0.686
11_Cd180	Infection	0.063
11_Cd180	Diet	0.498
11_Cd180	Diet-Infection	0.841
12_Cd4	Infection	0.220
12_Cd4	Diet	0.561
12_Cd4	Diet-Infection	0.817
13_Cd40	Infection	0.103
13_Cd40	Diet	0.120
13_Cd40	Diet-Infection	0.726
14_Cd74	Infection	0.000
14_Cd74	Diet	0.444
14_Cd74	Diet-Infection	0.916
15_Cd86	Diet-Infection	0.126
15_Cd86	Diet	0.479
15_Cd86	Infection	0.587
16_Cdk1	Infection	0.113
16_Cdk1	Diet	0.531
16_Cdk1	Diet-Infection	0.788
17_COMTKRT20	Infection	0.085
17_COMTKRT20	Diet-Infection	0.745
17_COMTKRT20	Diet	0.971
18_COX10	Infection	0.039
18_COX10	Diet-Infection	0.483
18_COX10	Diet	0.586
19_COX6A1	Infection	0.166
19_COX6A1	Diet-Infection	0.885
19_COX6A1	Diet	0.967
2_ACADL	Infection	0.294
2_ACADL	Diet	0.539
2_ACADL	Diet-Infection	0.584
20_COX7C	Infection	0.143
20_COX7C	Diet	0.809
20_COX7C	Diet-Infection	0.972
21_Crybb1	Infection	0.441

Gene	Effect	p-value
21_Crybb1	Diet-Infection	0.549
21_Crybb1	Diet	0.594
22_Csf1	Infection	0.021
22_Csf1	Diet-Infection	0.673
22_Csf1	Diet	0.915
23_Cxcl16	Infection	0.070
23_Cxcl16	Diet	0.616
23_Cxcl16	Diet-Infection	0.857
24_Cxcr2	Infection	0.075
24_Cxcr2	Diet-Infection	0.777
24_Cxcr2	Diet	0.821
25_CYBA	Infection	0.502
25_CYBA	Diet	0.588
25_CYBA	Diet-Infection	0.728
26_CYCS	Diet	0.317
26_CYCS	Infection	0.467
26_CYCS	Diet-Infection	0.700
27_DBH	Infection	0.031
27_DBH	Diet-Infection	0.184
27_DBH	Diet	0.221
28_DMT	Diet	0.022
28_DMT	Diet-Infection	0.047
28_DMT	Infection	0.124
29_Dnmt1	Infection	0.020
29_Dnmt1	Diet-Infection	0.693
29_Dnmt1	Diet	0.984
3_ACADM	Diet	0.349
3_ACADM	Diet-Infection	0.621
3_ACADM	Infection	0.816
30_Dnmt3a	Infection	0.002
30_Dnmt3a	Diet	0.580
30_Dnmt3a	Diet-Infection	0.585
31_DRD1	Diet	0.278
31_DRD1	Diet-Infection	0.646
31_DRD1	Infection	0.703
32_DRD2	Infection	0.057
32_DRD2	Diet-Infection	0.502
32_DRD2	Diet	0.618
33_Egr1	Diet-Infection	0.140
33_Egr1	Diet	0.448
33_Egr1	Infection	0.967
34_FADS6	Infection	0.034

Gene	Effect	p-value
34_FADS6	Diet-Infection	0.659
34_FADS6	Diet	0.919
35_Fcrlg	Diet	0.335
35_Fcrlg	Diet-Infection	0.725
35_Fcrlg	Infection	0.969
36_Fcgr2b	Infection	0.018
36_Fcgr2b	Diet	0.400
36_Fcgr2b	Diet-Infection	0.929
37_FTH	Infection	0.190
37_FTH	Diet	0.711
37_FTH	Diet-Infection	0.763
38_FTL	Infection	0.176
38_FTL	Diet	0.412
38_FTL	Diet-Infection	0.743
39_GABARAP	Infection	0.042
39_GABARAP	Diet	0.450
39_GABARAP	Diet-Infection	0.463
4_ACO2	Infection	0.042
4_ACO2	Diet	0.620
4_ACO2	Diet-Infection	0.715
40_GABBR1	Infection	0.041
40_GABBR1	Diet	0.201
40_GABBR1	Diet-Infection	0.296
41_GLRB	Infection	0.030
41_GLRB	Diet	0.168
41_GLRB	Diet-Infection	0.858
42_HDAC3	Infection	0.303
42_HDAC3	Diet	0.645
42_HDAC3	Diet-Infection	0.883
43_HIF1A	Infection	0.035
43_HIF1A	Diet-Infection	0.848
43_HIF1A	Diet	0.890
44_HTR1B	Diet	0.183
44_HTR1B	Infection	0.545
44_HTR1B	Diet-Infection	0.962
45_HTR1D	Infection	0.294
45_HTR1D	Diet	0.556
45_HTR1D	Diet-Infection	0.706
46_HTR2A	Infection	0.456
46_HTR2A	Diet	0.527
46_HTR2A	Diet-Infection	0.537
47_HTR2C	Diet	0.287

Gene	Effect	p-value
47_HTR2C	Infection	0.339
47_HTR2C	Diet-Infection	0.619
48_HTR4	Diet-Infection	0.039
48_HTR4	Infection	0.125
48_HTR4	Diet	0.255
49_HTR7	Infection	0.317
49_HTR7	Diet-Infection	0.334
49_HTR7	Diet	0.334
5_Bdnf	Diet-Infection	0.467
5_Bdnf	Infection	0.685
5_Bdnf	Diet	0.904
50_Ifit3	Infection	0.000
50_Ifit3	Diet-Infection	0.472
50_Ifit3	Diet	0.917
51_Ifng	Infection	0.000
51_Ifng	Diet-Infection	0.654
51_Ifng	Diet	0.818
52_Ifngr1	Diet	0.164
52_Ifngr1	Infection	0.457
52_Ifngr1	Diet-Infection	0.987
53_Il-10	Infection	0.064
53_Il-10	Diet-Infection	0.652
53_Il-10	Diet	0.911
54_Il-1b	Infection	0.018
54_Il-1b	Diet-Infection	0.064
54_Il-1b	Diet	0.479
55_Il-6	Infection	0.132
55_Il-6	Diet-Infection	0.523
55_Il-6	Diet	0.665
56_Il-8	Diet	0.009
56_Il-8	Infection	0.021
56_Il-8	Diet-Infection	0.237
57_Itgam	Infection	0.354
57_Itgam	Diet-Infection	0.671
57_Itgam	Diet	0.994
58_Itgb5	Diet	0.203
58_Itgb5	Diet-Infection	0.542
58_Itgb5	Infection	0.640
59_Ly86	Infection	0.035
59_Ly86	Diet	0.710
59_Ly86	Diet-Infection	0.780
6_C1qa	Infection	0.521

Gene	Effect	p-value
6_C1qa	Diet-Infection	0.936
6_C1qa	Diet	0.960
60_MAOA	Infection	0.110
60_MAOA	Diet	0.460
60_MAOA	Diet-Infection	0.466
61_MAOB	Infection	0.044
61_MAOB	Diet	0.686
61_MAOB	Diet-Infection	0.887
62_MBP	Diet	0.219
62_MBP	Diet-Infection	0.602
62_MBP	Infection	0.670
63_Mip2	Diet	0.047
63_Mip2	Diet-Infection	0.542
63_Mip2	Infection	0.906
64_MTOR	Infection	0.309
64_MTOR	Diet-Infection	0.582
64_MTOR	Diet	0.683
65_NDUFS5	Infection	0.002
65_NDUFS5	Diet	0.549
65_NDUFS5	Diet-Infection	0.937
66_Nos2	Diet-Infection	0.201
66_Nos2	Diet	0.295
66_Nos2	Infection	0.589
67_NR3C1	Infection	0.318
67_NR3C1	Diet	0.565
67_NR3C1	Diet-Infection	0.718
68_NTRK2	Diet	0.212
68_NTRK2	Infection	0.372
68_NTRK2	Diet-Infection	0.830
69_P2ry12	Infection	0.432
69_P2ry12	Diet	0.680
69_P2ry12	Diet-Infection	0.727
7_C5	Infection	0.211
7_C5	Diet-Infection	0.415
7_C5	Diet	0.968
70_P2ry6	Infection	0.109
70_P2ry6	Diet-Infection	0.587
70_P2ry6	Diet	0.952
71_PFKFB1	Diet-Infection	0.185
71_PFKFB1	Diet	0.288
71_PFKFB1	Infection	0.954
72_PLP1	Infection	0.009

Gene	Effect	p-value
72_PLP1	Diet-Infection	0.321
72_PLP1	Diet	0.522
73_Rel	Infection	0.258
73_Rel	Diet-Infection	0.411
73_Rel	Diet	0.827
74_RPL19	Diet	0.092
74_RPL19	Infection	0.092
74_RPL19	Diet-Infection	0.092
75_SCD	Infection	0.002
75_SCD	Diet	0.059
75_SCD	Diet-Infection	0.294
76_SCD5	Infection	0.013
76_SCD5	Diet	0.361
76_SCD5	Diet-Infection	0.813
77_SDHB	Infection	0.043
77_SDHB	Diet	0.340
77_SDHB	Diet-Infection	0.501
78_Selplg	Diet-Infection	0.338
78_Selplg	Diet	0.911
78_Selplg	Infection	0.939
79_SLC2A1	Infection	0.016
79_SLC2A1	Diet	0.124
79_SLC2A1	Diet-Infection	0.606
8_Ccr5	Infection	0.026
8_Ccr5	Diet	0.529
8_Ccr5	Diet-Infection	0.880
80_SLC2A3	Diet	0.038
80_SLC2A3	Diet-Infection	0.468
80_SLC2A3	Infection	0.851
81_SLC2A4	Diet-Infection	0.064
81_SLC2A4	Infection	0.220
81_SLC2A4	Diet	0.812
82_Slc2a5	Diet	0.328
82_Slc2a5	Infection	0.737
82_Slc2a5	Diet-Infection	0.989
83_TACO1	Diet	0.108
83_TACO1	Infection	0.330
83_TACO1	Diet-Infection	0.855
84_TFRC	Diet	0.007
84_TFRC	Infection	0.085
84_TFRC	Diet-Infection	0.437
85_Tgfb	Diet	0.551

Gene	Effect	p-value
85_Tgfb	Diet-Infection	0.728
85_Tgfb	Infection	0.891
86_Tgfb1	Diet	0.276
86_Tgfb1	Infection	0.429
86_Tgfb1	Diet-Infection	0.651
87_Tlr2	Infection	0.300
87_Tlr2	Diet-Infection	0.688
87_Tlr2	Diet	0.860
88_Tnfa	Infection	0.057
88_Tnfa	Diet-Infection	0.178
88_Tnfa	Diet	0.296
89_Tnfrsf1b	Diet	0.347
89_Tnfrsf1b	Infection	0.723
89_Tnfrsf1b	Diet-Infection	0.929
9_Ccr12	Infection	0.079
9_Ccr12	Diet	0.535
9_Ccr12	Diet-Infection	0.679
90_Tpi1	Infection	0.055
90_Tpi1	Diet	0.075
90_Tpi1	Diet-Infection	0.795
91_Tyrobp	Diet	0.555
91_Tyrobp	Diet-Infection	0.817
91_Tyrobp	Infection	0.951
92_UCP2	Infection	0.515
92_UCP2	Diet	0.647
92_UCP2	Diet-Infection	0.793
93_VAMP4	Infection	0.141
93_VAMP4	Diet	0.498
93_VAMP4	Diet-Infection	0.914
94_VAPB	Infection	0.009
94_VAPB	Diet-Infection	0.380
94_VAPB	Diet	0.438